Toxicology Reports 3 (2016) 21–28

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

Metallothionein's role in PCB126 induced hepatotoxicity and hepatic micronutrient disruption

W.D Klaren[a,b], S. Flor[b], K.N. Gibson-Corley[c], G. Ludewig[a,b], L.W. Robertson[a,b,*]

[a] Interdisciplinary Graduate Program in Human Toxicology, University of Iowa, Iowa City, IA, United States
[b] Department of Occupational and Environmental Health, College of Public Health, University of Iowa, Iowa City, IA, United States
[c] Department of Pathology, University of Iowa, Iowa City, IA, United States

ARTICLE INFO

Article history:
Received 25 November 2015
Accepted 2 December 2015
Available online 10 December 2015

Chemical compound studied in this article:
3,3′,4,4′,5-pentachlorobiphenyl PCB126 (PubChem CID63090)

Keywords:
Metallothionein
Micronutrients
Metals
PCB
AhR
Hepatotoxicity

ABSTRACT

Polychlorinated biphenyls (PCBs), industrial chemicals and persistent environmental pollutants, are found in rural and urban settings. Rodent studies have shown that exposure to PCB126, a dioxin-like PCB, causes a significant disruption of hepatic micronutrient homeostasis and an increase in metallothionein (MT), an antioxidant protein and metal carrier. A MT knockout mouse strain was used to assess metallothionein's role in micronutrient disruption and overall hepatotoxicity. Twenty four 129S male mice (12 wild type (WT) and 12 MT knockout (MTKO)) were placed on a purified diet (AIN-93G) for 3 weeks to achieve hepatic metal equilibrium. Mice were then given a single IP injection of either vehicle or 150 μmol/kg PCB126 in vehicle. The animals were sacrificed 2 weeks later and organs processed for analysis. Liver histology, hepatic lipids, gene expression, micronutrient and ROS status were investigated. Liver weights, liver lipids, ROS, and hepatocyte vacuolation were increased with PCB126 exposure along with AhR responsive genes. The MTKO animals had more severe histological changes in the liver and elevated liver lipids than their wild type counterparts. Hepatic and renal metals levels (Cu, Zn, Se and Mn) were mostly reduced by PCB126 treatment. Renal micronutrients were more affected by PCB126 treatment in the MTKO animals. This research suggests that MT may not be the sole/primary cause of the metal disruption caused by PCB126 exposure in mice, but may provide protection against overall hepatotoxicity.

1. Introduction

Polychlorinated biphenyls (PCBs) are persistent environmental and industrial chemicals that continue to pose a threat to human health because of their toxicity and recurrent exposure [2]. The recent elevation by IARC of these chemicals to group I carcinogens exemplifies this threat [17]. Of the 209 congeners, the dioxin-like PCBs, in particular PCB126 (3,3′,4,4′,5-pentachlorobiphenyl), affect multiple targets through activation of the aryl-hydrocarbon receptor (AhR) [1]. This activation drives the induction of a multiplicity of genes including xenobiotic metabolizing enzymes (e.g., cytochrome P450s (CYPs)) as well as antioxidant proteins, like paraoxonases and metallothionein [15,33]. In addition, studies have shown that PCB126 can alter the micronutrient status of the liver causing hepatic copper to increase whereas hepatic zinc, selenium and manganese decrease [13]. The extent to which micronutirent alterations exacerbate the ongoing liver damage is not fully understood as is the mechanism by which these micronutrients are being altered.

Metallothionein is an important protein family that has several roles alongside metal transport and reactive oxygen scavenging [31]. The metallothionein family consists of 4 isoforms in mammals. Two main metallothioneins are ubiquitously expressed, MTI and MTII, with especially high levels seen in the liver and kidney [38]. They consist of a 6 kDa cytosolic protein with a large percentage of cysteine residues (≈30%) which mainly chelates intracellular copper and zinc, but can also bind other metals [28]. The high thiol content results in its antioxidant property and allows it to interact with several metal ions at a time, in particular 7 zinc atoms or 12 copper atoms [4,28]. Given the molar equivalence, a small change in its expression can result in a very marked change in the levels of the metals bound to metallothionein. Metallothionein expression is altered by many different inducers, including cytokines, hormones, specifically glucocorticoids, and some metals [19,26]. Sato and

* Corresponding author at: Department of Occupational and Environmental Health, The University of Iowa, College of Public Health, 100 Oakdale Campus #219 IREH, Iowa City, IW 52242-5000, United States. Fax: +1 319 335 4290.
E-mail address: larry-robertson@uiowa.edu (L.W. Robertson).

http://dx.doi.org/10.1016/j.toxrep.2015.12.001

co-workers have shown that activation of the AhR induced changes in metallothionein expression through interaction with the glucocorticoid receptor which corroborates work showing PCB126 can alter metallothionein expression [12,32]. Aside from metal binding, metallothionein has been shown to mitigate the toxicity of some chemicals, including carbon tetrachloride and cadmium, and is believed to facilitate zinc's abrogative properties in alcohol induced liver damage [7,11,39]. Overall, metallothionein is a versatile protein that positively contributes to different aspects of cellular and organ health and whose properties may be involved in the dynamics of PCB126 mediated liver damage.

The liver injury characteristic of PCB126 exposure is believed, in part, to be the result of reactive oxygen species (ROS) generated by idle CYPs, among other mechanisms [36]. Given the ROS scavenging aspects of metallothionein and its metal binding ability, metallothionein could be central to the hepatic toxicity of PCB126 in the context of micronutrient alterations and ROS. The hypothesis of this study is that loss of metallothionein will result in increased hepatotoxicity with PCB126 exposure with alterations in micronutrient homeostasis. The role of metallothionein in micronutrient alteration and hepatic injury caused by PCB126 is addressed using a metallothionein knockout mouse line.

2. Materials and methods

2.1. Chemicals

Unless stated otherwise, all chemicals were obtained from Sigma–Aldrich Chemical Company (St. Louis, MO). The synthesis of PCB126 followed the Suzuki coupling of 3,4-dichlorophenyl boronic acid and 3,4,5-trichlorobenzene using a palladium catalyzed cross coupling reaction [24]. The product was purified using an aluminum oxide column with flash silica gel column chromatography, finally being recrystallized with methanol. GC/MS determined the final product purity to be >99.8% and ^{13}C NMR was used for confirmation of structure. Caution: PCBs and their metabolites should be handled as hazardous compounds in accordance with NIH guidelines.

