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Influence of Developmental Lead Exposure on Expression of DNA Methyltransferases and Methyl Cytosine-Binding Proteins in Hippocampus

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Abstract

Developmental exposure to lead (Pb) has adverse effects on cognitive functioning and behavior that can persist into adulthood. Exposures that occur during fetal or early life periods may produce changes in brain related to physiological re-programming from an epigenetic influence such as altered DNA methylation status. Since DNA methylation is regulated by DNA methyltransferases and methyl cytosine binding proteins, this study assessed the extent to which developmental Pb exposure might affect expression of these proteins in the hippocampus. Long Evans dams were fed Pb-containing food with or without added Pb acetate (0, 150, 375, 750 ppm) prior to breeding and stayed on the same diet through weaning (perinatal exposure group). Other animals were exposed to the same doses of Pb but exposure started on postnatal day 1 and continued through weaning (early postnatal exposure group). All animals were euthanized on day 55 and hippocampi were removed. Western analyses showed significant effects on DNMT1, DNMT3a, and MeCP2 expression, with effects often seen at the lowest level of exposure and primarily modified by sex and developmental window of Pb exposure. These data suggest potential epigenetic effects of developmental Pb exposure on DNA methylation mediated at least in part through dysregulation of methyltransferases.

Keywords

lead; epigenetics; methylation

1. Introduction

Gene regulation during development and in adulthood is regulated by the binding of a variety of regulatory proteins to gene promoter regions and through epigenetic modification of chromatin by post-translational histone modifications and DNA methylation. DNA methylation is catalyzed by a family of DNA methyltransferases (DNMTs) that include the maintenance enzyme DNMT1 and the *de novo* methyltransferase DNMT3a (Bestor, 2000; Feng et al., 2005; Robertson and Wolffe, 2000). Recent studies have suggested an important role for DNA methylation in the development and function of brain (Feng et al., 2005) and

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in particular, DNMT1 has been suggested to play an important role in brain development considering its high expression pattern in the developing brain. In contrast, DNMT3a is expressed during development and continues to be expressed, albeit at relatively low levels, in the adult brain (Feng et al., 2007), suggesting a role for DNMT3a in brain maturation as well as adult brain function. In addition, DNA methyltransferase expression is subject to active regulation under both physiologic or pathologic conditions and appears to play an important role in the regulation of synaptic plasticity in the mature CNS (Feng et al., 2007). Dysregulation of DNMTs has been associated with a variety of cognitive disorders such as schizophrenia, Rett syndrome, and Fragile X mental retardation (Levenson, 2007; Levenson et al., 2006) and in particular, DNMT activity, and particularly DNMT3a, appears to be required for normal hippocampal synaptic plasticity in the adult brain (Levenson et al., 2006).

Developmental exposure to even low levels of lead (Pb) adversely impacts a variety of cognitive and behavioral functions and neurochemical systems resulting in deficits in learning, memory, attention, and executive function in children that persist into adulthood (Nigg et al., 2010; Surkan et al., 2007; Mazumdar et al., 2011; Cecil et al., 2008; Yuan et al., 2006). Persistent effects from early-life Pb exposure are consistent with a model of fetal/early-life basis of adult disease/disability in which early insults produce changes that arise from physiological re-programming (Cottrell and Seckl, 2009). The mechanisms underlying the effects of Pb on disparate cognitive and behavioral functions and its effects on the developmental programming of cortical-subcortical systems subserving key aspects of cognition are not well understood but possibly involve epigenetic mechanisms. A possible way in which developmental Pb exposure may influence cognitive development and later life cognitive functioning is through epigenetic modifications. Previous work (Hanna et al., 2012; Pilsner et al., 2009; Wright et al., 2010) has linked epigenetic modification of DNA methylation to elevated blood Pb levels in humans but effects of Pb on DNA methyltransferases and closely related methyl cytosine-binding proteins such as Methyl-CpG-binding protein 2 (MeCP2) and Methyl-CpG binding protein 1 (MBD1), which also play important roles in transcriptional regulation have only been reported in cerebral cortex from aged non-human primates that had Pb exposure as infants (Bihaqi et al., 2011). The present study was conducted to investigate the effects that Pb exposure, at different levels during different developmental periods, has on expression of DNMTs and methyl cytosine-binding proteins MeCP2 and MBD1. DNA methylation in mammals is catalyzed by DNMT1, DNMT3a and DNMT3b. DNMT1 and DNMT3a were chosen for study as they are both expressed in neurons from embryogenesis through adulthood (Goto et al., 1994) and represent two functionally distinct DNMTs: DNMT1 is a maintenance methyltransferase and DNMT3a is involved in *de novo* methylation (rather than the maintenance of existing methylated sites).

