Effect of mercury (Hg) dental amalgam fillings on renal and oxidative stress biomarkers in children

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ABSTRACT

We examined the effect of mercury (Hg) associated with dental amalgam fillings on biomarkers of renal and oxidative stress in children between the ages of 5–15.5 years. Urine samples were analyzed for N-acetyl-β-D-glucosaminidase (NAG), α1-microglobulin (α1-MG), β2-microglobulin (β2-MG), retinol binding protein (RBP), albumin (ALB), 8-hydroxy-2-deoxyguanosine (8-OHdG) and malondialdehyde (MDA). The level of urinary Hg (UHg-C) was calculated as µg/g creatinine. Multiple regression analyses revealed that the excretion of urinary NAG was significantly associated with the presence of dental amalgam fillings (β = 0.149, P = 0.03) and the levels of UHg-C (β = 0.531, P = 0), with an interaction between the two (P = 0). The increase in urinary NAG in relation to UHg-C levels had a dose-effect pattern. The lowest observed effect was seen at UHg-C levels above 1.452 µg/g creatinine, which is lower than previously reported. In contrast, α1-MG was negatively associated with the presence of dental amalgam fillings (β = −0.270, P = 0), but positively with UHg-C levels (β = 0.393, P = 0). There were 7 children without, and one child with, dental amalgam fillings with urinary α1-MG levels above the reference limit of > 7 mg/g creatinine. Even though α1-MG seems to be a reliable biomarker for early changes in renal functions, it might exert its effect only at a higher level of exposure. An inverse relationship was also observed between urinary 8-OHdG levels and the presence of dental amalgam fillings. This might suggest that the dental amalgam does not increase DNA damage but reduces the capacity to repair DNA, leading to lower urinary excretion of 8-OHdG. On the other hand, we found that Hg affected the excretion of urinary 8-OHdG in a dose-related pattern that was mostly associated with long-term exposure to low Hg levels. Urinary NAG levels were positively associated with urinary MDA levels (β = 0.516, P = 0) but not with 8-OHdG (β = 0.134, P = 0.078) after adjustment for potential confounders. Both UHg-C and the presence of dental amalgam fillings remained predictors of the NAG model. Our data provide evidence that low exposure to Hg from dental amalgam fillings exerts an effect on kidney tubular functions in children. Oxidative stress may have played a role in this mechanism. The results of this study would also suggest that urinary NAG is the most sensitive of all the investigated renal biomarkers. These results should be confirmed with further investigation.

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1. Introduction

Most previous studies showed that mercury (Hg) could be toxic when inhaled, ingested or absorbed through the skin (Goldman et al., 2001). Its absorption and relative toxicity depend on its chemical form (Clarkson, 1997). The sources of exposure are also markedly different for the various forms of Hg. Diet, especially fish and other seafood is the main source of exposure of the general public to organic Hg that has the greatest potential neurotoxicity (WHO, 1990). Both elemental and inorganic mercury are nephrotoxic (WHO, 2003). Clinical health implications were seen in both occupational and accidental exposures (Paruchuri et al., 2010; Garetano et al. 2008). Hg has been used in tooth fillings for more than 150 years (Fung and Molvar, 1992). Although the safety of Hg-containing dental amalgam has been strongly debated for a long time for its association with many health conditions (Martin and Woods, 2006), amalgam remains popular because of its relative cheapness, durability and ease of use (Newman, 1991). It has been also widely utilized to restore posterior teeth in pediatric dentistry (Fuks, 2002). There have been few studies assessing the safety of dental amalgam restorations in children. Bellinger et al. (2006) found no statistically significant differences in adverse neuropsychological or renal effects observed over a 5-year period in children whose caries were restored using dental amalgam or composite materials. Another study (DeRouen et al., 2006) found that children who received dental restorative treatment with amalgam did not, on average, have statistically significant differences in neurobehavioral assessments or in nerve conduction velocity
when compared with children who received resin composite materials without amalgam. The authors still believe, though as a precaution, that future use of amalgam should be avoided since, it does involve some level of mercury exposure. According to Needelman (2006), both studies by Bellinger et al. and DeRouen et al. represent thoughtful and important contributions to understanding the question of dental amalgam risks to children, but the question of the more subtle effects remains open. Although Ye et al. (2009) found that urinary Hg levels were slightly elevated among children with amalgam fillings, no evidence of adverse effects on the outcomes evaluated was found. A recent study by Geier et al. (2011) restated samples collected from children with and without dental amalgam fillings previously collected by DeRouen et al. (2006) for urinary porphyrins as a biomarker of mercury toxicity (Woods, 1996). The authors found that cumulative exposure to Hg from dental amalgam might distort porphyrin metabolism.

