

Thiamine Supplementation Attenuated Hepatocellular Carcinoma in the Atp7b Mouse Model of Wilson's Disease

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Abstract. *Background:* Wilson's disease is caused by a genetic defect in P-type Cu^{2+} -ATPase (Atp7b), resulting in Cu^{2+} accumulation in the liver, toxicity, and hepatocellular carcinoma. Exposure of HepG2 cells, and livers of Atp7b mutant mice to toxic Cu^{2+} resulted in oxidation, (KGDH) and (PDH) enzyme inhibition, and death that was attenuated by thiamine. *Materials and Methods:* The effect of oral thiamine supplementation (2%) on hepatocellular carcinoma induced by Cu^{2+} accumulation in the livers of Atp7b animals at 4, 6, 9, 12, 16, and 21 months was demonstrated using gross morphology and multi-nucleate analysis. *Results:* By 16 months of age, untreated Atp7b animals became moribund, their livers were >180% the weight of controls and >75% of their liver was cancerous. At 16 months the livers of thiamine treated Atp7b mice were <130% the weight of controls and <30% cancerous, and at 21 months the mice were still active. However thiamine was ineffective in a subcutaneous xenograft model. *Conclusion:* This study suggests that thiamine may constitute a prophylactic for Wilson's disease-induced hepatocellular carcinoma.

The essential trace metal copper is highly redox-active, a property required for its co-factor function in electron transfer by key proteins: cytochrome c oxidase for electron transport in the mitochondrial respiratory chain, $\text{Cu}^{2+}/\text{Zn}^{2+}$ superoxide dismutase for defense against free radicals, and ceruloplasmin for iron homeostasis. Although this redox property of Cu^{2+} is essential for life, the same property can lead to toxicity and cause deleterious oxidation of lipids,

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DNA, and proteins by formation of free radical species (1). Cu^{2+} preferentially facilitates the formation of hydroxyl free radicals via the Fenton reaction, and copper redox cycling through glutathione, cysteine, or ascorbic acid can potentiate reactive oxygen species (ROS) generation (2-4).

Disturbances in copper homeostasis and resultant cellular toxicity are responsible for damage to the liver and central nervous system in Wilson's disease, a rare autosomal recessive disorder affecting approximately 1 in 50,000 people. It is caused by loss of function mutations in the gene for a P-type copper ATPase, ATP7B, that is localized primarily in the liver and responsible for moving copper into golgi for incorporation into ceruloplasmin or excretion into bile. Copper overload in hepatocytes induces liver failure, releasing the copper which is redistributed into other tissues, damaging in particular basal ganglia as well as cerebral cortex, white matter, and thalamus (5).

Both the Atp7b mouse and the Long Evans Cinnamon (LEC) rat have spontaneous autosomal recessive mutations in their respective Atp7b genes, and serve as animal models for Wilson's disease. The loss of Cu^{2+} ATPase prevents liver excretion of Cu^{2+} , causing increased Cu^{2+} buildup, overloading the capacity of its chaperone and thus allowing free Cu^{2+} . Free Cu^{2+} initiates a catalytic generation of hydroxyl radicals which cause lipid peroxidation, mitochondrial dysfunction, and damage to DNA and proteins (reviewed in (6, 7)). Increased free radical generation, lipid peroxidation, damage to DNA and proteins, and mitochondrial dysfunction also occurs in the livers of people affected by Wilson's disease (8). Cu^{2+} -induced ROS-mediated DNA damage (9) in the liver may have consequences as regards malignant transformation, as hepatocellular carcinomas are common in patients with Wilson's disease, LEC rats, and Atp7b mice (10, 11).

