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Tooth manganese as a biomarker of exposure and body burden in rats☆

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Abstract

Background—Neonates and children are particularly vulnerable to the toxic effects of excess manganese (Mn), but studies of Mn exposure during these developmental periods are hampered by a lack of validated biomarkers. Deciduous teeth may be used to assess Mn exposure during these developmental periods but require further validation to determine the relationship between tooth Mn, Mn in target tissues, and exposure.

Objectives—To determine the relationship of tooth Mn concentrations with: (i) exposure dose, (ii) the timing/duration of exposure, and (iii) with Mn concentrations in blood, brain and bone.

Methods—Rats in different treatment groups were orally exposed to 0, 25 or 50 μg/g/day Mn either from postnatal day (PND) 1 – 21 and culled at PND 24, from PND 1 – 21 and culled as adults (>PND 290), or from PND 1 – throughout life and culled at >290 PND. Mn was measured in second molars, femurs, brain and blood by ICP-MS.

Results—Tooth Mn increased significantly with dose in rats exposed for 21 PND and culled at 24 PND ($p < 0.001$). In rats culled at >290 PND, tooth Mn increased with exposure duration ($p < 0.001$) and reflected exposure duration. A significant, positive association between tooth Mn and Mn levels in blood (Spearman's *rho* 0.69, $p < 0.01$) brain (*rho* 0.59, $p < 0.05$) and bone (*rho* 0.69, $p < 0.01$) was observed in animals with lifelong exposure. Tooth Mn and Mn levels in bone were also significantly positively associated in animals exposed only early in life (*rho* 0.76, $p < 0.001$).

Conclusions—Teeth are a sensitive biomarker of active and past Mn exposure and Mn burden in tissues. Unlike blood, teeth retain information on exposure history over the short and long-term.

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The authors declare they have no actual or potential competing financial interests.

Keywords

biomarker; blood; bone; brain; exposure; manganese; teeth

Introduction

Manganese (Mn) is an essential element that comes under strict homeostatic control with age but deficiency or excess in early life can cause neurotoxic effects and increase the risk of lower birth weight infants (Claus Henn et al., 2010; Zota et al., 2009). Neonates are particularly susceptible to neurotoxicity as Mn can cross the blood-brain barrier, is actively transported across the placenta, and neonates do not have well developed Mn regulation (Aschner and Aschner, 1991; Krachler et al., 1999; Santos-Burgoa et al., 2001).

Details of the exposure-effect relationship of Mn are not well defined, partly due to the lack of well recognized and validated biomarkers of Mn exposure (Smith et al., 2007). The majority of studies reviewed recently by (Rodriguez-Barranco et al., 2013) reported a significant negative effect on neurodevelopment and behavioral disorders but only two studies specifically addressed prenatal exposure, illustrating a pronounced lack of knowledge of the potential impacts of Mn exposure during this critical developmental period.

Studies addressing prenatal or early childhood Mn exposure are particularly hampered by a lack of suitable biomarkers. Measures of fetal exposure through maternal biomarkers are limited by complex kinetics that obscure the relationship with environmental exposures. The tight homeostatic control of Mn in adults and short half-life in blood and urine may give unreliable estimates of fetal exposure (Schroeter et al., 2011). Cord blood was used to demonstrate negative associations with early psychomotor development (Takser et al., 2003) and neurodevelopment (Lin et al., 2013), but the collection of fetal blood at different stages of pregnancy is not feasible for studies attempting to pinpoint critical exposure time windows. Epidemiological studies assessing the health outcomes of fetal and early childhood Mn exposure would benefit from a retrospective biomarker that indicates exposure during these critical development periods.

In humans, deciduous teeth mineralize in an incremental pattern that spans from the 14th gestational week to 11 months postnatal, depending on the tooth type (Nelson and Ash, 2010). Metals and other trace elements are incorporated into the mineralizing tissue, creating a timeline of exposure. Several studies have used teeth as a biomarker for lead (Pb) exposure (Arora et al., 2006; Bellis et al., 2008; Needleman et al., 1974; Shepherd et al., 2012). Mn in prenatally formed parts of teeth has been linked to neurodevelopment (Claus Henn and Coull, 2015; Gunier et al., 2015; Mora et al., 2015), childhood behavioral outcomes (Ericson et al., 2007) and environmental levels (Arora et al., 2012; Gunier et al., 2013; Gunier et al., 2014). However, there is limited data to evaluate the relationship of tooth Mn concentrations in response to different doses and timing/duration of Mn exposure, and body burden in other tissues.