Metal toxicity usually involves the production of reactive oxygen species (ROS) that in return damage lipids in membranes, proteins or enzymes in tissues, and DNA (Valko et al., 2005). Normally, ROSs are balanced by natural anti-oxidant enzymes (Ercal et al., 2001; Gobe and Crane, 2010). The imbalance between these ROSs and natural anti-oxidants creates the condition of oxidative stress that can play a vital role in disease pathogenesis (Roberts et al., 2010). Recent studies (Sabloič, 2006; Jan et al., 2011) have described how inorganic Hg induces the production of ROSs that plays a role in its nephrotoxic effect. Stacciotti et al. (2009) reported that exposing experimental animals to subcytotoxic doses of inorganic Hg increased ROS, reactive nitrogen species (RNS) and the expression of metallothionein that can lead to nephrotoxicity. Monitoring urinary Hg is useful for controlling the nephrotoxic risk of overexposure, particularly to its inorganic form. It should not exceed 50 μg/g creatinine in order to prevent cytotoxic and functional renal effects (Roels et al., 1999). Urinary N-acetyl-β-D-glucosaminidase activity (NAG), α₂-microglobulin (α₂-MG), and β₂-microglobulin (β₂-MG) were used as markers of renal tubular damage, especially for nephrotoxic assessment of non-occupational exposures to Hg (Ohno et al., 2007). A study by Jarosińska et al. (2008) found that exposure to Hg had no effects on the kidney markers (particularly NAG) in occupationally exposed workers. In recent years, 8-hydroxy-2′-deoxyguanosine (8-OHdG) has been widely used in many studies as a biomarker for the measurement of endogenous oxidative DNA damage as well as a risk factor for many diseases, including cancer (Valavanidis et al., 2009). Chen et al. (2005) recommended the use of urinary 8-OHdG as a marker of oxidative DNA damage in mercury-exposed populations. However, they also mentioned that one should take into account the anti-oxidative repair systems that might minimize DNA lesions caused by Hg. It has been shown that Hg can oxidize the unsaturated fatty acids in the membrane lipid bilayer leading to an increase in lipid peroxidation (Milaeva, 2006). Hg and/or other heavy metals might induce vascular effects due to increased oxidative stress and lipid peroxidation (Houston, 2007). Malondialdehyde (MDA) is one of the major secondary oxidation products derived from polyunsaturated fatty acids in body fluids or tissues and has been widely used as a biomarker of lipid peroxidation (Del Rio et al., 2005; Lykkefseldt, 2007).

In Saudi Arabia, high rates of dental caries (94%) among primary-school children have been reported (Al Dosari et al., 2004; Al-Malik and Rehbbini, 2006). This might lead to the increased use of dental amalgam fillings. Data on the use of amalgam in children, though, are still not available. Mahmood et al. (2004) conducted a cross-sectional study of 10 polyclinics within the Riyadh metropolitan area and found that amalgam was the most commonly used restorative material (53%).

The main objective of this study was to test the effect of Hg-associated dental amalgam on renal and oxidative stress biomarkers in children between the ages of 5 and 15.5 years.

2. Materials and methods

2.1. Study design

A survey was conducted in three pediatric dental clinics from three major hospitals in Taif City that provide medical care, mainly for military staff and their families, between July 2007 and March 2008. A total of 182 healthy Saudi children, aged 5.1–15.5 years were enrolled. Written informed consent was obtained from parents before the study, which was approved by the Ethics Committee of King Faisal Specialist Hospital and Research Centre. All children come from a homogenous socioeconomic background, because the study was limited to the children of military staff.

2.2. Questionnaire

The parents or the guardian of each child was interviewed by a trained data collector and completed a questionnaire on demographic, lifestyle, health and environmental data as described previously (Al-Saleh and Al-Sedairi, 2011). From the questionnaire, information on location of current residence (Western/Northern/Southern/Eastern/Central); duration of residence, in years; attendance at dental clinics at three major hospitals, designated as A, B, and C, in Al-Taif City; age; gender; body weight; height; smoking status of household (yes/no); seafood intake (yes/no); frequency of seafood intake (1 = daily, 2 = once a week, 3 = twice a week, 4 = once a month, 5 = irregular, 6 = don’t remember), use of medical ointment (yes/no), and vaccination within the last year (yes/no) were obtained. Information on the number of amalgam fillings was obtained at the time of participation by a dentist.

