Tetrathiomolybdate Protects Against Liver Injury from Acetaminophen in Mice

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ABSTRACT

Acetaminophen toxicity has become a leading cause of both accidental and intentional poisoning, and is now the most common cause of acute liver failure in the United States. Acetaminophen poisoning is thus a significant burden on the health care system and a cause of considerable morbidity and mortality. Tetrathiomolybdate is a fast acting anti-copper drug developed for Wilson's disease. However, if copper levels are lowered with tetrathiomolybdate, anti-cancer effects are obtained both in animal models and human patients. Inhibition of angiogenic cytokines by tetrathiomolybdate is the hypothesized mechanism. Tetrathiomolybdate has also proven to be effective against organ damage in the bleomycin mouse model of pulmonary damage, and in concanavalin A and carbon tetrachloride models of liver damage. Again, the hypothesized mechanism is cytokine inhibition, in this case inhibition of profibrotic and proinflammatory cytokines. In the present work in mice, we show that tetrathiomolybdate protects against liver damage from acetaminophen. Further, we show that the drug can be started after acetaminophen administration, and still be effective, indicating a possible role in treating human acetaminophen poisoning.

INTRODUCTION

Acetaminophen (ACAP) poisoning, rare in the United States prior to 1980, is now the most common cause of acute liver failure.[1] A multicenter Acute Liver Failure group collecting data on acute liver failure since 1997 found 160 out of 308 total cases (51.9%) due to ACAP.[1] The incidence of ACAP toxicity as a cause of acute liver failure is even higher (73%), in the United Kingdom. In the United States, in the multicenter study, 41% of ACAP patients took the drug with suicidal intent, while 55% accidentally took an overdose (the other 4% are unknown). In another study, at a single institution, ACAP accounted for 7.5% of all poisonings, with 80 of 93 patients classified as suicidal and 13 as accidental.[2] It is clear that ACAP toxicity leading to liver toxicity is a significant burden on the health

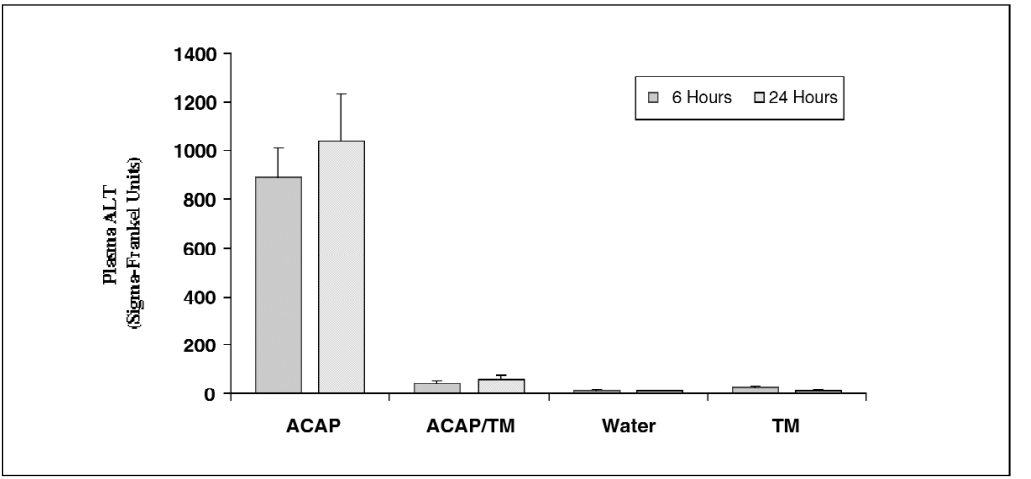

Figure 1. This figure shows the mean and SE for plasma ALT levels at 6 and 24 hours in the 4 groups of mice of Experiment 1, each group containing 5 mice. Mice in the TM groups were pretreated for 7 days with TM.

