Published in final edited form as:

Environ Res. 2018 October ; 166: 507–515. doi:10.1016/j.envres.2018.06.028.

Multiple-metal exposure, diet, and oxidative stress in Uruguayan school children

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Abstract

Oxidative stress (OS) is an important consequence of exposure to toxic metals but it is unclear to what extent low-level metal exposures contribute to OS in children. We examined the cross-sectional association between urinary concentrations of arsenic (As), cadmium (Cd), and lead (Pb) and urinary markers of OS: F₂-8 α isoprostane and 8-hydroxy-2-deoxy-guanosine (8-OHdG). We also tested effect modification by dietary intakes. Of the 211 children aged 6–8 y living in Montevideo who were eligible for the study because they had at least one OS marker measured via ELISA, 143 were included in a complete-case analysis. Urinary metals were measured with inductively coupled plasma mass spectrometry (ICP-MS: Pb, Cd) and high-performance liquid chromatography online with hydride generation ICP-MS (As-metabolites); concentrations were log₂-transformed. All urinary markers were adjusted for specific gravity of urine. Two 24-hour dietary recalls were conducted to estimate children's dietary intakes, including total fruit and vegetable consumption and vitamin C, zinc and fiber intake. Ordinary least square (OLS) and weighted quantile sum (WQS) regressions were used to estimate the association between metals and each OS marker as outcome. Metal exposure was generally low: median urinary As, Cd, Pb 9.6 μ g/L, 0.06 μ g/L and 1.9 μ g/L, respectively. Median 8-isoprostane concentration was 1.1 and 8-OHdG 39.6 ng/mL. Log₂-transformed urinary As concentrations were positively associated with 8-OHdG concentrations (10.90 [3.82, 17.97]) in covariate-adjusted OLS models which also took account of exposure to Cd and Pb. In WQS, a mixture index was also associated with 8-OHdG (8.71 [1.12, 16.3] for each 25% increase in index value), mostly driven by As exposure. There was

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Declarations of conflict of interest

All authors declare having no conflicts of interest.

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little evidence of effect modification by dietary antioxidants. In sum, even at low-level, As exposure is associated with detectable oxidative damage to the DNA.

Keywords

metals; arsenic; child; oxidative stress

INTRODUCTION

There is increasing evidence that children are exposed to multiple toxic metals concurrently (Kordas et al. 2010), and that these toxicants act together to produce adverse effects on functional outcomes, such as cognition and behavior (Claus Henn et al. 2014). Individual effects of metals on molecular processes, like oxidative stress (OS), thought to underlie metal toxicity, have also received attention. To date, few studies have measured the association between the exposure to metal mixtures and OS, particularly in children, a population group that bears much of the burden of metal toxicity.

Among studies of pediatric metal exposure and OS, Thai children, either exposed to As *in utero* or belonging to a non-exposed control group, had total inorganic As concentrations in saliva and water that were positively associated with urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG¹) concentrations (Hinhumpatch et al. 2013). Among Mongolian children aged 9–15 years with high As exposure from water, proportions of inorganic As (%iAs) and monomethylarsonic acid (%MMA) in urine were positively associated with urinary 8-oxodG and negatively associated with antioxidant glutathione (GSH) concentrations in blood (Xu et al. 2008). There is one report of Cd exposure and OS in children, showing that both urinary and breast milk Cd concentrations were positively associated with urinary 8-oxodG in very young infants (Kippler et al. 2012). On the other hand, in the general population of Brazilian adults, low-level blood Cd was not associated with levels of malondialdehyde (MDA), a marker of lipid peroxidation (Almeida Lopes et al. 2017). On the other hand, a recent review showed inconsistent associations between Pb exposure and a number of OS biomarkers in children (Roy and Kordas 2015).

Studies of multiple metal exposures and OS are scarce, and have been conducted mostly in adults, with mixed findings. For example, among occupationally-exposed adults, higher exposure to metals was associated with elevated levels of some but not all biomarkers of oxidative stress (Garçon et al. 2007; Yoshioka et al. 2008). Among pregnant Bangladeshi women, urinary Cd and As, and %MMA in urine were independently associated with higher urinary concentrations of 8-oxodG (Engström et al. 2010a). In the same population, urinary and breast milk Cd but not As concentrations were associated with 8-oxodG concentrations in young, predominantly breastfed infants (Kippler et al. 2012).

In addition to the dearth of evidence on the relationship between exposure to multiple metals and OS in children, other questions remain. These include understanding the effects of single versus multiple metals and potential effect modification by diets rich in antioxidants.

¹8-OHdG and 8-oxodG are equivalent markers of OS.

We examined these questions in a cross-sectional study of 6–8 year-old children from Montevideo, Uruguay. Previously, we had shown a modest inverse association between low blood lead concentrations (BLL) and F₂-8α isoprostane in these children (Roy et al. 2015). Our objectives here were to determine: 1) whether each arsenic (As), cadmium (Cd), and lead (Pb), is associated with higher oxidative stress markers, as measured by urinary F₂-8α isoprostane and 8-OHdG; 2) whether combined exposure to metals is associated with oxidative stress; 3) and the extent to which dietary intake of nutrients thought to promote oxidative balance (vitamin C, zinc, fiber) or the consumption of fruits or vegetables modify the association between metal exposure and OS.

