J. Vet. Sci. (2004), **5**(1), 19–28

JOURNAL OF
Veterinary
Science

Inherited canine copper toxicosis in Australian Bedlington Terriers

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Inherited copper toxicosis in Bedlington Terriers (CT-BT) is a copper associated hepatopathy caused by an autosomal recessive genetic defect of gene involving copper metabolism. To compare clinical and histopathological findings with previous reports and to expand our knowledge for future genetic studies, 18 terriers were clinically and histopathologically examined in this study. Pedigree information and dietary history were obtained from the owners before a thorough clinical examination was undertaken. Following the examination, a blood sample was collected for haematology, biochemistry and genetic analysis and a urine sample for urinalysis. Seven dogs were also liver biopsied for histopathology, histochemistry and electron microscopy. In this study, plasma alanine transaminase (ALT) activity was highly concordant with DNA marker test results and was the most reliable and sensitive biochemical test measured. Also clinical and biochemical copper toxicosis-affected states were noticed in a genotyped carrier dog. Histopathological and electron microscopy findings showed that the severity of the lesion was more closely correlated to the presence of clinical signs than to hepatic copper concentration. In addition, the involvement of apoptosis and *p53* gene was observed in electron microscopy. The general findings related to CT-BT in this study was similar to those previously reported except few differences in histopathology and electron microscopy.

Key words: Canine copper toxicosis, Bedlington Terriers, ATP7B, Wilsons disease, p53, apoptosis

Introduction

Inherited copper toxicosis in Bedlington Terriers (CT-BT) is due to autosomal recessive genetic defect an was first reported in the United States of America [8] and later reported in other countries [6,21,24,31,33]. A similar disease has been reported in West Highland White terriers [39], Doberman Pinschers [22] and Skye terriers [13]. However, the mechanism of copper accumulation, the onset and severity of clinical signs and the hepatic copper concentration which causes toxicity vary considerably between breeds [32]. In Bedlington Terrier's, the main cause of hepatic copper accumulation is due to reduced biliary excretion of stored copper, brought about by a genetic derangement in copper excretion [12].

Due to several similarities in pathogenesis and clinical signs, CT-BT has been investigated as a possible animal model for human Wilson's disease [36]. Because initial clinical signs seen in inherited copper toxicosis are usually nonspecific, detailed history taking, careful physical examination and several laboratory tests are required**.** The clinical signs seen with CT-BT vary and when present suggest hepatic involvement. Clinical signs have been grouped, based on the age of onset and progression [11,15]. Group 1 dogs are asymptomatic, young and up to a third of affected dogs may have normal biochemical profiles. Group 2 dogs are usually 2 to 6 years of age, have an acute onset and nonspecific clinical signs including jaundice, enlarged liver and acute haemolytic anaemia. Group 3 dogs are middle aged and older with a chronic, progressive, debilitating form of the disease. Their clinical signs are similar to Group 2 dogs but less severe.

Haematology is usually normal. However, abnormal blood panels such as mild anaemia and haemolysis and abnormal coagulation profiles can be observed [8,16]. Plasma biochemistry usually indicates liver disease but varies with the stage of the disease. Plasma alanine transaminase (ALT) activity has been commonly used for screening Bedlington Terriers for copper toxicosis and compared to other biochemical liver tests, is the most reliable [10,11]. In dogs with haemolytic crisis, hyperbilirubinaemia and bilirubinuria can occur [42]. Blood copper levels are usually normal to elevated unlike humans with Wilsons disease (WD) [41]. Plasma ceruloplasmin oxidase activity is normal or slightly increased in affected dogs, because of relatively smaller ceruloplasmin copper

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pool and larger transcuprein pool in this species [28]. In man, plasma ^{64}Cu, 24 hours after oral administration, is measured. However, in dogs because this test does not readily differentiate between affected and normal dogs, measuring faecal ^{64}Cu levels, 48 hours after parenteral administration, is a better method for differentiating between affected and unaffected dogs [3]. Hepatic copper content in Bedlington Terrier may exceed 50 times normal without significant morphological and functional liver changes [41]. Clinical signs related to CT-BT have not been obvious until liver copper levels exceed 2,000 ug/g D.W [41]. Elevated copper levels have been reported in the kidney, brain and cornea of Bedlington Terriers with copper toxicosis [16]. However, clinical signs attributed to copper accumulation at these sites have not been reported [16]. Although necropsy findings are not pathognomonic for CT-BT, findings of chronic hepatic disease are suggestive in this [9]. Liver biopsies have traditionally been the only definitive method of diagnosing CT-BT [6,9]. Although wedge biopsy is commonly preferred, needle biopsy and impression smear can provide reliable results [41]. Histopathological findings in CT-BT are mainly confined to the liver [16,25]. Histochemical staining for hepatic copper such as rubeanic acid stain [17] or rhodanine stain [23] is also useful diagnostically.