Lipid peroxidation, 4-hydroxy nonenal (HNE) generation, and DNA damage have been demonstrated to occur in Wilson's disease and aceruloplasminemia (8, 12), and Cu^{2+} induces lipid peroxidation-dependent dysfunction of mitochondria preferentially, *in vitro* and *in vivo* (7, 13-17). Alimentary copper overload in rats or Bedlington terriers induces lipid peroxidation marked by formation of HNE, mitochondrial dysfunction, and

hepatocellular carcinoma (18). Oxidative stress and HNE inhibit pyruvate dehydrogenase (PDH) and α -ketoglutarate dehydrogenase (KGDH), perhaps by covalently modifying the lipic acid moiety of these enzymes; oral thiamine restores the activity of these enzymes (15, 19, 20). PDH has also been demonstrated to be preferentially sensitive to other insults that induce the formation of oxygen free radicals, such as ischemia, and exposure to hydrogen peroxide (21-24).

Here it is hypothesized that free radical-induced DNA damage due to mitochondrial dehydrogenase dysfunction is a prominent mechanism of Cu^{2+} -induced hepatocellular carcinogenesis. The purpose of the present study was to test the hypothesis that oral thiamine supplementation would attenuate the Cu^{2+} -induced hepatocellular carcinogenesis in the Atp7b mouse model of Wilson's disease.

Materials and Methods

Cell culture and toxicity studies. HepG2 or Hepa (ATCC, Manassas, Virginia) cultures were grown in Dulbecco's modified minimum essential medium (DMEM) with 10% fetal bovine serum, glutamine, and non-essential amino acids and passaged every 2-3 days by trypsin/EDTA digestion. Cultures were grown to 40-75% confluence before exposure to thiamine, or harvesting for subcutaneous xenografts.

Toxicity was initiated by exposure to 1-10 mM thiamine in growth medium. Cell death was assayed at different times. Cell death was assessed by lactate dehydrogenase efflux to the bathing medium, or by staining with propidium iodide, and cell proliferation was measured by 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) staining (25), and confirmed visually.

Colony maintenance and trials. The Atp7b inbred mouse strain (Jackson Labs, Bar Harbor, ME, USA), and the LEC inbred rat strain (Charles River, Japan) were maintained in our barrier facility, and treated in accordance with our animal protocol and institutional guidelines. Pups of the inbred Atp7b mouse strain were foster parented by pseudo-pregnant C57/B16 females to provide Cu^{2+} -proficient milk. Atp7b, and wild-type C57/B16 control mice were used in a double blind trial, where the animals in each group were age and sex matched. Thiamine treatment of 24 Atp7b animals began at 5 weeks of age and continued for the duration of the study by *ad libitum p.o.* administration in the drinking water of 2% thiamine. Water and food ingestion was monitored weekly and did not vary between these two groups. At the time of sacrifice, groups of 4 mice were heavily anesthetized (150 mg/kg), cervically dislocated, and the liver was removed, weighed, photographed, and macroscopic tumors were dissected and weighed, and tissue was fixed, and processed for sectioning and staining. The percentage of liver tissue that was cancerous was determined by removing and weighing the large cancerous nodules, followed by multi-nucleate morphologic microscopic examination of the remaining liver tissue.

Histochemistry. After removing and weighing large cancerous nodules, the remaining liver tissue was rinsed in phosphate buffered saline (PBS), and fixed in 10% formalin for at least 24 h. The tissue was then processed for cryostat sectioning as previously described (26). Briefly, tissue was washed in PBS, cryopreserved overnight in

30% sucrose/PBS, frozen on dry ice, and embedded in OCT medium (Sakura Finetek, Torrance, CA, USA). Five sets of sections were cut at 10 μm , separated by 300 μm for each liver. These sections were stained with hematoxylin and eosin before being graded for percentage of carcinogenic tissue by the Histology Core at Washington University. The percentage of carcinogenic tissue was averaged from these slides which were based upon a multi-nucleated cellular phenotype.

Subcutaneous cancer xenograft model. Two groups of five C57/B16/J mice were injected with 1,000,000 cells of the Hepa mouse liver cancer line subcutaneously into the right flank. One group received normal drinking water, and the other group received water supplemented with 2% thiamine for two weeks prior to Hepa cell injection. Chronic exposure to thiamine supplementation is not detrimental to the animal, and has been shown to attenuate stroke lesions and copper toxicity in mouse models (15, 27). Tumor weight was measured after 4 weeks with or without continuous thiamine supplementation.

