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The Effects of 3,3',4,4'-tetrabromobiphenyl on Rats Fed Diets Containing a Constant Level of Copper and Varying Levels of Molybdenum

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Abstract

Copper (Cu) metabolism is altered in rats fed diets high in molybdenum (Mo) and low in Cu. This 10-week study was carried out to examine the effects of supplemental Mo (7.5–240 µg/g diet) on male Sprague Dawley rats fed diets adequate in Cu (5 µg/g diet); and to determine the susceptibility of Mo-treated animals to the environmental pollutant 3,3',4,4'-tetrabromobiphenyl (TBB). After seven weeks of dietary treatment, half the rats in each group received a single IP injection of TBB (150 µM/Kg bw), while the other half received the corn oil vehicle. Rats sacrificed at 10 weeks showed no effects of Mo on growth, feed efficiency, or selected organ or tissue weights. Dose-dependent effects on plasma Mo (0 – 5.1 µg/mL), Cu (0.95 – 0.20 µg/mL) and bone Cu (3.4 – 10 µg/g), in control through the high-dose were found. Cu sequestration in bone of Mo-treated rats is a new finding. TBB treatment resulted in dramatic weight loss and loss of absolute organ mass. Relative organ weights were increased, except for the thymus. TBB altered amino acid concentrations of certain amino acids. Compared to control rats, this polybrominated biphenyl congener significantly decreased plasma Cu and ceruloplasmin at higher concentrations of dietary Mo, and promoted the process of plasma Cu decrease by Mo, suggesting a combined effect.

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Keywords

Copper; Molybdenum; Diet; Rats; PBBs; PCBs

Introduction

Copper (Cu) is an essential trace element for animals and a critical component of Cu-containing enzymes necessary for energy metabolism (cytochrome c oxidase), antioxidant activity (CuZn Superoxide Dismutase, (Leary et al. 2009)), and intermediary metabolism (amine oxidase, (Largerone 2011)). Cu also functions as a pro-oxidant through participation in a Fenton-like reaction that generates reactive hydroxyl radicals (Brewer 2008). The control of tissue Cu levels and factors that interfere with its control are of considerable relevance. Molybdenum (Mo), also an essential trace element, is found in enzymes such as xanthine oxidase (Schwarz et al. 2009). The metabolism of these two essential elements is interrelated so that an increase in dietary Mo can interfere with the bioavailability of Cu (Nederbragt 1980). This may be due to a decrease in Cu absorption and/or interference by Mo with Cu utilization. The severity of this Cu:Mo antagonism is species dependent (Suttle 1974).

Hazardous waste disposal processes, especially waste incineration and electronic waste recycling, may give rise to occupational and environmental exposures to metals. These processes are also a potential source of persistent organic pollutants. Emissions from incineration and reprocessing facilities, as well as fly ash and sludge from incinerators, may therefore result in co-exposures to both metals and organic pollutants (Bagnoli et al. 2005). Metals, such as Cr, Pb, Cd and Zn (Badsha et al. 1986, Williams et al. 1988), and organics, such as polyaromatic hydrocarbons, polychlorinated biphenyls (PCBs) and polychlorinated dibenzodioxins (Bagnoli et al. 2005, H.-Jones et al. 1993) were found in high levels in the pastures adjacent to waste incinerators. Several cases of livestock death were reported after exposure to toxic wastes from incinerators (Lloyd et al. 1991, Williams et al. 1988). Electronic waste, known as “e-waste”, is currently the fastest growing co-exposure pathway for metals and organics. Recycling operations and leaching from e-waste facilities cause contamination of the local environment, and lead to heavy exposure of workers to heavy metals and organics, including PBBs. High levels of metals such as As, Cd, Cr, Cu Hg, Pb were reported in rice grown near an e-waste recycling area (Fu et al. 2008). In Zhejiang, China the levels of PBBs, PCBs and polybrominated diphenyl ethers in the hair of residents and in tissues of cancer patients living around an e-waste disassembly site were found to be significantly elevated (Zhao et al. 2008, Zhao et al. 2009). Notably, the cancer risk was reported to be increased among the residents around the e-waste disassembly site (Zhao and Wang 2009).