Therapy is aimed at reducing any further copper absorption from the gastrointestinal tract and enhancing copper excretion [32]. This is achieved by decreasing the copper content of the diet, using drugs that either prevent the absorption of ingested copper or promote copper excretion. Because most commercial diets contain high levels of copper, homemade diets using low copper containing foods such as fresh fruits, vegetables, most meats (not organ meats), eggs, fish (not shellfish or crustaceans), dairy products, processed cereals (white rice, white flour) are a good alternative [1,19]. Copper free vitamins and water should be provided. To lower the intestinal absorption of copper, oral zinc therapy is beneficial. Recommends dosage of zinc is initially, 10 mg of zinc acetate/kg, twice a day for 3 months followed by a maintenance dose of 5 mg/kg, twice daily [2]. Zinc supplements have a low palatability and can cause haemolytic anaemia and gastrointestinal signs due to gastric irritation, such as anorexia, vomiting, diarrhoea and abdominal pain [2].

Ascorbic acid supplementation, given with food, is claimed to reduce intestinal copper absorption at dosages of 500-1000 mg/kg but there are no studies in dogs to support this claim [32]. Copper chelators play an important part in the treatment for copper toxicosis in dogs. D-penicillamine (Penamine®) is an effective chelator [26]. Besides having anti-inflammatory and immune suppressive properties, its also has an inhibitory effect on collagen synthesis by disrupting disulphide bonds thus reducing fibrosis associated with liver cirrhosis [32]. Because D-penicillamine should be used for several years to treat copper toxicosis, a sepecific treatment has been advocated [8,34]. The recommended oral dose of D-penicillamine is 10-15 mg/kg, twice daily and should be given 20 to 30 minutes before feeding on an empty stomach to improve absorption. Common side-effects reported in dogs is gastric discomfort such as vomiting and anorexia [26]. Supportive care such as anti-inflammatory drugs and vitamin E should be used for alleviating clinical signs related to copper toxicosis [11,32].

Regular health checks at 4 to 6 month intervals and liver biopsies should be used to monitor the state of the patient and to identify carrier state dogs. Prognosis is depending on the stage of disease [11,32]. Because dogs with mild to moderate hepatic signs usually respond to supportive care, so an early diagnosis and continuous monitoring make prognosis better [32].

The general pathology of CT-BT is well described in the literature [17,32]. However, only three short reports have been documented involving the Australian Bedlington Terrier population [7,31,42]. This study was done to further extend our knowledge of the disease condition in the Australian Bedlington Terrier population and obtain preliminary data and genetic material for further genetic studies.

Materials and Methods

Animals

Eighteen Bedlington Terriers were used in this study. On presentation, pedigree information and dietary history were obtained from the owners before a thorough clinical examination was undertaken. Following the examination, a blood sample was collected for haematology, biochemistry and genetic analysis and a urine sample for urinalysis. Seven dogs were also liver biopsied.

Sample collection

A blood sample (11 ml) was collected from each dog and immediately subdivided into EDTA tubes for haematology (1 ml) and genetic analysis (4 ml), lithium heparin for biochemistry (4 ml) and sodium citrate for a coagulation profile (2 ml). A blood smear for cytology was also made and stained with Wright's stain using the Ames Hema-tek 1000 slide stainer. The heparinised blood sample was centrifuged within 10 min of collection at 350 g (2,000 rpm) for 5 min using a IECHN-SII centrifuge (Damon/IEC Division) and the plasma separated, refrigerated and analyzed the same day.

Blood and urine analysis

Haematology was measured using a Roche ABX blood cell counter (Cobas Minos Vet, Roche Diagnostic Systems) and ABX Minoton LMG diluent. Differential white cell

count was done manually. Blood biochemistry was done on a Cobas Mira (Roche Diagnostic Systems) using Boehringer Mannheim (Germany) reagents for lipase estimation, trace reagents (Trace Scientific, Australia) for calcium, phosphorus, aspartate transaminase (AST), alanine transaminase (ALT), creatine kinase (CK), amylase and creatinine estimations, and Roche reagents (Roche Diagnostic Systems, Australia) for sodium, potassium, chloride, bicarbonate, alkaline phosphatase (AP), urea, bilirubin, protein, albumin, cholesterol and glucose estimation. Plasma globulin was derived by subtracting plasma albumin from plasma protein concentration. Fibrinogen estimation was determined using a heat precipitation method [4]. Activated partial thromboplastin time and prothrombin time were done manually using the Simplastin Exels kit (Cat. No. 52182, Organon Teknika, USA). Blood copper was analysed by atomic absorption spectrophotometry. Genetic analysis for CT-BT was performed on each blood sample using a microsatellite marker (C04107) as previously described [18,20].

Urine samples (10 ml) were collected by antepubic cystocentesis and analyzed within 30 min of collection. Chemical analysis was done using Multistix 10SG reagent strips (Bayer, Australia) and the specific gravity of the urine was determined by refractometry (Atago, Japan) at 37°C. The urine samples were centrifuged at 350 g (2,000 rpm) for 10 min using an IECHN-SII centrifuge (Damon/IEC Division) and the sediment examined by light microscopy. Urine copper concentration was estimated as for blood copper.