METHODS

Study design

The study methodology, including the setting, assessments and laboratory assays, has been described in detail (Kordas et al. 2016; Roy et al. 2015). We provide a brief overview of these methods here, with specific details regarding the sampling and measurement of urinary metal concentrations. The study was conducted in Montevideo, Uruguay, in a number of urban neighborhoods selected because of documented or suspected risk of metal exposure, particularly Pb. We recruited first-grade children (~7 y of age) and their primary caregivers through a two-stage process, first inviting schools in the neighborhoods of interest. Once school principals agreed to participate, we invited parents to attend informational meetings regarding the study's purpose and procedures. All first-grade children who regularly attended the participating schools were eligible and 357 children were enrolled into the study. There were two exclusion criteria for the present analysis. First, children ought to be excluded if they had a previous BLL >45 µg/dL, which would have necessitated medical treatment. None of the children were excluded based on this criterion. Second, due to availability of funding, children enrolled into the study in the latter years (2012–13) were ineligible to have their oxidative stress biomarkers measured. Consequently, 211 children met both eligibility criteria, and in comparing children who were included and excluded from the study, we use this pool of eligible children, rather than the overall study sample.

The study was approved by the Ethics Committee for Research Involving Human Participants at the Catholic University of Uruguay, the Ethics Committee of the Faculty of Chemistry at the University of the Republic of Uruguay, and the Office of Research Protections at the Pennsylvania State University.

After providing written consent, parents completed several questionnaires regarding their children and families. A phlebotomy nurse took a venous blood sample at a morning clinic, after an overnight fast, and children received yogurt and sweet rolls following the blood draw. Blood was collected into a tube containing no anti-coagulant, to separate out serum (tube left out to stand for 45 minutes, centrifuged for 10 min at 3000 rpm, an aliquot stored at –20°C). Serum was used for the determination of ferritin and C-reactive protein concentrations (see “Covariates” below).

Exposure measurements

The children's exposure to As, Cd and Pb was assessed based on the concentrations of these metals in spot urine samples. First morning urine was collected into polyethylene cups that had been rinsed repeatedly with 10% HNO₃ and deionized water prior to distribution to the children. Parents received instructions for urine collection at home, that included capturing mid-stream urine. When children brought the urine samples to school, study nurse placed them on ice and transported them to a laboratory for processing and storage at -80°C.

Urinary As concentrations were measured using high-performance liquid chromatography (HPLC) online with hydride generation (HG) and inductively coupled plasma mass spectrometry (HPLC-HG-ICP-MS), as described previously (Kordas et al. 2016). Inorganic As (iAs), MMA and dimethylarsinic acid (DMA) concentrations were assayed in urine and summed to represent total urinary concentrations of metabolites of inorganic As (UAs), a specific marker of exposure to inorganic As. This method does not include the much less toxic organic As compounds originating from various seafood. The intra- and inter-assay coefficients of variation were both approximately 4%.

Measurements of other elements (Cd and Pb) in urine, diluted 1:10 with 1% nitric acid (65% w/w, Scharlau, Scharlab S.L., Sentmenat, Spain), were performed on an Agilent 7700x ICP-MS (Agilent Technologies, Tokyo, Japan), equipped with the collision/reaction cell technology to minimize spectral interferences, as described elsewhere (Kippler et al. 2007; Kippler et al. 2012). The limit of detection for Pb was 0.005 ng/g in batch 1 and 0.0009 ng/g in batch 2. No batch differences were detected. There was one value below the limit of detection and the actual reported value was used in statistical analysis. The limit of detection for urinary Cd was 0.0005 ng/g for batch 1 and 0.001 ng/g for batch 2; there were no batch differences. There were no values below the LOD.

All urine concentrations of metals were adjusted for specific gravity of urine using formula: $(\text{biomarker} \times 0.024) / (\text{specific gravity} - 1)$, to account for differences in urine dilution hydration levels of the first-morning urine samples (Nermell et al. 2008). Specific gravity of each urine sample was measured on the day of the collection, using a portable specific gravity refractometer (PAL 10S, Atago Inc, USA). Metal concentrations were log-transformed for analysis to approximate normal distributions.

Oxidative stress measurements

The urine samples for OS measurements were stored at -80°C and did not undergo any freeze-thaw cycles prior to the OS analysis. The treatment of urine samples for the analysis of OS biomarkers was described previously (Roy et al. 2015). We measured two OS biomarkers (8-isoprostane and 8-OHdG) in duplicate via enzyme immunoassay kits (Cayman Chemical, Ann Arbor, MI), with an inter-assay CV of 5.4% and 7.0%, respectively. OS biomarker concentrations were adjusted for specific gravity of urine as described above. The two biomarkers were chosen because they are well understood and frequently used, show consistent association with chronic disease, and reflect different aspects of oxidative stress (in fatty acids and the DNA). Additional biomarkers were not considered due to limited resources.

Dietary intake measurement

At the time of the morning clinic, parents completed a 24-hour dietary recall, with a second recall occurring at least 2 weeks later, by phone. As previously reported, a number of visual aids and probing questions was used to elicit detailed a record of the child's diet, with the child also assisting, particularly with foods consumed at school (Kordas et al. 2016). Foods were entered into a database consisting of 342 unique items representing individual foods and preparations commonly encountered in the Uruguayan diet. Calculations of nutrient intake (vitamin C, zinc, fiber) were derived from these data for each 24-hr recall. The intakes were then averaged over the two days and expressed on a per 1000 kcal basis. Each child's consumption of any fruits (whole fruit, fruit salad, and fruit juices) was grouped to create a "fruit" variable; similarly, dark leafy vegetables, red-orange vegetables, beans/peas or other vegetables, fresh, frozen or canned were combined to represent the child's vegetable consumption. Furthermore, as described previously (Kordas et al. 2016; Kordas et al. 2017), the consumption of foods was grouped into like categories, including: meats (red and white meats), grain and wheat-based foods (flours, rice, oats, granola, cereal bars, polenta, grain-based dishes, pasta, breads, pizza, dinner pies), dairy (milk, cheese, yogurt), and fruit. A principal component analysis was applied to these food groups. Two food patterns with eigenvalues >1 were identified and only items with loadings > 0.3 were retained: 1) "processed foods"—higher consumption of breads, processed meats, fats and oils, and sweetened beverages, but also of yogurt; reduced intake of milk, pastries and pizza dinners; 2) "nutrient dense"—higher consumption of dark leaf and red-orange vegetables, higher consumption of eggs, beans & peas, potatoes; reduced consumption of pasta and sauces/condiments. Factor scores were dichotomized at the median. All dietary variables (nutrients, food groups, and the nutrient-dense dietary pattern) were split at the median to represent low vs. high dietary intake.