2.2. Animals

All animal procedures and experimental designs were performed with the approval of the Institutional Animal Care and Use Committee of the University of Iowa. Twelve male wild type [129S1/SvlmJ] mice (WT) and twelve male double knockout (MTI and MTII) MT−/− mice [129S7/SvEvBrd-Mt1-Mt2] (MTKO), 8–10 weeks of age, were purchased from Jackson Laboratories (Sacramento, CA). The mice were housed three per cage and assigned a treatment to effectively age match the different groups. The age of the mice was chosen given recent evidence of adolescent PCB exposure and the sensitivity/induction potential of that age of animal [9,10]. Access was provided to water and a pelletized AIN-93G diet (Harlan Teklad; Madison, WI) ad libitum throughout the study. The animals were acclimatized for three weeks to reach hepatic micronutrient equilibrium [29]. Mice were then given a single i.p. injection (5 ml/kg) of either vehicle (tocopherol stripped soy oil) or a dose of 150 μmol/kg PCB126 in vehicle. This dose and route of administration was chosen based on previous studies with similar AhR nonresponsive mouse strains that showed comparable hepatic effects to earlier rat studies [30]. The resulting four groups of six animals each (WT—vehicle; WT—PCB126; MTKO—vehicle; MTKO—PCB126) were monitored for two weeks following the injections, then mice were euthanized with carbon dioxide followed by cervical dislocation; blood, liver and other organs were removed and processed for further analysis. The genotype of the animals was confirmed from a small piece of liver with the help of Transnetyx (Cordova, TN).

2.3. Histology

A small piece of liver was fixed in 10% neutral buffered formalin. Formalin fixed tissue was routinely processed and embedded in paraffin followed by sectioning at 4 μm. Sections were then stained with hematoxylin and eosin (H&E) and examined by a board-certified veterinary pathologist (KN G-C).

2.4. Micronutrient analysis

0.5 g of liver and kidney were weighed and placed into acid washed 15 ml polyethylene tubes. Acid digestion was conducted with a 4:1 ratio of nitric acid and 30% hydrogen peroxide followed by heat block treatment at >110 °C for 2 h. Once the samples were thoroughly digested, a Thermo X-series II inductively coupled plasma–mass spectrometer (ICP-MS) with collision cell and Cetac autosampler was used to determine micronutrient status. Analysis was performed at the Iowa Trace Element Analysis Laboratory at the University of Iowa with the assistance of Dr. David Peate.

2.5. Lipid extraction

0.25–0.75 g pieces of liver were homogenized with diatomaceous earth with a mortar and pestle and extracted using Accelerated Solvent Extraction (Dionex, Sunnyvale, CA), as described previously, utilizing chloroform:methanol (2:1 v/v) [5]. Extracted samples were initially concentrated and placed in preweighed vials followed by evaporating and heating to dryness. Dried samples were placed in a desiccator and weighed several times until constant weight was achieved, usually taking several days. Weights were expressed relative to weight of the liver piece used.

2.6. Gene expression

The gene expression of several proteins was assessed using a two-step RT-PCR method utilizing an Eppendorf Realplex Mastercycler. First mRNA was isolated from a small piece of liver using a Qiagen (Hilden, Germany) RNeasy Mini kit, as per the manufacturer's instructions. Total mRNA concentration and purity was confirmed by measuring absorbance at 260 nm and 280 nm. A High Capacity cDNA Reverse Transcription Kit from Applied Biosystems (Waltham, MA) was used to reverse transcribe the mRNA to cDNA. 10 ng cDNA was used along with 300 nM primers and a SYBR green PCR master mix (Applied Biosystem) for the real time RT-PCR. The reaction conditions for all primer sets were optimized and two technical replicates were used. Primer sequences were obtained from references and are given in Supplementary Table 1 [18,20]. All primers were purchased from Integrated DNA Technologies (IDT) (Coralville, IA). GAPDH was used as a reference gene and the wild type vehicle group functioned as the biological control.

2.7. ROS determination

An OxiSelect InVitro ROS/RNS Assay Kit from Cell Biolabs (San Diego, CA) was used to determine the level of ROS in the liver. Briefly, liver samples were homogenized with PBS and diluted to a uniform concentration. Samples were added to both catalyst and a fluorescein based dye in a 96 well plate. The plate was incubated at room temperature for 45 min. The fluorescence of the dichlorofluorescein dye (DCF) was determined at 480 nm excitation and 530 nm emission. Samples were analyzed in triplicate. A DCF stan-

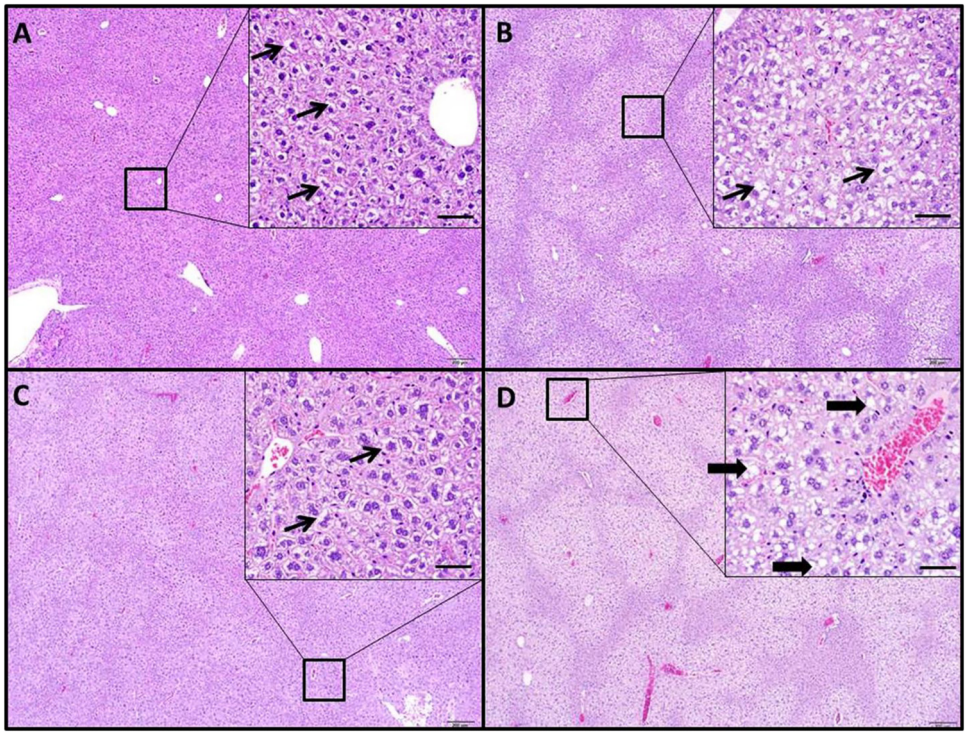

Fig. 1. Increased hepatotoxicity seen with PCB126 in MTKO animals.
WT and MTKO animals were treated once, intraperitoneally, with 150 μmol/kg PCB126. After 2 weeks the animals were sacrificed. Liver sections were stained with hematoxylin and eosin and analyzed by a pathologist. (A) WT animal treated with vehicle. (B) WT animal treated with PCB126. (C) MTKO animal treated with vehicle. (D) MTKO animal treated with PCB126. Differences in hepatocellular vacuolation was observed between genotypes and was exacerbated with PCB126 treatment. Thin arrows indicate microvesicular hepatocellular vacuolation and thick arrows indicate macrovesicular hepatocellular vacuolation. Hepatic injury was relegated to the centrilobular to midzonal region. These are representative images taken from each group. (Bars = 200 μm; Inset bars = 50 μm).

dard curve was established to convert the raw fluorescence and finally the values were adjusted to tissue weight.