Histopathology, histochemistry and electron microscopy

A 10 g wedge-biopsy of the quadrate lobe of the liver was taken under general anaesthesia using a midline laparatomy approach. The liver sample was divided into 4 pieces. One piece was immediately fixed in 2% cold gluteralderhyde, post-fixed in 1% phosphate-buffered osmium tetraoxide and embedded in Epon. For standard transmission electron microscopy (TEM), ultrathin sections were stained with uranyl acetate and lead citrate, laid on nickel grids and examined in a JEOL 1010 transmission electron microscope. The second piece of liver tissue was fixed in 10% buffered neutral formalin, embedded in paraffin wax and 4-µm thick sections were cut and stained with haematoxylin and eosin (H&E), Perls stain and periodic acid Schiff stain (PAS). For histochemical staining for copper, the third piece of liver tissue was fixed in 0.1% rubeanic acid with ethanol for 24 h. Stained sections were rinsed in several changes of distilled water and then counter stained with H&E stain. The remaining piece of liver tissue was placed in a plain, sterile plastic bottle for copper analysis. For hepatic copper analysis, the liver was dried at 65°C for 24 h to constant weight, digested in a mixture of nitric and perchloric acids and diluted to 25 ml with water. The digest was analyzed by atomic absorption spectroscopy using 324.7 nm wavelength. Based on literatures [10,41], less than 500 µg/g DW (dry weight) of hepatic copper level was used for standard for clear state of copper toxicosis and higher than 1000 µg/g DW (dry weight) was used for standard for affected state of copper toxicosis.

Results

Signalment, history and clinical signs

Signalment, DNA microsatellite marker test results, hepatic copper levels, history and clinical signs of 18 Bedlington terriers are outlined in Table 1. Ten were females aged between 0.6 and 11 years (3.9 ± 2.85) and eight were males aged between 1 and 4.5 years (2.5 ± 1.12). The mean body weight for 7 males and 6 females was 10.5 ± 0.86 and 8.8 ± 1.57 kg, respectively. One dog was normal for CT-BT, 5/18 were carriers and 12/18 were affected. However, only 5/18 dogs had a history of either hepatic disease or haemolytic crisis prior to presentation and no dog showed clinical signs of CT-BT at presentation. The mean value ± SD for hepatic copper concentration in 5 affected dogs was 2,638.8 ± 688.95 µg/g DW (dry weight) compared to 267 µg/g DW in a normal dog. The 18 dogs in this study came from 11 households, designed A to K and all dogs were mainly fed a low copper, low protein and low fat homemade diet (Table 2).

Blood and urine analysis

Haematology was done in 16 dogs in this study and no significant hematological changes were observed. Blood coagulation profiles and plasma fibrinogen concentration measured in 10 dogs were normal. Plasma sodium, potassium, chloride and bicarbonate concentrations were normal. Elevated plasma calcium concentrations were seen in 3 dogs while low plasma phosphorus concentrations were seen in 9 dogs (Table 3). However, only dog 3 and 17 had both an elevated calcium and low phosphorus level. Blood copper levels, except in dog 16, were lower than normal. Plasma ALT activity was elevated in 14 dogs. Increased ALT activities were noticed in 11/12 affected dogs in this study. Of 11 dogs with increased ALT activity, a greater than two-fold increase was noticed in 8 dogs. In 12 affected dogs, AST activity was increased in 7 dogs. Except for dog 13, all dogs that had increased AST activity also had increased ALT activities. In five carrier dogs, ALT and AST activity were elevated in 3/5 and 1/5 dogs, respectively. In only one of these carrier dogs was both ALT and AST elevated; this dog (dog 4) also had a history of haemolytic disease and liver disease. Plasma alkaline phosphatase activity was elevated in two dogs, 10 and 16 which also had elevated AST and ALT activity. Plasma total bilirubin level was elevated in dog 4. Plasma creatine phosphokinase activity was normal. In dog 13, plasma amylase and lipase activity

Table 1. Signalment, genetic analysis for copper toxicosis (DNA test), hepatic copper levels (μg/g, DW), history and clinical signs of 18 Bedlington Terriers

Dog No.	Sex	Age (year)	DNA Test*	Hepatic copper (μg/g DW)	History and clinical signs
1	F	0.6	Normal	267	
2	M	1.7	Carrier	ND**	Chronic upper respiratory tract infection, coughs after exercise, mild productive bronchotracheitis
3	M-	3	Carrier	ND	Dermatitis
4	M	2	Carrier	ND	Recent haemolytic crisis, liver disease
5	F	1.7	Carrier	ND	Pseudopregnancy , 3 litters (8/8, 7/7, 8/8 pups)
6	F	3.5	Carrier	ND	1 litters (4/4 pups), ear mite infestation, dermatitis
7	M	3	Affected	ND	No significant finding
8	M	1	Affected	ND	No significant finding
9	M	4.5	Affected	3,171	Liver disease
10	M	3.5	Affected	3,373	Gastic discomfort, aggressive behaviour
11	M	2	Affected	ND	Recurrent pyoderma, heart murmur
12	F-	5	Affected	ND	2 litters (3/3 and 4/4 pups)
13	F	3	Affected	2,360	Mated 4 weeks earlier; becomes depressed during oestrus and lost coat condition
14	F	4.5	Affected	ND	3 litters (5/4, 5/5, 1/0 pups), liver disease, overshot jaw
15	F-	11	Affected	ND	Severe haemorrhagic diarrhoea 7 year age; left sided systolic murmur; incontinence
16	F-	3.5	Affected	2,847	Anorexia, incontinence, polydipsia/polyuria for 3-5 days prior to presentation, haemolytic crisis
17	F	2	Affected	1,443	One litter (3/2 pups), undershot jaw
18	F-	5	Affected	ND	Skin lump on back, vaginal discharge