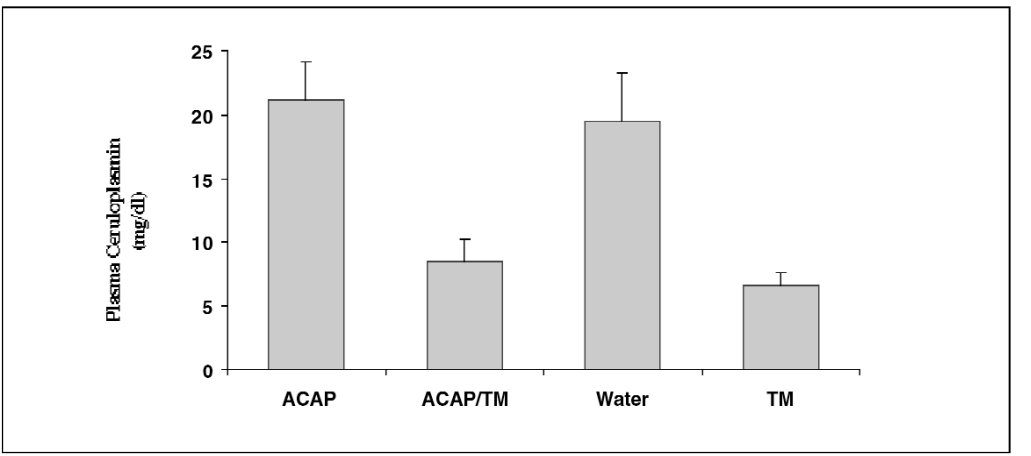

Figure 2. This figure shows the mean and SE for plasma ceruloplasmin at 24 hours in the 4 groups of mice of Experiment 1.

care system and a significant cause of morbidity and mortality.[1,2] Treatment with N-acetylcysteine is partially effective,[3] but still many patients do not survive unless transplanted.[1]

Tetrathiomolybdate (TM) is a new, fast-acting, anticopper drug, recently shown to be effective against organ damage in rodent models of lung and liver injury.[4,5] Given this background, in the current work, we chose to study possible TM efficacy on ACAP toxicity in a mouse model. TM was originally developed for the initial treatment of Wilson's disease[6-9] and has a unique mechanism of action. It forms a stable tripartite complex with copper and protein. When given with food it complexes dietary copper and food protein, preventing absorption of copper. Given away from food, it is absorbed into the blood and complexes available copper and serum albumin. This quickly eliminates further copper toxicity in Wilson's disease.

Subsequently, we began to evaluate TM as an antiangiogenic agent for can-

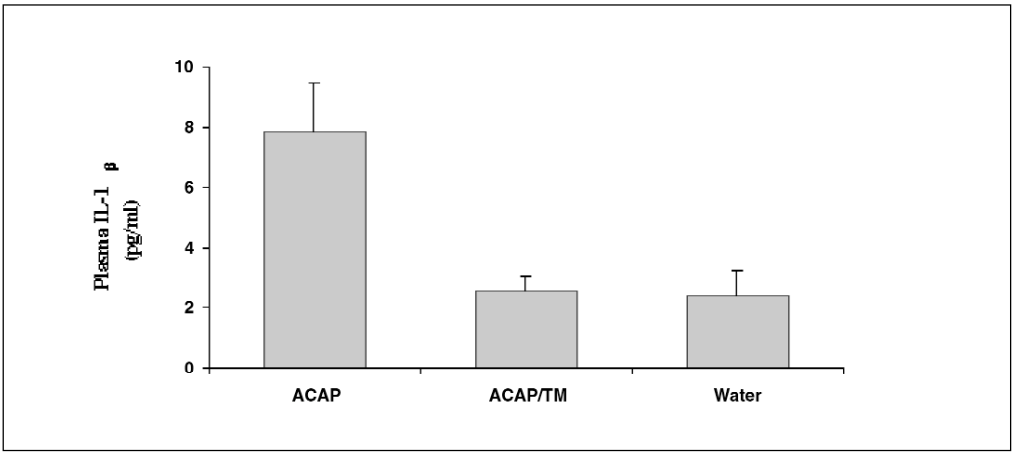

Figure 3. This figure shows the mean and SE for plasma IL-1$_{\beta}$ at 24 hours in the 3 groups of mice of Experiment 2. There were 8 mice in each of the ACAP and ACAP/TM groups, and 4 mice in the water control group. Mice received ACAP and TM exactly as in Experiment 1.