3,3',4,4'-Tetrabromobiphenyl (TBB) is a polyhalogenated aromatic hydrocarbon that is a toxic industrial product of environmental concern. TBB is prototypical of the acutely toxic, dioxin-like halogenated biphenyl congeners whose effects include hepatomegaly, thymic involution, and a wasting syndrome (Lai et al. 2010, Robertson et al. 1983, Robertson et al. 1984). Our previous studies have shown that treatment with dioxin-like PCBs alters the

hepatic levels of copper, even at a single dose as low as 1 $\mu\text{mol/kg}$ body weight (Lai et al. 2010; Lai et al. 2011).

The purpose of the present study was 1) to determine the effects of excess dietary Mo on Sprague-Dawley rats and 2) to investigate the susceptibility of Mo-treated rats to TBB. Growth, feed efficiency, effects on organ weights, histology, and effects on mineral metabolism in blood and bone were evaluated.

Materials and Methods

Chemicals

TBB was prepared via benzidine rearrangement of the related hydrazobenzene, followed by a Sandmeyer reaction, as previously described (Hoefler et al. 1988). TBB was purified by Florisil (Macherey-Nagel) and alumina (Aluminiumoxid 90, Merck) chromatography followed by recrystallization from methanol. Structural assignments were confirmed by nuclear magnetic resonance spectrometry and mass spectroscopy. The final purity was measured by GC-MS analysis to be > 99% (Supplementary Figure S1).

Animal Treatment and Diets

Forty-eight male weanling Sprague Dawley rats (Harlan Sprague Dawley, Indianapolis, IN), were placed into hanging stainless steel cages with wire bottoms on arrival. All rats were acclimatized with the control diet (no added molybdenum) for the first week. All diets during acclimation and during the study, as well as distilled water, were provided *ad libitum*. The rats were maintained on a 12-hour light/dark cycle, and were housed individually. At the end of acclimatization, the rats were divided into six groups (n=8) based on body weight; the mean body weight being 83 ± 0.2 g.

The AIN-76A purified diet (Catalog number 170481, Lot number 980164) was purchased from Teklad, Madison WI (Supplementary Table S1) and contained 5 ppm Cu. During the treatment phase, sodium molybdate was added to the purified diets to provide molar ratios of Mo:Cu ratios of 1, 4, 8, 16, or 32:1 (equivalent to 7.5, 30, 60, 120 and 240 mg Mo/kg diet, respectively). The feeding of the Mo diets was carried out for seven weeks, during which time the feed consumption was measured twice/week and body weights were determined once/week. At the end of the feeding period, each group of rats was divided in half. Four rats in each group were given a single IP injection of TBB (150 $\mu\text{M/Kg}$) in corn oil (10 mL/Kg). The remaining four rats in each group received the vehicle. Dietary treatment was continued for an additional three weeks.

At the end of the study, the rats were given a single IP injection of pentobarbital (120 mg/Kg) and exsanguinated via cardiac puncture. The blood was placed into heparinized plastic tubes and centrifuged at 3,000 rpm for 10 minutes. The plasma was transferred to metal-free plastic tubes and frozen at -80°C until analysis. The following organs were excised and weighed: liver, kidney, thymus, heart, spleen, lung and testes. Samples of the liver, kidney, thymus, spleen, and lung were placed into neutral buffered formalin for histologic examination. The tibias were defleshed and frozen until analysis.

All procedures and diets were approved by the University of Kentucky IACUC.

Chemical Methods

Diets, plasma, and bone were digested by standard wet digestion methods and analyzed by atomic absorption spectrometry (AAS) as previously described (Stuart et al. 1987). Plasma was analyzed as follows: one mL of plasma was mixed with 2 mL 15% trichloroacetic acid while vortexing and centrifuged to separate the protein precipitate from the supernatant. Zn, Cu, and Fe concentrations were determined in the supernatant on a PE 5000 atomic absorption spectrometer as previously described (McClain et al. 1980). Plasma Mo concentrations were determined on a PE 560 atomic absorption spectrometer equipped with a HGA graphite furnace (PerkinElmer Instruments, Norwalk, CT).

