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Toxicity biomarkers among US children compared to a similar cohort in France: a blinded study measuring urinary porphyrins

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The purpose of this blinded study was to evaluate potential environmental toxicity in a cohort of neurotypical children ($n=28$) living in a suburban area of north-central Texas in the United States (US) with a comparable age- and gender-matched cohort of neurotypical children ($n=28$) living in a suburban area of southeastern France using urinary porphyrin testing: uroporphyrin (uP), heptacarboxyporphyrin (7cxP), hexacarboxyporphyrin (6cxP), pentacarboxyporphyrin (5cxP), precoproporphyrin (prcP), and coproporphyrin (cP). Results showed significantly elevated 6cxP, prcP (an atypical, mercury-specific porphyrin), and cP levels, and increasing trends in 5cxP levels, among neurotypical children in the USA compared to children in France. Data suggest that in US neurotypical children, there is a significantly increased body-burden of mercury (Hg) compared to the body-burden of Hg in the matched neurotypical children in France. The presence of lead contributing to the higher levels of cP also needs to be considered. Further, other factors including genetics can not be completely ruled out.

Keywords: mercury; heavy metal; porphyrins; biomarkers; xenobiotic; lead; toxicity

Introduction

For many years, measuring heavy metal toxicity in children involved a direct measure of the metals in the blood, urine, hair, or fecal matter. A more recent approach is to use urinary porphyrins as a measure of toxic metal body-burden. Previous studies showed that urinary porphyrins (heme precursors formed in the heme synthesis pathway) afford a measure of xenobiotic exposure, particularly mercury (Hg) (Woods 1996; Pingree et al. 2001a; Pingree, Simmonds, and Woods 2001b). Specific patterns of porphyrins suggest the presence of Hg exposure. Mercury toxicity was demonstrated to be associated with

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elevations in urinary coproporphyrin (cP), pentacoproporphyrin (5cxP), and by the expression of an atypical porphyrin – precoproporphyrin (prcP; also known as ketoisocoproporphyrin) not found in urine in unexposed controls (Woods 1996; Woods et al. 2005; Heyer et al. 2006). Woods (1996) noted that these distinct changes in urinary porphyrin concentrations were observed as early as 1–2 weeks after initiation of Hg exposure, and that porphyrin levels rose in a dose- and time-related fashion with the concentration of Hg in the kidney, a principal target organ of Hg compounds. In addition, urinary porphyrin profiles were also found to correlate significantly with Hg body-burden and with specific neurobehavioral deficits associated with low-level metal exposure. Woods (1996) concluded that urinary porphyrin profiles are a useful biomarker for Hg exposure and its potential adverse health effects on human subjects. Several studies used urinary porphyrins to examine Hg body-burden in children (Geier and Geier 2006, 2007; Nataf et al. 2006; Kern et al. 2007; Austin and Shandley 2008; Youn et al. 2010).

The purpose of the present, blinded study was to examine potential xenobiotic toxicity, using urinary porphyrin testing, in a cohort of neurotypical children living in a suburban area of north-central Texas, USA and a matched cohort of age- and gender-matched neurotypical children living in a suburban area of southeastern France. France was chosen as a site to compare to the USA because it has a profile of environmental exposures that differs from the USA profile.

Methods

The study was conducted on USA and French neurotypical children ($n = 56$). The study protocol received Institutional Review Board (IRB) approval from Liberty IRB, Inc. (Deland, Florida). The Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale (CCPPRB) that gives approval for the use of biomedical materials in research in France was informed of the study and there were no objections. The consent from the US IRB was translated into French for the French parents. All parents signed a consent and Health Insurance Portability and Accountability Act (HIPAA) form and all received a copy. Each child was assigned a study number. After an evaluation on each participant in the study, a first-morning-urine sample was collected and submitted to Laboratoire Philippe Auguste for a standard urinary porphyrin profile assessment. Specimens were submitted using the study number; no names were used. The urine porphyrin results reported by this lab were tabulated and statistical analysis was used to quantitatively assess the differences between the two groups of children.