Covariates

At the clinic, anthropometric measurements (height, weight) were taken according to established methods. To indicate if iron deficiency (ID) was present, serum ferritin (SF) was measured in duplicate with an immunoradiometric assay (Coat-A-Count Ferritin IRMA; SIEMENS Diagnostic Products, USA), according to manufacturer instructions. Intra- and inter-assay coefficients (CV) were 4.2% & 9.5%. Serum C-Reactive Protein (CRP) was analyzed in duplicate using an ELISA technique (Erhardt et al. 2004). Serum control samples (Liquicheck, Bio-Rad) were used as standards. Intra-assay and inter-assay CV were 4.9% and 8%, respectively. Based on the age of the study children (~7 y) and WHO guidelines (WHO 2001), ID was defined as CRP-adjusted serum ferritin of less than 15 ng/mL.

Caregivers of the participating children completed a questionnaire about socio-demographic characteristics of the family, the child's medical history and the home environment. To assess socio-economic status (SES), caregivers reported on possession of 12 common household (ex., TV, washer, cellular phone, and car), from which an SES index was computed. In a factor analysis of these household assets, a single factor (eigenvalue 1.4) was initially identified. Following an oblimin rotation, 5 salient items were identified based on their factor loadings: computer (0.47), car (0.34), freezer (0.34), washing machine (0.52),

landline phone (0.49). The parental responses regarding the possession of these assets were summed to create an index score with a range of values between 0 and 5. This score was used in further analysis. Household occupant density was calculated based on the number of people living in the house divided by the number of rooms.

Statistical methods

This study aimed to address three questions: 1) whether each As, Cd, and Pb, is associated with higher oxidative stress markers, as measured by urinary F₂-8 α isoprostane and 8-OHdG; 2) whether all metals together were associated with markers of oxidative stress; 3) and the extent to which dietary intake of nutrients thought to promote oxidative balance (vitamin C, zinc, fiber) or the consumption of fruits or vegetables modified the association between metal exposure and OS.

To answer the first question, separate OLS regression models were carried out entering each metal separately (Models 1–3) as a log₂-transformed continuous independent variable. Each OS marker was tested as a separate dependent variable, first in unadjusted and then covariate-adjusted models (see below for covariate selection).

To address the second question, two analyses were conducted. First, a multi-metal regression (Model 4) was fit with all the log-transformed metal concentrations entered into the same model. Again, separate models were carried out for 8-isoprostane and 8-OHdG. Due to low sample size, only the main effects of metals (no interactions among metals) are presented. Second, weighted quantile sum (WQS) regression analysis (Model 5) was used to calculate a weighted multi-metal exposure index (MEI) and its association with oxidative stress markers. The direction of the association between MEI and the OS concentrations was constrained to be negative. WQS modeling has been employed in a number of environmental exposure studies (Brunst et al. 2017; Czarnota et al. 2015) to examine the effects of chemical mixtures on health-related outcomes. Separate models were run for F₂-8 α isoprostane and 8-OHdG, initially without and later with covariate-adjustment. To construct the MEI, log₂-transformed urinary metal concentrations were used. Study data were split randomly into a training set (40% of observations) and a validation set (60% of observations). Within the training dataset the three metals were scored as ordinal variables (quartiles) and summed across 100 bootstrapped samples. Metal weights were estimated for each dependent variable and the index was defined using average weights across the bootstrap samples. The association of the MEI with OS markers was tested in the validation set. The analyses were repeated with the metals scored as deciles. Because the findings were consistent across percentiles, the quartile analysis is presented.

Finally, for the third question, dietary variables were tested as predictors of each OS biomarker in unadjusted and covariate-adjusted models. Separate models were fit for each dietary predictor. We also explored potential confounding by dietary exposure to metals by adjusting for log₂-transformed urinary metal concentrations. Because of our small sample size (and to reduce the number of multiple comparisons), only select models were carried forward to test effect modification by dietary intakes. Specifically, the association between 8-OHdG and urinary As was statistically significant in models 1–4 described previously and was explored further in models of diet-metal interaction. This analysis was conducted by

repeating unadjusted and covariate-adjusted regression Model 1 (urinary As as predictor) within the low and high dietary strata.

The covariate-adjusted analyses included variables selected on the basis of published literature and previous experience with our data: household possessions, household density, parental smoking status (a categorical variable indicating whether either of the parents currently smokes), the child's sex, BMI, and CRP-adjusted ferritin concentrations.

The Mann-Whitney test was used to investigate differences between girls and boys on all untransformed biomarker variables. Spearman's rho was used to determine the degree of correlation among metals. Statistical analyses were conducted in STATA 14 (College Station, TX) and R with the gWQS package (Renzetti et al. 2016).