2.8. COMET assay

The alkaline COMET assay was performed following the protocol by Speit et al. with slight modifications to detect a broad spectrum of DNA lesions [35]. Whole blood was collected from the heart and 10 μL were mixed with 40 μL low melting point (LMP) agarose (37 °C, 0.5% in PBS) and added onto a microscope slide covered with 1.5% agarose. Slides were then transferred to 4 °C until agarose hardened and cold lysis buffer was added for 1 h. Slides were then placed in an alkaline buffer (pH > 13) to unwind the DNA for 1 h. The slides were transferred to an electrophoresis apparatus and exposed to 24–25 V and 300 mA for 30 min. After staining with ethidium bromide, images of cells were taken using an Axio A1 Imager epifluorescence microscope with a 40-fold objective, and an Axio Cam MRm camera (Zeiss, Jena, Germany). Images of 200 randomly selected cells were analyzed from each coded slide with COMETScore pro (TriTek; Sumerduck, VA) and the% of DNA in the tail was calculated as a measure for DNA migration.

2.9. Statistics

To assess statistical significance, a 2-way ANOVA was conducted along with a Tukey's multiple comparison test to confirm differences between groups. Statistics were calculated in SAS 9.3 or GraphPad Prism 6 and significance of p-value < 0.05 is notated by an *.

3. Results

3.1. Histological examination

A hallmark of exposure to dioxin-like compounds is pathology in the liver of exposed animals. H&E liver sections were prepared for the determination of hepatotoxicity of PCB126 and the contribution of metallothionein expression. Within the vehicle control groups, the MTKO had increased hepatocellular vacuolation, cytomegaly and karyomegaly in the centrilobular to midzonal regions compared to the wild type controls (Fig. 1A, C). Upon PCB126 treatment, the wild type animals had marked microvesicular and scattered macrovesicular hepatocellular vacuolation which was more pronounced in the MTKO animals (Fig. 1B, D). This hepatic injury was predominantly seen in the centrilobular to midzonal hepatocytes. In addition, the MTKO PCB126 treated group exhibited the most extensive inflammation of all the groups. This inflammation presented as mixed inflammatory cell infiltration by neutrophils, with fewer macrophages, eosinophils, and lymphocytes. Overall, the absence of metallothionein contributed to an increase in PCB126 liver injury.

3.2. AhR activation

Canonical metrics for AhR activation were investigated to confirm the traditionally seen pathologies associated with PCB126 exposure and the effects of metallothionein deficiency. Relative liver weight was significantly increased following PCB126 exposure; genotype appeared to have no effect (Fig. 2). Absolute liver weights were increased as well (data not shown). As a means of quantifying the extent of liver damage, particularly hepatocellular vacuolation, hepatic lipids were extracted and measured. In agree-

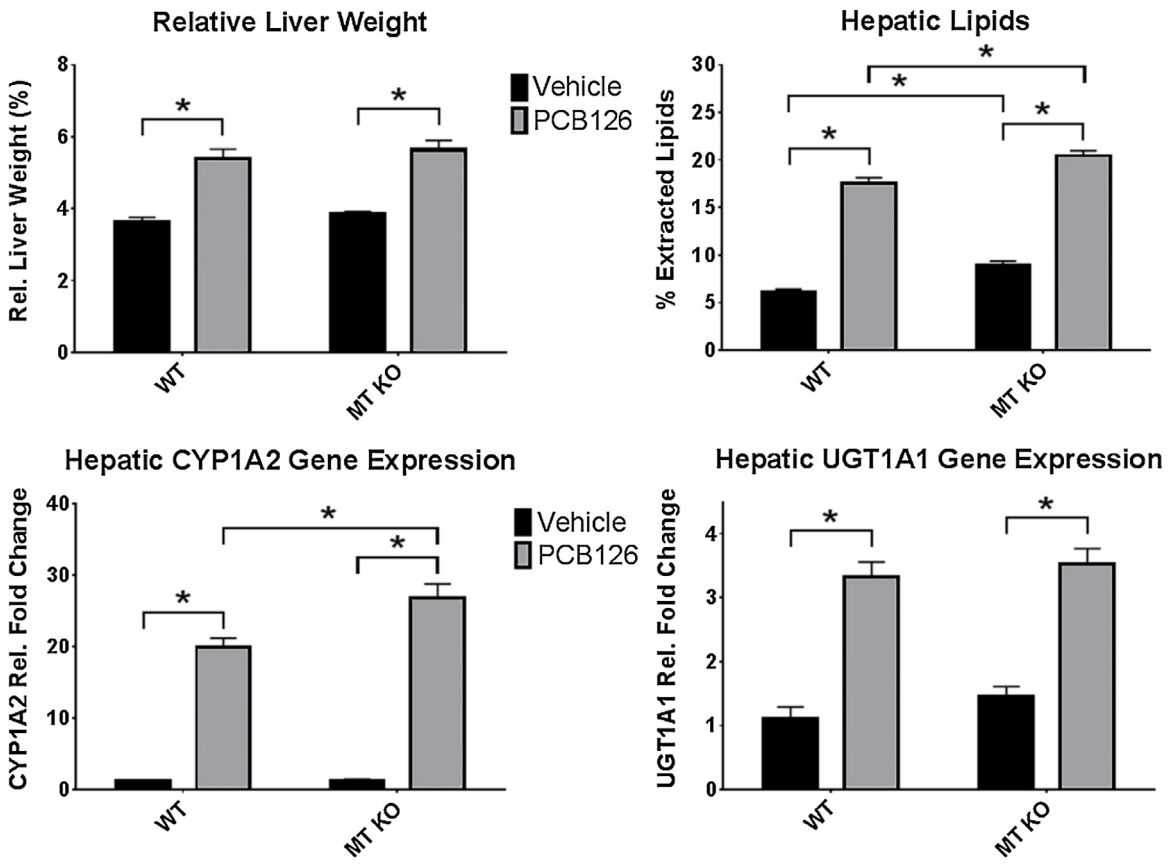

Fig. 2. Canonical AhR activation observed in both WT and MTKO mice with exposure to PCB126.
Hepatomegaly is seen in both WT and MTKO animals. Hepatic lipids shows an increase in lipid accumulation by treatment of PCB126 and a more pronounced effect is seen in the MTKO animals. Representative AhR activated genes were induced following PCB126 administration with some effect of genotype. (Bars are standard error; *represents $p < 0.05$; two-way ANOVA; $n = 6$).