The timing of developmental Pb exposure influences a variety of outcomes including gene expression patterns in the hippocampus (Schneider et al., 2012a). Thus, exposures that occur during different developmental periods may have different functional implications. For example, effects on early development and organization of the brain that might occur with gestational exposures may have different outcomes than exposures that occur later in development that might more directly affect functional mechanisms particularly related to synaptic transmission and plasticity. The ways in which exposures that occur during different developmental periods affect the molecular architecture of the brain are not well understood. Considering that different DNMTs and methyl cytosine-binding proteins may have different roles during early and later developmental periods (Feng et al., 2005; Feng et al., 2007; Goto et al., 1994), the current study examined effects from Pb exposures during two developmental time periods previously shown by us to differentially influence gene expression patterns in the hippocampus.

2. Material and Methods

2.1 Animals

The use of animals was in compliance with NIH Guidelines for the Care and Use of Laboratory Animals and the study was approved by the institutional animal care and use committee at Thomas Jefferson University. Long Evans dams (Harlan Laboratories) were food (RMH 1000 chow) with or without added Pb acetate: 0 ppm, 150 ppm, 375 ppm or 750 ppm for ten days prior to breeding and remained on the same diet through weaning. Litters were culled to equal numbers of pups to standardize litter size, with an aim of having eight pups per litter. These animals were exposed to Pb from gestation through lactation (i.e., to postnatal day 21) and comprised the perinatal (Peri) exposure group. Other animals were exposed to the same levels of Pb added to food but exposure started on postnatal day 1 and continued to postnatal day 21 (early postnatal exposure group (EPN)). In all instances, equal numbers of males and females were maintained wherever possible. One male and 1 female was taken from each litter and combined with animals from other litters to form experimental cohorts. Rats were all housed 4 to a standard cage (47.6 × 25.9 cm) and were exposed to a 12h:12h light:dark cycle for the duration of the study. All animals were euthanized on day 55 and hippocampi were removed fresh, frozen and stored until processed. Blood was collected at the time of euthanasia and analyzed for Pb levels by graphite furnace atomic absorption with Zeeman background correction (ESA Labs, MA). Blood Pb levels were also obtained from a sample of pups at day 21 (weaning).

2.2 Protein Expression Studies

One hippocampus from each animal was weighed and protein was extracted using the NE-PER™ (Pierce, Inc., Rockford, IL) cytoplasmic and nuclear protein extraction kit according to manufacturer's instructions. Samples were homogenized in lysis buffer containing HALT™ protease inhibitor (Pierce, Inc.) and incubated on ice for 10 min. Cytoplasmic protein was isolated by centrifugation and the resulting pellet was extracted for nuclear proteins with a second lysis buffer for 40 min on ice. Samples were centrifuged and the resulting supernatant containing nuclear protein was quantified using the BCA reaction (Pierce Inc.) and stored at –80°C for use in Western blot analysis.