Our overall objective is to validate teeth as a biomarker of Mn exposure and body burden in a controlled rat experiment. We determined the relationship of tooth Mn concentrations with: (i) dose, (ii) the timing/duration of exposure, and (iii) with Mn concentrations in blood, brain and bone.

Methods

Animals

Details on the subjects and methods used in this study have been previously reported (Beaudin et al., 2013; Beaudin et al., 2016). Briefly, the animals were born into the study over a 2 day period from primiparous pregnant Long–Evans rats (gestational day 18, Charles River, Hollister, CA, USA). Twelve - 24 hours after parturition (designated PND 1, birth = PND 0) litters were sexed, weighed and culled to eight pups per litter such that each litter was comprised of five to six males and the remainder females. Only one male per litter was assigned to a particular treatment condition. Animals (dams and weaned pups) were fed Harlan Teklad rodent chow #2018 (reported by the manufacturer to contain 118 mg/kg Mn) and housed in polycarbonate cages at a constant temperature of 21 ± 2 °C. At PND 22, all pups were weaned and pair-housed (two rats/cage) with an animal of the same treatment group and maintained on a reversed 10:14 hr light/dark cycle. All animal care and treatments were approved by the institutional IACUC, and followed the guidelines outlined in the Guide for the Care and Use of Laboratory Animals (Council, 2011).

Animal Exposure

In the short term experiments, rats were orally exposed to 0, 25 or 50 μg/g/day Mn over PND 1 – 21 and culled at PND 24. In long term experiments, rats were orally exposed to 0, 25 or 50 μg/g/day Mn over PND 1 – 21 (early life, E), or PND 1 – throughout life (lifelong, L) and culled at PND > 290. Dosing over PND 1 – 21, was administered using a 225 mg/ml Mn stock solution prepared from $MnCl_2$ diluted with 2.5 % (w/v) stevia (a natural sweetener) delivered directly into the mouth of the pups (~25 μL/dose) using a micropipette fitted with a flexible polyethylene tip (Fisher Scientific, Santa Clara, CA, USA). Control animals received only the stevia vehicle. After weaning starting on PND 22, Mn was administered via the drinking water at levels of ~210 μg/mL or ~420 μg/mL Mn for the 25 or 50 μg/g/day Mn exposure groups, respectively; actual water Mn levels were adjusted weekly if needed to maintain target exposure levels based on water intake. Water bottle weights were recorded at refilling to determine water intake per cage, and daily Mn intake per kg body weight was estimated based on daily measured body weights of the two post-weaned rats housed per cage. These Mn exposure regimens are relevant to children exposed to elevated Mn via drinking water, diet, or both; pre-weaning exposure to 25 and 50 μg/g/day Mn produces relative increases in Mn intake that approximate the increases reported in infants and young children exposed to Mn-contaminated water or soy-based formulas (or both) (Kern et al., 2010). Chronic oral exposure to the same Mn doses were maintained after weaning via drinking water, since children may continue to suffer chronic elevated Mn exposures from a variety of environmental sources (e.g., contaminated well water, dust, etc.) (Bouchard et al. 2011; Lucas et al. 2015; Oulhote et al. 2014). Rates of drinking water intake were not significantly different between treatment groups.

Mn determination in blood, brain and bone

Animals were euthanized by sodium pentobarbital overdose and exsanguination. Whole blood (2 – 3 mL) was collected from the left ventricle of the surgically-exposed heart and stored in EDTA vacutainers at −20 °C. Tissue preparation procedures for Mn determination were previously described (Kern et al., 2010). Whole brain was removed immediately and bisected into hemispheres. The hind-brain regions (~200 mg wet wt) were collected and stored at −80 °C. For bone, whole femur was dissected from the animal, cleaned of all adhering tissue and periosteum, bisected and the marrow flushed using Milli-Q water. Brain and bone samples were dried to a constant weight and digested with hot 16 M nitric acid. The digestate was evaporated and re-dissolved in 1 M nitric acid for Mn determination.

Rhodium was added to samples as an internal standard and Mn was determined in the samples using a Thermo Element XR inductively coupled plasma-mass spectrometer (ICP-MS). Certified SPEX standards (Spex Industries, Inc., Edison, NJ) were used for quantification and NIST SRM 1577b (bovine liver) was used to assess analytical performance (Mn recovery 98 – 102 %). The Mn analytical detection limit ranged from 0.015 to 0.04 ng/mL.