ment with the vacuolation seen in the histology, hepatic lipids were increased with PCB126 exposure. A significant difference was also seen between the WT and MTKO groups, both vehicle or PCB126 treated groups. This gives additional weight to the proposed function of metallothionein in protecting the liver. The gene expression of two AhR responsive genes, CYP1A2 and UGT1A1, was also investigated to confirm AhR activation. CYP1A2, a family 1 cytochrome P450 enzyme, was strongly induced with PCB126 treatment in both WT and MTKO. However, PCB126 treated MTKO had a significantly higher induction of CYP1A2 (Fig. 2). Uridine 5′-diphospho-glucuronosyltransferases (UGT) are important enzymes in phase II metabolism. UGT1A1, a known AhR target, was increased in both WT and MTKO with PCB126 exposure, however no difference was seen between genotypes (Fig. 2).

3.3. Presence of ROS

PCB126 exposure is known to increase ROS. Several metrics were investigated to determine the presence of ROS and the effect of metallothionein, a ROS scavenger. Hepatic ROS was increased, regardless of genotype, with PCB126 treatment (Fig. 3). ROS are known to induce DNA strand breaks. A genotoxic endpoint, the COMET assay, was used to investigate the contribution of PCB126 exposure and/or metallothionein deficiency to systemic genotoxicity. The amount of fragmented DNA in whole blood lymphocytes was significantly elevated after PCB126 exposure as seen in the increased percentage of DNA in the COMET tails. No difference was observed between the WT and MTKO (Fig. 3). Transcriptional activation of genes in a ROS sensitive pathway has been reported and therefore the gene expression of two genes, nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and NAD(P)H quinone oxidoreductase 1 (NQO1), involved in that pathway, was investigated. The expression of both genes was elevated in the PCB126 groups, irrespective of genetic background (Fig. 3). In sum, these data suggest an increase of ROS after PCB126 exposure with little effect of the presence of metallothionein.

3.4. Micronutrient analysis

A major function of metallothionein is in regulating the micronutrient homeostasis in tissues. Micronutrient status was determined using ICP-MS to assess the role of metallothionein in the normal physiological homeostasis of micronutrients and PCB126 mediated modulation in the liver. A small decrease was seen in hepatic copper with PCB126 treatment regardless of genotype but this decrease was statistically significant only in the MTKO group (Fig. 4). Hepatic zinc was decreased in both the wild type and MTKO animals following PCB126 exposure (Fig. 4). Although not significant, hepatic Se was decreased with PCB126 exposure, similar to zinc. Neither Cu, Zn, nor Se was significantly influenced by the absence of metallothionein I and II in the liver. The only hepatic metal that appears to be affected by the loss of metallothionein is manganese which was seen to increase in the MTKO mice; however PCB126 abrogated that increase (Fig. 4).

The kidneys are an organ with high MTI and II expression. Renal copper significantly decreased in the MTKO PCB126 treated animals while all other groups were unaffected (Fig. 5). Renal zinc was unaffected in the vehicle treated MTKO groups compared to the WT controls. A decrease was seen in the PCB126 treated MTKO animals which was significant compared to the corresponding WT group

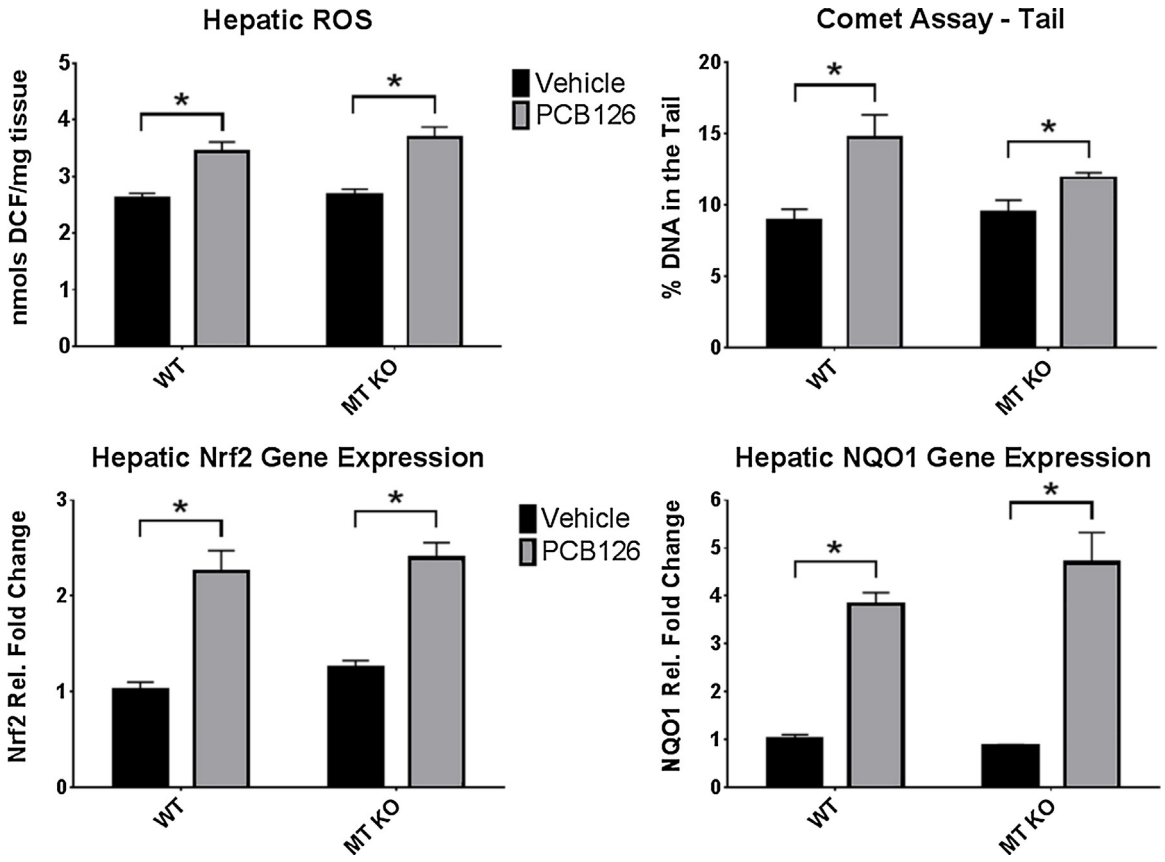

Fig. 3. ROS metrics are increased with PCB126 treatment with no difference observed between WT and MTKO mice.
Hepatic ROS was increased with PCB126 as was the fragmented DNA in whole blood lymphocytes as measured by the COMET assay in both WT and MTKO animals. ROS responsive genes were induced with PCB126 exposure but no difference observed between genotypes. (Bars are standard error; * represents $p < 0.05$; two-way ANOVA; $n = 6$)