Samples (5µg for MeCP2, 10µg for all other proteins) were mixed with loading buffer and reducing agent (Invitrogen Inc. Carlsbad, CA) and heated to 70 °C for 10 min prior to being loaded onto 4–12% Bis-tris gels (Invitrogen, Inc.). Gels were run at 200V for either 1hr (MBD-1 and MeCP2) or 1:50 (DNMT1 and DNMT3a) in a MOPS SDS running buffer (Invitrogen, Inc.). Proteins were then transferred to a 0.2 µm nitrocellulose membrane using a semi-dry transfer apparatus at 15V for 15 min per membrane. Membranes were then washed in TBS containing 1% tween-20 (TBS-T), blocked in 5% milk with Tween-20 (5%NFM-T) and then incubated in primary antibody as follows: MeCP2 (ABE171 Millipore), 1:1000 for 1hr at room temperature; MBD-1 (ab3753 Abcam), 1:1000 in 5%NFM-T overnight; DNMT1 (1–248, BioAcademia), 1:1000 in 5%NFM-T overnight; DNMT3a (ab14291 Abcam), 1:1000 in 5%BSA 1hr at room temperature; and β-actin (Imgenex), 1:2000 in 5%NFM-T overnight. Following incubation with primary antibody, blots were washed in T-TBS and exposed to horseradish peroxidase conjugated secondary antibody (anti-rabbit 1:20,000, anti-chicken 1:50,000; Pierce Inc.) for 1hr hour at room temperature. Finally, membranes were washed in T-TBS to remove excess antibody and exposed to either Pico or Dura chemiluminescent substrate (Pierce Inc.) and developed. Densitometry was used to determine expression levels of target proteins and normalized relative to β-actin for each sample (MCID Basic V7.2). Data were then expressed as Normalized Relative Optical Density (ROD) and analyzed by analysis of variance followed by post hoc comparisons using Newman-Keuls Multiple Comparison Test.

3. Results

3.1 Blood Lead Levels

Mean blood Pb levels for control animals was $<1.0 \mu\text{g/dl}$ at both weaning and at the end of the study. Mean blood Pb levels for all Pb-exposed groups were significantly increased compared to controls. At weaning, blood Pb levels were higher than at the end of the study for all groups, with day 55 blood Pb levels close to control levels. Blood Pb levels within sex and developmental exposure period are shown in Table 1.

3.2 Dnmt1 Expression

In males, there were no significant differences in Dnmt1 expression in animals with perinatal Pb exposure at any level compared to control animals (Figure 1A). In contrast, there was a significant effect of Pb on DNMT1 expression in males with early postnatal Pb exposure ($F_{(3,32)} = 11.37$, $p < 0.0001$). There were significant decreases in DNMT1 expression with exposure to 150 ppm ($-23.2\% \pm 2.6$, $p < 0.01$) and 375 ppm Pb ($-18.0\% \pm 7.0$, $p < 0.05$) and increased expression of DNMT1 in animals with 750 ppm Pb exposure ($+20.0\% \pm 4.8$, $p < 0.01$), compared to control animals with no Pb exposure (Figure 1B). In females, there was a significant effect of Pb on DNMT1 expression in animals with perinatal Pb exposure ($F_{(3,37)} = 4.20$, $p = 0.012$), with significant decreases in expression seen at all levels of exposure ($-26.0\% \pm 10.1$, $-24.2\% \pm 9.5$, and $-25.3\% \pm 8.6$ for 150, 375, and 750 ppm exposure groups, respectively; $p < 0.05$ for each vs. control) (Figure 1C). In females, there were no significant differences in DNMT1 expression in animals with early postnatal Pb exposure at any level compared to control animals (Figure 1D). Representative Western blot images are shown in supplemental figure 1.

3.2 Dnmt3a Expression

In perinatal Pb-exposed males, there was an effect of Pb exposure on DNMT3a expression ($F_{(3,36)} = 4.36$, $p = 0.01$) with increased expression of DNMT3a in animals with the lowest and highest levels of Pb exposure ($+14.2\% \pm 2.9$ and $+14.7\% \pm 5.1$, respectively; $p < 0.05$ each compared to control) (Figure 2A). There was no significant effect of Pb exposure on DNMT3a expression in males with early postnatal Pb exposure (Figure 2B). In females, there was no significant effect of Pb exposure on DNMT3a expression in animals with perinatal Pb exposure (Figure 2C) but there was a small but statistically significant effect of Pb exposure in females with early postnatal exposure ($F_{(3,35)} = 4.59$, $p = 0.008$) (Figure 2D). There were significant decreases in DNMT3a expression with exposure to 150 ppm ($-11.2\% \pm 2.8$, $p < 0.05$) and 375 ppm Pb ($-11.8\% \pm 2.7$, $p < 0.05$) compared to control animals with no Pb exposure but not in animals with 750 ppm Pb exposure ($p > 0.05$). Representative Western blot images are shown in supplemental figure 1.

