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Chronological changes in tissue copper, zinc and iron in the toxic milk mouse and effects of copper loading

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Abstract

The toxic milk (*tx*) mouse is a rodent model for Wilson disease, an inherited disorder of copper overload. Here we assessed the effect of copper accumulation in the *tx* mouse on zinc and iron metabolism. Copper, zinc and iron concentrations were determined in the liver, kidney, spleen and brain of control and copper-loaded animals by atomic absorption spectroscopy. Copper concentration increased dramatically in the liver, and was also significantly higher in the spleen, kidney and brain of control *tx* mice in the first few months of life compared with normal DL mice. Hepatic zinc was increased with age in the *tx* mouse, but zinc concentrations in the other organs were normal. Liver and kidney iron concentrations were significantly lower at birth in *tx* mice, but increased quickly to be comparable with control mice by 2 months of age. Iron concentration in the spleen was significantly higher in *tx* mice, but was lower in 5 day old *tx* pups. Copper-loading studies showed that normal DL mice ingesting 300 mg/l copper in their diet for 3 months maintained normal liver, kidney and brain copper, zinc and iron levels. Copper-loading of *tx* mice did not increase the already high liver copper concentrations, but spleen and brain copper concentrations were increased. Despite a significant elevation of copper in the brain of the copper-loaded *tx* mice no behavioural changes were observed. The livers of copper-loaded *tx* mice had a lower zinc concentration than control *tx* mice, whilst the kidney had double the concentration of iron suggesting that there was increased erythrocyte hemolysis in the copper-loaded mutants.

Introduction

The dependency of physiological processes on the activity of copper-containing enzymes creates the need for a continued supply of copper. If copper intake exceeds the nutritional requirements, excess copper is excreted from the liver in bile (Linder *et al.* 1998). The copper transport disorder, Wilson disease, results from defective biliary excretion of copper which causes a massive copper accumulation in the liver (Danks 1995). Wilson disease is caused by mutations in the copper transporting ATPase, ATP7B. This protein is located in the *trans*-Golgi network of hepatocytes when copper concentrations in the cells are relatively low, and in this location ATP7B supplies copper to the serum copper protein ceruloplasmin. When copper levels in the hepatocyte rise, ATP7B traffics to cytoplasmic vesicles and these subsequently mediate copper excretion from the cell into bile (Schaefer *et al.*

1999). The principle methods for treatment of Wilson disease are the elimination of excess mobilised copper by chelating agents such as d-penicillamine (Walshe 1956) or with orally ingested zinc salts which upregulate metallothioneins thereby blocking the absorption of copper via the gut (Yuzbasiyan-Gurkan *et al*. 1992).

The *tx* mouse was established as a model of Wilson disease when the point mutation of a highly conserved methionine to valine (M1356V) in the murine *Atp7b* orthologue was described (Theophilos *et al*. 1996). The mutant ATP7B was subsequently shown to have minimal copper transport activity (Voskoboinik *et al*. 2001). As with patients with Wilson disease, the *tx* mouse accumulates massive quantities of hepatic copper.

Other rodent models of Wilson disease include the LEC rat (Wu *et al*. 1994), a knock-out *tx* mouse in which the murine WND gene has been completely disrupted (Buiakova *et al*. 1999) and a mouse with another autosomal recessive mutation in the *Atp7b* gene designated tx^J, derived at the Jackson's laboratory (Coronado *et al*. 2001). The tx^J mouse model has a glycine to aspartic acid (G712D) mutation in ATP7B, which results in a similar phenotypic disorder to *tx* mice.

Copper, zinc and iron are all essential trace elements for humans and animals. This study aimed to assess the effect of copper toxicity on the zinc and iron concentration in the normal DL and *tx* mice as models of normal and abnormal copper metabolism, respectively. We then further stressed the copper metabolic pathways by providing a diet high in copper through copper-loaded drinking water over 3 months.

Methods

Mouse husbandry

The *tx* mutation spontaneously arose in the inbred DL strain and has subsequently been maintained on this background (Rauch 1983). Pups of *tx* mice require fostering in order to survive and were routinely cross fostered within 5 days of birth to BALB/C dams that had produced litters at a similar time. All mice used in this study were kept and cared for in the mouse house facility at the Murdoch Childrens Research Institute. Both water and food were provided *ad libitum*. The Royal Children's Hospital Animal Ethics Committee approved the experimental protocols (#A332).

