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Heterozygous *tx* mice have an increased sensitivity to copper loading: Implications for Wilson's disease carriers

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Abstract

Wilson's disease carriers constitute 1% of the human population. It is unknown whether Wilson's disease carriers are at increased susceptibility to copper overload when exposed to chronically high levels of ingested copper. This study investigated the effect of chronic excess copper in drinking water on the heterozygous form of the Wilson's disease mouse model – the toxic milk (*tx*) mouse. Mice were provided with drinking water containing 300 mg/l copper for 4–7, 8–11, 12–15 or 16–20 months. At the completion of the study liver, spleen, kidney and brain tissue were analyzed by atomic absorption spectroscopy to determine copper concentration. Plasma ceruloplasmin oxidase activity and liver histology were also assessed. Chronic copper loading resulted in significantly increased liver copper in both *tx* heterozygous and *tx* homozygous mice, while wild type mice were resistant to the effects of copper loading. Copper loading effects were greatest in *tx* homozygous mice, with increased extrahepatic copper deposition in spleen and kidney – an effect absent in heterozygote and wild type mice. Although liver histology in homozygous mice was markedly abnormal, no histological differences were noted between heterozygous and wild type mice with copper loading. *Tx* heterozygous mice have a reduced ability to excrete excess copper, indicating that half of the normal liver Atp7b copper transporter activity is insufficient to deal with large copper intakes. Our results suggest that Wilson's disease carriers in the human population may be at increased risk of copper loading if chronically exposed to elevated copper in food or drinking water.

Introduction

Copper is an essential element required for a range of important enzymes such as cytochrome oxidase, superoxide dismutase (CuZnSOD; SOD1), dopamine β-hydroxylase and lysyl oxidase (Scheinberg & Sternlieb 1996). The reactive nature of copper ions, however, can lead to free radical generation and tissue damage. Homeostatic mechanisms have been developed by organisms to ensure the safe supply of copper to essential enzymes and removal of excess copper.

In mammals, most copper absorbed from the diet is initially taken up by the liver where a copper ATPase, ATP7B, is involved in copper transport. In the autosomal recessive genetic

copper disorder, Wilson's disease (WD), a range of mutations in the *Atp7b* gene inactivates the copper pump, leading to excessive copper accumulation in the liver that can result in liver failure if untreated. Patients also accumulate excess copper in the brain, possibly due to inappropriate release of copper from liver damage, which can lead to neurological disease.

The prevalence of WD in most populations is around 1:30,000 suggesting the carrier frequency is about 1:90 (Scheinberg & Sternlieb 1996). In some populations the frequency of WD is much higher and the carrier frequency may reach 4% (Loudianos *et al*. 2005). Although carriers are usually asymptomatic, a study of WD families revealed that some siblings of undetermined genotype had elevated copper concentrations in the liver, which raises the possibility that some of these individuals may have been heterozygous for a WD mutation (Yuzbasiyan-Gurkan *et al*. 1991). This observation has raised concerns that WD carriers may be at risk of developing copper toxicity if exposed to high copper levels. If proven, this issue would pose a considerable public health problem in areas where populations are exposed to high levels of copper in drinking water (Commission on Life Sciences 2000). There is currently limited data available to address this concern.

Using an animal model of WD, the toxic milk (*tx*) mouse, we investigated the susceptibility of heterozygotes to chronic copper loading. The *tx* mouse is an accurate genetic model of WD as it has a missense mutation in the murine orthologue of ATP7B that inactivates the copper transport activity of the protein (Theophilos *et al*. 1996; Voskoboinik *et al*. 2001). Phenotypically, the mouse displays many similarities to the human disease, with massive copper accumulation in the liver leading to pronounced histological abnormalities.

Materials and methods

All animal studies were performed with approval of the Royal Children's Hospital animal experimentation ethics committee. The mice were housed in the Murdoch Childrens Research Institute's animal facilities with a 12 h light/dark cycle, and standard mouse chow (Barastoc, Australia – approximately 0.06 mg Cu/day) *ad libitum*. Breeding pairs received normal drinking water (0–0.05 mg/l copper). Eight-month-old non-copper loaded *tx* homozygous and wild type mice from our previous study were used as age-matched controls (Allen *et al*. 2004).

Copper acetate (Merck, Germany) was dissolved in deionized water; the copper concentration was calculated from its elemental percentage in copper acetate. Animals were placed on copper water immediately after weaning at 3 weeks of age. An acclimatization process with step-wise increases in copper was used to allow the mice to adjust to the taste of copper in the drinking water and ensured appropriate intakes (Allen *et al*. 2006). The protocol was as follows: 100 mg/l Cu acetate in drinking water for one week; 200 mg/l for the second week; 300 mg/l Cu until completion of the study.