Mn Determination in Whole Teeth

Pulp and soft tissue adherent to the external surface of each tooth was removed by scraping with a dental instrument before washing in Milli-Q water and drying prior to weighing. Teeth were digested with 100 μL of concentrated nitric acid (Baseline® grade, Seastar, Canada) at 80 °C for half an hour. Solutions were allowed to cool to room temperature before dilution to 1.5 mL with Milli-Q water.

Samples were analyzed on an Agilent Technologies 7500cs ICP-MS. Yttrium was used as an internal standard, added via t-piece to the sample introduction tubing. Quantification was performed using a blank and external standards covering the ranges of 0.1–100 ng/g and 0.5 – 10 ng/mg prepared from a certified ICP-MS Mn standard (Choice Analytical). A certified multi-element standard (TraceCert®, Fluka Analytical) was used to confirm analytical accuracy. The method detection limit was 0.05 ng/g Mn.

Statistical Analysis

Spearman's correlation coefficient was used to measure associations between Mn concentrations in different biological media (teeth, blood, brain and bone). When displaying bivariate associations as scatter plots with regression lines, we $\log_e$ transformed our data to achieve normally distributed variables. To avoid producing negative values after $\log_e$ transformation, we multiplied values between 0 and 1 by 10. Treatment groups were compared using the non-parametric Mann-Whitney U test and the Jonckheere-Terpstra test was used to determine if there was a significant increasing trend in measured Mn concentrations in teeth, blood, brain or bone with increasing ordinal dose or duration of exposure. Results were considered statistically significant at $p < 0.05$. All statistical data analysis was performed using IBM SPSS software v19.

Results

To examine the relationship between tooth Mn and level of exposure we measured Mn in whole molars from rats exposed to 0, 25 or 50 μg/g/day Mn over PND 1 – 21 and culled at PND 24. Tooth Mn increased significantly with dose (Jonckheere-Terpstra $p < 0.001$) (Figure 1). Tooth Mn in animals exposed to 25 or 50 μg/g/day Mn was significantly different from control animals (Mann-Whitney U $p < 0.01$). Similar trends for blood, brain and bone Mn levels were observed (Figure 1).

The relationship between tooth Mn and cumulative dose was also assessed within a longer term study using rats exposed over PND 1 – 21 (early, E) or over PND 1 – throughout life (lifelong, L), and culled at PND > 290. For rats exposed throughout life, median tooth Mn was higher in the 50 μg/g/day Mn group than the 25 μg/g/day Mn group but this was not statistically significant ($p < 0.181$). Tooth Mn increased with total cumulative dose (Figure 2a). For the same daily dose, rats exposed for a limited period (PND 1 – 21) early in life had significantly lower tooth Mn than rats exposed throughout life (Mann-Whitney U $p < 0.001$ for 25 μg/g/day Mn and $p < 0.01$ for 50 μg/g/day Mn).

These same rats were used to assess the relationship between tooth Mn and duration of exposure. In rats exposed over PND 1 – 21 (early, E) or throughout life (lifelong, L), and culled at PND > 290, tooth Mn increased with the duration of exposure at 25 and 50 μg/g/day Mn dosage (Jonckheere-Terpstra $p < 0.001$) (Figure 3).

The relationship between tooth Mn and Mn levels in blood, brain and bone was assessed by measuring the association between Mn levels using Spearman's ρ. Strong, positive, significant associations were observed between Mn in teeth and blood, brain, and bone in animals that were exposed over PND 1 – 21 and culled at PND 24 (Table 1). Scatter plots showing the association between tooth Mn and Mn levels in blood, brain and bone are provided in Figure 4.

When all rats from the long term experiments were pooled (exposed over PND 1 – 21 or throughout life, and culled at PND > 290), associations between Mn in teeth and blood/brain Mn were weakened compared to rats exposed over PND 1 – 21 and culled at PND 24 but remained significant (Table 1, see Supplemental Material, Figure S1).

Associations between tooth Mn levels and other tissues were stronger when restricted to animals exposed throughout life and culled at PND > 290 (Table 1). Tooth Mn was significantly, positively associated with Mn in blood ($rho = 0.72$, $p < 0.01$), brain ($rho = 0.59$, $p < 0.05$) and bone ($rho = 0.74$, $p < 0.001$). For rats only exposed early in life (over PND 1 – 21 and culled at PND > 290), tooth Mn was only significantly associated with bone Mn ($rho = 0.76$, $p < 0.001$) (Table 1).