The following number of DL mice were in each age group: 35 mice 5 days old (35 male); 9 mice 2–4 months (1 male, 8 female); 21 mice 5–7 months (4 male, 17 female); 20 mice 8–11 months (10 male, 10 female); 5 mice 12–21 months (2 male, 3 female). The following number of *tx* mice were in each age group: 26 mice 5 days old (12 male, 14 female); 17 mice 2–4 months (11 male, 6 female); 18 mice 5–7 months (12 male, 6 female); 18 mice 8–11 months (13 male, 5 female); 9 mice 12 – 21 months (2 male, 7 female).

Copper-loading experiment

In a pilot assessment of copper administration to *tx* mice, copper in the form of copper acetate (Merck) was dissolved in deionized drinking water at varying concentrations (100–1000 mg/l of copper as copper acetate) and given to mice for 3 months. This range of concentrations was chosen to reflect doses given to rats in copper-loading experiments (Sokol *et al*. 1990; Myers *et al*. 1993). All mice survived the pilot study, but the mice did not reliably consume water with 1000 mg/l copper and so a concentration of 300 mg/l was chosen for longer-term copper-loading studies. A copper-loading protocol was developed whereby mice received a gradually increasing concentration of copper in drinking water at 8 weeks of age, with incremental increases (100 mg/l of copper per week) over a period of 3 weeks, which were then maintained at 300 mg/l for 3 months. Nine DL mice were copper-loaded (2 male, 7 female), whilst 12 *tx* mice were copper-loaded (8 male, 4 female). Copper-loaded mice received, on average, 1.2 mg of elemental copper in drinking water per day (approximately 50 mg copper/kg bodyweight/day). Control animals received a normal diet, that is, no additional copper in the drinking water. The drinking water had an average copper content of 0.05 mg/l, whilst the irradiated mouse pelleted food (Barastoc Stockfeeds Pty Ltd) contained an average of 0.02 mg/g.

Collection of tissue and trace metal analysis

Liver, kidney, spleen and brain samples were collected from all experimental animals. Samples of spleen and brain were not collected from 5 day

old pups. The concentration of copper, zinc and iron were determined by atomic absorption spectroscopy. Briefly, tissue was dried at 50 °C for 1 week, weighed and transferred into an acid-washed container to which 0.5 ml of nitric acid had been added. The samples were incubated at room temperature for 1 h, then at 65 °C for 4 h; after this, 2.5 ml of deionized water was added, the samples were centrifuged and the supernatant analysed with a Varian SpectraAA-880 with direct aspiration. All results are expressed as μg/g dry wt of tissue.

Serum ceruloplasmin oxidase activity

Ceruloplasmin oxidase activity in mouse sera was analysed using a modified method of Schosinsky *et al.* (1974). Human serum was used as a positive control for the assay.

Liver histology

Tissue collected for histology was placed in an overnight processor on the same day. Paraffin wax sections were cut (5 μm) and placed onto glass slides. All slides were stained by hematoxylin and eosin for histological assessment.

Data analysis

Analysis of data by one-way ANOVA and two-way ANOVA was performed using the SPSS (Statistical Package for Social Sciences – Version 11.5) program. Data more than two standard deviations away from the mean were considered outliers and removed. Data was considered significant when $p < 0.05$. Significance was determined by "Tukey's b" *post-hoc* analysis. All data presented as mean ± SE.

Results

Tissues were collected at various ages from *tx* and DL (normal) mice, and the concentration of copper, zinc and iron were determined by atomic absorption spectrometry.

Metal concentrations in the liver

The copper concentrations in *tx* mouse liver were significantly lower than normal at 5 days of age