Participants

This study examined consecutive qualifying neurotypical participants living in a suburban area of north-central Texas, USA ($n = 28$) and a comparable group of age- and gender-matched neurotypical participants living in a suburban area of southeastern France ($n = 28$). Age matching was defined as less than 12 months apart in age. These participants were prospectively recruited from their communities. Children included in this study were between 2 and 13 years of age. All of these children received routine childhood vaccinations and none of the children received chelation therapy. Each qualifying child had a Childhood Autism Rating Scale (CARS; Schopler, Reichler, and Renner 1994) score ≤ 18 (the lowest possible CARS score is 15), and no history of neurological, learning, medical, or developmental problems.

Table 1. Characteristics of children examined.

Parameter	US neurotypical children ^a (<i>n</i> = 28)	French neurotypical children ^a (<i>n</i> = 28)
Male/female (ratio)	25/3 (8.3 : 1)	25/3 (8.3 : 1)
Mean age in years \pm SD (range)	6.3 \pm 2.8 (2–13)	6.2 \pm 3.1 (2–13)
Mean fish consumption per week \pm SD	0.6 (0.7)	1.4 (0.8) ^b

Notes: SD, standard deviation; US children and French children were matched for age and gender. No study subjects had previously received chelation therapy. All children received childhood vaccines.

^aSubjects had CARS scores \leq 18; ^bsignificant ($p < 0.01$) increase in mean fish consumption per week was observed among French neurotypical children in comparison to US neurotypical children.

Clinical evaluation

As a baseline, investigators obtained information regarding demographics, any formal diagnoses, medical issues, medications, fish consumption, whether the child had been vaccinated or received chelation treatment, or had any neurological, learning, or developmental problems. A baseline CARS evaluation was performed on all of the children by interviewing the parents and observing the child. Table 1 summarizes the pertinent demographics of the participants included in this study.

Childhood Autism Rating Scale

CARS is a recognized 15-item behavioral rating scale developed to identify autism as well as to quantitatively describe the severity of the disorder (Schopler, Reichler, and Renner 1994). CARS was used in this study to help ensure that only neurotypical children were examined because evidence suggests that children with autism or autism spectrum disorder may have more difficulty excreting heavy metals, and so it was considered that they may have elevated toxicity levels due to intrinsic reasons (e.g., genetic, metabolic) rather than exposure (Holmes, Blaxill, and Haley 2003; Kern et al. 2007). CARS addresses the neurodevelopmental characteristics of autistic symptoms, such as problems with socialization, emotional response, abnormal body movements, abnormal use of objects, insistence on sameness, visual and auditory responses, sensory issues, anxiety and nervousness, language problems, activity level, and any learning or intellectual problems that child might have. The acceptable CARS score in this study was set very low (at \leq 18) in order to eliminate children with any significant issues in any of the aforementioned areas. As mentioned, the lowest possible score on a CARS evaluation is 15.

For CARS evaluation, a total score of 15–29.5 is considered non-autistic; a score of 30–36.5 is considered mild-to-moderate autism; a score from 37 to 60 is considered moderate-to-severe autism. The CARS is a well-established measure. The internal consistency reliability alpha coefficient is 0.94; the inter-rater reliability correlation coefficient is 0.71; and the test–retest correlation coefficient is 0.88. CARS scores have high criterion-related validity when compared to clinical ratings during the same diagnostic sessions, with a significant correlation of 0.84 (Schopler, Reichler, and Renner 1994).

Lab evaluation

Following the intake evaluation, parents were given a urinary porphyrin collection kit and instructions. The lab specimens were all collected as first morning urine samples following an overnight fast. Specimens were shipped to Laboratoire Philippe Auguste (Paris, France). The lab used in this study was blinded and received no information regarding the clinical status of the participants examined. Urine specimens underwent routine urinary porphyrin profile testing at Laboratoire Philippe Auguste, Paris, France (ISO-approved). The profile includes the following: uroporphyrin (uP), heptacarboxyporphyrin (7cxP), hexacarboxyporphyrin (6cxP), 5cxP, prcP, and cP.