RESULTS

Study sample characteristics, exposures, and OS biomarkers

The study sample consisted of 143 children who had complete data on urine concentrations of metals, 8-isoprostane or 8-OHdG, and the relevant covariates (Table 1). The children, aged approximately 6.7 ± 0.5 years (42% girls), came from low-middle income households, with approximately 20% of the mothers having only primary education, 36% being unemployed or stay-at-home, and 22% living in crowded households. Whereas 38% of the children were overweight/obese, 59% had ID. Of the 211 children eligible for the measurement of urinary OS markers, 143 had complete information. The complete-case sample did not differ in terms of household or child characteristics or biomarkers in terms of household or child characteristics or biomarkers from children who were eligible for the study but excluded on the basis of missing data (Table 1).

Table 2 and Supplementary Figure 1 show the distributions of urinary concentrations of metals and OS biomarker in the study children. Children's metal concentrations in urine indicated low-level exposure to As, Cd, and Pb, with no sex differences (Table 2). The correlation among urinary metal concentrations was low, with Spearman correlation coefficient less than 0.30 for all metal pairs. There was no difference between boys and girls in terms of urinary 8-OHdG, but boys had slightly higher median [5%, 95%] concentrations of urinary 8-isoprostane than girls (1.3 [0.3, 3.5] vs. 1.0 [0.3, 3.8], $p=0.079$).

Associations of metal concentrations with OS biomarkers

In single-metal models (Table 3, **Models 1–3** and Supplemental Figure 2), we found no associations of urinary As, Cd or Pb concentration with 8-isoprostane, either prior or following covariate adjustment. Similar lack of association was observed in the multiple-metal (urinary As, Cd & Pb entered into a single regression, **Model 4**) and MEI (WQS-derived mixture index, **Model 5**) analyses. On the other hand, each doubling in urinary As concentration was associated with ~10 ng/mL higher 8-OHdG in single (Model 1) and multiple-metal (Model 4) analyses. These associations remained unchanged after covariate-adjustment. Similarly, an increase across the value of the MEI was associated with higher 8-OHdG concentrations. Specifically, for each change in MEI quartile, 8-OHdG concentration was higher by 8.7 [1.12, 16.3] ng/mL in the covariate-adjusted model (Table 3, **Model 5**,

and Figure 1). The three metals contributed differentially to the weighted index modeled in the 8-OHdG analysis: urinary As (78.7%), urinary Pb (14.4%), urinary Cd (6.9%).

Metal exposure and OS by dietary categories

The median consumption of any fruit was 105 [0, 402] g/d and vegetables, 5 [0, 130] g/d. The median [5%, 95%] intake of vitamin C was 20.1 [2.0 – 68.1] mg/1000 kcal/d, of fiber 1.3 [0.5 – 4.2] g/1000 kcal/d, and of zinc 2.2 [0.9, 4.6] mg/1000 kcal/d. No dietary nutrient or food intakes were associated with 8-isoprostane or 8-OHdG concentrations, although it appears that additional adjustment for metals attenuated some of the estimates. In particular, the association between the intake of zinc or vegetable consumption and 8-OHdG was attenuated when models were adjusted for metals (Table 4).

Because only urinary As was associated with 8-OHdG in single and multiple-metal models, only this association was carried forward to test for effect modification by dietary intakes. There was little evidence of an effect modification by nutrient intakes or diet (Figure 2) in that most regression estimates were similar across strata (particularly for vitamin C, vegetable consumption and nutrient-dense pattern score, or the confidence intervals overlapped considerably (observed for zinc and fiber intake). The confidence intervals were also generally wide, indicating imprecise estimates, possibly due to small sample size. Of note, in the analysis stratified by fruit consumption, the association between urinary As and 8-OHdG concentration was stronger and statistically significant among children with low fruit consumption (β [95% CI: 17.8 [8.4, 27.1]). A more attenuated association was observed among high fruit consumers (3.9 [–6.7, 14.6]). Nevertheless, the CIs for both these groups were overlapping (Figure 2).

DISCUSSION

In this cross-sectional study of 6–8 y old children, we found that low-level exposure to inorganic As was positively associated with urinary 8-OHdG, a marker of higher OS to the DNA, even when accounting for markers of exposure to Cd and Pb. Similarly, an analysis using an index of a mixture effect found strong association with 8-OHdG, mostly driven by the contribution of urinary As to the mixture. None of the exposure markers was associated with 8-isoprostane, indicating little impact of low metals exposure on lipid peroxidation. Our study is the first to test the associations between exposure to multiple metals and OS markers in children in the context of diets with varying levels of antioxidant nutrients, but we found limited evidence of effect modification by dietary factors. One possible exception is the stronger association between urinary As and 8-OHdG among children who consumed lower vs. higher amounts of fruit. Oxidative stress is an imbalance between pro- and anti-oxidant elements in cellular processes (Jones 2006). It is a phenomenon that occurs naturally, and the levels of some markers of oxidative stress, like isoprostanes, appear to be stable day-to-day (Cracowski et al. 2002), but the balance can be tipped by a number of environmental factors, contributing to cellular damage.

Among the principal products of oxidative damage to the DNA, 8-OHdG is the most frequently measured and best understood (Cooke et al. 2006; Wu et al. 2004). Isoprostanes are prostaglandin-like compounds produced by the peroxidation of arachidonic acid and are

considered reliable markers of oxidant injury; 8-isoprostane is one of the isomers generated during this process (Morrow and Roberts 1997). Because there is great variability in the methods for biomarker measurement (for example, ELISA kits, gas chromatography-mass spectrometry) and presentation (for example, with adjustment for creatinine excretion or specific gravity of urine), it is difficult to compare the level of oxidative stress of the Uruguayan children with other population groups. However, 8-isoprostane concentrations were also low (median [5%, 95%]: 1.43 [0.30, 4.20] pg/mL) among 8–14 year old Canadian children with asthma who were exposed to air pollution (Liu et al. 2009).