and compared to the MTKO vehicle control group (Fig. 5). Although not significant, a similar pattern was seen with renal selenium, a difference because of PCB126 treatment and MTKO genotype. Also, renal manganese was only affected in the MTKO group where PCB126 treatment significantly decreased the Mn levels. Thus a significant consistent decrease was seen with renal Cu, Zn, Mn and Se (not significant) in the MTKO animals following PCB126 exposure.

4. Discussion

Metallothionein has been shown to be protective against many difference toxic agents including acetaminophen, cadmium, cisplatin, and carbon tetrachloride [7,11,22,23,25]. Given its role in both metal transport and oxidant scavenging, it seems a prime candidate for potentially mediating a well observed phenomenon associated with PCB126, the disruption of micronutrient homeostasis in rodent liver. Early studies have shown that animals treated with PCB126 have disrupted hepatic micronutrients yet a clear explanation of how this is occurring and how this contributes to the broader hepatotoxicity are unknown [15]. This study sets out to address the involvement of metallothionein in PCB126-mediated hepatic micronutrient disruption and its potential protective capacity.

In micronutrient investigations, it is important to consider the changes that occur with body weight to ensure that the effect is due to the toxic agent itself and not from dietary or wasting mechanisms. Body weight was monitored over the course of the study and was uniform before and after PCB126 treatment (Supplementary Fig. 1). Interestingly, the MTKO animals had a marginal increase in body weight however this was not significant and has been observed before [3]. Elsenshans et al. established the hypophagia/cachexia independent effects of TCDD, a PCB126-like compound, by using a pairwise feeding model with rats which confirms that the effects on micronutrients seen are unlikely hypophagia/cachexia mediated [8].

The histology analysis showed a marked difference between the WT and the MTKO animals, even in the vehicle treated animals, suggesting that the loss of metallothionein alone alters the liver (Fig. 1). Interestingly, in many studies with similar mouse strains (MTI/II knockouts), this alteration in liver histology was not observed [7,22,23]. However, Beattie et al. have described a natural partitioning of MT-null mice by body weight into distinct lean, average, and obese groups [3]. Of the MTKO mice, the obese MTKO group had a higher hepatic lipid content and liver weight, compared to controls. Unfortunately this research group did not investigate the liver histologically. The MTKO animals in the present study were slightly heavier than the WT controls and displayed increased hepatic lipids (Supplementary Fig. 1; Fig. 2). This increased weight may have played a role in the difference in liver histology and lipids observed between the genotypes. However, there was also a clear increase in liver injury with PCB126 treatment which was more pronounced in the MTKO mice, not only with respect to vacuolation, which was confirmed with the increase in hepatic lipids (Fig. 2), but also in the form of increased inflammation.

PCB126 produced classical AhR activation effects. Relative liver weight, CYP1A2 and UGT1A1 expression were increased with PCB126 in WT and MTKO animals (Fig. 2). Interestingly, CYP1A2 was significantly increased in the MTKO animals compared to its PCB126 treated WT control. This has not been observed before in vivo but work in cell culture has shown that low levels of cop-

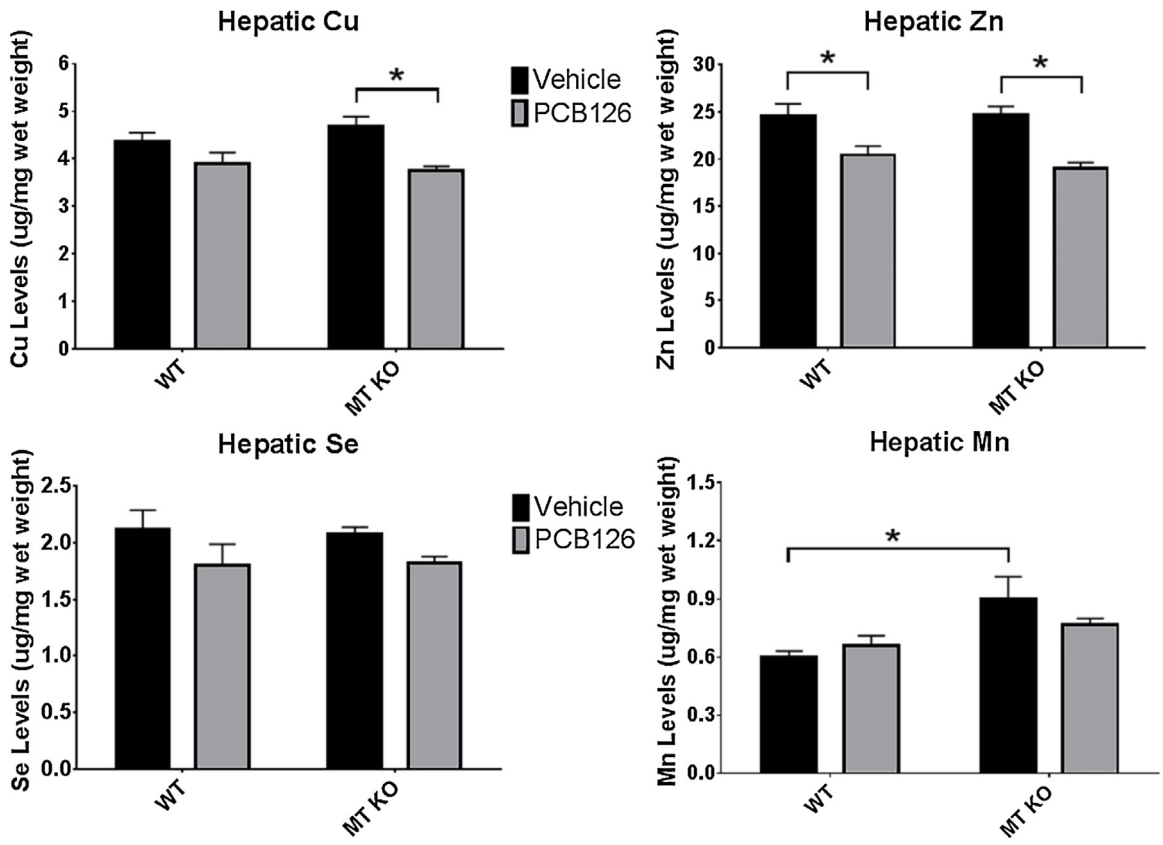

Fig. 4. Hepatic micronutrients are altered following PCB126 exposure with only Mn showing a genotypic effect.
Hepatic Cu is decreased with PCB126, a rather novel finding that differs from previous studies in rats. Hepatic Zn is decreased in both WT and MTKO animals suggesting that alterations in zinc are metallothionein independent. Hepatic Se is unchanged with exposure however slight decreases are observed. Mn is unaffected by PCB126 but is increased between WT and MTKO mice in the liver. (Bars are standard error; * represents $p < 0.05$; two-way ANOVA; $n = 6$)