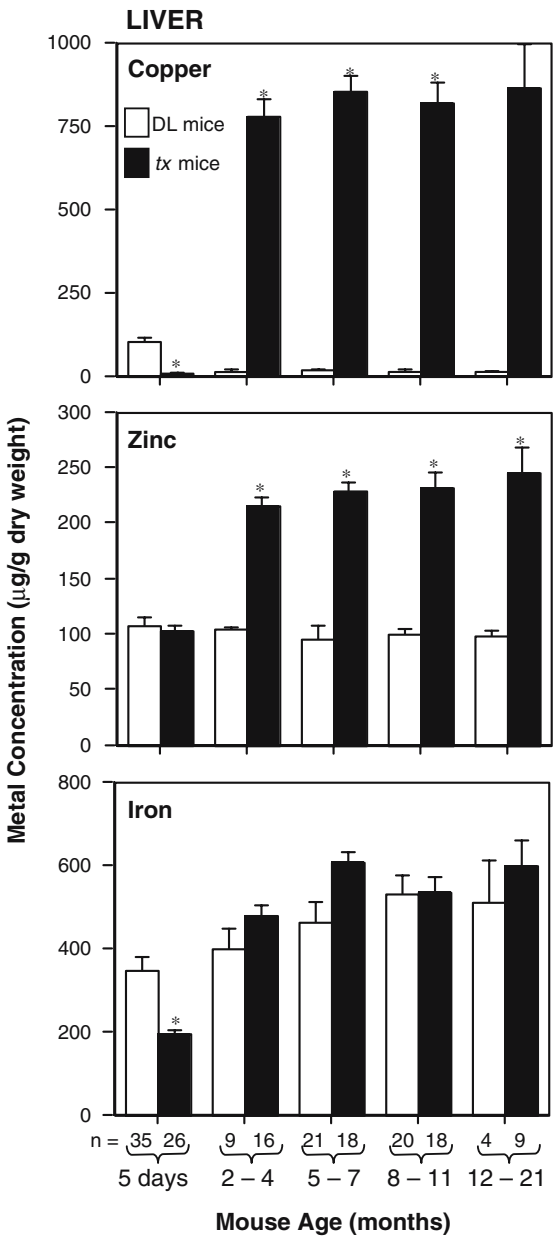

Figure 1. Comparison of liver copper, zinc and iron levels in normal (DL) and *tx* mice in relation to mouse age. Concentration of copper, zinc and iron were determined by atomic absorption spectroscopy. Data are presented as mean ± SE.

($p < 0.05$), but by 2–4 months copper levels had dramatically increased (Figure 1). DL mouse liver copper concentration dropped significantly during the same period. Liver copper concentrations plateaued at about 50-fold higher in *tx* mice (approximately 850 μg/g dry wt) and remained relatively constant over a 19-month period

(Figure 1). Control mice maintained a copper concentration of approximately 15 μg/g dry wt in the liver over this time period. At 5 days of age the zinc levels in the *tx* mouse liver were normal, however by 2 months of age there was a 2-fold increase compared to controls, correlating to some extent with the increase in liver copper concentration (Figure 1). Iron concentrations were significantly lower ($p < 0.05$) 5 days after birth in *tx* mice, then increased by 2 months of age. *Tx* mouse liver iron concentration was then maintained at the normal level (Figure 1).

Metal concentrations in the kidney

At 5 days after birth, copper levels in both DL and *tx* mouse kidney were lower than adult levels (Figure 2). In normal mice the concentration of kidney copper had plateaued by 2–4 months at approximately 15 μg/g dry wt, but in *tx* mice the concentrations increased with age reaching about 28 μg/g dry wt by 12 months (Figure 2). Kidney zinc concentration was higher in the 5-day old mice than older groups, but there was no difference between mutant and normal animals at any age. Iron concentration in *tx* mouse pups at 5 days was significantly lower than normal pups, but increased to normal levels by 2 months. In older animals iron concentrations were tending higher in the *tx* group, but this increase was not statistically significant.

Metal concentration in the spleen

Splenic copper and iron concentrations (Figure 3) were higher in *tx* mice at each age group analysed (2–21 months) compared to normal mice, and increased with age until one year old. Whilst zinc concentration did not show any significant changes in relation to mouse age or strain (Figure 3).

Metal concentrations in the brain

Copper concentration in *tx* mouse brain was increased significantly at all ages examined (2–21 months) and the difference between normal and *tx* animals increased with age, such that the 12–21 month *tx* group had a mean brain copper concentration approximately 2-fold higher than normal mice (Figure 4). Brain zinc was higher in *tx* mice in each age group, but the difference was not statistically significant, whilst iron did not show significant strain or age changes.