Total hemoglobin was determined by a standard cyanmethemoglobin technique (Sigma Diagnostics; Procedure No. 525) while plasma ceruloplasmin was measured colorimetrically at 530 nm by following the oxidation of p-phenylenediamine (PPD) to a colored product (all reagents from Sigma). Sodium azide, a specific inhibitor of ceruloplasmin, was used to both stop the enzyme reaction and to correct for the non-specific oxidation of PPD (Sunderman and Nomoto 1970). Under the conditions of the assay, the formation of the colored product was proportional to ceruloplasmin concentrations. The assay was standardized against human standards assayed by immunochemical methods for human ceruloplasmin. The later assay was unsuitable for the determination of rat ceruloplasmin since antibody against human ceruloplasmin did not cross react with rat ceruloplasmin.

Histology

Tissues examined histologically included the thymus, liver, kidney, spleen and lung. After weighing, the tissue samples were fixed in neutral buffered formalin, embedded in paraffin, sectioned at 5 μ m, and stained with hematoxylin and eosin. Selected sections of the spleen were stained for iron using the Prussian blue method (Luna LG 1968, Thompson SW 1966).

Amino Acid Metabolism Assay

Concentrations of 26 amino acids and metabolites (alanine (ALA), alpha-aminobutyrate (AMB), asparagine (ASN), arginine (ARG), aspartic acid (ASP), citrulline (CIT), cysteine (CYS), glutamine (GLN), glutamic acid (GLU), glycine (GLY), histidine (HIS), isoleucine (ILE), leucine (LEU), lysine (LYS), 3-methylhistidine (3MH), methionine (MET), ornithine (ORN), phenylalanine (PHE), serine (SER), taurine (TAU), threonine (THR), tryptophan (TRP), tyrosine (TYR), valine (VAL), 1-methylhistidine (1MH), ethanolamine (ETA)) were determined in plasma after deproteinization with methanol and subsequent derivatization with o-phthalaldehyde and mercaptoethanol. They were quantitatively measured using reverse-phase HPLC with fluorescence detection (Schmidt and McClain 1991).

Statistics

The normality and homogeneity of the data were checked with Shapiro-Wilk's test and Bartlett's test in SAS. The assumptions about normal distribution and homogenous variance were violated. Interaction term was checked and there was no interaction between diet and TBB treatment. Between treatments differences were examined with nonparametric two-

way ANOVA. Comparison of each Mo diet group which was not TBB treated with a control group (no added Mo), comparison of each Mo diet group which was TBB treated with a control group (no added Mo) and comparison of TBB treated and control values (no TBB) within each dietary group, were examined by the pairwise Wilcoxon test with Bonferroni adjusted p values ($P < 0.05$).

Plasma concentrations of 25 amino acids were measured for the 24 control and 24 TBB-treated rats. The statistical analyses for these two groups were based on the mean and standard error values for each amino acid. Standard cross correlation calculations of the two groupings, TBB-treated and controls, were carried out to study possible linkages between levels of various amino acids across the populations of these groups. In the case of TBB rats, severe dehydration was noted which could have led to increased amino acid concentrations in plasma. The concentration in TBB-treated rats was adjusted by the ratio of the individual rat's hematocrit divided by the average hematocrit of the control rats. This resulted in a moderate decrease in the elevation of amino acids observed in the TBB-treated rats. Statistical analysis was carried out within each group for each amino acid, as well as with the other physiological parameters such as organ weights, metals in plasma and bone in this study.

Results

Animal Growth, Feed Efficiency and Organ Weights

Weight gain and feed efficiency were not statistically influenced by increasing dietary Mo for either the control or TBB-treated rats (data not shown). Body weight of these two groups is shown in Figure 1. Feed efficiency was calculated by dividing the feed consumed (g) by weight gained (g) $\times 100$, and is shown in Figure 2. Before the TBB injection, no effect of increasing dietary Mo was seen on body weight gain or feed efficiency (data not shown). Also, no effect of increasing dietary Mo was found on the relative organ weights of liver, kidney, thymus, spleen, lung, testes or tibia (Table 1). Absolute organ weights are provided in Supplemental Table S2. The relative organ weights of heart at several levels of dietary Mo were significantly decreased in non-TBB treated rats ($p < 0.05$). TBB treatment decreased body and thymus weights (Table S2), while many relative organ weights, such as the liver, kidney and lung, were increased, especially at the highest Mo treatment (Table 1).