3.3 MeCP2 Expression

In males, MeCP2 expression was not significantly affected by Pb exposure in either the perinatal or early postnatal exposure groups (Figure 3A and 3B). In females, perinatal Pb exposure had a significant effect on MeCP2 expression ($F_{(3,33)} = 14.44$, $p < 0.0001$) with significant decreases observed at all levels of exposure ($-18.4\% \pm 2.8$, $p < 0.01$, $-20.7\% \pm 2.9$, $p < 0.001$, $-24.1\% \pm 2.8$, $p < 0.0001$, for 150, 375, and 750 ppm exposure groups, respectively) (Figure 3C). MeCP2 expression was significantly affected in females with early postnatal Pb exposure only at the highest exposure level ($-15.7\% \pm 1.2$, $p < 0.05$ vs control) (Figure 3D). Representative Western blot images are shown in supplemental figure 1.

3.4 MBD1 Expression

MBD1 expression was not significantly affected by Pb exposure in either males or females in either exposure period at any exposure level (Figure 4A, B, C, D). Representative Western blot images are shown in supplemental figure 1.

4. Discussion

The results of this study suggest that developmental Pb exposure can affect DNMT1, DNMT3a, and MeCP2 expression in the hippocampus. These effects of Pb may in turn influence the dynamic modulation of DNA methylation that occurs during development of the brain as well as during a variety of neuronal processes including learning and memory (Feng et al., 2007). Interestingly, the effects of Pb on DNA methyltransferases and DNA binding proteins appear to be different in males and females and are further differentiated by the period during development when the exposure to Pb occurred. Although the reasons for this sex-related difference in response to Pb are not entirely clear, sex differences in the response to Pb have been reported in other contexts (Cory-Slechta et al., 2004). Sex-related differences in gene expression patterns have been observed in hippocampus (Schneider et al., 2011) and frontal cortex (Schneider et al., 2012b) and in the expression of learning and memory deficits in Pb-exposed animals (Anderson et al., 2012), and these effects are expressed differently depending on the developmental period of Pb exposure (Anderson et al., 2012; Schneider et al., 2012a). Little is known in general regarding sex differences in the regulation of the genome and epigenome. Sex does influence genomic methylation status although the mechanisms underlying this effect are not completely known (Liu et al., 2010). It is possible that an interaction between genes and sex hormones during development, modified by Pb exposure, may make specific brain regions such as the hippocampus differentially susceptible to methylation-associated modulation of transcriptional regulation. Pb-induced effects on epigenetic mechanisms may contribute to a differential susceptibility between males and females that is characteristic of a spectrum of neurological and psychiatric disorders (Qureshi and Mehler, 2010) including perhaps some of the cognitive and behavioral sequelae of developmental Pb exposure (Bouchard et al., 2009).

We also observed for some proteins a non-traditional dose-response effect. Non-linear dose-response effects are a common observation in toxicology and particularly in the area of Pb neurotoxicity. For example, synaptic release of glutamate, and calcium dependent release of GABA follow a “U -shaped” dose-response curve in hippocampus of Pb-exposed rats ((Lasley and Gilbert, 2002; White et al., 2007)). Also, the effects of low levels of Pb on IQ scores in Pb-exposed children are greater than the impact at higher levels of exposure (Lanphear et al., 2005). Different mechanisms may be affected by low and high levels of Pb exposure than are affected at intermediate and levels of exposure. At low levels of exposures, Pb (in the nanomolar and picomolar ranges) may interfere with physiological processes that are acutely sensitive its presence (ex., Braga et al., 1999; Markovac and Goldstein, 1988) and may overwhelm some processes such that no additional effects are seen with higher exposures. At higher (intermediate) levels of exposure, homeostatic adjustments may occur leading to an apparent loss of toxic effect that may more likely reflect compensatory responses becoming active. At still higher exposure levels, compensatory responses may fail and additional toxic responses may be observed (Davis and Svendsgaard, 1990).

Both DNMT1 and DNMT3a play roles in the development, maturation, and function of the nervous system. During development, deletion of *Dnmt1* causes hypomethylation in postmitotic neurons resulting in defects in neuronal maturation and synaptic transmission (Golshani et al., 2005; Fan et al., 2001). DNMT3a is also important for neural development and function and there is evidence for overlapping roles for DNMT1 and DNMT3a in the

mature brain. DNMT1 and DNMT3a deficiency in forebrain neurons causes defects in synaptic plasticity as well as learning and memory and DNMT1 may cooperate with DNMT3a to maintain DNA methylation (Feng et al., 2010). Decreased DNMT1 in hippocampus, as found in perinatal Pb-exposed females and early postnatal Pb-exposed males, may lead to deficient synaptic plasticity in these animals as may altered expression of DNMT3a in perinatal-exposed males and early postnatal-exposed females. The current data suggest that just as under normal physiologic conditions, the regulation of DNMTs under pathologic conditions is complex and may play different roles in neural development and function in males and females exposed to Pb and may have different influences on different genes resulting in different functional outcomes.