Blood from rats that were only exposed early in life (21 PND) and culled at > 290 PND, had Mn levels similar to control rats (Figure 2b). Blood Mn in rats exposed throughout life (> 290 PND) was significantly higher than control rats and rats exposed early in life (21 PND) at the same dose (Mann-Whitney U $p < 0.05$).

Finally, rats exposed over PND 1 – 21 and culled at PND 24 had significantly higher tooth Mn than rats exposed over PND 1 – 21 and culled at PND > 290 (Mann-Whitney U $p<$ 0.001) (Figure 5).

Discussion

There is increasing evidence that at high exposures, Mn is a neurotoxicant that poses an increased threat during prenatal and early childhood development (Coetzee et al., 2016; Sanders et al., 2015). Measures of Mn exposure during these critical periods are either not feasible to obtain (fetal blood) or may be limited in their validity due to complex kinetics that obscure the relationship of Mn in blood and urine to environmental exposures (Schroeter et al., 2011; Smith et al., 2007). Teeth are an opportune biomarker, particularly for studying Mn exposure in key early life development windows when vulnerability is greatest. The aim of this study was to remedy the distinct lack of well recognized, validated biomarkers of Mn exposure by assessing the relationships between tooth Mn and dose, timing/duration of exposure and with Mn concentrations in blood, brain and bone.

The assessment of a dose-response relationship is essential for the validation of teeth as an exposure biomarker. The significant increase in tooth Mn with dose (Figure 1 and 2) and duration of exposure (Figure 3) demonstrates a clear dose-response relationship in rats orally exposed to Mn. The rate at which teeth mineralize changes over time and therefore the uptake of Mn in the tooth will also vary. Uptake in primary dentine is expected to be faster than secondary dentine which forms at a much slower rate. In this study, depending on the duration of exposure and PND at the time of collection, some teeth contained only primary dentine (short term experiment), while others contained primary and secondary dentine. In addition, for the long term experiments, secondary dentine formed with or without Mn exposure. These factors make it difficult to assess the shape of the relationship between tooth Mn and dose.

Mn in blood and brain media only demonstrated a dose-response relationship when exposure was active at the time of sampling. Mn levels in blood and brain from rats exposed over PND 1 – 21 and culled at PND > 290 had returned to levels observed in control animals by the time of sampling (Figure 2b, c). This was expected given the rapid clearance of Mn in blood and the immaturity of the blood-brain barrier (BBB) in young rats. The half-life of Mn in human blood is about 4 days (Mahoney and Small, 1968). If sampling of rats exposed early in life (PND 1 – 21) did not occur until the animals were culled at PND > 290, the time lapse would have ensured the excess Mn had been largely eliminated from blood. The biliary excretion pathway and BBB are relatively immature in the preweanling rat, leading to high absorption but also relatively unimpeded elimination of Mn in the brain (Rehnberg et al., 1981).

Mn in bone appeared to reflect recent exposure, in combination with some history of prior exposure. Bone Mn was statistically different between the two groups exposed throughout life (L25, L50) but not the groups only exposed early in life. Yet the groups exposed early in life had significantly different bone Mn compared to the control group, indicating there remained some signal from past exposure later in life.

The strong correlation between bone and tooth Mn across all treatment groups was expected given the similarities in Mn accumulation and storage between these media. The slope of the correlation between tooth and bone Mn was much greater than 1 (slope = 374, Supplemental Figure S1), indicating that teeth accumulate Mn with dose at a higher proportion than bone. This could reflect the inclusion of the enriched enamel sub-surface in the whole tooth data (discussed below) but is more likely due to the turnover of bone reducing the accumulation of Mn in this tissue. Dentine remodels to a much lesser extent than bone (~ 1 % for dentine and ~ 8 % per year for trabecular bone in humans (Gulson and Gillings, 1997)). The effect of bone remodeling on the validity of bone as a biomarker of Mn exposure is the subject of another study.

Strong positive associations between Mn levels in teeth and blood, brain, and bone Mn were observed in rats that were exposed throughout life (exposed over PND 1 – 21 and culled at PND 24, or exposed over PND 1 – >290), demonstrating that tooth Mn is a good measure of body burden (Figures 4 and S1). As expected, tooth Mn was poorly associated with blood and brain Mn in animals that were only exposed early in life (exposed over PND 1 – 21 and culled at PND > 290).