*__DNA test__ is base on the assumption that the *Murr* 1 gene is responsible for copper toxicosis in Bedlington Terries (CT-BT). "Normal" refers to dogs with two copies of intact exon 2 of *Murr* 1. "Affected" refers to dogs lacking exon 2 of *Murr* 1. Recent finding indicates that CT-BT is not entirely caused by the exon 2 deletion of *Murr* 1 gene [20].
**__ND__: Not determined.

was increased to 2,098 and 1,292 respectively. Plasma cholesterol concentrations varied and were elevated in four dogs and lower than normal in three other dogs. Dogs 3 and 4 had elevated plasma protein and albumin concentrations. Plasma urea levels were reduced in 3 dogs.

Urinalysis was done in seven dogs and found to be normal. Urine copper levels (normally less than 10.0 μmol/l) measured in dog 16 and 18 were 44.7 and 3.3 μmol/l, respectively and the creatinine/copper ratio was 262 and 3,313, respectively.

Histopathology, histochemistry and electron microscopy

Liver biopsies for histopathology and histochemistry were performed in one normal dog (dog 1) and 5 affected dogs (dog 9, 13, 14, 16, 17). The liver of the normal dog was grossly and histologically normal and was negative for special stains. In the affected dogs, the liver grossly varied in severity. In dog 13, it was normal except for a few focal pale areas over its surface. The livers of dog 14 and 16 were smaller than normal and showed several focal, pale areas over their surfaces and in dog 16, a surface depression was also evident in the center of a relatively large pale area. The appearance of the liver in dogs 9 and 17 was not recorded.

Microscopically, the liver of dog 13 and 17 were less affected than in dogs 9, 14 and 16. In dog 13 and 17 with hepatic copper concentrations of 2,369 and 1,440 μg/g DW, respectively, hepatocyte pathology was largely absent. Copper laden hepatocytes were seen scattered throughout the liver especially in the periacinar region. Histochemical staining was positive for copper and lipofuscin but negative for haemosiderin.

The livers in dog 9, 14 and 16 were similarly affected. In dog 14, necrotic foci of various sizes were scattered throughout the liver, especially in the periportal area. Most of the necrotic foci consisted of macrophages, Kupffer cells and dead hepatocytes with variable degree of fibrosis. Marked copper deposition in hepatocytes was clearly visible with rubeanic acid staining. The hepatocytes around the periacinar region were PAS positive, suggesting copper bound lipofuscin. Perl's stain demonstrated haemosiderin in macrophages and Kupffer cells, especially in the periportal area (Fig. 1-C).

Table 2. Diet fed to 18 Bedlington Terriers from 11 different households

Household	Dog No.	Diet history
A	2, 12, 14	Home made (chicken, carrot, pumpkin, garlic, rice, pasta, wheatbix & milk, honey), Life system®, Missing link®
	5	Raspberry leaf®, Missing link®, Dolomite®(calcium)
	1	PAL ®(adult performance)
B	13	Low copper/ low protein diet; Filtered copper free water, St Mary's thistle® (1ml/day)
	9	Zinc, iron, vitamin
C	7,15, 17	Low fat, low salt diet (raw meat, chicken, rice & vegetables) Supa cote® & Dog biscuits, Liver tonic
D	8	Lucky Dog®, Nature's Gift®, homemade diet (chicken mince, carrot, pumpkin, garlic, flour, pasta, rice), Balanced CAL®, St. Mary's thistle®, Supa Cote®
E	16	Homemade diet (chicken, mince, rice, pasta) with dry food (Eukanuba® & Bonnie lite®), dog sausage and chocolates
F	10	Oxy-Vital®, creamed cottage cheese, chicken, zinc acetate, boiled rice with chicken breast, Filtered water, boiled egg and fruit
G	11	PAL,® Friskees® (Trusty), minced steak, processed bran, oatmeal, rice, lamb bones
H	3	Chicken and rice, Filtered copper free water, dry food, beef mince, Supa Cote®
I	4	Unknown
J	18	My dog Tin food®, Raw chicken wing, beef mince with vegetables, pasta with brown rice, chocolates (1-2 block) Supa Cote®
K	6	PAL® (Super performance), rice, chicken, milk, pasta, minced beef, bones (weekly)