Tx heterozygous mice were derived from mating *tx* homozygous and wild type (DL) mice. The *tx* mouse strain is a natural mutation derived from DL background mice (Rauch 1983). These initial *tx* heterozygous mice were used as breeders to produce the experimental animals. The genotypes of the offspring (*tx* homozygous, *tx* heterozygous or wild type) were determined from tail biopsies, as previously described (Allen *et al*. 2004). *Tx* homozygous dams produce copper-deficient milk due to a lack of placental Atp7b (Michalczyk *et al*. 2000). The copper status of milk produced by heterozygous dams is unknown; therefore, all pups were fostered onto lactating Swiss females regardless of genotype.

Five groups of mice were employed in the study, with each group consisting of *tx* homozygotes, *tx* heterozygotes and wild type mice that were as closely age-matched as possible. One group was culled at weaning prior to the introduction of copper and the remaining 4 groups received Cu-containing water for 4–7, 8–11, 12–15 or 16–20 months. At study completion all mice were culled.

Copper concentrations in the liver, brain, spleen and kidney collected at the completion of the study were determined by atomic absorption spectroscopy as previously described (Michalczyk *et al*. 2000). Briefly, the tissues were dehydrated in an 85 °C oven and dissolved in 0.5 ml of 65% suprapure nitric acid (Merck, Germany) at room temperature for 1 h, then at 65 °C for 3 h before being diluted with 3 ml deionized water, for analysis on a Varian SPECTRAA-800 instrument in the flame

mode. Copper concentrations for organs are presented as μg Cu/g dry tissue weight.

Mouse blood was collected in heparinized blood tubes, centrifuged at 3000 *g* for 10 min, after which the top plasma layer was stored at −80 °C. Ceruloplasmin oxidase activity in the resultant plasma was analyzed using a modified method of Schosinsky *et al*. (1974). Normal human plasma was used as a positive control for the assay.

Liver for histological analysis was collected and immediately fixed in phosphate buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin. All liver sections were mixed and assessed by the pathologist without any study information and ranked in order according to the degree of histological abnormality. This rank was then correlated with the genetic status and copper loading period.

Statistical analysis was performed by one-way ANOVA with significance accepted at $P < 0.05$ using SPSS version 12.01. Values are presented as mean ± standard error (SE).

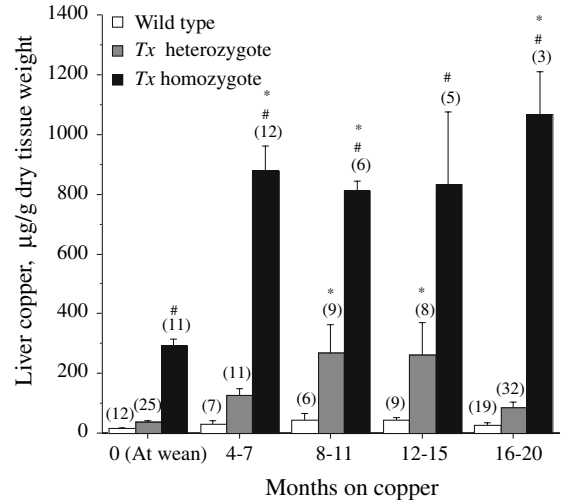

Figure 1. Comparison of liver copper concentration in chronically copper loaded mice. #*Tx* homozygote values are significantly different ($P < 0.05$) to wild type and *tx* heterozygous mice for each copper loaded group. *Values are significantly different ($P < 0.05$) to other values in their respective mouse strains. Sample size for each group given in parenthesis. Liver copper concentrations in non-copper loaded wild type and *tx* homozygous mice at 8 months of age were 16 ± 1 and 910 ± 103 μg/g dry tissue weights, respectively.

Results

Mice were categorized into 5 groups based on copper exposure time: no exposure (collected at weaning age); 4–7 months exposure; 8–11 months exposure; 12–15 months exposure; and 16–20 months exposure. Eight-month-old non-copper loaded *tx* and wild type mice were used as age-matched controls.

Organ copper concentrations in chronically copper-loaded mice

Liver

As expected liver copper was several-fold higher in *tx* homozygous mice than either heterozygotes or wild type, both at baseline and at all time exposures to copper (Figure 1). Wild type mice were resistant to chronic copper loading with no significant increase in liver copper even after 20 months of excess copper exposure (Figure 1). Heterozygote mice were less resistant to copper loading and after 4 months of copper loading there was a rise in liver copper concentration compared to wild type copper loaded mice that became significant by 8 months (Figure 1).