Rats in TBB-treated groups began to lose weight after the TBB injection ($p < 0.05$, Figure 1) with a concomitant general loss in organ mass at the highest level of Mo (Table S2). The feed efficiency of rats in TBB-treated groups decreased significantly following TBB injection ($p < 0.05$, Figure 2).

Plasma and Metal Results

Increasing dietary levels of Mo showed no effect on hematocrit, ceruloplasmin, plasma iron, or plasma and bone zinc concentrations with or without TBB treatment (Table 2 and Figure 3). Hemoglobin was increased slightly (not significantly) in all Mo-treated groups. A stepwise increase in plasma Mo and bone Cu was seen, while plasma Cu decreased (Figure 3). TBB treatment consistently increased plasma iron content. Plasma Cu and ceruloplasmin

were diminished by TBB in the highest Mo group. Most other parameters were also affected at the highest dietary Mo level indicating the greatest influence of TBB in those rats.

Amino Acid Metabolism

Increasing dietary levels of Mo did not affect the serum concentrations of any measured amino acid except in the 32:1 group. There, HIS, TYR, VAL, PHE, ILE, LEU and LYS were significantly reduced (compare those values designated with the superscript “a” in Supplementary Table S3).

TBB treated rats showed altered plasma amino acid concentrations, with some means highly elevated. Notably, the coefficient of variation (S.D. relative to mean) was also larger for some amino acids, as displayed in Table 3 where the amino acids are grouped by these considerations. By inspection, it could be observed that several individual rats had extremely elevated means for the same group of amino acids, designated group I in the table. This was reflected in the high cross-correlation calculated for amino acids in this group. Elevation of these amino acids in the other TBB-treated rats was more moderate. There is a negative correlation as follows, for plasma zinc with ALA (−0.85), AMB (−0.75), ARG (−0.80), ASN (−0.81), GLN (−0.74), GLY (−0.784), HIS (−0.797), SER (−0.818), THR (−0.847), and ethanolamine (−0.715). Plasma Mo was negatively correlated with CYS (−0.766). Other metal ion correlations with amino acids were of absolute value less than 0.7, and considered statistically insignificant for this sample size (N = 24).

Branched chain amino acids LEU, ILE, VAL were not elevated and due to the hematocrit correction appear to be slightly lowered (Supplementary Table S3 and Table 3), but not to the extent seen in advanced liver disease. Unlike those in Group I, the S.D. of the levels of branched chain amino acids is only moderately larger than that of the controls. The extreme elevation noted for certain animals persisted among most of these excepting LYS which was highly elevated for all TBB-treated individuals.

Histology

There were no apparent differences in the livers of non-TBB-treated rats on varying levels of dietary Mo. Microscopically, the hepatocytes in livers of control rats and non-TBB treated rats contained cytoplasmic organelles in a vacuolated background. The most severe liver lesions were in TBB-treated rats where the spectrum of severity of hepatic lesions was unrelated to dietary levels of Mo. The patterns of change started with minimal vacuolation and early diffuse necrotic changes that were easily distinguishable from livers of control rats. This change progressed to a diffuse necrotic change with some lipid accumulation and cytoplasmic hyaline body formation. There was no apparent preferential lobular pattern with viable hepatocytes remaining in a field of dying hepatocytes in various stages of necrosis. A further progression of these changes resulted in more focal changes in which individual hepatocytes, or clusters of hepatocytes, were missing with some lipid accumulation and cytoplasmic hyaline body formation in remaining degenerating hepatocytes. These changes appeared to be a continuous spectrum of response to TBB with the focal hepatocellular dropout being the later stage. There was no apparent change in bile ducts.