There has only been a handful of other studies investigating the effects of multiple metal exposures on OS in humans, and only one in children, conducted among predominantly breastfed infants from Bangladesh (Kippler et al. 2012). In that study, breastmilk and urinary Cd, but not As, was associated with elevated 8-oxodG concentrations. In pregnant women from Bangladesh, both urinary Cd and As concentrations were associated with higher urinary 8-oxodG (Engström et al. 2010a). To note, because rice is a staple food in Bangladesh and the plant takes up Cd from the soil, Cd exposure is generally higher in studies conducted in Bangladesh than in Uruguayan children (median 0.30–0.58 vs. 0.06 µg/L). Also the As concentrations in maternal urine, placenta and cord blood of newborns from Bangladesh were positively associated with cord blood 8-OHdG concentrations (Ahmed et al. 2012). Other studies investigating the effects of As exposure in children on OS also revealed higher DNA oxidative damage. Consistent with these studies, urinary As was significantly associated with 8-OHdG in Uruguayan children, even when exposure to the other metals was factored in. When investigating a mixture effect, we also found associations, but it appears they were largely driven by As exposure. Interestingly, the level of As exposure among Uruguayan children is much lower than in other studies investigating effects on OS (median urinary As in Uruguayan children—9.9 µg/L; mean in Thai children—73–146 µg/L (Hinhumpatch et al. 2013); mean in children from Inner Mongolia—58 µg/g creatinine (Xu et al. 2008); median in women from Bangladesh—100 µg/L (Engström et al. 2010b)). Nevertheless, it is important to point out that food, particularly rice, was a source of As exposure (Kordas et al. 2016) in this population, thus confounding by other pro-oxidant factors related to the same food sources is possible.

We found little evidence of an association between dietary intakes and OS markers in Uruguayan children, contrary to previous evidence, where diet overall and the intake of specific nutrients was associated with lower OS. For example, the consumption of fruits and vegetables, as well as typical intake of fiber and vitamin C, was adversely associated with OS markers in Brazilian men (Cocate et al. 2014). The consumption of fruits, vegetables, and fiber was positively associated with GPx activity and total antioxidant capacity (TAC) in young adults from Brazil and Spain (Hermsdorff et al. 2012). Finally, among adolescents, total fruit and vegetable consumption was associated with lower urinary F₂-isoprostane concentrations (Holt et al. 2009). Most of these reports are based on adult populations, and it is unclear whether it is age, the quality of the diet, or other factors that contribute to the discrepancy between our study and others. Our findings may also be explained by overall low level and variability in fruit and vegetable consumption in our study sample or confounding by metal exposure from these foods.

We also found no effect modification by dietary factors in the association between As exposure and OS, with the possible exception of fruit intake. This contrasts with other research on the role of diet in OS among environmentally or occupationally-exposed individuals. For example, among pregnant Korean women, in covariate-adjusted models, polycyclic aromatic hydrocarbon (PAH) levels were positively associated with MDA concentrations when women had low but not high consumption of fruits and vegetables, or vitamin C (Kim et al. 2011). Xie and colleagues (Xie et al. 2015) examined the association between quartiles of a fruit-vegetable (FV) dietary pattern and concentrations of superoxide dismutase (SOD), 8-OHdG and MDA among coke oven workers and controls. Among workers but not controls, higher scores of the FV pattern were associated with lower OS. In Korean adults, the intakes of vitamin C and E were negatively associated with 8-OHdG concentrations, but only in those individuals who also had low BLL (Hong et al. 2013). Similar patterns of findings were reported for the association between vegetable consumption and 8-OHdG (Hong et al. 2013). Finally, antioxidant nutrient intake (vitamin A) and a biomarker of exposure to PAH were independently associated with urinary 8-OHdG concentrations in 3–6 y old Japanese children (Mori et al. 2011), although it does not appear that effect modification was tested.

We found no associations between urinary Cd or Pb and OS markers in the Uruguayan children. Pb produces free radical damage via the formation of reactive oxygen species and the depletion of cellular antioxidant pools (Jomova and Valko 2011). It also inhibits the activity of glutathione reductase (GR), an enzyme responsible for recycling the oxidized glutathione disulfide to glutathione (Jomova and Valko 2011). Nevertheless, a recent comprehensive review of the literature revealed little consistent evidence on OS in Pb-exposed children, particularly at low levels of exposure (Roy and Kordas 2015). A previous report on OS in relation to BLL of the Uruguayan children (Roy et al. 2015) showed a weak positive association with 8-isoprostane but not 8-OHdG concentrations; it was included in the above-mentioned review (Roy and Kordas 2015). In contrast to that study, here we used urinary Pb concentrations, which reflect circulating Pb in plasma and short-term exposure, likely a couple of weeks (Berghdal and Skerfving 2008). Blood Pb has a half-life of ~2 months, and measures Pb bound to red blood cells. Importantly, we re-analyzed the data on BLL and 8-isoprostane and 8-OHdG in the present sample, controlling for urinary As and Cd. We observed findings consistent with the original report—that BLL was associated with 8-isoprostane levels but not 8-OHdG (Roy et al. 2015)—and suggesting that the choice of biomarker of exposure (short vs. longer term) matters.