Spleen and kidney

At wean *tx* homozygous mice had significantly lower splenic copper levels than both heterozygous and wild type mice (Table 1). This low value dramatically reversed with copper loading and homozygotes developed significantly higher copper levels in both the spleen and kidney throughout the study. Copper concentrations in the kidney and spleen were not significantly different between heterozygotes and wild type as a result of copper loading and the results from the different time points have been combined for each genotype.

Brain

At weaning, brain copper levels in homozygous *tx* mice were significantly lower than wild type and heterozygous mice ($P < 0.01$) (Figure 2). With age, homozygotes developed significantly higher brain copper concentrations compared to wild type and heterozygous animals. Values reached 36.2 ± 3.5 μg/g after 4–7 months of copper loading compared with 18.9 ± 2.6 μg/g dry tissue weight and 20.1 ± 0.8 μg/g dry tissue weight in wild type and heterozygous groups, respectively, and did not significantly change with copper loading. Brain copper

Table 1. Spleen and kidney copper concentration (μg/g dry tissue weight) prior to and following copper loading (4–20 months).

Mouse Strain	Spleen		Kidney	
	At wean	Copper loaded	At wean	Copper loaded
Wild type	7.5 ± 1.8 (11)	5.9 ± 0.5 (28)	16.7 ± 1.8 (12)	14.3 ± 0.4 (31)
Tx heterozygote	7.0 ± 1.0 (19)	6.1 ± 0.5 (56)	12.2 ± 1.2 (24)	15.3 ± 0.4 (57)
Tx homozygote	5.3 ± 1.5 (10)	137 ± 13.9*,# (24)	14.4 ± 1.3 (11)	220.9 ± 27.4*,# (25)

Sample size for each group is given in parenthesis. Copper levels in the spleen of non-copper loaded wild type and *tx* homozygous mice at 8 months of age, were 7.5 ± 0.7 and 20.2 ± 3.0 μg/g dry tissue weight respectively and in the kidney, 14.0 ± 0.5 and 30.6 ± 6.4 μg/g dry tissue weight, respectively. *Values are significantly different ($P < 0.05$) to 'At wean' values in their mouse strain. #Values are significantly different ($P < 0.05$) to wild type and *tx* heterozygote values.

concentrations in non-copper loaded wild type and homozygous mice at 8 months of age were 20.2 ± 2 and 32.7 ± 0.6 μg/g dry tissue weights, respectively. Brain copper was not significantly different between heterozygote and wild type animals.

Ceruloplasmin oxidase activity

At wean, there was a significant difference in ceruloplasmin activity between all three genotypes with heterozygotes midway between low levels for *tx* homozygotes and normal values for wildtype (Table 2). With copper loading there were no significant differences in plasma ceruloplasmin oxidase activity between homozygote and wild type groups until 16 months of copper loading when there was a marked increase in the *tx* animal. Ceruloplasmin oxidase activity in non-copper loaded wild type and homozygous mice at 8 months of age were 36.3 ± 4.4 and 15.4 ± 2.9 IU/l, respectively. Ceruloplasmin oxidase activity in wild type mice was decreased to approximately 30% of the non-copper loaded wild type animals.

Liver histology

All three genotypes had normal liver histology at wean prior to copper loading (data not shown). In the older copper loaded mice, the *tx* homozygotes were clearly separated by ranking from mice of the other 2 genotypes. Wild type and *tx* heterozygous mice that were copper loaded showed a moderate degree of nuclear enlargement, often involving

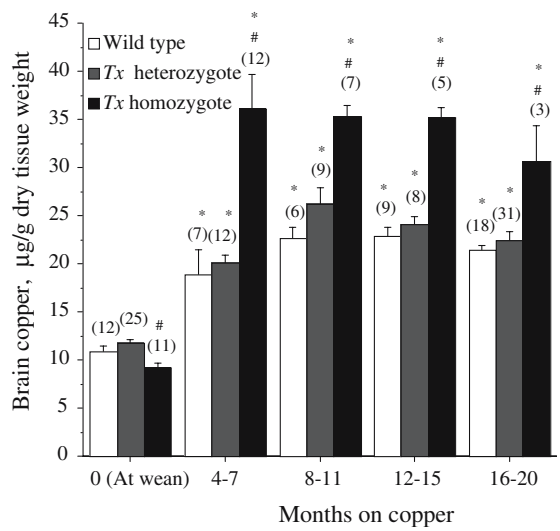

Figure 2. Comparison of brain copper concentration in chronically copper loaded mice. #*Tx* homozygote values are significantly different ($P < 0.05$) to wild type and *tx* heterozygous mice for each copper loaded group. *Values are significantly different ($P < 0.05$) to the 'At wean' values. Sample size for each group given in parenthesis.