The most significant pathologic change in the thymus of TBB-treated rats was extensive destruction of thymic tissue. Only stromal remnants of the thymus remained, as reflected in organ weight data (Table 1 and Supplemental Table S2). The thymuses of non-TBB treated rats retained the usual lobular structures, including a smooth capsule and distinct separation of medullary and cortical tissue in appropriate ratios. There were little to no indications of thymic involution in these rats. In spite of careful dissection and histologic techniques, thymic tissue was not present in histologic preparations in 8 of 24 TBB-treated rats. When present, only a meager stroma and a few degenerating thymic cells remained.

There were no apparent microscopic cellular changes in the spleens of any rats, despite significant changes in levels of copper and iron in plasma as seen in Table 2 and Figure 3. There was an apparent increase in iron-storage in TBB-treated rats. Ten spleens were stained for iron [4 non-TBB treated and 6 TBB treated]. The degree of iron storage in the spleens of non-TBB treated rats was minimal while iron storage in TBB treated rats was minimal to moderate.

There were no apparent treatment related microscopic changes in the kidneys of any rats. Observations that were not dose-related included minimal amounts of protein in tubules, occasional vacuolation of renal tubules and minimal dilation of renal tubules. There was minimal to mild chronic pneumonia in the lungs of these rats.

Discussion

Copper (Cu) is an essential trace element, and also a cofactor for many different enzymes. Copper can participate in a Fenton-like reaction generating reactive hydroxyl radicals (Barber and Cousins 1988). Molybdenum (Mo) is also an essential element. An increase of dietary Mo may interfere with Cu absorption and/or utilization. By modulating doses of Mo in the diet, we examined the severity of Cu:Mo antagonism and the role of Mo in TBB toxicity.

Dietary Mo in relation to copper, as high as a 32:1 molar ratio of Mo:Cu, had no significant influence on weight gain or feed efficiency for either TBB-treated or control rats. The daily consumption of Mo in this experiment was lower than 40 mg/kg bw/day. (The daily consumption was calculated based on weekly consumption of Mo divided by body weights and days. Data were not provided) Lower doses of Mo had no effect on weight gain, as has been reported by other investigators in cattle (Gengelbach et al. 1994, Ward and Spears 1997), rabbits (Arrington and Davis 1953) and rats (Bompart et al. 1990). Other research reported that when rats were fed sufficient dietary Cu supplementation, dietary Mo did not alter the body weight gain of rats (Jeter and Davis 1954).

In this study, TBB treatment dramatically decreased body and thymus weights while many relative organ weights, such as the liver, kidney and lung, were increased, especially at the highest Mo treatment. The TBB-related decrease of relative thymus weight in each Mo dietary group is a well-described toxic effect for this group of compounds (Lai et al. 2011, Lai et al. 2010).

Increasing dietary Mo caused increased plasma Mo and bone Cu, while plasma Cu decreased. It has been reported that increased dietary Mo can decrease the plasma Cu in ruminant animals (Arthington et al. 1996, Humphries et al. 1983). It has previously been shown that increasing Mo intake should increase the excretion of Cu in urine (Deosthale and Gopalan 1974). Cu is a component of lysyl oxidase, which catalyzes the cross-linkage of collagen in organic bone matrices (Linder and Hazeigh-Azam 1996). As with Cu, Mo and sulfate can form a complex with the molar ratio of Cu to Mo at 4:3 (Dowdy and Matrone 1968). It is possible that the complex is taken up through lymphatic circulation and accumulated in the bone marrow.

Ceruloplasmin is considered an indicator of plasma copper concentration (Milne and Johnson 1993) and is an acute-phase protein increased with acute or chronic infection (Barber and Cousins 1988) or inflammation (Denko 1979). The plasma Cu was significantly different from control groups at the dietary Mo levels of 30, 60, 120 and 240 mg/kg in TBB treated groups, and at 60, 120 and 240 mg/kg in non-TBB treated group. However, the concentration of ceruloplasmin was significantly different only at the highest Mo level in TBB treated group. The ceruloplasmin concentration of non-TBB treatment was slightly changed without any statistical differences. Compared to the significant change of plasma copper, the change of ceruloplasmin was slight. The induction of ceruloplasmin by IL-1 (Ramadori et al. 1985) and IL-6 (Geiger et al. 1988), induced by liver necrosis, may be the cause the nonparallel effects. TBB treatment significantly decreased plasma Cu and ceruloplasmin in the presence of the higher levels of dietary Mo. TBB treatment may facilitate the decrease of plasma Cu with increased dietary Mo. The significant decrease of plasma Cu ultimately will reduce synthesis of ceruloplasmin.