Cd does not generate OS directly, but likely through the superoxide radical, hydroxyl radical, nitric oxide or hydrogen peroxide (Jomova and Valko 2011). In rats treated with high levels of Cd, altered catalase enzyme activity and MDA concentrations (Nasiadek et al. 2014). It is possible that the Cd exposure (median urinary Cd concentration: 0.06 µg/L) in our study sample was not high enough to provoke oxidative damage. Also, urinary Cd reflects the accumulation of this metal in the kidneys, which is not very high at this age in areas without particular pollution. Both breastmilk and urinary Cd levels were associated with elevated 8-OHdG concentrations in infants and pregnant women from Bangladesh, which may reflect higher gastrointestinal absorption in infancy and higher exposure from rice-based diets of the mothers (Engström et al. 2010a; Kippler et al. 2012), as well as

potential increased susceptibility. However, a study of adult kidney donors, reported no association between kidney Cd concentrations and urinary 8-oxodG (Hossain et al. 2014). Thus, identification of potential susceptibility factors is warranted.

We have already alluded to the fact that biomarker choice may influence the measurement of the association between exposure to environmental toxicants and health outcomes or early biomarkers of effect, such as OS. For example, BLL is thought to represent exposure over several weeks, urinary Pb over last few weeks (Bergdahl and Skerfving 2008). Similarly, blood As levels reflect longer-term and urinary As levels short-term (days) exposure, but the interpretation of biomarkers may also depend on whether exposure was high or low-level, and whether source of exposure is food or water (Marchiset-Ferlay et al. 2012). In contrast, urinary Cd represents long-term exposure (Tellez-Plaza et al. 2008). However, the interpretation of metal biomarkers may be particularly challenging for low-level exposure scenarios, where, presumably, some equilibrium has been achieved among body pools, where biomarkers represent endogenous re-exposure as much as or more than exogenous sources, and where low or infrequent new exogenous inputs are occurring.

The relative rates of excretion of metals into urine may also influence the strength of associations observed with other biomarkers measured in urine. For example, similar rates of excretion of As and oxidative stress markers may explain the positive association we observed between urinary As and 8-OHdG. The variation in urinary (metal or OS) biomarker concentrations will depend on the volume of the urine sample, unless properly adjusted for hydration level (Nermell et al. 2008), as well as the way (glomerular filtration or passive secretion) in which the metabolite arrives in urine (Barr et al. 2005). We have adjusted all urinary biomarkers for specific gravity, thus accounting for the volume of the sample. It is also noteworthy that an association was observed between urinary As and 8-OHdG but not isoprostane, which suggests that co-excretion is not the explanation for our findings. As other studies investigate OS in young children, the relationships with metal exposures should get clearer.

Our study had several strengths, including the use of two widely accepted and well characterized markers of OS, the measurement of these markers in urine, which is a more stable matrix than serum when it comes to the oxidation of circulating lipids (Morrow and Roberts 1997), the testing of metal mixture effects, the use of acceptable markers of recent (urinary As, Pb) and long-term (urinary Cd) metal exposure, the inclusion of foods as well as nutrients as dietary predictors, careful collection of dietary information, and the inclusion of two non-consecutive 24-hr recalls to better capture typical diets.

On the other hand, our findings should be considered in the context of the study's limitations: 1) the cross-sectional nature of the study precludes any statements regarding causality; 2) we were only able to determine OS marker concentrations on a sub-sample of study children and our sample size was further limited by missing data on a number of model covariates; 3) due to limited resources, we only measured two markers of OS and this limited characterization of OS may be one of the reasons we saw few associations with metal exposures. There is a variety of ways to assess oxidative stress, including levels of oxidants and anti-oxidants, activity of antioxidant enzymes, antioxidant/pro-oxidant balance,

and oxidative damage (Roy and Kordas 2016). However, it is also important to highlight the general lack of consistency within the field of environmental epidemiology with respect to the use of OS biomarkers (Roy and Kordas 2016). These inconsistencies make cross-study comparisons difficult. 4) We used ELISA kits to measure OS, and these have sometimes been criticized as inferior to chromatography-based methods (Barregard et al. 2013; Garratt et al. 2010); 5) A recent study of methodological sources of variation in 8-oxodG also revealed that the excretion of this biomarker in first void urine samples is higher than throughout the rest of the day, even after adjustment for specific gravity was performed (Barregard et al. 2013). This raises the possibility of higher co-excretion of both metals and biomarkers of OS in morning urine samples (Barregard et al. 2013). However, our finding of positive association between urinary As, but null association between urinary Cd and Pb, and OS biomarkers reduces this possibility. Furthermore, because all children provided a first void urine, the variability in excretion rates resulting from differential timing of spot urine samples collected throughout the day was not a problem in our sample; 6) The 24-hr diet recall methodology may not represent typical dietary preferences and intakes; 7) The contribution of measurement error to our findings also needs to be acknowledged. While we used laboratory techniques with low limits of detection and implemented quality control measures in assessing metal and OS marker concentrations, measurement error, and therefore, misclassification of “status” is possible. Random measurement error would result in greater “noise” in the data and effect estimates tending toward the null. The impact of systematic error on regression estimates would depend on which (exposure or outcome) was over or under-estimated (for example, under-estimating exposure and over-estimating effect could produce stronger estimates). The concern over measurement error is the greatest in our dietary data, which are prone to error in recall or desirability bias. Desirability bias with respect to our hypothesis is unlikely and the low consumption of the “desirable” fruits and vegetables suggests that recalls reflect real diets.