A sub-surface layer of enamel approximately 20 – 40 μm thick was reported in several studies to be enriched in Mn and Pb (Arora et al., 2005; Arora et al., 2011; Budd et al., 1998). The higher abundance of Mn and Pb in this layer may be a result of hypermineralization of the enamel surface during the maturation phase of enamel mineralization (Smith, 1998), or the result of enamel surface exchanges with ions in saliva accumulating over time. An analysis of whole teeth will include this enriched enamel layer which may impact the estimation of cumulative dose, particularly if the enriched layer is due to surface exchanges with ions in saliva, an exposure source that never entered the blood stream and therefore cannot contribute to neurological toxicity. In this study, all animals were exposed orally, providing a high amount of ions in saliva available for enamel surface exchange after the dosing event. Despite this, teeth demonstrated a clear dose-response relationship and significant strong, positive correlations with other tissues while exposure was active.

The whole teeth analyzed in this study proved to be a good measure of cumulative exposure but temporal information was lost. For example, no distinction would be possible between animals exposed at a high dose for a short period of time and animals exposed at a low dose for a longer period of time if total cumulative dose were the same. Additionally, tooth Mn levels varied with age at sampling even when comparing animals with the same total cumulative dose. Animals exposed over PND 1 – 21 and culled at PND 24 had higher tooth Mn levels than those exposed to the same daily dose and duration but culled at PND > 290 despite timing of exposure also being the same (Figure 5). The difference in Mn levels is likely due to a dilution effect in the molars from rats culled at PND > 290. Molars from rats culled at PND 24 contain only dentine (and enamel) that has formed during the period of exposure (PND 1 – 21), whereas molars from rats culled at PND > 290 also contain secondary dentine that has formed after the period of exposure (PND 1 – 21). When analyzing whole teeth, this secondary dentine would result in a lower value for the Mn mass fraction.

The results presented here agree with another recent animal study of Mn accumulation in hair and teeth (Liang et al., 2016). A significant positive correlation between Mn in the hippocampus, teeth and hair was reported in male rats exposed for 18 weeks by intraperitoneal injection from PND 28–35. Mn in the hippocampus, teeth and hair were also significantly correlated with learning and memory function tests, while serum Mn was not. The Liang *et al.* and our study presented here make significant contributions towards filling a crucial gap in the understanding of teeth as a biomarker of Mn exposure.

Teeth form in an incremental pattern that can be followed by counting daily lines left behind, much like growth rings in trees. Micro-spatial sampling techniques can reconstruct exposure history in terms of level and timing of exposure over a fine temporal scale which will allow us to examine critical developmental periods. We have shown previously that associations between tooth Mn and environmental samples were stronger when the tooth Mn data collected was restricted to the same time period as when the environmental data was collected (Arora et al., 2012). While this study has shown that whole tooth analysis will provide a good indication of cumulative exposure, techniques which enable spatial analysis of teeth will be more informative regarding timing of exposure.

Conclusion

The lack of appropriate validation of teeth as a biomarker of Mn exposure has hindered the application of this technique to the study of health outcomes. This study demonstrates a clear Mn dose-response relationship in whole rat molars, sensitive to level and duration of exposure. Correlation of tooth Mn with blood, brain, and bone Mn levels in rats demonstrates that teeth are a good measure of Mn body burden, particularly in which case exposure occurred prior to sampling when Mn may be removed from other biological media.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

BBB	blood-brain barrier
ICP-MS	inductively coupled plasma-mass spectrometry
Mn	manganese
Pb	lead
PND	postnatal days

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Highlights

- Tooth biomarkers have been used to study manganese exposures in humans
- Whether tooth manganese is associated with organ levels has not been studied
- We show how tooth manganese can be used to study short- and long-term exposure, and body burden