Life system (liver tonic) contains milk thistle, dandelion, globe artichoke and garlic. **Missing Link** contains flax seed, sunflower seed, rice bran, alfalfa, carrot, apple, hesperidin, brocolli, licorice root, cherry, parsley, kelp, garlic and rosemary. **Dolomite & Balanced CAL** contains ionic calcium supplement. **St Mary's thistle** contains iron supplement. **Supa Cote** contains vitamin E and essential fatty acid supplements. **Lucky dog**, **Eukanuba & Bonnie lite** are commercial diets and treats, **Nature's Gift** contains beef, chicken, vegetables, garlic and vitamins and minerals.
Copper content in diets (mg/100g W/W): Boiled rice, boiled pasta -0.02; plain flour, beef mince, chicken -0.2; Milk -0.03 (skim), 0.02 (full cream); Honey -0.05; Boiled pumpkin -0.1; Carrot -0.1 [38].
All owners attempted to fed dogs a low copper diet although the copper content of some portions of the diets, for example, commercial dog foods was unknown. Several supplements, for example, St. Marys thistle was included in the diet based on naturopathic beliefs.

In dog 16 with 2,847 µg/g DW hepatic copper, the characteristic histological changes attributed to high copper accumulation were present. Focal and piecemeal necrosis with infiltration of neutrophils, macrophages and lymphocytes were seen throughout the liver (Fig. 1-A). Heavy copper-positive lipofuscin granules in the hepatocytes were visible throughout the liver, especially in the periacinar region. The number of Kupffer cells and macrophages containing large amounts of haemosiderin pigment were markedly increased. Mild periportal fibrosis, deposits of copper within some clusters of macrophages and occasional extramedullary haematopoiesis and erythophagocytosis were also observed in this dog. Histochemical stains for copper (Fig. 1-B), lipofuscin and haemosiderin were strongly positive.

The liver of dog 9 contained 3,171 µg/g DW copper and showed typical generalized brown granular pigmentation of the hepatocytes. Focal accumulation of large macrophages containing coarse brown granular pigment and usually surrounded by mononuclear inflammatory cells was seen throughout the liver. Smaller portal areas show evidence of early fibroplasia and cholangiolar hyperplasia. Histochemical staining was not performed in this dog.

Electron microscopy of the liver was done in and one normal dog (dog 1) and four affected dogs (dog 13, 14, 16, 17). In the normal dog, the hepatocytes were normal although heterogeneous electron dense material was observed in a few hepatocytes. The liver in dog 17 with the lowest copper content was the least affected. Irregular shaped electron dense material was observed throughout the hepatocytes, macrophages and Kupffer cells. Mild condensation of hepatic micro-organelles, bi-nucleated hepatocytes and mitochondria of various shapes and sizes but with intact membrane were also seen.

In dog 13, the liver showed increased electron dense deposits in the hepatocytes, macrophages and Kupffer cells, irregular shaped lysosomes and shrunken nucleus with irregular membranes. Variable sized mitochondria with loss of cristae containing electron dense material were also seen. Vacuolation and swelling of the smooth endoplasmic

Table 3. Mean values ± SD for biochemical parameters measured in 18 Bedlington Terriers

Test	Range	1	2	3	4	5	6	7	8	9	10
Copper	15.7-19.0 µmol/L	**8**	**8.3**	**9.4**	ND	**6.7**	**10.4**	**13.6**	ND	**13.4**	**10.1**
Calcium	1.9-2.8 mmol/L	2.6	2.5	**3.4**	1.9	2.8	ND	2.7	**3.0**	2.2	2.7
Phosphorus	1.2-3.9 mmol/L	**0.7**	1.4	**1.1**	2.0	1.7	ND	**1.1**	1.2	1.3	1.2
AST	13-40 U/L@37C	24	24	37	**451**	25	35	24	34	37	**114**
ALT	10-50 U/L@37C	30	23	**72**	**191**	29	**96**	**72**	**115**	**71**	**406**
T. bilirubin	1.2-10.4 µmol/L	ND	1.4	ND	**26**	1.6	ND	ND	ND	2.5	ND
Protein	54-71g/L	58	64	**73**	**89**	**74**	ND	64	60	61	68
Albumin	23-32 g/L	**34**	32	**36**	**44**	31	ND	**34**	32	30	30
Globulin	27-44 g/L	ND	32	37	**45**	43	ND	30	28	31	38
Urea	4.3-6.4 mmol/L	4.7	4.8	**10.2**	**6.9**	5.1	ND	6.1	4.1	2.2	**10.4**
Creatinine	20-177µmol/L	69	92	75	92	82	ND	84	51	92	96
Cholesterol	3.2-6.5 mmol/L	**6.8**	4.4	3.9	8	**8.5**	ND	5.5	3.0	**2.9**	3.8
Test	Range	11	12	13	14	15	16	17	18	Mean ± SD	
Copper	15.7-19.0 µmol/L	**14.5**	**8.5**	**11**	**6.5**	**11**	**20.1**	**10.7**	**12**	10.8 ± 3.40	
Calcium	1.9-2.8 mmol/L	2.7	2.3	2.4	2.5	2.6	2.7	**2.9**	2.8	2.6 ± 0.32	
Phosphorus	1.2-3.9 mmol/L	**1.1**	1.3	**0.7**	1.6	**1.1**	**1.0**	**1.0**	**0.7**	1.2 ± 0.34	
AST	13-40 U/L@37C	**93**	**53**	**43**	39	**58**	**204**	36	**49**	77 ± 103	
ALT	10-50 U/L@37C	**184**	**63**	9	**99**	**194**	**1378**	**123**	**96**	181 ± 313	
T. bilirubin	1.2-10.4 µmol/L	ND	1.8	1.8	1.6	ND	ND	ND	ND	5.2 ± 9.16	
Protein	54-71g/L	60	**76**	63	66	58	62	61	56	65 ± 8.4	
Albumin	23-32 g/L	31	29	30	31	28	31	**36**	30	32 ± 3.8	
Globulin	27-44 g/L	29	**47**	33	35	30	31	25	26	34 ± 6.6	
Urea	4.3-6.4 mmol/L	**7.4**	4.8	**2.76**	4.4	5.9	4.9	5.5	**8.2**	5.8 ± 2.25	
Creatinine	20-177µmol/L	83	101	98	103	79	150	71	41	86 ± 23.6	
Cholesterol	3.2-6.5 mmol/L	4.9	4.7	4.0	3.9	**6.6**	5.6	4.0	**0.1**	4.7 ± 2.03	