Hemoglobin was slightly increased in all Mo-treated groups, though only the highest dietary Mo group increase was statistically significant. For cattle and sheep, excess dietary molybdenum can decrease hemoglobin concentration resulting in anemia (Arthington et al. 1996, Dowdy and Matrone 1968). However, the effects on rats fed dietary molybdenum were different from ruminant animals. Jeter and Davis reported that no change of hemoglobin concentration in rats treated with dietary molybdenum (Jeter and Davis 1954). We found a slight increase of hemoglobin with increasing dietary molybdenum in this study.

TBB treatment produced an increase in plasma and splenic iron. Increased plasma iron was also reported in rats following treatment with TCDD (Nishimura et al. 2001). Increased iron transfer from mucosa to bloodstream may be stimulated by TCDD and related compounds (Manis and Kim 1979). Increases of hemoglobin concentration were found in the TBB-treated group, though increases were not statistically significant in all levels of Mo. TBB-induced increases in plasma hemoglobin can be explained partly by the increase in plasma iron, but also by dehydration.

The extreme elevations of several amino acids only in plasma of a small number of animals could be caused by a sudden release of several metabolites from organ damage occurring just before the time of sacrifice.

We have no physiological explanation for the particular amino acids' concentrations varying upon TBB treatment, while others appear normal, relative to the controls. In humans with serious liver disease, there are notably decreased concentrations of the branched chain amino acids (LEU, VAL, ILE) (Schmidt et al. 1990; Exhizen et al. 1990). The decrease did not reach statistical significance in this rat study even if the TBB treatment induced drastic changes observed with TBB treatment. The correction due to hydration is an approximation which produces some uncertainty in this decrease, even though the TBB treatment produces striking changes in other amino acids and other endpoints. With the branched chains the change in dispersion with TBB is much less than that observed with several of the other amino acids. It is interesting that in both a human liver disease study and in this study, the three branched chain amino acids are positively cross-correlated (Schmidt and McClain, personal communication).

Conclusions

The addition of dietary Mo did not alter growth, feed efficiency, organ weights, organ histology, or several blood parameters of male Sprague-Dawley rats. Increased dietary Mo diminished plasma Cu levels, while increasing bone Cu. TBB treatment dramatically decreased body and thymus weights while many relative organ weights were increased. TBB treatment also significantly increased plasma and spleen iron content. Compared to non-TBB treated groups, TBB significantly decreased plasma Cu and ceruloplasmin at higher concentrations of dietary Mo, and promoted the process of plasma Cu decrease by Mo, suggesting a combined effect. The most severe lesions seen microscopically were in the liver and thymus of TBB-treated rats. The hepatotoxicity of the TBB treatment significantly altered serum amino acid concentrations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Growth curve of control and TBB-treated rats. The rats in TBB-treated groups began to lose weight after TBB injection ($P<0.05$).

Fig. 2. Feed Efficiency curve of control and TBB-treated rats. Feed Efficiency was generated from feed consumed (g)/ weight gained (g)×100. The feed efficiency of rats in TBB-treated groups decreased significantly after TBB injection ($P < 0.05$).

Fig. 3.

Plasma Copper(A), Plasma Ceruloplasmin(B), Bone Copper(C) and Plasma Molybdenum(D) of control and TBB-treated rats with increasing dietary Mo.

^acomparison among Mo diets, groups which were not TBB treated, means which were significantly different from the control mean (no Mo) by pairwise Wilcoxon test with Bonferroni adjusted p values ($P < 0.05$).

^bcomparison among Mo diets, groups which were TBB treated, means which were significantly different from the control mean (no Mo) by pairwise Wilcoxon test with Bonferroni adjusted p values ($P < 0.05$).