MeCP2 is a DNA binding protein that preferentially binds to methylated CpG dinucleotides and is involved in transcriptional regulation of a multitude of genes (Yasui et al., 2007). Alterations in MeCP2 expression and/or methylation have been associated with Rett syndrome (Amir et al., 1999) as well as a variety of cognitive and behavioral disorders including autism, mental retardation, ADHD, and mild learning disabilities (Nagarajan et al., 2006). MeCP2 also appears to play a role in synaptic plasticity, with the degree of MeCP2 expression in cortex and hippocampus appearing to correlate with synaptogenesis in both regions (Johnston et al., 2001; Mullaney et al., 2004). The phenotypic expression of altered MeCP2 (as well as perhaps altered DNMT1 and DNMT3a) may be dose dependent, with different alterations in methylation and protein expression leading to different severity cognitive dysfunction. Animals with impaired but residual MeCP2 protein function show milder abnormalities including heightened levels of anxiety, aberrations in social interactions, impaired nest building and diurnal activities as well as impaired cerebellar learning and hippocampal/amygdala-based cognition with degree of behavioral abnormality positively correlated with degree of protein dysfunction or absence (Pelka et al., 2006). MeCP2 expression was decreased in females with perinatal Pb exposure similarly at low and high exposures, suggesting that in females, MeCP2 expression is particularly at-risk during the perinatal period.

The functional significance of the present findings are not entirely clear at this time. Although the results indicate that developmental Pb exposure can influence the expression of proteins in the hippocampus “at rest” that are involved in the regulation of DNA methylation, recent studies have shown that DNA methylation changes in the adult brain are highly dynamic, driven by experience, and regulated by DNMTs (Miller and Sweatt, 2007). Dynamic regulation of methylation in the hippocampus appears to play an integral role in memory formation (Miller and Sweatt, 2007). The effects of Pb on this system “at rest” may alter the potential for the system to be properly dynamically regulated, leading to deficits in learning and memory.

A potential limitation of this work is that whole hippocampus homogenates were used for analysis and it is possible that in doing so, there may be some dilution of signal due to different epigenetic responses and protein expression changes in different hippocampal subregions. For example, the CA1 subregion has been shown to be particularly sensitive to the effects of developmental Pb exposure (ex., Guilarte and McGlothan, 1998; Hsiang and Diaz, 2011; Zaiser and Miletic, 1997) may also be particularly sensitive to changes in regulation of histone acetylation (Levenson et al., 2004) and methylation (Gupta et al., 2010). Additional work is needed to examine any potential subregional sensitivity in the hippocampus in epigenetic response to Pb exposure and to better appreciate how epigenetic modifications consequent to developmental Pb exposure functionally impact learning, memory, and plasticity and outcomes later in life.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Dnmt1 and Dnmt3a in hippocampus are affected by developmental lead exposure
- MeCP2 is affected by lead exposure only in females
- Lead-induced changes in Dnmts and MeCP2 are modified by sex and timing of exposure
- Developmental Pb exposure may alter the dynamic modulation of DNA methylation

Figure 1.

Effects of developmental Pb exposure on hippocampal DNMT1 expression. A) Perinatal Pb exposure in males had no significant effects on DNMT1 expression but males with early postnatal Pb exposure showed decreased DNMT1 expression with 150 (N = 7) and 375 (N = 7) ppm Pb exposures and increased DNMT1 expression with 750 ppm (N = 7) Pb exposure (Control N = 17) (B). In contrast, perinatal Pb exposure in females decreased DNMT1 expression at all exposure levels (C: Control N = 17; N = 8 for each Pb exposure group) but had no significant effects on DNMT1 expression with early postnatal Pb exposure (D: Control N = 20; N = 6 for each Pb exposure group). *p<0.05, **p<0.01 vs. control. ROD = relative optical density.