per resulted in increased expression of CYP1A2 [6]. In addition, the MTKO group treated with PCB126 had significantly lower hepatic Cu levels and indeed were the lowest Cu levels of all 4 groups (Fig. 4), which may suggest a combined effect of PCB126 and metallothionein on CYP expression.

Reactive oxygen species are commonly observed following PCB126 exposure or AhR activation [36]. Hepatic ROS and ROS responsive genes were all increased with PCB126 treatment as were DNA strand breaks (COMET assay) however genotype had little effect (Fig. 3). It is believed that the protective properties of metallothionein against other toxicants is through the increased availability of zinc and not solely by ROS scavenging itself, particularly in cases of carbon tetrachloride and alcohol induced liver damage [7]. Altered zinc status was not observed in untreated MTKO animals (Fig. 3) so the compensatory storage mechanism by which zinc levels are maintained is more rigid than the metallothionein system. This rigidity hinders the availability of zinc needed for abrogating liver injury. Additional studies looking into any compensatory storage system as a result of metallothionein loss and to zinc homeostasis in general are needed.

Our lab has shown previously the effect of PCB126 on hepatic micronutrients in rats [13,14,16]. Similar trends were observed in this study using mice with the notable exception of copper (Fig. 4). In the rat, hepatic copper increases with PCB126 treatment which was therefore initially hypothesized to be a response to increased metallothionein [15]. In this study, hepatic copper is basically unchanged in WT animals and significantly decreased in MTKO animals following exposure to PCB126. Decreased hepatic copper has been observed before in MTKO animals, when compared to control animals, but was seen much later, around 29–33 weeks of age [3]. It is noteworthy that differential gene expression has been observed between rats and mice following TCDD exposure [27]. In particular, transcription factors, i.e., the Sp family, are modulated differently. These transcription factors have been shown to be important for copper homeostasis [34,37]. Additional research is warranted to further dissect the interspecies differences observed. Other micronutrients in the liver, Zn, Se, and Mn, were observed to change as previously seen (Fig. 4) [13]. In particular, Zn and Se were decreased with PCB126 exposure suggesting alterations in zinc and selenium status appear to be metallothionein independent in the mouse. Interestingly, Mn increased in MTKO animals and is relatively unchanged with PCB126 treatment. MTKO animals have been shown to alter the expression of superoxide dismutase enzymes, decreasing Cu/ZnSOD and slightly increasing MnSOD, which may be contributing, in part, to the increases seen here [21]. The increase in hepatic Mn and the effect of metallothionein loss on SODs may indicate elevated antioxidant proteins to counterbalance the loss of metallothionein; this is corroborated with the activation of the Nrf2 pathway.

Renal micronutrients remained stable in the WT group after PCB126 treatment. This is different from what has been seen previously in rats, where increases in copper, selenium, and manganese were observed (Fig. 5) [15]. This is possibly explained by interspecies differences that have been discussed previously [27]. Interestingly, PCB126 appears to have an effect in the MTKO animals in the context of renal micronutrients. Cu, Zn, and Mn were all significantly decreased as well as Se, although not significantly. These changes are unlikely due to altered excretion, since copper, zinc, and manganese are traditionally excreted via bile, not in the urine. Previous studies in wild type animals show minor effects in

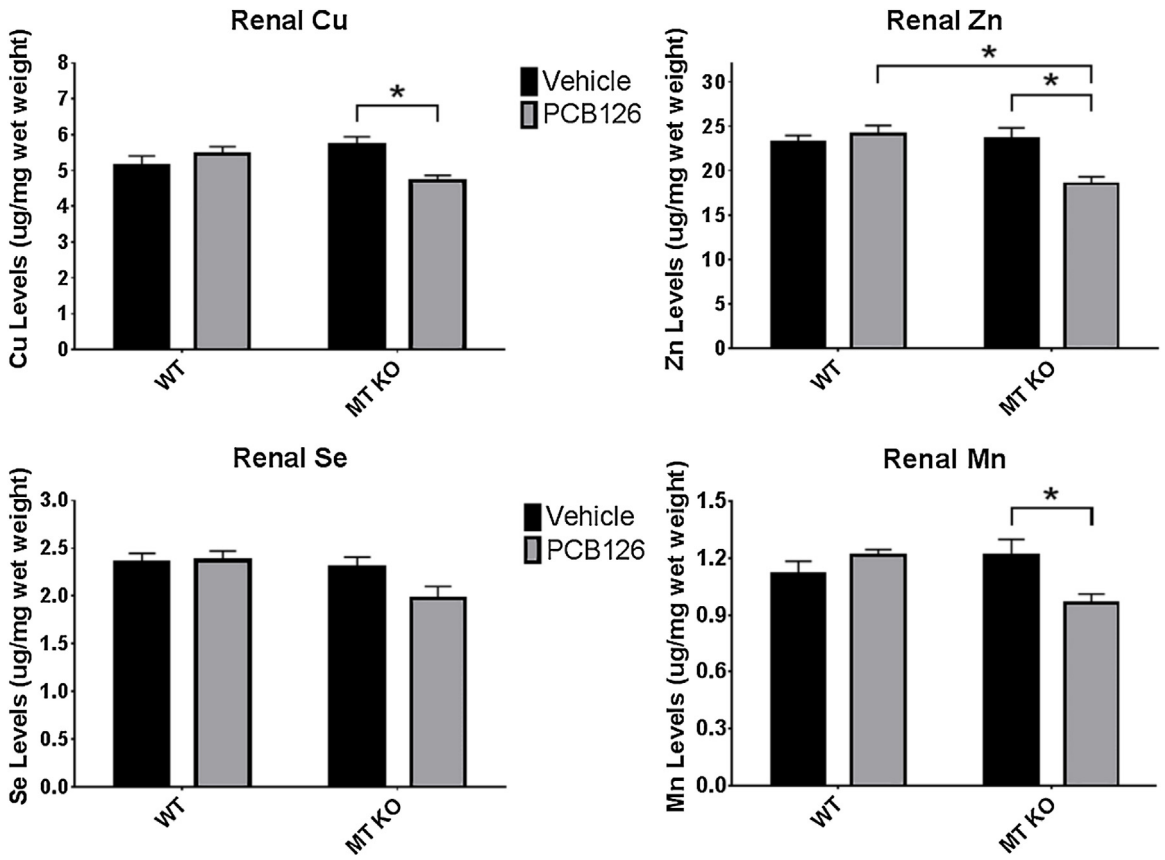

Fig. 5. MTKO mice are susceptible to renal micronutrient disruption caused by PCB126.
Renal copper, zinc, and manganese are all decreased in the MTKO animals following PCB126 administration. Renal Se is unaffected by both loss of metallothionein and PCB126 treatment. (Bars are standard error; * represents $p < 0.05$; two-way ANOVA; $n = 6$).