Reagents. Unless otherwise stated all reagents are from Sigma Chemical Co (St. Louis, MO, USA).

Results

The morphology of the livers from untreated mice began to change at 6-9 months of age, with tumors developing as demonstrated by characteristic multi-nucleated cells. The large nodes of hepatocellular carcinoma that developed by 9-12 months grew both within the liver tissue, and separate from it, and constituted the majority of the difference in carcinogenic content. In contrast, the animals receiving 2% thiamine in the drinking water had dramatically reduced hepatocellular carcinoma, and large exogenous cancer nodules never developed (Figure 1). In addition, the four remaining untreated animals became moribund and had to be sacrificed at 16 months of age, whereas the four remaining thiamine-treated animals were still alive and robust at 21 months of age. These effects of thiamine in reducing carcinoma were also demonstrated in the LEC rat model at 6 months (data not shown). We previously showed that lifespan expansion by thiamine is even more robust in the LEC rat model of Wilson's disease, where early mortality is a more prominent feature (14).

At 6 months of age, there was no difference in weight of the livers of treated or untreated Atp7b mice. However by 9 months, the livers of the untreated animals showed clear hepatocellular carcinoma, and increased weight which continued until 16 months, at which point ~80% of the untreated livers were cancerous, and they weighed >140% that of the thiamine-treated livers, which were <30% cancerous and <130% the weight of C57/B16 control livers as determined by gross morphologic and microscopic examination (Figure 2A and B).

The death of the mouse and human hepatocellular carcinoma cell lines, Hepa and HepG2 respectively, were unaffected by 10 mM thiamine as determined by PI staining

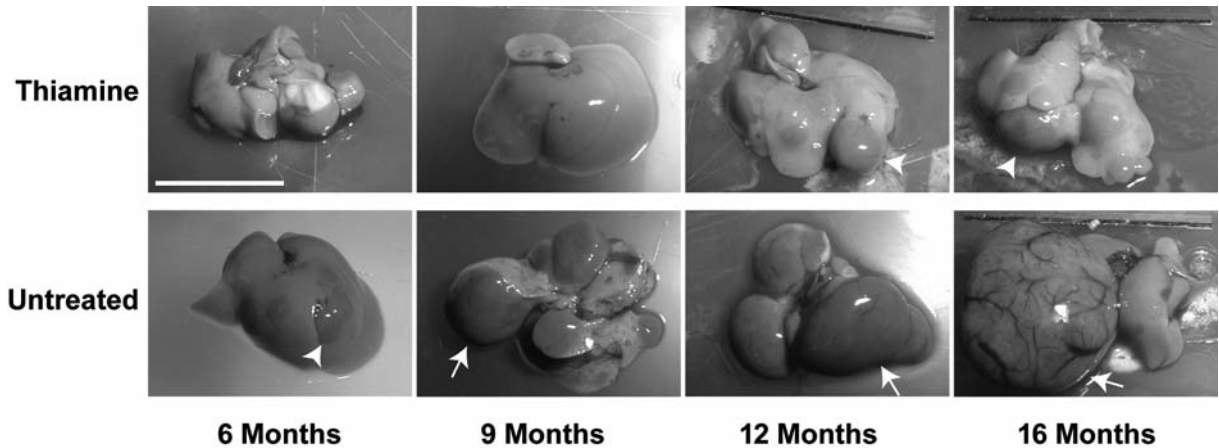


Figure 1. Thiamine reduces hepatocellular carcinoma in the *Atp7b* mouse by gross morphology. The livers of groups of four *Atp7b* mice treated with or without 2% thiamine in the drinking water were isolated and photographed at 6, 9, 12, or 16 months. The animals were sacrificed and livers isolated and processed at the times indicated. Arrows show large cancerous nodules. Arrowheads show smaller tumors. Bar represents 2 cm.