Note: ND; Not determined
Bold: abnormal range; T. bilirubin: Total bilirubin, AST: Aspartate transaminase, ALT: Alanine transaminse.

reticulum (SER), reduced amounts of rough endoplasmic reticulum (RER) and disaggregation of ribosomes were also present.

In dog 14, the electron dense deposits were more abundant and some nuclei showing membrane rupture. The mitochondria were varied in size and shape, had lost their cristae and their matrix heterogeneity was increased. Some mitochondria showed granulation, suggestive of more severe degeneration (Fig. 1-D). Vacuolation and swelling of SER and disaggregation of ribosomes, extramedullary hematosis and apoptosis were also noticed in this dog (Fig. 1-E).

In dog 16, the liver showed numerous electron dense deposits in the hepatocytes, macrophages, Kupffer and endothelial cells (Fig. 1-F). Most of the hepatocytes were shrunken and their nuclei misshapen. The hepatocyte microorganelles were tightly packed and condensed. The hepatocyte mitochondria varied in size and shape and their number increased due to all shrinkage. The mitochondrial cristae were mostly lost and replaced by a homogeneous uniformly dense matrix. Some mitochondria contained small electron dense floccular material. Swelling and vacuolation of SER, absence of RER and disaggregation of ribosomes were also observed in the hepatocytes.

Discussion

Australian Bedlington Terriers are descendents of 6 dogs imported from the United Kingdom between 1963 and 1967 and today number 200 to 300 dogs [31]. Although the 18 Bedlington Terriers used in this study were related, they were not genetically tightly linked. Two dogs had heart disease which has been previously reported in this breed [31]. However, the relationship between heart disease and copper toxicosis, if any, is unknown. Polydipsia and polyuria were seen in one dog, a finding previously reported [42]. One dog had behavioural problems, which has been reported in human Wilson's disease due to neurological disease [36] but not in CT-BT, although a mild histologically degenerative lesion has been reported in the cerebrum of one dog [16]. Three affected dogs and one carrier dog had a history of haemolytic disease. Intravascular haemolysis in CT-BT is occasionally reported and resulted in