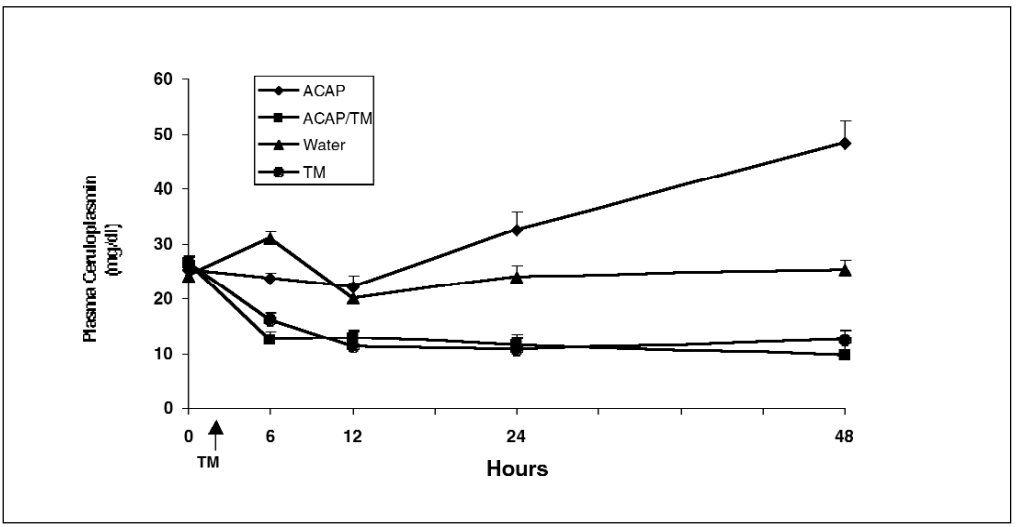

Figure 4. This figure shows the mean and SE for plasma ceruloplasmin for the 48 hour period after ACAP administration (Experiment 3) in the 4 groups of mice. TM was started by IP injection beginning 2 hours after ACAP (see arrow) and continued at intervals for 24 hours (see methods for details). Cp was significantly reduced by TM at the 6 hour point and thereafter.

cer therapy. We found that lowering copper levels to a midrange inhibits many angiogenic cytokines while not producing toxicity from copper deficiency. TM has been shown to have anticancer activity in 5 rodent models,[10-13] in pet dogs with spontaneous cancer,[14] and very encouraging effects in a phase I/II clinical study of patients with a variety of metastatic and advanced cancers.[15]

Recently, we have examined TM inhibition of organ damage from agents that produce inflammatory and fibrotic damage. In the bleomycin mouse model of pulmonary fibrosis, we can almost completely abrogate the disease by TM therapy.[5] In this model, tumor necrosis factor alpha (TNFα), a major inflammatory cytokine, peaks at about 7 days after bleomycin. Pulmonary fibrosis then