Figure 2.

Effects of developmental Pb exposure on hippocampal DNMT3a expression. A) Perinatal Pb exposure in males increased DNMT3a expression at the lowest and highest exposures (150 (N = 6) and 750 (N = 7) ppm, respectively) but not at the intermediate exposure (375 ppm, N = 8) and had no effect in males with early postnatal Pb exposure (B: Control: N = 20; 150 and 375 ppm, N = 7; 750 ppm, N = 8). In contrast, perinatal Pb exposure in females had no effect on DNMT3a expression (C: Control N = 20; N = 8 for each Pb exposure group) but females with early postnatal Pb exposure had decreased DNMT3a expression with 150 (N = 7) and 375 (N = 7) ppm exposures (D: Control N = 14; 750 ppm N = 7)).

*p < 0.05 vs. control. ROD = relative optical density.

Figure 3.

Effects of developmental Pb exposure on hippocampal MeCP2 expression. There was no effect from Pb exposure in males with perinatal (A: Control: N = 18; 150 and 375 ppm, N = 7; 750 ppm, N = 8) or early postnatal (B: Control: N = 15; 150 ppm, N = 7; 375 and 750 ppm, N = 8) Pb exposures. Decreased MeCP2 expression was observed in females with perinatal Pb exposures (C: Control N = 16; N = 7 for each Pb exposure group) but not in females with early postnatal Pb exposures (D: Control: N = 16; 150 ppm, N = 7; 375 ppm, N = 8; 750 ppm, N = 7). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. control. ROD = relative optical density.

Figure 4.

Developmental Pb exposure had no significant effect on expression of MBD1 in the hippocampus. ROD = relative optical density. Male perinatal exposure: Control N = 19; N = 8 for each Pb exposure group; Male early postnatal exposure: Control: N = 19; 150 ppm, N = 7; 375 ppm, N = 8; 750 ppm, N = 8; Female perinatal exposure: Control N = 19; N = 8 for each Pb exposure group; Female early postnatal exposure: Control: N = 19; 150 and 375 ppm, N = 7; 750 ppm, N = 8.

Table 1

Blood lead levels of all treatment groups.

	Lead Exposure*	Male		Female	
		Blood Lead Level (µg/dl) at Weaning	Blood Lead Level (µg/dl) at Day 55	Blood Lead Level (µg/dl) at Weaning	Blood Lead Level (µg/dl) at Day 55
Perinatal	Control	<1.00 ± 0.00	<1.00 ± 0.00	<1.00 ± 0.00	<1.00 ± 0.00
	150ppm	15.38 ± 3.79	1.25 ± 0.25	17.23 ± 3.49	1.33 ± 0.14
	375ppm	20.50 ± 4.46	2.42 ± 0.38	25.67 ± 3.53 ^h	2.12 ± 0.13 ⁱ
	750ppm	28.97 ± 1.88 ^a	2.47 ± 0.17 ^e	27.35 ± 3.31 ⁱ	2.08 ± 0.15 ^m
Early Postnatal	150ppm	8.73 ± 0.14 ^b	1.56 ± 0.22	9.67 ± 1.87	1.91 ± 0.34 ⁿ
	375ppm	12.53 ± 0.91 ^c	1.90 ± 0.23 ^f	12.45 ± 1.35 ^j	2.00 ± 0.21 ^o
	750ppm	15.75 ± 0.90 ^d	2.83 ± 0.21 ^g	15.65 ± 1.12 ^k	3.09 ± 0.21

Significant differences within a sex and developmental exposure period between lead exposure levels are indicated (a = p<0.01 vs. 150ppm, b = p<0.001 vs. 375ppm, c = p<0.01 vs. 750ppm, d = p<0.001 vs. 150ppm, e = p<0.01 vs. 150ppm, f = p<0.001 vs. 150ppm, g = p<0.001 vs. 150ppm, h = p<0.05 vs. 150ppm, i = p<0.01 vs. 150ppm, j = p<0.05 vs. 750ppm, k = p<0.001 vs. 150ppm, l = p<0.001 vs. 150ppm, m = p<0.001 vs. 150ppm, n = p<0.01 vs. 750ppm, o = p<0.01 vs. 750ppm). All values are expressed as mean ± s.e.m.

* N = 6 per all exposure groups.