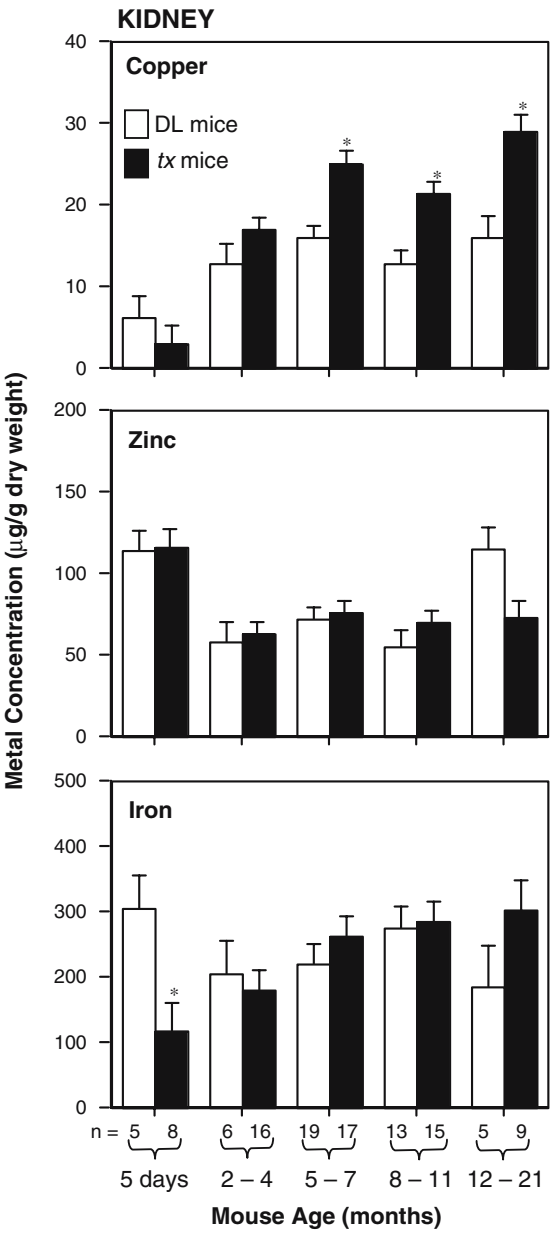

Figure 2. Comparison of kidney copper, zinc and iron levels in DL and *tx* mice in relation to mouse age. Concentration of copper, zinc and iron were determined by atomic absorption spectroscopy. Data are presented as mean ± SE.

Serum ceruloplasmin

Serum was obtained from *tx* and normal mice (5–8 months old) and ceruloplasmin oxidase

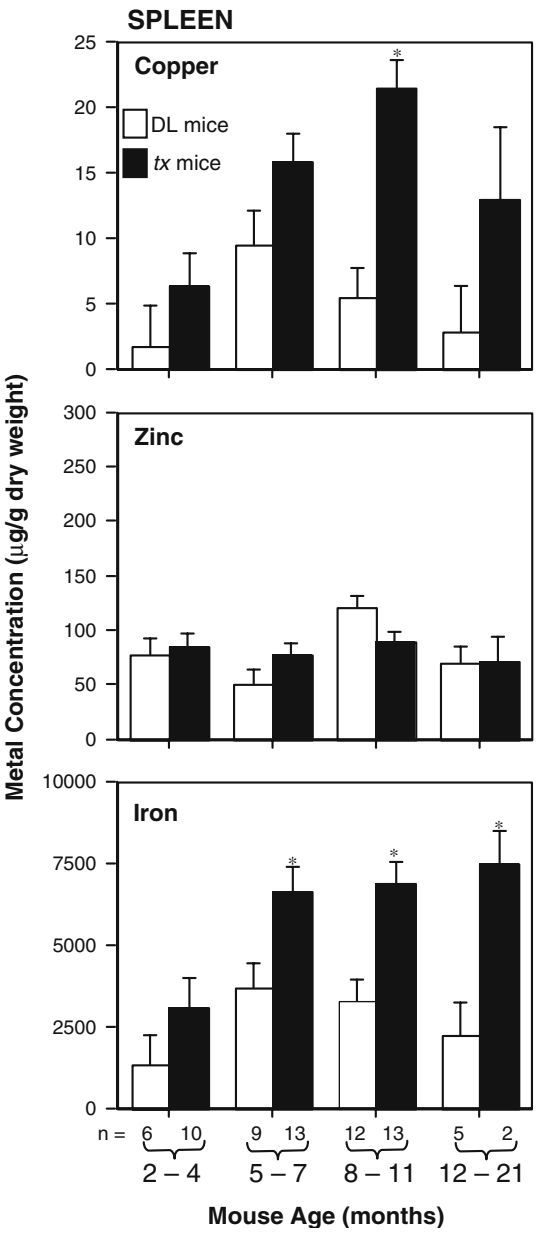

Figure 3. Comparison of spleen copper, zinc and iron levels in DL and *tx* mice in relation to mouse age. Concentration of copper, zinc and iron were determined by atomic absorption spectroscopy. Data are presented as mean ± SE.