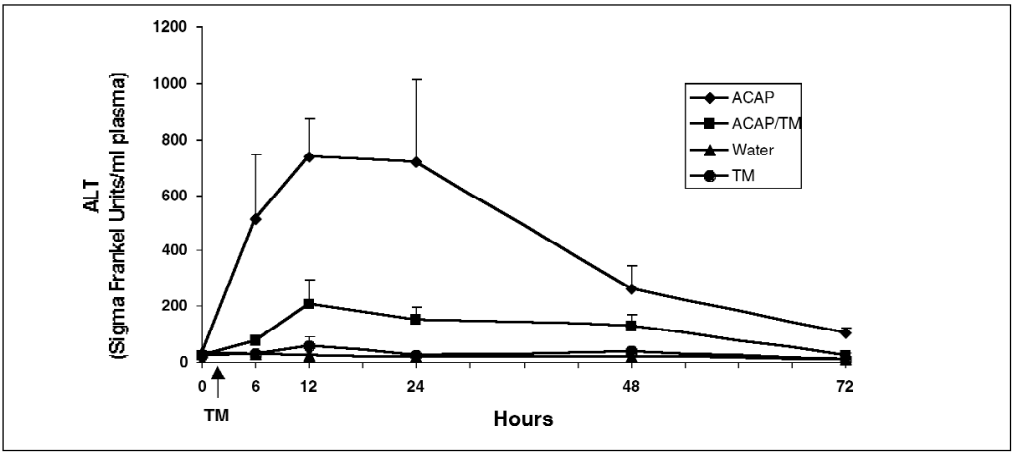

Figure 5. This figure shows the mean and SE for plasma ALT over the 72 hour period after ACAP administration (Experiment 3) in the 4 groups of mice. TM markedly inhibited the ALT increase brought about by ACAP.

ensues, is well established by 21 days, and is dependent on the fibrotic cytokine, transforming growth factor beta (TGF□). In subsequent studies we have shown that TM therapy strongly inhibits TNFα at 7 days and TGF□ at 21 days (submitted for publication). We have shown that the effects on fibrosis are independent from those on inflammation by starting TM therapy after the inflammatory and TNFα peak and still inhibit fibrosis and TGF□ expression at 21 days.

TM also prevents concanavalin A induced hepatitis as shown by its protection against elevated amino leucine transaminase (ALT) levels in the mouse liver model.[4] We have also shown that serum interleukin one beta (IL-1□), another major inflammatory cytokine, is lower in TM treated concanavalin A animals, than in concanavilin A controls.[4] TM also strongly inhibits the cirrhosis produced by 12 weeks of carbon tetrachloride therapy, and this is associated with a lower serum TGF□ level.[4]

Thus, TM inhibits the lung damage produced by bleomycin, and the liver damage from the two hepatotoxins, concanavilin A and carbon tetrachloride. In the present work, we are trying to determine whether it will also protect against the acute hepatotoxicity of ACAP. In the mouse model used,[16] a few hours after ACAP gavage, the mouse develops an acute hepatitis, manifested by elevated levels of ALT in the serum. In studies reported here, we administered TM before ACAP (TM pretreatment studies), and in a separate experiment, after ACAP (TM posttreatment study), to determine if the ACAP induced hepatitis could be prevented by TM.

MATERIALS AND METHODS

Acetaminophen and the assay kit for ALT were obtained from Sigma Chemical Company, St. Louis, Mo. Polyclonal antibody to murine IL-1□ and TNF□ were obtained from R&D Systems Inc, Minneapolis, Minn. ALT was measured according to the method in the Sigma diagnostic manual. IL-1□ and TNF□ were measured according to the R & D manual. Ceruloplasmin (Cp) was measured by an oxidase method as previously described.[11]

Female C57/BL mice (Jackson laboratory), weight 20 to 25 g, 80 to 100 days old, were housed at 21± 2˚C on a 12 hour light/dark cycle in polycarbonate cages containing hardwood chip bed-

ding. Animals for the TM Pretreatment Studies were provided normal mouse chow (501 mouse chow from Purina, St Louis, Mo, containing about 8 mg/Kg copper) and tap water ad libitum. Animals for the TM Posttreatment Study were given mouse chow with a lower amount of copper. Details will be provided in that section (Experiment 3). In all studies the animals were randomly assigned to groups by weight, such that there were no statistical differences in weight groups.