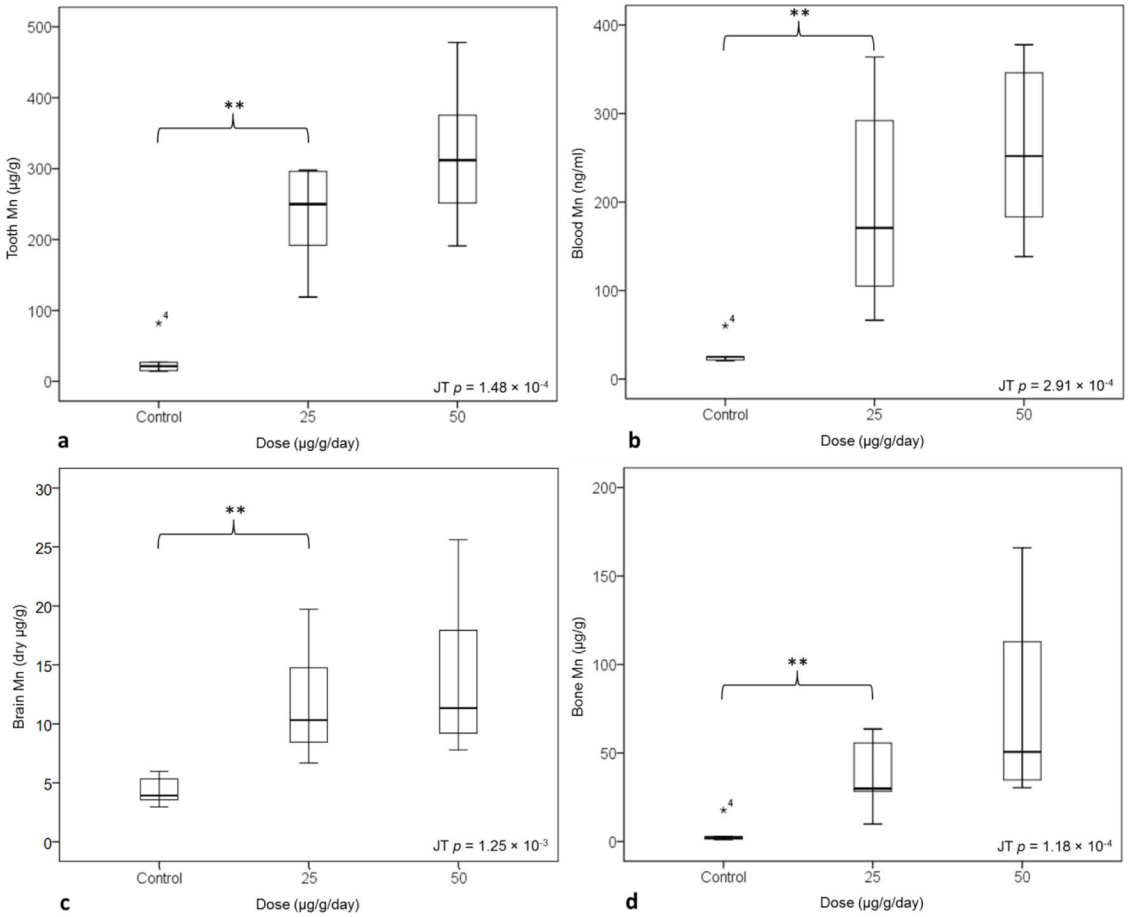

Figure 1.

Box plots of (a) tooth, (b) blood, (c) brain and (d) bone with dose for rats exposed over PND 1 – 21 and culled at PND 24. The Mann-Whitney U test was used to compare treatment groups. Mn in all biological media from animals dosed at 25 µg/g/day Mn was significantly higher than control groups (** $p < 0.01$) but the difference between 25 and 50 µg/g/day Mn groups was not significant. The increase in Mn with dose in all biological media was significant according to the Jonckheere-Terpstra (JT p).