the kidney following PCB126 exposure [13]. This rather novel finding suggests a specific renal sensitivity to PCB126 toxicity caused by a lack of metallothionein. The mechanism for this sensitivity is unknown and could be the result of a compensatory system which maintains levels but is unable to respond to a stress response. Studies are warranted to investigate the metallothionein-independent mechanisms of micronutrient homeostasis as is suggested above in the context of zinc.

Given that the only significant differences seen between vehicle treated WT and MTKO animals were hepatic lipid content and hepatic Mn, the increased toxicity that is seen with PCB126 in MTKO compared to WT mice with respect to histological findings and renal metal content could be additive to the underlying deficits that lacking metallothionein causes. The true protective potential of metallothionein against PCB126 needs further investigation using an overexpression model. Potential use of a zinc supplementation model would ensure sufficient and labile pools of zinc which has shown to be important for mitigation of other hepatotoxicants. In addition, the difference seen histologically, particularly with respect to hepatocyte vacuolation, and the lack of difference seen in ROS metrics, suggests that PCB126 hepatotoxicity is not exclusively ROS mediated. This is an important consideration for future studies investigating potential therapeutic interventions. The mechanisms by which the combination of metallothionein deficiency makes the liver more susceptible to damage by PCB126 needs further studies. However it is clear that metallothionein plays a minor role in the disruption of hepatic and renal micronutrient status caused by PCB126.

Transparency document

The Transparency document associated with this article can be found in the online version.

Acknowledgements

This research makes up a portion of the dissertation work of W. Klaren and was supported by funding from NIH (P42 ES013661). The opinions given are solely those of the authors and not any granting agency. The authors greatly appreciate the help of Dr. David Peate from the Iowa Trace Element Analysis Laboratory at the University of Iowa for assistance in micronutrient analysis. The authors thank Dr. Gregor Luthe for the synthesis of PCB126 and Dr. Hans Lehmler and Dr. Iza Korwel for assistance with the lipid analysis. In addition, the authors thank Mrs. Sabah Hassain Enayah for assistance with the COMET assay and the rest of the lab members for help with the study. Finally, W. Klaren gratefully recognizes support from the Training Core of the Iowa Superfund Research program (P42 ES013661).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxrep.2015.11.009.