TM Pretreatment Studies

Experiment 1: Twenty mice were separated into 4 groups, each group with 5 mice. Group 1 received only ACAP, Group 2 received TM and ACAP, Group 3 received only water, and Group 4 received only TM. Groups 2 and 4 were given 0.7 mg TM per mouse per day by oral gavage for 7 days of TM pretreatment plus one day of treatment after ACAP. At time zero (the time when ACAP was given) groups 1 and 2 were treated with 300 mg/Kg body weight of ACAP by oral gavage. Groups 3 and 4 were given a water gavage in lieu of ACAP. At 6 and 24 hours after ACAP, mice were bled by tail vein to measure plasma ALT and Cp. Plasma Cp levels were used as a surrogate marker of copper status as previously reported.[5,15]

Experiment 2: Twenty mice were separated into 3 groups: 8 mice in group 1 were treated only with ACAP, 8 mice in group 2 were treated with ACAP and TM, and 4 mice in group 3 were treated by water gavage as control. TM and ACAP were given exactly as in Experiment 1. Mice were bled at 24 hours after ACAP for serum TNF□ and IL-1□ studies.

TM Posttreatment Study

Experiment 3: The methodology was changed considerably for this study. We wished to model the clinical situation, where the patient takes an overdose of ACAP, becomes ill, and needs treatment. If TM is to be useful clinically, it must work *after* the toxic ACAP has been ingested. In Experiments 1 and 2, several days of pretreatment with TM occurred in order to lower Cp into the target range. Here, we needed a much faster induction of a lowered Cp. Two modifications of the above experiments (Experiment 1 and 2) were made. One was to lower the copper level in the mouse food. The second was to give TM intraperitoneally (IP), rather than orally, and to use a high enough dose to quickly lower Cp.

Regarding the food, mouse chow contains an excess of copper, about 8 mg/Kg of food. (Human food contains 1-2 mg/Kg). In preliminary experiments with low copper chow purchased from Harlan-Teklad, Madison, Wis, we added back copper to establish requirements. At 1.0 mg/Kg of food, mice slowly became copper deficient over a few weeks as judged by a slowly lowering Cp. At 1.5 mg/Kg of food a normal copper status was maintained for several weeks. We decided to use 2.0 mg/Kg of food in these experiments to provide a safety margin.

In additional preliminary experiments, with mice on the 2 mg copper/Kg of food, we studied various IP doses of TM with the objective of lowering Cp into the target range (about 50% of baseline) within 2 to 4 hours. The dose we ended up choosing was 0.5 mg at time 2 hours (2 hours after ACAP), and 0.1 mg at 2.5, 4, 6, 8, and 12 hours, all by IP injection.

Twenty mice were divided into 4 groups, group 1 (6 mice) to receive ACAP, group 2 (6 mice) to receive ACAP and TM, group 3 (4 mice) to receive neither agent, and group 4 (4 mice) to receive TM only. ACAP was given in a dose of 300 mg/Kg body weight by oral gavage at time 0, and TM

was given IP as discussed above.

STATISTICAL ANALYSIS

For comparisons of means, we used ANOVA, followed by Scheffé test for multiple comparisons when appropriate.

RESULTS

TM Pretreatment Studies

Experiment 1: The mean and standard errors for ALT at 6 and 24 hours are shown in Figure 1. The single dose of ACAP at time 0 causes a marked increase in serum ALT at the 6 and 24-hour points. At both times, TM markedly inhibited the ALT increase from ACAP (compare the ACAP to the ACAP/TM columns in Figure 1), essentially maintaining a near normal ALT level. These differences are significant at $P<0.0001$ at both time points.

The Cp values at the 24-hour point from Experiment 1 are shown in Figure 2. As shown by the lower values in columns 2 and 4, TM treatment lowered Cp values into the desired range (25-50% of control values). The differences between the means of ACAP and TM/ACAP were statistically significant ($P<0.03$).

Experiment 2: This experiment was designed to see if a difference could be detected in plasma levels of the inflammatory cytokines TNF□ and IL-1□ as a result of ACAP injury and TM therapy. TM and ACAP were administered exactly as in Experiment 1. At the 24-hour post ACAP time point, mice were bled for plasma TNF□ and IL-1□ protein assays. TNF□ could not be detected. However, IL-1□ was detected and the results are shown in Figure 3. ACAP caused a marked and significant ($P=0.01$) increase in serum IL-1□ levels. TM markedly and significantly ($P<0.005$) inhibited this increase, keeping IL-1□ levels similar to control levels.