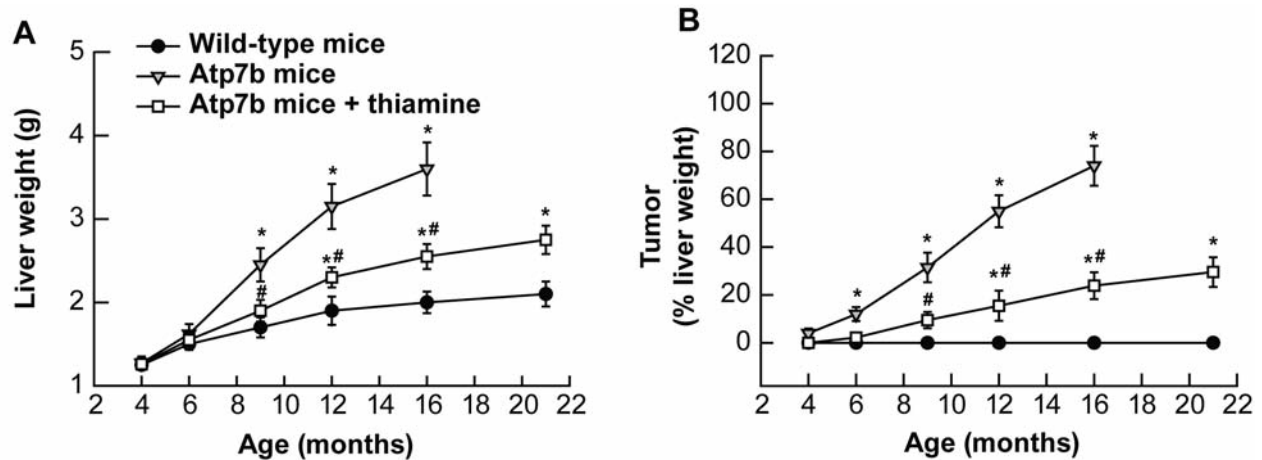


Figure 2. Thiamine prevents liver weight gain, and reduces the amount of hepatocellular carcinoma in the *Atp7b* mouse. Groups of animals ($n=4$) either C57/B16, *Atp7b* untreated, or *Atp7b* treated with oral thiamine (2%) were sacrificed at 4, 6, 9, 12, 16, and 21 months. A: The livers were weighed, and data was plotted (mean \pm SEM). B: The major carcinogenic nodes were macroscopically dissected and weighed, and the remaining liver tissue was assessed for percentage of carcinogenic content (multi-nucleate analysis) by microscopic examination by a blinded observer. These numbers were used to determine percentage of carcinogenic tissue (mean% \pm SEM). * Signifies a significant difference from wild-type, # signifies a significant difference from untreated *Atp7b* animals at $p>0.05$ by a Bonferroni test.

in vitro ($0.5\pm 0.2\%$ death untreated versus $0.9\pm 0.6\%$ 10 mM thiamine-treated, $n=9-12$). In addition, the proliferation rate of Hepa (and HepG2, not shown) was unaffected by 1-10 mM thiamine (Figure 3A). Finally, the tumor growth of subcutaneous xenografts of Hepa cells into C57/B16 mice was unaffected by 2% thiamine in the drinking water (Figure 3B).

Discussion

In these studies we demonstrate that oral thiamine supplementation reduces the amount of hepatocellular carcinoma in the *Atp7b* mouse (and LEC rat), but not in the

subcutaneous xenograft model. This allows more normal liver function and weight in *Atp7b* mice, and prolongs the lifespan in this model of Wilson's disease.