References

[1] J. Abel, T. Haarmann-Stemmann, An introduction to the molecular basics of aryl hydrocarbon receptor biology, Biol. Chem. 391 (2010) 1235–1248.
[2] M.D. Ampleman, A. Martinez, J. DeWall, D.F. Rawn, K.C. Hornbuckle, P.S. Thorne, Inhalation and dietary exposure to PCBs in urban and rural cohorts via congener-specific measurements, Environ. Sci. Technol. 49 (2015) 1156–1164.
[3] J.H. Beattie, A.M. Wood, A.M. Newman, I. Bremner, K.H.A. Choo, A.E. Michalska, J.S. Duncan, P. Trayhurn, Obesity and hyperleptinemia in metallothionein (-I and -II) null mice, Proc. Natl. Acad. Sci. 95 (1998) 358–363.
[4] I. Bremmer, Involvement of metallothionein in the hepatic metabolism of copper, J. Nutr. 117 (1987) 19–29.
[5] R.P. Bunaciu, J.C. Tharappel, H.J. Lehmler, I. Kania-Korwel, L.W. Robertson, C. Srinivasan, B.T. Spear, H.P. Glauert, The effect of dietary glycine on the hepatic tumor promoting activity of polychlorinated biphenyls (PCBs) in rats, Toxicology 239 (2007) 147–155.
[6] W.S. Darwish, Y. Ikenaka, S. Nakayama, M. Ishizuka, The effect of copper on the mRNA expression profile of xenobiotic-metabolizing enzymes in cultured rat H4-II-E cells, Biol. Trace Elem. Res. 158 (2014) 243–248.
[7] S.R. Davis, D.A. Samuelson, R.J. Cousins, Metallothionein expression protects against carbon tetrachloride-induced hepatotoxicity, but overexpression and dietary zinc supplementation provide no further protection in metallothionein transgenic and knockout mice, J. Nutr. 131 (2001) 215–222.
[8] B. Elsenhans, W. Forth, E. Richter, Increased copper concentrations in rat tissues after acute intoxication with 2,3,7,8-tetrachlorodibenzo-p-dioxin, Arch. Toxicol. 65 (1991) 429–432.
[9] F.A. Grimm, D. Hu, I. Kania-Korwel, H.J. Lehmler, G. Ludewig, K.C. Hornbuckle, M.W. Duffel, A. Bergman, L.W. Robertson, Metabolism and metabolites of polychlorinated biphenyls, Crit. Rev. Toxicol. 45 (2015) 245–272.
[10] R. Kato, A. Takanaka, Effect of phenobarbital on electron transport system, oxidation and reduction of drugs in liver microsomes of rats of different ages, J. Biochem. 63 (1968) 406–408.
[11] C.D. Klaassen, J. Liu, Metallothionein transgenic and knockout mouse models in the study of cadmium toxicity, Toxicol. Sci. 23 (1998) 97–102.
[12] W.D. Klaren, G.S. Gadupudi, B. Wels, D.L. Simmons, A.K. Olivier, L.W. Robertson, Progression of micronutrient alteration and hepatotoxicity following acute PCB126 exposure, Toxicology 338 (2015) 1–7.
[13] I. Lai, Y. Chai, D. Simmons, G. Luthe, M.C. Coleman, D. Spitz, W.M. Haschek, G. Ludewig, L.W. Robertson, Acute toxicity of 3,3',4,4',5-pentachlorobiphenyl (PCB 126) in male Sprague-Dawley rats: effects on hepatic oxidative stress, glutathione and metals status, Environ. Int. 36 (2010) 918–923.
[14] I. Lai, K. Dhakal, G. Gapdupudi, L. Miao, G. Ludewig, L.W. Robertson, A. Olivier, *N*-acetylcystiene (NAC) diminishes the severity of PCB126-induced fatty liver in male rodents, Toxicology 302 (2012) 25–33.
[15] I. Lai, W.D. Klaren, M. Li, B. Wels, D. Simmons, A.K. Olivier, W.M. Haschek, K. Wang, G. Ludewig, L.W. Robertson, Does dietary copper supplementation enhance or diminish PCB126 toxicity in the rodent liver? Chem. Res. Toxicol. 26 (2013) 634–644.
[16] I.K. Lai, Y. Chai, D. Simmons, W.H. Watson, R. Tan, W.M. Haschek, K. Wang, B. Wang, G. Ludewig, L.W. Robertson, Dietary selenium as a modulator of PCB 126-induced hepatotoxicity in male Sprague-Dawley rats, Toxicol. Sci. 124 (2011) 202–214.
[17] B. Lauby-Secretan, D. Loomis, Y. Grosse, F.E. Ghissassi, V. Bouvard, L. Benbrahim-Tallaa, N. Guha, R. Baan, H. Mattock, K. Straif, L.F. International Agency for Research on Cancer Monograph Working Group Iarc, Carcinogenicity of polychlorinated biphenyls and polybrominated biphenyls, Lancet. Oncol. 14 (2013) 287–288.
[18] C.H. Lee, Y. Ito, Y. Yanagiba, O. Yamanoshita, H. Kim, S.Y. Zhang, M. Kamijima, F.J. Gonzalez, T. Nakajima, Pyrene-induced CYP1A2 and SULT1A1 may be regulated by CAR and not by AhR, Toxicology 238 (2007) 147–156.
[19] D.K. Lee, J. Carrasco, J. Hidalgo, G.K. Andrews, Identification of a signal transducer and activator of transcription (STAT) binding site in the mouse metallothionein-I promoter involved in interleukin-6-induced gene expression, Biochem. J. 337 (Pt. 1) (1999) 59–65.
[20] Y. Li, Y. Huang, Y. Piao, K. Nagaoka, G. Watanabe, K. Taya, C. Li, Protective effects of nuclear factor erythroid 2-related factor 2 on whole body heat stress-induced oxidative damage in the mouse testis, Reprod. Biol. Endocrinol. 11 (2013) 23.
[21] Y. Lian, J. Zhao, P. Xu, Y. Wang, J. Zhao, L. Jia, Z. Fu, L. Jing, G. Liu, S. Peng, Protective effects of metallothionein on isoniazid and rifampicin-induced hepatotoxicity in mice, PLoS One 8 (2013) e72058.
[22] J. Liu, Y. Liu, S.S. Habeebu, C.D. Klaassen, Metallothionein (MT)-null mice are sensitive to cisplatin-induced hepatotoxicity, Toxicol. Appl. Pharmacol. 149 (1998) 24–31.
[23] J. Liu, Y. Liu, D. Hatley, C.D. Klaassen, S.E. Shehin-Johnson, A. Lucas, S.D. Cohen, Metallohthionein-I-II knockout mice are sensitive to acetaminphen-induced hepatotoxicity, J. Pharmacol. Exp. Ther. 289 (1999) 580–586.
[24] G.M. Luthe, B.G. Schut, J.E. Aaseng, Monofluorinated analogues of polychlorinated biphenyls (F-PCBs): synthesis using the Suzuki-coupling, characterization, specific properties and intended use, Chemosphere 77 (2006) 1242–1248.
[25] Y. Lv, B. Zhang, G. Xing, F. Wang, Z. Hu, Protective effect of naringenin against acetaminophen-induced acute liver injury in metallothionein (MT)-null mice, Food Funct. 4 (2013) 297–302.
[26] B.J. Murphy, G.K. Andrews, D. Bittel, D.J. Discher, J. McCue, C.J. Green, M. Yanovsky, A. Giaccia, R.M. Sutherland, K.R. Laderoute, K.A. Webster, Activation of metallothionein gene expression by hypoxia involves metal response elements and metal transcription factor-1, Cancer Res. 59 (1999) 1315–1322.
[27] R. Nault, S. Kim, T.R. Zacharewski, Comparison of TCDD-elicited genome-wide hepatic gene expression in Sprague-Dawley rats and C57BL/6 mice, Toxicol. Appl. Pharmacol. 267 (2013) 184–191.
[28] T.T. Ngu, M.J. Stillman, Metalation of metallothioneins, IUBMB Life 61 (2009) 438–446.
[29] P. Reeves, L. DeMars, Signs of iron deficiency in copper-deficient rats are not affected by iron supplements administered by diet or by injection, J. Nutr. Biochem. 17 (2006) 635–642.
[30] L.W. Robertson, A. Parkinson, S. Bandiera, I. Lambert, J. Merrill, S. Safe, PCBs and PBBs: Biological and Toxic Effects on C57BL/6 J and DBA/2J, Toxicology 31 (1984) 191–206.
[31] M. Sato, I. Bremner, Oxygen free radicals and metallothionein, Free Radic. Biol. Med. 14 (1993) 325–337.
[32] S. Sato, H. Shirakawa, S. Tomita, M. Tohkin, F.J. Gonzalez, M. Komai, The aryl hydrocarbon receptor and glucocorticoid receptor interact to activate human metallothionein 2A, Toxicol. Appl. Pharmacol. 273 (2013) 90–99.
[33] H. Shen, L.W. Robertson, G. Ludewig, Regulation of paraoxonase 1 (PON1) in PCB 126-exposed male Sprague Dawley rats, Toxicol. Lett. 209 (2012) 291–298.
[34] I.S. Song, H.H. Chen, I. Aiba, A. Hossain, Z.D. Liang, L.W. Klomp, M.T. Kuo, Transcription factor Sp1 plays an important role in the regulation of copper homeostasis in mammalian cells, Mol. Pharmacol. 74 (2008) 705–713.
[35] G. Speit, A. Rothfuss, The comet assay: a sensitive genotoxic test for the detection of DNA damage and repair, Methods Mol. Biol. 920 (2012) 79–90.
[36] S.J. Stohs, Oxidative stress induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), Free Radic. Biol. Med. 9 (1990) 79–90.
[37] G. Suske, The Sp-family of transcription factors, Gene 238 (1999) 291–300.
[38] B.L. Vallee, The function of metallothionein, Neurochem. Int. 27 (1995) 23–33.
[39] Z. Zhou, Zinc and alcoholic liver disease, Dig. Dis. 28 (2010) 745–750.