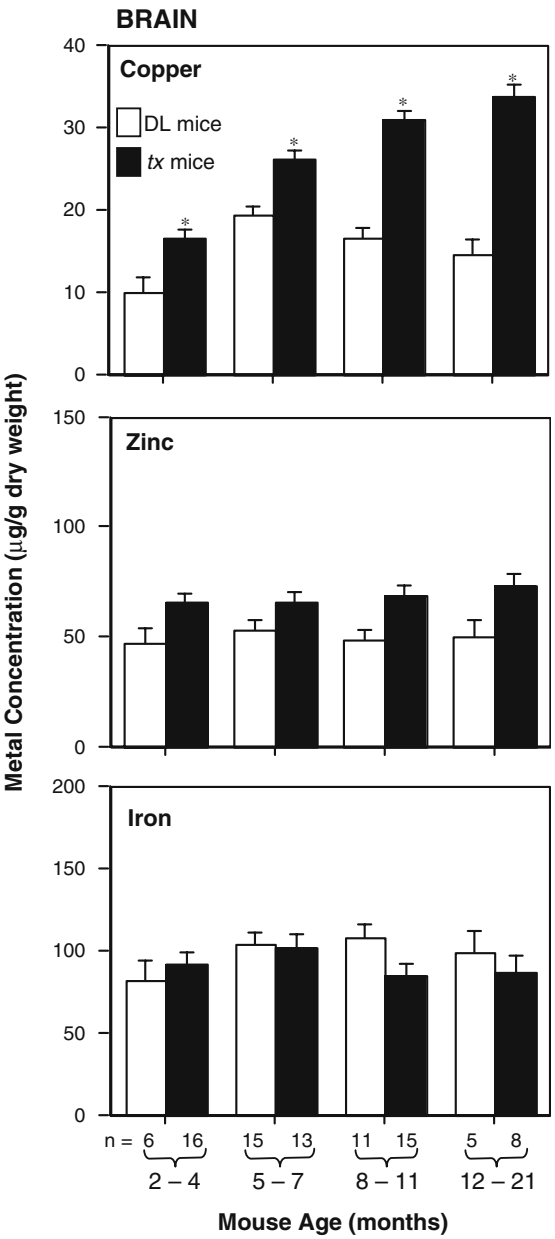

Figure 4. Comparison of brain copper, zinc and iron levels in DL and *tx* mice in relation to mouse age. Concentration of copper, zinc and iron were determined by atomic absorption spectroscopy. Data are presented as mean ± SE.

activity was determined. In *tx* mice the ceruloplasmin oxidase activity was 15.9 ± 1.8 IU/l whereas the equivalently aged DL mice had an activity of 40.3 ± 3.2 IU/l.

An activity of 102 ± 5 IU/l was calculated for the normal human positive control, which is within the normal range of ceruloplasmin oxidase activity in humans of 62–140 IU/l (Schosinsky *et al.* 1974).

Effect of copper-loading

Normal and *tx* mice were placed on the copper-loaded drinking water for 3 months as described in the Methods. Even during the extended loading period of 3 months both *tx* and normal mice appeared in good health. Table 1 shows the tissue

Table 1. Copper levels in copper-loaded DL and *tx* mice.

Mouse Strain	Diet	Liver	Kidney	Spleen	Brain
DL	Normal	19 ± 1 (21)	16 ± 1 (19)	9 ± 2 (9)	19 ± 1 (15)
	Cu-loaded	16 ± 1 (9)	14 ± 1 (9)	1.4 ± 0.2*(9)	19 ± 0.4 (9)
Tx	Normal	853 ± 47 (18)	25 ± 2 (17)	16 ± 2 (13)	26 ± 1 (13)
	Cu-loaded	964 ± 54 (12)	24 ± 1 (10)	46 ± 7* (9)	31 ± 2* (10)

*Significant ($p < 0.05$) increase in copper concentration in copper-loaded mice compared to normal. Mice were supplied with drinking water containing 300 mg/l copper as copper acetate for 3 months. Data is shown as the mean concentration (μg/g dry wt) ± SE with number of animals in each group in parenthesis.

Table 2. Zinc levels in copper-loaded DL and *tx* mice.

Mouse Strain	Diet	Liver	Kidney	Spleen	Brain
DL	Normal	95 ± 12 (21)	71 ± 3 (16)	50 ± 8 (7)	53 ± 9 (12)
	Cu-loaded	92 ± 1 (9)	68 ± 1 (9)	92 ± 1* (9)	69 ± 1 (9)
Tx	Normal	228 ± 8 (18)	75 ± 3 (17)	78 ± 4 (12)	66 ± 1 (11)
	Cu-loaded	176 ± 13* (11)	64 ± 5 (12)	66 ± 5 (10)	56 ± 3 (10)

*Significant ($p < 0.05$) change in zinc concentration in copper-loaded mice compared to normal. Mice were supplied with drinking water containing 300 mg/l copper as copper acetate for 3 months. Data is shown as the mean concentration (μg/g dry wt) ± SE with number of animals in each group in parenthesis.

Table 3. Iron levels in copper-loaded DL and *tx* mice.