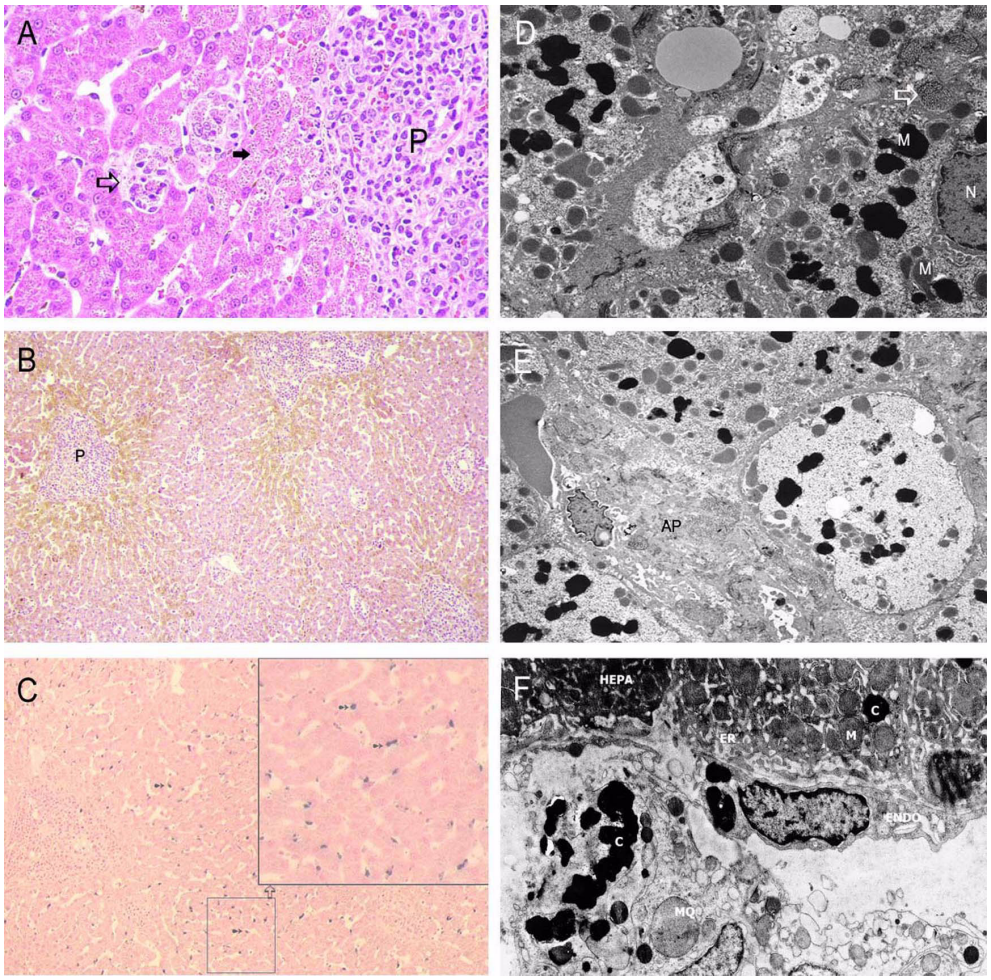

Fig. 1. Histopathology, histochemistry and electron microscopy in copper toxicosis in Bedlington Terriers.
A: Liver of dog 16. Piecemeal necrosis (blank arrow), heavy copper laden hepatocytes (arrow) and marked inflammatory cell infiltration in the periacinar area (A). H&E stain, ×400.
B: Liver of dog 16. Heavy copper accumulation in periacinar region (P), H&E stain, Rubeanic acid stain, ×100.
C: Liver of dog 14. Heavy haemosiderin deposition in Kupffer cells and macrophages (pin-pointed arrow). Perl's stain, ×100, ×200, (Inset).
D: Hepatocyte of dog 14 showing deposition of irregular shaped electron dense materials. The nucleus was misshapen and its membrane ruptured. Mitochondria were variable in size and shape and had lost their cristae and increased matrix heterogeneity. Some mitochondria showed granulation (arrow) ×4,000.
E: Liver of dog 14. Apoptotic body (Ap) in liver ×4,000.
F: Liver of dog 16. The microorganelles of the hepatocyte are tightly packed and the adjacent endothelial cell contains some electron dense material. Endothelial cells (ENDO), hepatocyte (HEPA), macrophage (MQ), mitochondria (M), endoplasmic reticulum (ER), nucleus (N), electron dense materials (C). ×5,000.

haemoglobinaemia, haemoglobinuria and polychromasia [8,42]. However, haematological and clinical evidence of haemolysis may not be present unless sampling is done during the crisis [8,16]. Nevertheless, histological evidence of haemolysis such as hepatic haemosiderosis and erythrocytophagosis was seen in the liver of one affected dog in this study. Although dog 4 had a history of acute haemolytic disease and an elevated AST activity, it was a carrier suggesting that carrier dogs may also show clinical manifestation. Epstein and Sherlock [5] suggested that the delayed recovery from a high copper state in human WD carrier neonates is because one controller gene would be normal and the other defective in the carrier state. This delayed recovery from a high copper state has been observed in Bedlington Terrier carrier pups [29,30]. Unfortunately a liver biopsy was not available for this dog to confirm the possible aetiology of the haemolysis. However, only affected dogs have been reported to show clinical signs of CT-BT.

Dietary influences on plasma biochemistry were observed in this study. Dogs fed a low copper diet as a preventive measure had low plasma copper levels. This finding

confirms that dietary management in affected dogs is important and effective, but needs to be considered when using plasma copper levels for screening CT-BT. Most dogs on a low protein diet had decreased phosphorus and urea concentrations. The elevated plasma urea concentrations in dog 3 and 4 may have been due to dehydration, because plasma total protein and albumin concentrations were also elevated. The high plasma calcium and low phosphorus levels in some dogs were most likely due dietary calcium supplementation and reduced protein intake.

The reliability of plasma ALT activity in screening Bedlington Terriers for copper toxicosis has been well documented in the literature [11], despite some controversies [17,31,41]. In this study, ALT activity found to be more sensitive indicator of liver disease than the AST activity. This is not surprising that AST activity increases after ALT activity in liver disease because it requires more severe injury due to its distribution in cytosol and mitochondria whereas ALT is solely in the cytosol [27]. The sensitivity of ALT in CT-BT has been reported as 34% [9] and 45% [41]. In this study, although it was 91%, the number of dogs tested was small. The elevated ALT activity in some carrier dogs suggests that liver disease is present and that normal copper excretion in these dogs is also mildly affected [11]. In CT-BT, the hepatic damage by copper accumulation seems to be slowly progressed and it is suddenly triggered by stress such as dog show, shipping according to this study, similarly to others [9,11].