TM Posttreatment study (Experiment 3)

As shown in Figure 4, we accomplished our goal of lowering Cp quickly with the new 2.0 mg copper/Kg of food diet, and the use of IP TM beginning 2 hours after ACAP. Cp levels were in the target range by time 6 hours of the experiment (time 0 being when ACAP was given) and by 4 hours after starting TM. The TM regimen used also kept the Cp in target range for the 48 hours of the experiment.

As shown in Figure 5, the single dose of ACAP markedly increased serum ALT levels over the first 24 hours, and they remained modestly elevated through 48 hours. This increase in ALT was strongly and significantly inhibited by the post ACAP TM therapy, particularly during the first 24 hours ($P=0.03$ to 0.0007), but some effect was probably still seen at 48 hours ($P=0.07$).

DISCUSSION

These experiments clearly show that TM therapy, given either before or after ACAP administration, can strongly mitigate the hepatitis from ACAP as evidenced by suppression of plasma ALT levels. The hypothesized mechanism is that lowering copper to a mid range results in inhibition of inflammatory cytokines, such as IL-1□ and TNF□, which when present in excess in the liver, lead to additional hepatocellular damage. In these studies we did not measure liver levels of these cytokines. However, we were able to detect a significantly lower IL-1□ level in the plasma of TM treated, ACAP exposed animals than in ACAP controls. Presumably the elevated plasma IL-1□ levels from ACAP exposure are due to leakage of IL-1□ from the liver.

We believe that the alternative explanation that TM interferes more directly with the toxic action of ACAP is unlikely. TM started 2 hours after ACAP (and copper would not be lowered for

another period of time after TM, perhaps one to 2 hours) is almost as effective as TM pretreatment. Further, in the bleomycin study,[5] TM could be started 7 days after bleomycin was given, and still mitigate lung damage, showing that, at least in the case of bleomycin, the therapeutic effect of TM is on the aftermath of bleomycin damage rather than on the direct toxic effect of bleomycin.

The mechanism of ACAP toxicity is believed to be conversion of the drug to N-acetyl-p-benzoquinone imine (NAPQI) by cytochrome p450 in the liver.[17] This metabolite interacts with hepatic glutathione (GSH) leading to rapid GSH depletion. As GSH is depleted NAPQI binds to proteins causing cellular damage. Induction of nitric oxide synthesis and superoxide generation occurs subsequently, leading to peroxynitrite formation. This metabolite, too, is usually detoxified by GSH. However, with GSH depletion, this molecule also causes cellular damage.[17]

The current therapy of ACAP toxicity with N-acetylcysteine (NAC) takes advantage of the above mechanistic information.[3] NAC helps maintain GSH levels. Nevertheless, there is a significant mortality and requirement for transplantation in this group of patients in spite of NAC therapy. Since TM works through an entirely different mechanism, concomitant TM and NAC therapy might offer an excellent combination therapy.

As pointed out in the introduction, ACAP liver toxicity represents a major and growing cause of poisonings in the United States, United Kingdom, and other counties. NAC is helpful, but only partially so. Thus, there is a strong need for further effective medical therapy, both to save lives and to stave off liver transplantation. The experience with intravenous or subcutaneous TM administration in copper poisoned sheep, who normally die from acute liver failure, has been excellent.[18-20] This information has led us to experiment with parental TM in treating this mouse model of ACAP poisoning. The results reported here show that TM therapy can be started after ACAP poisoning, with good therapeutic results. This encourages us to believe that parenteral TM could be very useful in human ACAP poisoning. Oral TM has already been widely and safely used in the human, and is in the track for a New Drug Application (NDA) for Wilson's disease as well as other uses. This work suggests the need for a parenteral preparation and therapeutic trial in ACAP poisoning.

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