The increased Cu^{2+} resulting from the loss of the P-type Cu^{2+} ATPase in the *Atp7b* mouse and LEC rat causes increased ROS which we have shown inhibits the critical mitochondrial enzymes PDH, and KGDH. This inhibition results in mitochondrial dysfunction, loss of mitochondrial transmembrane potential, and increased ROS (15), effects that are postulated to potentiate ROS mediated DNA damage. This DNA damage has been implicated in the incidence of hepatocellular carcinoma in these disease

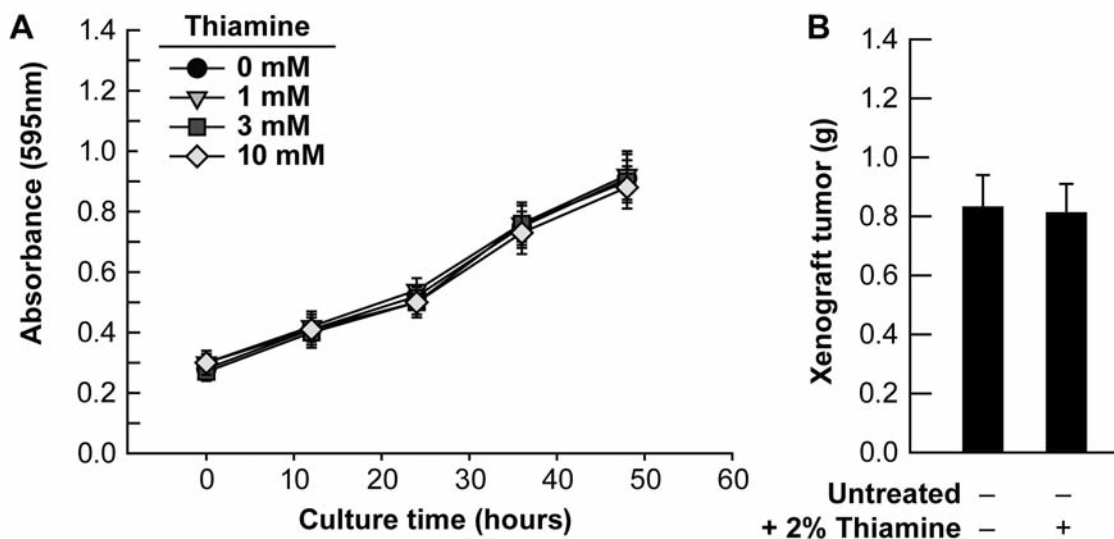


Figure 3. Thiamine does not cause Hepa cell death or reduce proliferation either *in vitro* or *in vivo*. A: Hepa cells were exposed to 1-10 mM thiamine for 48 hours as indicated. Cell proliferation was determined by MTT staining from three experiments (n=9-12), expressed as an increase in absorbance at 595nm. B: Groups of C57/Bl6 mice (n=5) either untreated, or pretreated with oral thiamine (2%) were injected with Hepa cells subcutaneously, and the resultant tumor weighed after 4 weeks (mean±SEM).

models. Oral thiamine given to these animal models was shown to largely restore the activity of PDH and KGDH, reducing ROS and increasing the lifespan of these animals (14, 15). Furthermore, we also showed that thiamine attenuated neuronal PDH and KGDH inhibition induced by compounds which cause oxidative stress, and attenuated the lesion induced by middle cerebral artery occlusion and reperfusion (27). Thiamine has not been reported to be a direct ROS scavenger, and has only 5 unsaturated bonds that are required to shield the extra electrons obtained from free radical species. Thiamine has not been reported to bind to Cu^{2+} with a high affinity (28). Furthermore, thiamine did not change the uptake of 64 Cu^{2+} into neuronal cultures as might be expected for a Cu^{2+} -binding protein (Sheline, unpublished observations). Furthermore, thiamine (1-10 mM) did not induce death of, or affect the proliferation rate of the Hepa or HepG2 hepatocellular carcinoma cell lines, either *in vitro* or *in vivo*. This suggests that thiamine may not affect the growth rate, or the survival of the carcinoma in Atp7b mice, but rather that it may delay the occurrence of or reduce the rate of incidence of the carcinoma.

These observations suggest that the mechanism of thiamine protection is through its ability to restore the functioning of the mitochondrion, thereby reducing mitochondrially derived ROS (15), and overall DNA damage that results. Thus, thiamine probably affects the rate of incidence of hepatocellular carcinoma in Wilson's disease, and should be considered as a safe, orally available prophylactic for treatment of this disease in humans.

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