Mouse Strain	Diet	Liver	Kidney	Spleen	Brain
DL	Normal	463 ± 46 (20)	219 ± 17 (20)	3664 ± 578 (9)	103 ± 6 (15)
	Cu-loaded	544 ± 30 (9)	213 ± 16 (9)	2508 ± 301 (9)	115 ± 5 (8)
Tx	Normal	606 ± 25 (18)	261 ± 30 (17)	6629 ± 962 (13)	102 ± 14 (13)
	Cu-loaded	748 ± 94 (12)	503 ± 50* (12)	7935 ± 732 (10)	78 ± 5 (11)

*Significant ($p < 0.05$) increase in iron concentration in copper-loaded mice compared to normal. Mice were supplied with drinking water containing 300 mg/l copper as copper acetate for 3 months. Data is shown as the mean concentration (μg/g dry wt) ± SE with number of animals in each group in parenthesis.

copper concentrations in the loaded and normal groups. Remarkably the normal mice that had been loaded showed essentially the same hepatic copper concentrations as the unloaded mice. The spleens of these animals showed a significant decrease in copper (Table 1). Both loaded and unloaded *tx* mice had extremely high copper concentrations in the liver. The copper-loaded *tx* animals had a slightly higher copper concentration in the liver, but the difference was not statistically significant. The effect of copper-loading on the *tx* mouse was more marked in the other organs with almost a three-fold increase in the spleen copper concentration and a 20% increase in the brain copper concentration compared with unloaded mice. The extra copper in the brain did not result in any obvious behavioural changes, suggesting that the mice had not suffered any neurological damage.

Zinc concentrations in the liver of copper-loaded *tx* mice showed a 23% decrease (Table 2). The spleen of copper-loaded normal mice showed a nearly two-fold increase to 92 μg/g dry wt, which is higher than normal, but within the range of results for splenic zinc concentrations of these mice (Figure 3).

Copper in the kidney of *tx* mice was higher than normal (Table 1), but was not increased by copper-loading, however *tx* mice had an increase in iron concentrations in the kidney (Table 3). The

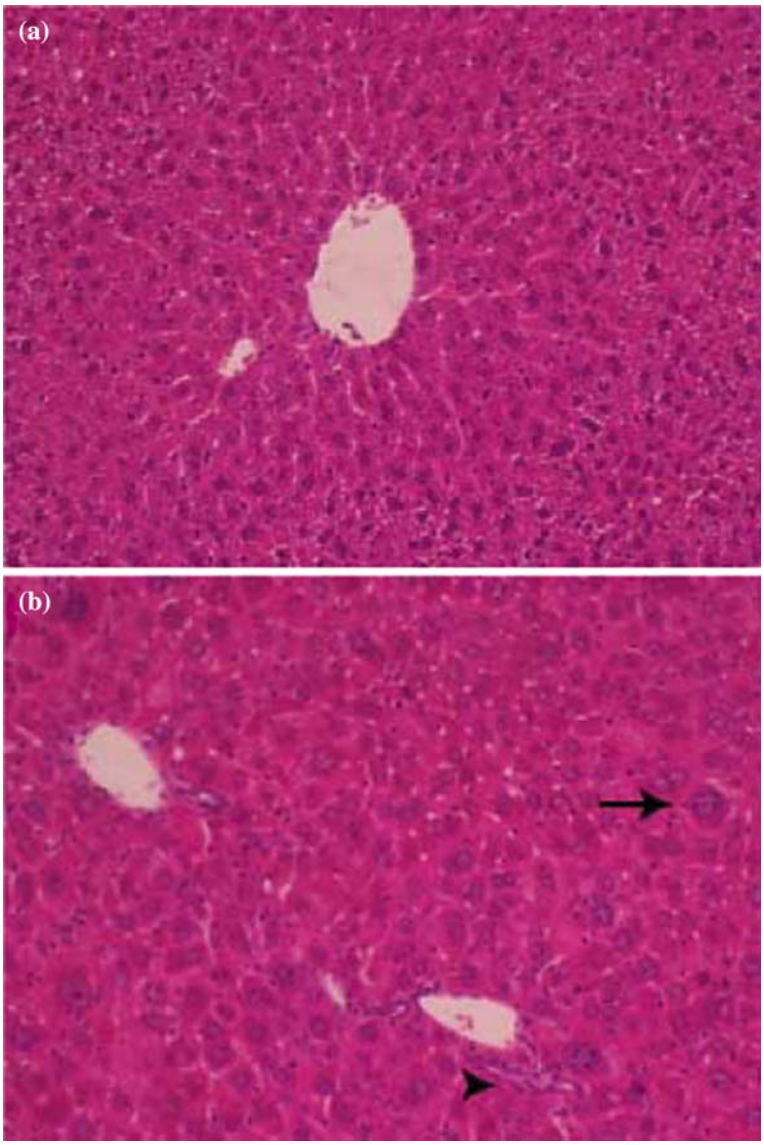

Figure 5. Liver histology of DL and *tx* adult mice (age 7–9 months) on normal diets (hematoxylin and eosin stain) (Magnification 20×). (a) The DL liver shows a homogenous array of hepatocytes with normal liver plate structure and no evidence of inflammation. Liver copper content of this mouse was 16 μg/g dry wt. (b) In contrast, the *tx* mouse liver shows hepatocyte disarray with megalocytotic nuclei (arrow), early bile duct proliferation (arrowhead) and abnormal liver plate structure reflective of response to liver injury. Liver copper content of this mouse was 735 μg/g dry wt.

iron concentration was not significantly changed in the other organs of either *tx* or control mice.