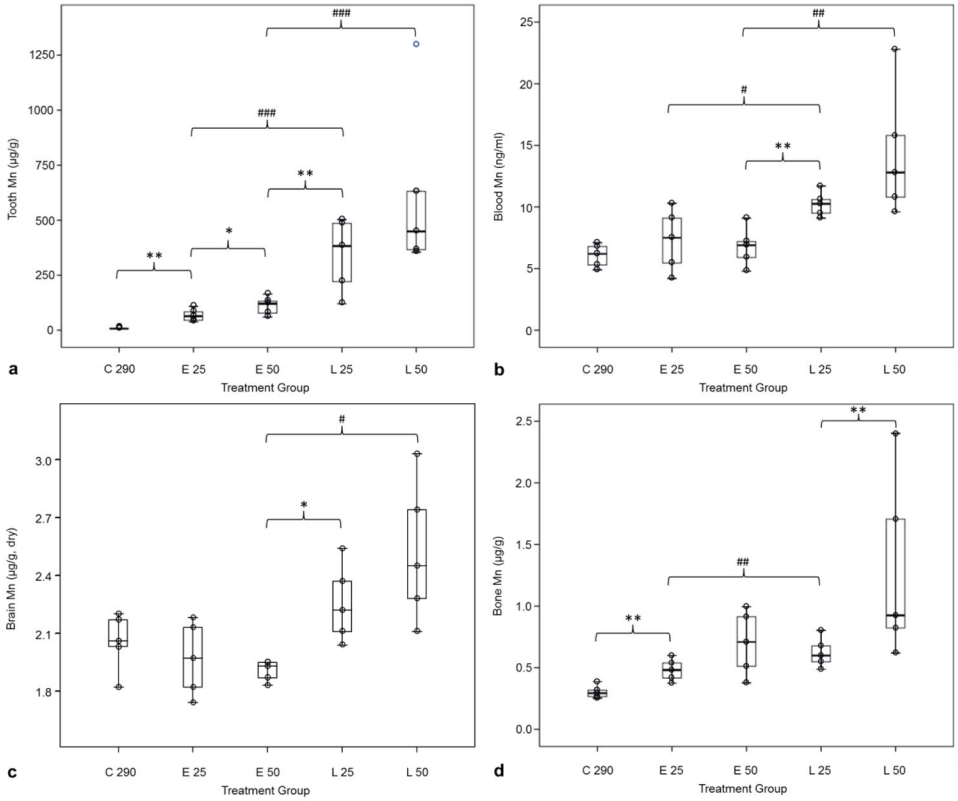

Figure 2.

Box plots of Mn in teeth (a), blood (b), brain (c) and bone (d) for each treatment group of rats culled at PND > 290; control (C 290), exposed to 25 µg/g/day Mn over PND 1 – 21 (E 25), exposed to 50 µg/g/day Mn over PND 1 – 21 (E 50), exposed to 25 µg/g/day Mn for PND > 290 (L 25) and exposed to 50 µg/g/day Mn for PND > 290 (L 50). Box plot constructed from median, 25th and 75th percentiles. Whiskers represent minimum and maximum. Total cumulative dose increases C 290 < E 25 < E 50 < L 25 < L 50 (E 25 = 0.525 µg/g, E 50 = 1.05 µg/g, L 25 = 9.7 – 12.6 µg/g and L 50 = 19.4 – 24.3 µg/g). * p< 0.05, ** p< 0.01, *** p< 0.001; between exposure durations (E vs L) # p< 0.05, ## p< 0.01, ### p< 0.001.

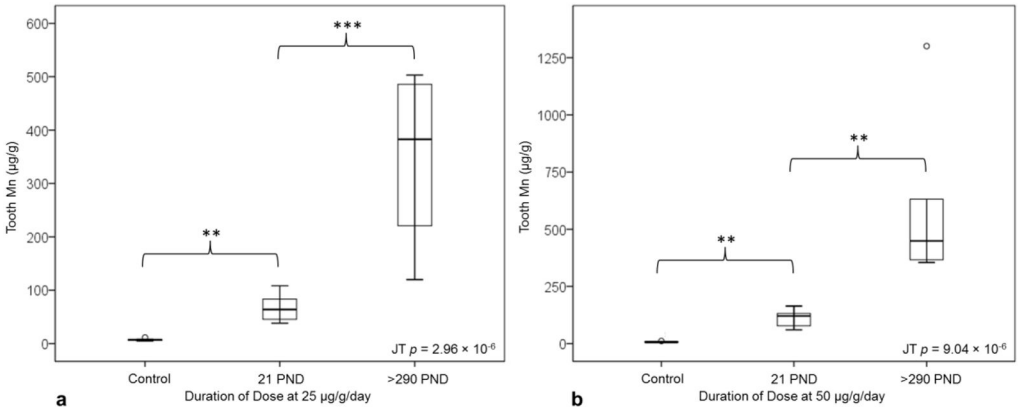

Figure 3.

Box plots of tooth Mn over different durations of exposure at (a) 25 and (b) 50 µg/g/day Mn in animals exposed over PND 1 – 21 or throughout life and culled at PND > 290. Mann-Whitney U ** $p < 0.01$, *** $p < 0.001$).