In summary, in this cross-sectional study of 6–8 y old children, we found evidence of elevated oxidative stress in children exposed to low-level inorganic As, even when accounting for co-exposure to other metals. However, given the uncertainty of the effects of the low-level As exposures, and the relatively low exposure from dietary sources compared to water in populations that do not consume rice as a staple food (Mantha et al. 2017), further studies are warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank nurses Delminda Ribeiro and Graciela Yuane for their help with sample collection and processing, nutritionists Valentina Baccino, Elizabeth Barcia, Soledad Mangieri and Virginia Ocampo for assistance with dietary recalls, and Jing Nie for assistance with data analysis. This study was funded by the National Institutes of Environmental Health Sciences/Fogarty International Center (1R21 ES16523, PI: Kordas and 1R21 ES019949, PI: Kordas).

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Highlights

- In 6–8 year-old children from Montevideo, Uruguay, we examined cross-sectional associations between urinary concentrations of arsenic, cadmium, and lead, and two urinary markers of OS: F₂-8 α isoprostane and 8-hydroxy-2-deoxy-guanosine (8-OHdG).
- We found that even at low-level, arsenic exposure was associated with detectable oxidative damage to the DNA, as indicated by ~10 ng/mL higher 8-OHdG concentrations for every doubling in urinary arsenic concentrations.
- An indicator of mixture of the three metals was associated with higher 8-OHdG concentrations.
- We found no effect modification by children's dietary antioxidant intake.

Figure 1. Lowess plot of the association between multi-metal exposure index (MEI) and 8-OHdG in ~7 y old Uruguayan children.

Figure 2. Association between urinary arsenic and urinary 8-OHdG concentrations in ~7 y old Uruguayan children with low and high dietary intakes

β [95% CI] derived from multi-metal, covariate-adjusted regressions modeling 8-OHdG as the dependent variable; $n=143$; all urinary biomarker concentrations were adjusted for specific gravity of urine (0.024) prior to analysis; urinary As concentration was $\log_2$ -transformed prior to analysis; low (black circles ●) and high dietary (gray squares ■) intakes were defined as the median split for vitamin C (20.1 $\mu\text{g}/1000 \text{ kcal/d}$), fiber (1.3 $\text{mg}/1000 \text{ kcal/d}$), zinc (2.2 $\mu\text{g}/1000 \text{ kcal/d}$), any fruit (105 g/d) and vegetables (5 g/d), and the nutrient-rich dietary pattern score (0.2); the **fruit group** includes the consumption of whole fruit, fruit salad, and fruit juices; the **vegetable group** includes the consumption of dark leafy, red-orange, beans/peas or other vegetables, either fresh or canned (but excludes potatoes); the **nutrient-dense pattern** consists of higher consumption of dark leaf and red-orange vegetables, higher consumption of eggs, beans, peas and potatoes, and reduced consumption of pasta and sauces/condiments; covariate adjustment included household possessions, household density, parent smoking (yes/no), child sex, BMI, and CRP-adjusted ferritin concentration.

Table 1

Household and demographic characteristics of the study participants.

Characteristic	Complete-case analysis N=143	Excluded from analysis	
		N	
Girls	42.0% ^a	65	47.7%
Age (months)	81.8 ± 6.2 (72 – 100) ^b	63	82.1 ± 7.1 (67 – 103)
Maternal education (years)	8.9 ± 2.8 (4 – 17)	52	8.8 ± 2.4 (4 – 14)
Mother unemployed/stay-at-home	36.4%	51	31.4% [*]
Parents with potential occupational metal exposure	26.6%	63	22.2%
Household crowded ^c	22.4%	37	24.3%
Parent currently smokes	52.4%	38	63.2%
BMI (kg/m ²)	16.9 ± 2.7 (12.8–26.6)	42	16.8 ± 2.5 (9.8–22.4)
Overweight or obese ^d	38.0%	42	47.6%
Obese ^d	21.8%	42	14.3%
CRP-adjusted ferritin (ng/mL)	12.4 [2.4, 43.8] ^e	28	10.2 [3.3, 35.6]
Iron deficiency ^f	59.4%	28	67.9%
Urinary 8-isoprostane (ng/mL) ^g	1.1 [0.3, 3.8]	42	1.5 [0.5, 3.6]
Urinary 8-OHdG (ng/mL)	39.6 [11.8, 115.2]	41	40.5 [15.1, 230.6]
Urinary As (µg/L)	9.6 [4.8, 24.0]	46	10.8 [5.0, 25.7]
Urinary Cd (µg/L)	0.06 [0.02, 0.2]	48	0.05 [0.03, 0.1]
Urinary Pb (µg/L)	1.9 [0.7, 3.9]	48	1.7 [0.4, 4.5]

^aValue given as percent (%);^bValue given as M±SD (range);^cHousehold crowded=more than 2 persons per bedroom living in house;^dOverweight or obese=BAZ > 1 SD, Obese=BAZ > 2 SD;^eValue given as median [5%, 95%];^fIron deficiency=CRP-adjusted serum ferritin concentration < 15 ng/mL;^gUrinary biomarker adjusted for specific gravity of urine;^{*} Different from the complete-case sample at p<0.05;^{**} Different from complete-case sample p<0.01.

Table 2

Urinary metal and oxidative stress marker concentrations among children from Montevideo, Uruguay (n=143).

Urinary biomarker ^a	Overall	Girls	Boys	P value ^c
As, µg/L	9.6 [4.8, 24.0] ^b	9.6 [4.3–21.8]	9.4 [4.9, 28.6]	0.886
Cd, µg/L	0.06 [0.02, 0.17]	0.06 [0.02, 0.14]	0.06 [0.03, 0.17]	0.907
Pb, µg/L	1.9 [0.7, 3.9]	1.9 [0.6, 4.5]	1.9 [0.7, 3.0]	0.875
8-Isoprostane, ng/mL	1.1 [0.3, 3.8]	1.0 [0.3, 3.8]	1.3 [0.3, 3.5]	0.079
8-OHdG, ng/mL	39.6 [11.8, 115.2]	39.6 [14.4, 106.7]	39.6 [11.0, 135.9]	0.526

^aConcentrations adjusted for the specific gravity of urine using formula: (biomarker*0.024)/(specific gravity-1);

^bValue given as median [5%, 95%];

^cMann-Whitney test was used to test the differences between girls and boys.