Table 2. Plasma ceruloplasmin oxidase activity (IU/l) in copper loaded mice.

Mouse Strain	Months on Copper				
	0 (At wean)	4–7	8–11	12–15	16–20
Wild type	29.9 ± 2.1*,# (11)	8.8 ± 2.3 (9)	9.5 ± 2.2 (10)	10.6 ± 1.7 (12)	6.3 ± 1.4 (19)
Tx heterozygote	15.5 ± 1.4*,# (20)	3.5 ± 0.5 (26)	3.8 ± 0.9 (22)	5.4 ± 1.3 (16)	4.8 ± 0.8 (32)
Tx homozygote	1.0 ± 0.4# (10)	8.2 ± 3.8 (13)	7.3 ± 5.3 (5)	12.1 ± 5.0 (3)	31.0 ± 12.2*,# (3)

Sample size for each group is given in parenthesis. *Values are significantly different ($P < 0.05$) to other values in their respective mouse strains. #Values are significantly different ($P < 0.05$) to other values from the same copper exposure period.

zone 2, (mosaic pattern deleted as not seen in these high power illustrations) (Figure 3B, C). There was no clear difference in histological severity that could be accounted for by genotype or length of copper loading. These sections were not distinguishable from age-matched non-copper loaded wild type mice (Figure 3A) suggesting that these mild changes were the result of aging rather than copper exposure. There was no histology available from age-matched non-copper loaded heterozygote mice.

Liver sections from age-matched *tx* homozygous copper loaded mice demonstrated marked nuclear enlargement and irregularity, many with inclusions, variable irregularity of the liver trabeculae and bile duct proliferation (Figure 3D). There was no clear difference between animals copper loaded for 4 months or longer. When compared with 8 month old, non-copper loaded *tx* homozygous mice, there was also no clear histological difference, again suggesting that the changes noted are more likely to be attributed to age rather than to copper exposure (data not shown).

Discussion

Our data is the first to show that heterozygous Wilson's disease mice are susceptible to chronic copper loading with an elevation in liver copper concentration. This result has implications for human individuals who are heterozygous for WD genetic mutations. Although studies of chronic copper ingestion in healthy human individuals report no ill effects aside from nausea and minimal alteration to the immune system (Olivares *et al.*

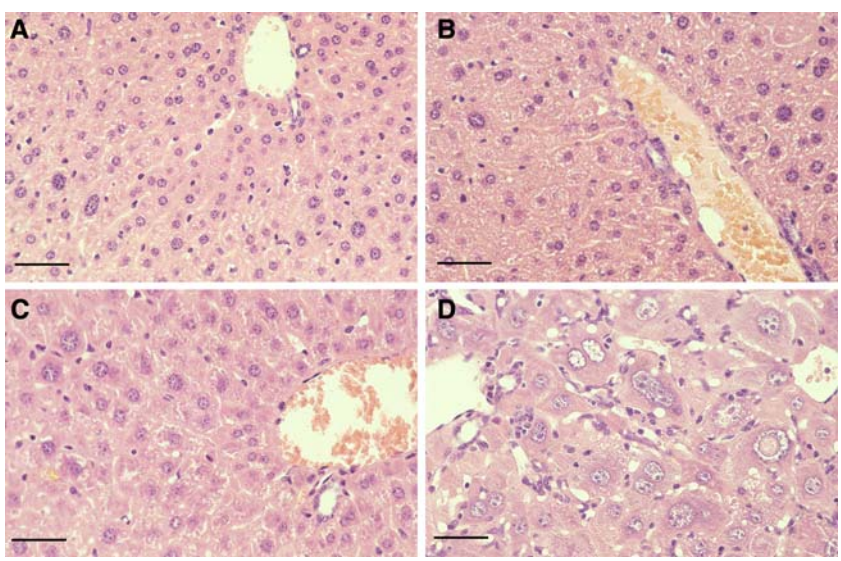

Figure 3. Liver histology of mice. A: wild type mouse, 8 months old, non-copper loaded. B: wild type mouse, 9 months old, copper loaded for 8 months. C: *tx* heterozygote mouse, 9 months old, copper loaded for 8 months. Panels A, B and C all show similar focal moderate nuclear enlargement. D: *tx* homozygote mouse, 9 months old, copper loaded for 8 months. Shows marked nuclear enlargement, nuclear inclusions and irregular trabeculae. Bar = 50 μm, magnification ×100.