Histology

There were marked differences in histology between DL and *tx* mice at all ages (Figure 5). *Tx* mice have mild parenchymal disarray and megocytotic changes in nuclei of hepatocytes from 2 months of age onwards. There was also varying degrees of lobular hepatitis, mitotic activity, Councilman bodies, portal inflammation, central vein phlebitis, bile duct proliferation and oval cell induction. Cholestasis was rarely seen and evidence of fibrosis was not demonstrated in any *tx* mouse. The severity of liver histology did not correlate with age or level of liver copper even in *tx* mice that were copper-loaded for more than

3 months. There was no evidence of hepatocellular carcinoma in any mouse studied, including mice that survived to 2 years of age and few animals displayed the regenerative nodules which were reported as common in the *tx* mouse by Biempica *et al.* (1988).

There were no consistent morphological changes by light microscopy in DL or *tx* mice that were copper-loaded for 3 months compared to control *tx* mice. These findings held true whether or not liver copper was increased in mice receiving a copper loaded diet.

Discussion

Our results confirm previous findings of the changes in copper concentrations in the tissues of the *tx* mouse, and extend these studies to zinc and iron. Moreover we provide the first analysis of the effects of copper loading on the accumulation of copper in this mutant model of Wilson disease. It is assumed in Wilson disease that once hepatic capacity for storage of the unexcreted copper is exceeded, copper ''spills over'' from the liver and is deposited extra-hepatically in tissues such as the kidney, spleen and brain. This has been postulated to be the mechanism underlying the progression from a ''hepatic'' presentation to a ''neurological'' presentation in patients (Danks 1995). The levels of copper in the kidney, brain and spleen are clearly elevated by 2 months of age in the *tx* mutants, but there were no obvious effects of this accumulating copper as the animals remained in good health.

Pups from *tx*/*tx* dams are born severely copper deficient (Figure 1) (Rauch 1983) and this is thought to be due to the disturbance of placental transport of copper, relating somehow to the mother's disturbed copper homeostasis (Michalczyk *et al.* 2000). ATP7B has been shown to be expressed in the human placenta (Hardman *et al.* 2004), however the decrease in the pups does not depend on pup genotype, and instead is controlled by the genotype of the dam (Mercer *et al.* 1992). Buiakova *et al.* reported an increase of copper in the placenta of ATP7B null mice (Buiakova *et al.* 1999). As ATP7B appears to traffic to the apical surface of the placental trophoblasts, at least in the human placenta, it would be expected to have a role in removing excess copper from the placenta, perhaps acting as a protective mechanism for the fetus (Hardman *et al.* 2004). The absence of ATP7B activity in the fetal placenta would therefore not be expected to prevent transfer of copper into the fetal circulation, as this step is carried out by ATP7A (Hardman *et al.* 2004). Thus, it remains unclear why copper in the placenta is not successfully transported into the fetus, so the apparent marked reduction in copper transport in *tx* dams remains unexplained.

We found in addition to a reduction of copper in the 5-day old animals, that iron was also significantly reduced. This is a new observation that points to a disturbance of iron homeostasis in the *tx* mice. The deficiency of iron is rapidly corrected as the mice age, and in older animals there is a tendency for increased iron in the kidney, and a significant increase in the spleen. Ceruloplasmin is a multicopper oxidase essential for normal iron homeostasis. It is possible that the neonatal deficiency of iron in the pups could be related to disturbed placental transport of iron due to a deficiency of ceruloplasmin in the placenta. Ceruloplasmin is expressed in the placenta of rats, and presumably mice (Aldred *et al.* 1987), and ATP7B may provide copper to this ferroxidase in the placenta. Thus, in a mutant placenta the ceruloplasmin protein may be copper deficient and impair mobilisation and transport of iron, as is found with ceruloplasmin knockout mice (Harris *et al.* 1999). Mating a *tx*/*tx* dam with a normal male could test this hypothesis. If the iron concentration in *tx* pups of this mating is still low, then this iron deficiency is determined by maternal genotype and not pup genotype.