*significantly different from control value (no TBB) by pairwise Wilcoxon test with Bonferroni adjusted p values ($P < 0.05$) within each dietary group.

TABLE 1
Effects of Dietary Mo and TBB on Relative Organ and Tibia Weight in the Male Sprague Dawley Rats

DIETARY Mo : Cu	TBB	Liver	Kidney	Thymus	Heart	Spleen	Lung	Testes	Tibia
0	-	3.90±0.08	0.66±0.02	0.19±0.01	0.37±0.01	0.24±0.02	0.46±0.02	0.85±0.04	0.15±0.01
0	+	3.90±0.21	0.84* ±0.06	0.02* ±0.00	0.36±0.01	0.23±0.03	0.51±0.04	1.10* ±0.05	0.21* ±0.01
1	-	3.96±0.11	0.64±0.02	0.21±0.02	0.32 <i>a</i> ±0.01	0.23±0.02	0.44±0.03	0.84±0.01	0.15±0.00
1	+	4.52±0.26	0.80* ±0.07	0.07* ±0.04	0.34±0.01	0.27±0.01	0.52±0.04	0.99* ±0.03	0.18* ±0.01
4	-	3.89±0.10	0.61±0.01	0.17±0.01	0.32 <i>a</i> ±0.01	0.23±0.01	0.39±0.01	0.82±0.02	0.14±0.01
4	+	4.21±0.55	0.79* ±0.02	0.03* ±0.01	0.34±0.02	0.23±0.01	0.48* ±0.03	1.03* ±0.02	0.20* ±0.01
8	-	4.20±0.08	0.65±0.02	0.16±0.02	0.31 <i>a</i> ±0.01	0.23±0.01	0.46±0.05	0.80±0.04	0.14±0.00
8	+	4.46±0.56	0.90* ±0.09	0.03* ±0.00	0.37±0.02	0.27* ±0.01	0.48±0.04	0.96±0.07	0.19* ±0.02
16	-	3.69±0.07	0.62±0.01	0.16±0.01	0.33 <i>a</i> ±0.01	0.22±0.01	0.39±0.01	0.80±0.04	0.14±0.00
16	+	3.82±0.20	0.79* ±0.02	0.03* ±0.00	0.37±0.03	0.26±0.01	0.45* ±0.01	1.04* ±0.02	0.18* ±0.01
32	-	3.75±0.12	0.62±0.01	0.19±0.01	0.32 <i>a</i> ±0.01	0.22±0.01	0.36±0.01	0.85±0.04	0.15±0.00
32	+	4.53* ±0.25	0.80* ±0.01	0.03* ±0.01	0.35* ±0.00	0.26* ±0.01	0.50* ±0.03	1.05* ±0.07	0.18* ±0.01

Relative organ weights were calculated according to 100*organ weight/ body weight. Relative tibia weight were calculated based on 100*tibia weight/ body weight.

^a comparison among Mo diets, groups which were not TBB treated, means which were significantly different from the control mean (no Mo) by pairwise Wilcoxon test with Bonferroni adjusted p values (P< 0.05).

^b comparison among Mo diets, groups which were TBB treated, means which were significantly different from the control mean (no Mo) by pairwise Wilcoxon test with Bonferroni adjusted p values (P< 0.05).

* significantly different from control value (no TBB) by pairwise Wilcoxon test with Bonferroni adjusted p values (P< 0.05) within each dietary group.

All values are Mean±SEM, n=4

Relationship of Added Dietary Mo and TBB Treatment on Mo: Cu Status and Related Parameters in Plasma (P) and Bone (B) in the Male Sprague Dawley Rat