Lab methods

Study participants' first morning urine samples (10 mL) were collected in vacuette containing a preservative (Greiner Bio-One, Les Ulis, France), maintained in the dark at ambient temperature during shipping to France (approximately 5–10 days), and then frozen (-20°C) until analysis (preserved samples collected in the Greiner Bio-One tubes used for porphyrin testing and held at room temperature were found to provide accurate/reproducible porphyrin result values for more than 20 days, as reported by Laboratoire Philippe Auguste). Porphyrins were determined by high performance liquid chromatography (HPLC) Spectrofluorimetric technique: after centrifugation ($3000 \times g$, 5 min), 500 μL urine were acidified with 50 μL of aqueous hydrochloric acid (37% w/v), re-centrifuged, and 20 μL of the resulting sample injected into Econosphere column (18, 5 μm particle size, 250 \times 46 mm, Alltech, Templemars, France). Elution from the column was effected using a gradient (Phase A: 50 mmol KH_2PO_4 , pH adjusted to 3.5 with CH_3COOH ; Phase B: CH_3OH). Eluant flow was 1 mL/min and the following A : B gradient: 0 min A/B 50 : 50, 2 min 35 : 65, 5 min 15 : 85, 15 min 1 : 99, and 28 min 50 : 50 was applied. Fluorescence detection and measurement (excitation 405 nm, emission 618 nm) was used for the porphyrins in system that had dual on-line capability (UV model 310, Fluorescence model 363 both from Varian, Les Ulis, France). The nominal porphyrin retention times (in min) were: 7.3, 8.6, 10.2, 11.7, 12.7, and 13.9 for uP, 7cxP, 6cxP, 5cxP, prcP, and cP, respectively. The HPLC separation method used by this lab does not separate the I and III isomers of uP and cP. The fluorescence responses from the samples were quantified against the corresponding responses from a mixed porphyrin reference sample (Porphyrin Products, Logan, Utah). The urinary creatinine level in each sample was measured by a spectrophotometric assay (Vitros, Ortho-Clinical Diagnostics, Johnson & Johnson). The porphyrin values reported were expressed in nmole/g of creatinine measured (Nataf et al. 2006).

Statistical analyses

The statistical package contained in StatsDirect (version 2.4.7) was employed in this study. In order to evaluate urinary porphyrin levels measured among children from the USA in comparison to matched children from France, the Wilcoxon matched-pairs signed-ranks test statistic was utilized. The null hypothesis was that there would be no difference in the data distributions for urinary porphyrin levels measured among US children in comparison to matched children from France. In addition, a statistical analysis was conducted using the non-parametric Wilcoxon's signed ranks comparing the fish consumption between USA and matched French children. For all statistical tests

Table 2. Comparison of the urinary porphyrin results reported for the groups of subjects tested.

Metabolic parameter	Mean \pm SD [range]		P-value ^b
	US neurotypical children ^a (n = 28)	French neurotypical children ^a (n = 28)	
nmol/gram creatinine			
Uroporphyrins	22.3 \pm 7.5 [9–39]	18.4 \pm 8.5 [8–46]	ns
Heptacarboxyporphyrins	4.2 \pm 1.4 [1.7–7.1]	3.9 \pm 1.6 [1.9–7.7]	ns
Hexacarboxyporphyrins	0.90 \pm 0.52 [0.1–2.2]	0.62 \pm 0.29 [0.0–1.4]	< 0.05
Pentacarboxyporphyrins	4.8 \pm 3.7 [1.8–22.2]	3.9 \pm 1.5 [1.9–7]	ns
Precoproporphyrins	18 \pm 10.0 [6–45]	13.5 \pm 7.9 [3–31]	< 0.05
Total coproporphyrins (I + III)	208 \pm 114 [74–544]	151 \pm 68 [70–340]	< 0.005
grams of creatinine/L	960 \pm 453 [308–2425]	953 \pm 504 [175–2760]	ns

Notes: SD, standard deviation; ns, not significant.

^aSubjects that were neurotypical (CARS score \leq 18). US and French children were matched for age and gender; ^bthe two-tailed non-parametric Wilcoxon matched-pairs signed-ranks test statistic was utilized.

employed in this study, a two-tailed p -value \leq 0.05 was considered to be statistically significant.

Results

Table 2 summarizes the urinary porphyrin levels observed among the US children in compared to matched cohort from France. US children were found to have significantly increased urinary porphyrins associated with elevated Hg body-burden (6cxP, precP, and cP) compared to matched children from France. Overall, on average, there was a 1.45-fold rise in 6cxP, a 1.3-fold elevation in precP, and a 1.4-fold increase in cP levels among US children. In addition, 5cxP, another urinary porphyrin levels associated with elevated Hg body-burden were noted to trend toward increased levels among the US children by a 1.2-fold amount. Among the US children compared to matched children from France, there were no significant differences found in the non-Hg associated urinary porphyrins of uP and 7cxP. Finally, similar urinary creatinine concentrations were observed among the USA and matched French children.