The cause of the increase in plasma lipase and/or amylase concentration in three dogs (dog 10, 12, 13) is unclear as they were clinically normal and did not appear to have pancreatic, renal and gastrointestinal abnormalities. Increased pancreatic enzyme activities has been reported in human Wilsons disease patient receiving large dose of zinc supplement in the absence of pathological changes in the pancreas [43]. Therefore, increased pancreatic enzyme activities in dogs 10 and 13 may have been due to zinc supplement.

The increased urinary copper excretion seen in this study was due to the compensation mechanism of reduced biliary copper excretion [37].

The hepatic copper content in the normal Bedlington Terrier in this study was within the normal range (206 ± 56 μg/g DW) reported by others [34,41]. The average hepatic copper level in 5 affected dogs was 2,638.8 ± 688.95 μg/g DW and lower than values previously reported [11,41], because all the dogs in this study were clinically normal when liver biopsies were taken. Clinical signs of CT-BT can appear when hepatic copper levels exceed 2,000 μg/g DW [21,40,41]. In this study, 4/5 dogs had levels greater than 2,000 μg/g DW of hepatic copper and 3/4 dogs had history and clinical signs before or after hepatic copper analysis. Histopathological evidences of liver disease without clinical signs was seen at 1,444 μg/g DW of hepatic copper (dog 17), while hepatocellular granules are visible when hepatic copper levels are greater than 850 μg/g DW [21,40,41]

Although a correlation between clinical signs and copper accumulation is not observed [16], there appears to be a close correlation between the severity of the liver lesion and the concentration of copper [14,17,35,41]. However, although hepatic copper content was similar in dogs 13 and 16, the liver was more severely affected in dog 16. Nevertheless, the severity is more closely correlated with the presence of clinical signs than with the hepatic copper concentration.

Due to the absence of clinical signs, the livers of dog 13 and 17 were macroscopically normal and histopathologically graded as stage 2 liver [17,41]. In these dog, liver pathology was largely absent except for the periacinar accumulation of copper positive lipofuscin granules. Unlike other works, haemosiderin deposition was not seen in these dogs [25,42].

Ultrastructural changes in the liver of dogs 13 and 17 were less obvious than those of dogs 14 and 16. Although ultrastructural changes such as shrunken nuclei, mitochondrial changes and accumulation of electron dense material were noticed in both dogs, as previously reported [14]. The severity of these changes between dog 13 and 17 was not obviously different despite the fact that the hepatic copper content between these two dogs was about 1,000 μg/g DW. It is re-confirmed that the severity of lesion is more correlated to the presence of clinical signs.

The liver pathology seen in CT-BT was categorized by the severity [17,41]. Liver pathology of two affected dogs (13, 17) in this study were categorized as stage 2, while the other affected dogs (9, 14 and 16) were included in stage 3. However, bridging necrosis which occasionally occurred in stage 3 was not observed in these dogs, while extramedullary haematopoiesis which is one of main findings in stage 4 was noticed in two dogs (14 and 16).

Although the accumulation of electron dense material in Kupffer cells, macrophages and hepatocytes has previously been reported [14,17,41], its presence in endothelial cells as in this study have not. The changes in mitochondria and SER were much severer than those of dog 13, 17 and the dog with 2,184 μg/g DW of hepatic copper reported in literature [14]. RERs were absent and most ribosomes were disaggregated from endoplasmic reticulum. However, electron dense material was not noticed in nucleus that was reported in dogs with 7,187 μg/g D.W of hepatic copper [14]. Haywood *et al.* [14] suggested that apoptosis and *p53* gene might be involved in the pathogenesis of CT-BT. Apoptotic bodies were seen in the liver of dog 14.

In summary, plasma ALT activity is a reliable and sensitive biochemical test for screening CT-BT. Possible clinical and biochemical CT- affected states were noticed in one genotypically carrier dog. Low copper diets can effectively maintain low plasma copper levels in affected dogs. The average hepatic copper level in 5 clinically normal

affected dogs was 2,638.8 ± 688.95 µg/g DW. The severity of the liver is more closely correlated with clinical signs than hepatic copper content. Although overall liver pathologies in affected dogs studied were similar to those previously reported, a few differences were observed in histopathological and ultrastructural studies. Apoptotic bodies possibly induced by *p53* were seen in affected dogs in ultrastructural studies.

Acknowledgment

This study was supported by research grants from the Canine Control Council and John and Mary Kibble Trust and stipends from Keith-Mackie Lucas scholarship and Graduate School Award of The University of Queensland. This project would not have been possible without the cooperation and support of the members of the Bedlington Terrier Club of Queensland and many veterinary practitioners. The authors appreciate the assistance provided by Drs. Jason Mouatt and Lindsay M. Freney (Gympie) for liver biopsies, Mr. Bryan Benon for hematology and biochemistry, Dr. Majid Ghoddusi for histopathology and electron microscopy in School of Veterinary Sciences, The University of Queensland, Australia.

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