TABLE 2

DIETARY Mo : Cu	TBB	HGB ¹	HCT ²	P-Zn ³	P-Fe ³	B-Zn ⁴
0	-	12.5±0.1	46.5±0.3	1.18±0.04	1.74±0.05	214±4
0	+	15.5*±0.9	58.5±6.2	1.34±0.20	3.69*±0.40	213±5
1	-	13.0±0.1	47.8±0.3	1.15±0.05	1.95±0.20	208±7
1	+	14.9*±0.8	55.0±3.1	1.58*±0.12	3.15±0.26	217±8
4	-	12.7±0.1	46.5±1.0	1.15±0.02	1.63±0.05	208±3
4	+	14.2*±0.5	51.0±1.8	1.53*±0.04	2.96*±0.20	219*±3
8	-	12.9±0.1	47.5±0.5	1.15±0.03	1.64±0.04	218±6
8	+	14.3±0.9	52.3±3.2	1.35±0.28	2.82*±0.33	240±10
16	-	13.1±0.3	47.3±0.6	1.22±0.02	1.53±0.03	219±7
16	+	14.0±0.6	51.0±2.5	1.40±0.11	3.65*±0.42	249±13
32	-	13.5 ^a ±0.2	48.5±0.6	1.07±0.03	1.68±0.07	200±7
32	+	14.9*±0.3	53.5*±1.0	1.61*±0.03	2.38 ^b *±0.11	231*±9

^a comparison among Mo diets, groups which were not TBB treated, means which were significantly different from the control mean (no Mo) by pairwise Wilcoxon test with Bonferroni adjusted p values (P< 0.05).

^b comparison among Mo diets, groups which were TBB treated, means which were significantly different from the control mean (no Mo) by pairwise Wilcoxon test with Bonferroni adjusted p values (P< 0.05).

* significantly different from control value (no TBB) by pairwise Wilcoxon test with Bonferroni adjusted p values (P< 0.05) within each dietary group.

All values are Mean±SEM, n=4

¹ hemoglobin, g/dl

² hematocrit, %

³ µg/ml

⁴ µg/g

TABLE 3

Summary of Plasma Amino Acid Statistics

Cross Correlation Group	Amino Acid	(TBB Treated vs. Control)			An Animal with Extreme Mean Exists for this Amino Acid
		Means Ratio (PBB/Control)	COV Ratio (PBB/Control)		
I	ALA	1.06		4.3	yes
I	ARG	1.65		2.9	no
I	ASN	1.74		5.2	yes
I	AMB	4.15		6.0	yes
I	CIT	1.22		2.9	no
I	GLU	0.80		3.1	no
I	GLY	1.19		3.3	no
I	GLN	1.33		6.5	yes
I	HIS	6.89		12.1	yes
I	SER	2.76		6.8	yes
I	THR	2.06		1.7	yes
I	TRP	1.36		3.0	no
I	ASP	1.00		1.0	no
I	ETA	2.23		7.3	yes
BC	LEU	0.84		1.3	no
BC	ILE	0.77		1.4	no
BC	VAL	0.85		1.3	no
None	IMH	0.85		1.9	no
None	3MH	1.37		1.2	no
None	MET	1.05		5.3	no
None	ORN	1.50		1.4	no
None	TYR	0.87		1.9	no
None	PHE	0.74		1.1	no
None	TAU	1.46		1.5	no
None	CYS	1.10		2.3	no
None	LYS	3.31		1.4	no

This table shows patterns of plasma amino acid levels in the TBB-treated and control rats, each group with 24 rats irrespective of Mo treatment. SYMBOLS: Cross Correlation Groups: I (principal group associated with ALA, etc.); BC (branched chain amino acids; None: weak correlations only, not mentioned, cross correlation < 0.8. Means ratio is: mean (TBB-treated) / mean (control); highly elevated (>

1.4) shown in bold. COV (coefficient of variation, or relative SD) ratio is: (SD /mean (TBB-treated)) / (SD /mean (control)); highly elevated (> 1.6) shown in bold. This column shows the dispersion of amino acid levels comparing TBB-treated and control groups. Bold cutoffs are arbitrary, for readability and emphasis of clustering assignments. In general, SDs are between 10 and 20% of the mean value for nearly all amino acids of control animals (not treated with TBB). Cases of extreme mean exist for a minor subset (5) of the 24 TBB-treated rats. This subset was included in statistics for the group of 24 TBB-treated rats (not analyzed separately). The extreme variation for certain amino acids within the TBB-treated animals has physiological implications, as well as for differences in the means.