Discussion

This study was designed to compare the body-burden of Hg and related environmental toxins in a cohort of USA to a matched cohort of French neurotypical children. The results of this study indicated increased urinary porphyrin metabolite levels associated

with elevated Hg body-burden in a cohort of participants from the USA compared to the corresponding levels in the matched participants from France.

Woods (1996) noted that urinary Hg-associated porphyrin concentrations increased in a dose- and time-related fashion with the concentration of Hg in the kidney, a principal target organ of Hg compounds. Elevated Hg in the kidney usually also suggests elevated metal in the brain, as studies show that the brain and kidneys are target organs for Hg following metal exposure. Pingree, Simmonds, and Woods (2001b) administered to rats methylmercury hydroxide (MMH; 10 ppm) in drinking water for 9 weeks and determined both inorganic (Hg^{2+}) and organic (CH_3Hg^+) Hg species levels in urine and tissues by cold-vapor atomic fluorescence spectroscopy (CVAFS). After treatment, Hg^{2+} and CH_3Hg^+ concentrations were 0.28 and 4.80 $\mu\text{g/g}$ in brain and 51.5 and 42.2 $\mu\text{g/g}$ in kidney.

In a review of the consequences of toxicity on the developing brain, Kalia (2008) argues, as others do, that the developing brain is more vulnerable than the adult brain (Blomgren and Hagberg 2006; Pullela et al. 2006). The developing brain is extraordinarily complex, develops rapidly, and periods of development cannot be fully retrieved once interrupted, lost, or delayed (Chen et al. 2006; Kalia 2008). This disruption in the normal developmental process produces a cascade of neurological events (or a domino effect) that results in both chronic, severe consequences. For example, disruption of critical neuron populations in one area may further compromise other areas of brain development (Barrett et al. 2007). Kalia (2008) also indicated that exposure to neurotoxins, such as toxic metals (e.g., lead (Pb), Hg, arsenic (As)), polychlorinated biphenyls, toluene, and other xenobiotics damaged the developing brain and produced neurodevelopmental disorders (Lidsky and Schneider 2003; Kisby et al. 2009).

Mercury is known to produce brain damage. However, what may be less known is that mechanisms for neuronal degeneration are comprehensive and wide-ranging. There does not appear to be single target effect, but a plethora of consequences and cascades of events. The following lists some of the neurological consequences following Hg exposure in the brain: (1) inhibition of the binding of GTP to tubulin, thereby preventing tubulin polymerization into microtubules (Leong, Syed, and Lorscheider 2001); (2) growth cone collapse (Leong, Syed, and Lorscheider 2001); (3) disruption of membrane structure and linear growth rates (Leong, Syed, and Lorscheider 2001); (4) disintegrated tubulin/microtubule structure (Leong, Syed, and Lorscheider 2001); (5) neuronal somata sprouting failer (Leong, Syed, and Lorscheider 2001); (6) increase in lipid peroxidation (LPO) or oxidative stress (Singh et al. 2007; Huang et al. 2008); (7) excitotoxicity, elevated cytosolic free calcium, and cell death (Olanow and Arendash 1994); (8) diminished insulin-like growth factor 1 (IGF-1), factor growth signaling, and methionine synthase activity in the brain (Waly et al. 2004); (9) disturbed postnatal development of the glutathione (GSH) antioxidant system in the brain (Stringari et al. 2008); (10) reduced GSH levels in brain and a decrease in the enzymatic activities of acetyl cholinesterase in different regions of the brain (Singh et al. 2007); and (11) immune-glutamatergic dysfunction (Blaylock 2008; Blaylock and Struneka 2009).

An assessment of sources of mercury exposure

Mercury is an environmental contaminant that has always been in the environment to a certain extent; however, modern industry has brought about an increase in prevalence in the environment (food, water, air, and medicine; Soares et al. 2003; Latshaw et al. 2006; Geier, Sykes, and Geier 2007). Mercury has been used in agriculture, as a preservative

in vaccines, and medicinally, and released into the environment from coal burning and the production of chlorine. According to the United States Department of Interior/US Geological Survey (2002), the USA has a growing Hg problem.