2001; Turnlund *et al*. 2004; Turnlund *et al*. 2005), the susceptibility of the heterozygous population has not been investigated. Therefore we postulate that some WD heterozygous individuals may not handle excess copper in the same manner as healthy individuals, which could become a compounding factor to their overall health. From a population health perspective a large number of people could be at risk from exposure to high copper as the incidence of WD in most population studies suggests that the frequency of heterozygotes is as high as 1% (Scheinberg & Sternlieb 1996).

Spleen and kidney copper levels were not significantly altered by copper ingestion in *tx* heterozygous and wild type mice, suggesting that relatively efficient mechanisms in the liver exist to prevent copper overflow into these organs or more likely that maximal loading had not been reached using our experimental protocol. *Tx* homozygous mice exhibited significantly increased levels of copper in both of these organs implying that they are sites for excess copper once the liver copper levels have been saturated. This is further supported by the fact that liver copper levels appeared to plateau in *tx* homozygous mice with chronic copper loading. The brain remained protected from the effects of copper loading despite elevated levels of liver copper in both *tx* heterozygous and homozygous mice.

Differences in plasma ceruloplasmin activity were apparent even prior to copper loading. Heterozygous animals had an intermediate value, consistent with half the amount of Atp7b delivering half the amount of copper needed for ceruloplasmin to be synthesized in the *trans*-Golgi network. The copper loaded animals consistently showed similar levels of ceruloplasmin oxidase activity between the homozygous and wild type animals, with the latter reduced to one-third normal activity. A possible interpretation of this reduction is that in the copper loaded liver Atp7b is constitutively trafficked to vesicles away from the *trans*-Golgi network and ceruloplasmin is not receiving copper as a result. The heterozygous animal has approximately half the normal ceruloplasmin oxidase activity, as noted in the 'at wean' group, suggesting that the Atp7b-independent mechanism only operates when copper concentrations exceed the maximum of 280 μg/g dry weight in the copper loaded heterozygotes.

The small group of *tx* animals that had been loaded for 16–20 months showed a massive increase in ceruloplasmin oxidase activity which may relate to an increase in synthesis of the apoprotein in response to inflammation resulting from liver damage, as increases of ceruloplasmin occur in the acute phase reaction (Turnlund *et al*. 2004). Alternatively, given the absence of Atp7b activity, this increase in ceruloplasmin activity suggests that in the copper loaded liver some copper becomes available by an Atp7b-independent mechanism, which could represent an increased permeability of the *trans*-Golgi network membranes to copper. Another theory of why the ceruloplasmin activity has increased is that there is another oxidase upregulated in the liver which is interfering with the ceruloplasmin oxidase assay.

Like iron, copper is a heavy metal that can induce the Fenton reaction via production of free radicals. A significant body of research has been undertaken to understand the role that the carrier state of hemochromatosis might have on modifying liver diseases such as non-alcoholic steatohepatitis and Hepatitis C (Powell *et al*. 2005). Until the present study it was unknown whether carriers of WD disease had a normal capacity to metabolize excess copper loads. Although hemochromatosis heterozygosity is significantly more common with an incidence of 10% versus only 1% for WD (Delatycki *et al*. 2005), estimations of WD heterozygosity might be valuable in explaining variations in liver pathology severity in diseases where generation of free oxygen radicals might be expected to exert a negative effect.

The maximum tolerable intake of copper in humans is estimated to be 0.5 mg/kg in adults (Olivares & Uauy 1996) and the World Health Organization limit for copper in drinking water is 2 mg/l. Although the mice in this study received significantly more copper than recommended for humans, certain plumbing conditions such as long standing time, copper pipes, and physico-chemico properties of water (e.g. pH and alkalinity) can considerably increase the copper content of water (Lagos 1997). We have noted high copper levels in first flush tap water in the order of 30 mg/l, which is 10% of that given to our mice (Mercer J, personal communication). Although these levels are only transient and copper accumulation in stagnant pipes can be addressed by discharging the tap for a few minutes (Lagos 1997), the level of

understanding by the public about flushing discoloured water from stagnant pipes has never been formally assessed.

Conclusions

The present study demonstrates that a partially functioning copper excretory pathway, as in *tx* heterozygotes, is not sufficient to prevent copper overload following chronic exposure to excess dietary copper. Although there does not seem to be any obvious associated histological liver damage, whether this moderate increase in liver copper predisposes heterozygote individuals to damage by an additional agent such as hepatic infections or toxins, remains to be tested. Patients who already have underlying disease affecting biliary excretion in general should also be considered at risk. Flushing of stagnant taps would be a simple health measure to recommend until further research clarifies the implications of these results.

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