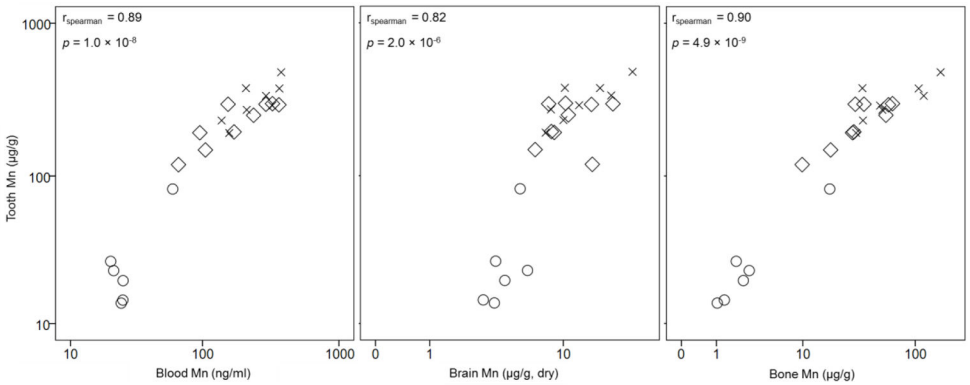

Figure 4.

Scatter plots showing association of tooth Mn and Mn levels in (a) blood, (b) brain and (c) bone on a $\log_{10}$ scale. Points are labeled according to treatment group: control (circle), and exposed to 25 (diamond) or 50 µg/g/day Mn (cross) over PND 1 – 21 and culled at PND 24. Spearman's r and corresponding *p* value are also reported.

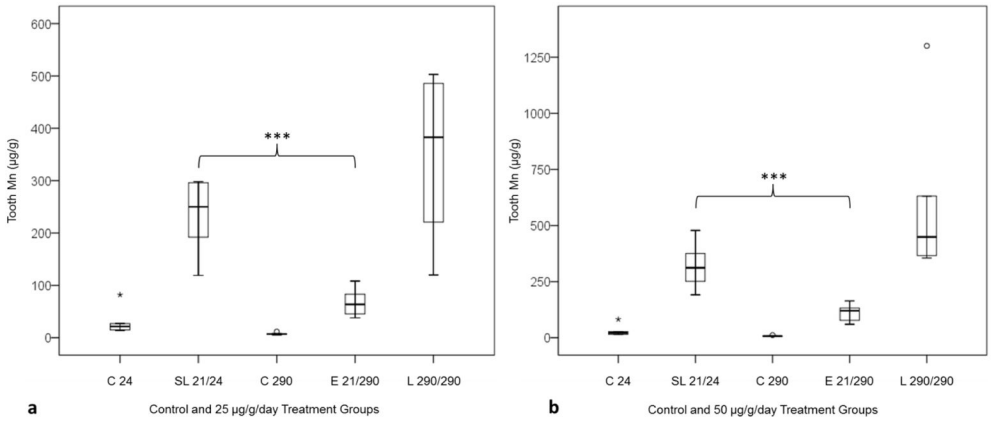

Figure 5.

Box plots of tooth Mn from control rats and rats exposed over different durations at (a) 25 and (b) 50 µg/g/day Mn. Mann-Whitney U *** $p < 0.001$. Groups are labelled as follows: control rats culled at PND 24 (C 24), rats exposed over PND 1 – 21 and culled at PND 24 (SL 21/24), control rats culled at PND > 290 (C 290), rats exposed over PND 1 – 21 and culled at PND > 290 (E 21/290), and rats exposed throughout life and culled at PND > 290 (L 290/290).

Table 1

Association of tooth Mn with Mn in blood, brain and bone in rats dosed over different durations in the short and long term experiment.

		Tooth Mn (μg/g)			
		Short Term[a]	**Overall[b]**	**Early Exposure[c]**	**Lifelong Exposure[d]**
Blood Mn (ng/ml)	Spearman's ρ	0.893***	0.675***	0.0704	0.724**
	n	23	29	18	16
Brain Mn (μg/g dry)	Spearman's ρ	0.817***	0.521*	0.00358	0.594*
	n	23	23	15	13
Bone Mn (μg/g)	Spearman's ρ	0.900***	0.727***	0.757***	0.742***
	n	23	34	21	18

* $p < 0.05$,

** $p < 0.01$,

*** $p < 0.001$

[a] Dosed at 0, 25 or 50 μg/g/day Mn till 21 PND and culled at 24 PND

[b] Dosed at 0, 25 or 50 μg/g/day Mn till 21 PND or throughout life and culled at > 290 PND

[c] Dosed at 0, 25 or 50 μg/g/day Mn till 21 PND and culled at > 290 PND

[d] Dosed at 0, 25 or 50 μg/g/day Mn throughout life and culled at > 290 PND