Table 3

Associations among urinary metals and oxidative stress markers in ~7 y old children from Montevideo, Uruguay (n=143).

Urinary metal ^{a, b}	Unadjusted, β [95% CI]		Covariate-adjusted ^c , β [95% CI]	
	8-isoprostane ^a	8-OHdG ^a	8-isoprostane ^a	8-OHdG ^a
Model 1: As	0.15 [-0.10, 0.41]	9.40 [2.33, 16.47]	0.13 [-0.12, 0.38]	10.31 [3.41, 17.21]
Model 2: Cd	0.18 [-0.04, 0.39]	-0.48 [-6.64, 5.68]	0.13 [-0.08, 0.34]	0.46 [-5.54, 6.47]
Model 3: Pb	0.10 [-0.10, 0.29]	0.07 [-7.855, 3.21]	0.08 [-0.24, 0.27]	-1.88 [-7.40, 3.66]
Model 4: multiple metals				
As	0.11 [-0.15, 0.38]	10.22 [2.98, 17.45]	0.10 [-0.16, 0.36]	10.90 [3.82, 17.97]
Cd	0.14 [-0.09, 0.38]	-0.84 [-7.36, 5.69]	0.10 [-0.13, 0.33]	-0.17 [-6.49, 6.14]
Pb	0.04 [-0.17, 0.25]	-3.22 [-9.06, 2.61]	0.03 [-0.18, 0.25]	-3.02 [-8.00, 2.76]
Model 5: weighted quantile sum regression				
MEI ^d	0.12 [-0.22, 0.46]	6.59 [-1.16, 14.34]	0.01 [-0.37, 0.39]	8.71 [1.12, 16.3]

^aConcentrations adjusted for the specific gravity of urine;

^bLog2-transformed values were used for all metals in regression models;

^cAdjusted for household possessions score, household density, parent smoking (yes/no), child sex, BMI, and CRP-adjusted ferritin concentration; MEI=multiple-metal exposure index.

Table 4

Associations between oxidative stress markers and dietary intakes among ~7 y old children from Montevideo, Uruguay.

	Unadjusted β [95% CI]			Covariate-adjusted ² β [95% CI]			Covariate and metals-adjusted ³ β [95% CI]		
	8-isoprostane ¹ N=140	8-OHdG ¹ N=143	8-isoprostane ¹ N=140	8-OHdG ¹ N=143	8-isoprostane ¹ N=140	8-OHdG ¹ N=143	8-isoprostane ¹ N=140	8-OHdG ¹ N=143	
Vit C intake (μg/1000 kcal/d)									
Low (< 20.1)	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	
High (>20.1)	-0.10 [-0.50, 0.29]	6.39 [-4.68, 17.47]	-0.14 [-0.52, 0.24]	6.84 [-3.88, 17.56]	-0.17 [-0.55, 0.22]			5.92 [-4.63, 16.47]	
Zinc intake (mg/1000 kcal/d)									
Low (< 2.2)	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	
High (>2.2)	0.13 [-0.26, 0.53]	-2.81 [-13.92, 8.31]	0.18 [-0.21, 0.57]	-2.39 [-13.34, 8.56]	0.24 [-0.16, 0.64]			-0.4 [-11.31, 10.44]	
Fiber intake (g/1000 kcal/d)									
Low (< 1.3)	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	
High (>1.3)	-0.15 [-0.54, 0.25]	7.50 [-3.55, 18.55]	-0.13 [-0.51, 0.26]	7.68 [-3.00, 18.36]	-0.11 [-0.50, 0.27]			7.29 [-3.19, 17.78]	
Fruit consumption⁴ (g/d)									
Low (< 105)	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	
High (>105)	-0.23 [-0.62, 0.17]	4.80 [-6.29, 15.90]	-0.18 [-0.57, 0.20]	4.66 [-6.16, 15.48]	-0.18 [-0.57, 0.20]			4.98 [-5.58, 15.54]	
Vegetable consumption⁵ (g/d)									
Low (< 5)	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	
High (>5)	-0.07 [-0.47, 0.32]	1.04 [-10.08, 12.16]	0.05 [-0.34, 0.44]	3.24 [-7.69, 14.18]	0.03 [-0.37, 0.43]			0.98 [-9.81, 11.77]	
Nutrient-dense pattern⁶ score									
Low (< 0.21)	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	
High (>0.21)	0.08 [-0.31, 0.48]	4.38 [-6.72, 15.48]	0.13 [-0.25, 0.52]	7.11 [-3.67, 17.89]	0.13 [-0.25, 0.52]			6.46 [-4.10, 17.01]	

¹Concentration adjusted for specific gravity of urine;

²Adjusted for household possessions score, household density, parent smoking, child sex, BMI, and CRP-adjusted ferritin concentration;

³Adjusted for covariates and log2-transformed urinary As, Cd and Pb concentrations;

⁴Fruit group included any consumption of whole fruit, fruit salad, and fruit juices;

⁵Vegetable group included any consumption of dark leafy, red-orange, beans/peas or other vegetables, either fresh or canned;

⁶The nutrient-dense pattern consists of higher consumption of dark leaf and red-orange vegetables, higher consumption of eggs, beans and peas and reduced consumption of pasta and sauces/condiments;

*
>0.50

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