As *tx* mice aged there was an increase of iron in the spleen, and a trend towards increased iron in the kidney. This iron is likely to be derived from erythrocyte hemolysis due to release of copper from the damaged liver, and we have previously demonstrated hemosiderin in the kidney of older *tx* mice (Howell & Mercer 1994). The LEC rat model of Wilson disease, which has a deletion of the C-terminal region of ATP7B, has an overall more severe phenotype and a marked elevation of iron in a range of tissues (Kato *et al.* 1996; Kim *et al.* 2005). In acerulopasminemia patients, iron accumulation occurs in the brain, pancreas, heart, thyroid gland as well as the liver, spleen and kidney (Richardson & Ponka 1997).

We found that the *tx* mouse liver zinc concentrations were twice the amount found in normal

mice. Much of the excess copper in the *tx* liver is bound to the small metal binding proteins, metallothioneins (MTs), and the levels of MTs are 100-fold higher than normal in the mutant liver (Koropatnick & Cherian 1993). In addition the levels of MT mRNA are also highly elevated, and it is presumed that the excess copper is inducing the synthesis of MTs (Mercer *et al.* 1992). It is possible the induced MTs also sequester zinc, thus raising the hepatic zinc concentrations. If so, this suggests that the amount of MTs induced in the *tx* mouse liver is more than that required to sequester the accumulating copper. The decrease in zinc in the copper-loaded mice can then be explained by the additional copper replacing the zinc on MTs. It is also possible that the increased zinc levels may have a protective effect on the liver. LEC rats accumulate iron as well as copper in the liver, which is postulated to contribute to the development of liver injury in these rats (Kato *et al.* 1993). In regard to iron, the *tx* mouse is closer to the human situation, as Wilson disease patients do not usually show increases in hepatic iron. Splenic iron levels were increased in *tx* mice compared to equivalently aged normal mice which is most likely due to erythrocyte hemolysis caused by high copper concentration.

As previously reported by Rauch (1983) *tx* mice had low ceruloplasmin oxidase activity in the serum, which correlates with findings in patients with Wilson disease where serum ceruloplasmin is reduced or absent, and is presumably due to a marked decrease in ATP7B activity in hepatocytes causing the failure of copper to be delivered to apoceruloplasmin.

Copper loading studies showed the normal mouse has a remarkable ability to remove excess copper. Even after 3 months of consuming water containing 300 mg/l of copper, DL mice were healthy and liver copper concentrations were not increased. It is instructive to consider that the US Environmental Protection Agency suggest a maximum level of copper in drinking water of 1.3 mg/day for humans, a value approximately 230-fold lower than used here. *Tx* mice showed increases in spleen and brain copper and kidney iron concentrations in response to copper-loading. The liver copper concentration in copper-loaded *tx* mice was increased, but this increase was not statistically significant, suggesting that the copper binding capacity of the liver had been reached even in the unloaded *tx* mice. Given this observation, it is perhaps surprising that the extrahepatic copper concentrations in the loaded *tx* mice were not more highly elevated and indeed that the mice appeared healthy after this extended loading period. It is possible that the copper-loaded mice had decreased the rate of copper absorption across the small intestine in response to the copper load as has been observed in humans exposed to high dietary copper (Turnlund 1998).

Copper accumulates in the liver, brain, kidney, and cornea in human Wilson disease patients. Liver disease is the most common initial manifestation in children, while older individuals often present with neuropsychiatric symptoms. The neurological effects associated with brain copper deposition are not detected in *tx* mice, even with the higher elevation associated with copper-loading for 3 months. It may be that longer term copper-loading experiments may result in neurological effects.

The brain of LEC rats was shown to have localised increases in iron concentration in older mice (Sugawara *et al.* 1992), which was not found in *tx* mice. However like the *tx* mouse, the LEC rat did not have an increase in zinc concentrations in the brain.

Although there is a clear difference in histological findings in the liver between the *tx* and DL mouse, this study did not reveal a reliable difference between copper-loaded and non-copper-loaded mice, irrespective of genotype.

Our results further the understanding of the disturbance of metal homeostasis in the *tx* mouse. The data supports and extends previous data from the *tx* mouse and patients with Wilson disease (Rauch 1983; Howell & Mercer 1994; Gow *et al.* 2000; Michalczyk *et al.* 2000; Allen *et al.* 2004). Our data provides further evidence of the remarkable ability of mice to control excess copper intake without resulting ill health, even when a major biliary excretion mechanism has been ablated by mutation. The results further illustrate the value of the *tx* mouse as a model for the study of mammalian copper homeostasis and for Wilson disease.

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