Laks (2009) recently reported on time trends on blood inorganic mercury (I-Hg) levels in 6174 women, ages 18–49, in the National Health and Nutrition Examination Survey (NHANES), 1999–2006 data sets. Laks (2009) found that in the US population, I-Hg rose sharply from 2% in 1999–2000 to 30% in 2005–2006 (Hg levels rose from 0.33 to 0.39 µg/L). I-Hg was significantly associated with age suggesting bio-accumulation.

In the USA, coal-burning power plants produce 51% of the nation's electricity and the largest amount of (human-caused) Hg emissions into the air. For example, in 2007, the US coal-burning power plants emitted 20 tons of toxic Hg into the air (Environmental News Service 2008). The Environmental Protection Agency (EPA) has given utilities until 2018 to cut Hg emissions to 15 tons a year from the currently allowed 47 tons (EPA 2008). The EPA's Toxics Release Inventory (TRI), using data from 2003 showed that Texas, Ohio, Pennsylvania, Indiana, and Alabama were the states with the highest Hg emissions from power plants and that 5 of 10 most polluting facilities were in Texas (EPA 2003).

One main difference between the USA (Texas) and France is coal-burning for energy. France's coal reserves are limited. Energy produced from coal in France in 2003, for example, was 6% (*versus* 51% in the USA and 36.5% in Texas; Encyclopaedia of the Nations 2007; US Energy Information Administration 2009).

In considering medicinal exposure to Hg, adults, infants, and children are exposed to Hg from Thimerosal, an ethylmercury-containing (49.55% Hg by weight) compound, which was added to many vaccines. Thimerosal was utilized as a preservative in vaccines, and was usually present at concentration of 0.01% (25 µg Hg) or 0.005% (12.5 µg Hg) per dose. In comparing the relative presence of Thimerosal in vaccines administered in the USA and France, Hessel (2003) reported that in 1999 in the USA, Thimerosal was present in approximately 30 different childhood vaccines, whereas there were only 2 in France. In the USA, Bigham and Copes (2005) estimated that the cumulative dose of Hg exposure some infants received from routinely recommended Thimerosal-containing childhood vaccines (187.5 µg Hg) was equal to about 50% of the cumulative dose of all Hg exposure during the first 6 months of life. Furthermore, the USA (unlike France) has seen since 2003 the rapid expansion of the recommendation to administer annual influenza vaccines to all pregnant women, infants and children, with formulations containing Thimerosal as a preservative (Hessel 2003; Geier et al. 2008; Institute for Vaccine Safety/Johns Hopkins Bloomberg School of Public Health 2008).

Strengths and limitations

This study compared a limited number of neurotypical children in Texas and in France. The results may vary for different parts of the USA and France. This study does have the limitation that it was not possible to directly measure or exactly identify the environmental toxins examined. However, urinary porphyrin patterns were examined to identify profiles well known to be associated with certain types of environmental toxins, including Hg. As a result, it is possible that there may be certain genetic or other environmental contributing factors to the effects observed. For example, as mentioned earlier, the pattern seen (increased 5cxP, prcP, and cP) is characteristic of Hg toxicity; however, Pb is known to elevate cP (Oskarsson and Fowler 1985). Therefore, the possibility of the presence of Pb contributing to the higher levels of cP needs to be considered.

Further, this study was not specifically designed to evaluate the potential population adverse consequences of increased body-burden of Hg present in US children. Given the inherent toxicity of Hg, future studies need to further examine the potential population adverse consequences of elevated Hg body-burden among US children.

The central strength of this study stems from its design as a prospective, blinded, matched study. As a result of this design, many of the common confounders/biases present in many studies were minimized, including effects of gender, age, or analyses of specimens in the context of known clinical status of subjects examined. Further, since consecutive study participants were examined in this study, it was not possible for investigators to directly or indirectly influence the collection of study participants examined. Data suggest that it would be useful to conduct a larger study over multiple geographical areas to more fully compare children in the two countries. Future studies further need to evaluate the relative body-burden of environmental toxins between children from different countries as well as the potential adverse consequences.

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