2.3. Collection of biological materials

Urine samples were collected from all participants in 50 mL polyethylene tubes that we routinely checked for possible Hg contamination. This is usually done by adding deionized water to randomly selected containers and then testing for possible contamination. Each sample was divided into five aliquots (1–4 mL each) according to the following: (1) an aliquot of 4 mL was stored immediately at −20 °C for Hg, MDA and creatinine analysis; (2) the pH of three aliquots of urine (1 mL each) was adjusted to 6–8 by adding one drop of 1 N sodium hydroxide and were stored at −20 °C in order to be used later for the measurement of NAG, α₂-MG, β₂-MG and RBP; and (3) a fifth aliquot of urine sample for the measurement of urinary 8-OHdG was treated with 0.05% sodium azide (w/v) (Sigma Chemical Co., USA) as a preservative after adjusting pH between the range of 6 to 8 and then stored at −70 °C.

2.4. Instrumentation

All urine Hg analyses were performed using a Varian AA-880 Zeeman atomic absorption spectrophotometer coupled to a Vapor Generation Accessory VGA-77 (Varian Techtron Pty. Ltd. Australia) and run by SpectraAA worksheet software (version 2.1 for AA-880). The gas used was argon. The reductant channel of the VGA-77 contained 25% stannous chloride in 20% hydrochloric acid. The acid channel contained 5 M hydrochloric acid. The flow rates were about 8 mL/min for the sample, 1.4 mL/min for the stannous chloride solution and 1.2 mL/min for the hydrochloric acid. The quantification of Hg was accomplished by constructing six-point calibration standards in the range of 0.5–8.0 μg/L that were prepared freshly in urine. A linear calibration graph was plotted by least-squares regression of concentration versus absorbance of the calibration standards, which showed an excellent linearity with a correlation coefficient (r) of 0.9994 ± 0.001 for 7 runs. The accuracy of the analysis was assessed by analyzing two sets of urine samples with known Hg concentrations that were obtained from Kaulson Laboratories (CONTOX Trace Metal Urine Control, West Caldwell, NJ, USA). These freeze-dried lyophilized urine samples were reconstituted with 5 mL of
deionized water, and 1-mL aliquots were placed into polypropylene tubes. All aliquots were then frozen until used. The values found for levels I and II were 7.54±1.57 μg/L and 23.24±2.66 μg/L, respectively, while the expected ranges for Hg were 2.0–8.0 and 12.0–24.0 μg/L, respectively. The analytical recoveries for in-house spiked urine samples with 7.5, 15.0 and 30.0 μg/L Hg were respectively 101.5±12.0%, 99.2±5.6% and 96.9±5.3%. The same-day precisions for urine samples spiked with 7.5, 15.0 and 30.0 μg/L Hg and expressed as the relative standard deviation (%RSD) for 8 runs were 5.2, 6.6 and 3.9%, respectively. The method detection limit (MDL) of Hg in urine was 0.39 μg/L (0.08 μg dry wt). Urinary Hg levels were calculated as micrograms per gram creatinine (μg/g creatinine).

2.5. Biochemical assays for renal and oxidative stress biomarkers

We used enzyme-linked immunosorbent assay kits (ELISA) as a diagnostic immunoassay to measure the excretion of 8-OhdG (Trevisen Inc., CT, USA), α1-MG, β2-MG, RET (Immudagnostik, Germany) and ALB (Alpha Diagnostic Int., USA) in urine samples. For the determination of NAG activity (Bio-Quant, USA) and creatinine (Oxford Biomedical Research, MI, USA) in urine, colorimetric assays were utilized. After the completion of each assay according to manufacturers’ instructions, the absorbance (ODsso) was read in an Anthos Zenyth 340 microplate multimode reader (Anthos Labtec Instruments GmbH, Wals, Austria), except for creatinine, which was measured with a microplate reader from Bio-Tek Instruments, USA. Urinary MDA levels were determined by a modified thiobarbituric acid method (Fukunaga et al., 1998) using the Alliance Waters high-performance liquid chromatography (HPLC) 2695 system and a multi-wavelength fluorescence detector, model 2475. The MDL was 0.28 nmol/mL. The calibration curve was linear over 0.5–15 mmol/mL (r>0.9993 for six runs). The analytical recoveries for spiked urine samples with 1.5, 3.0 and 6.0 mmol/mL MDA were 94.8%±7.1%, 98.8%±5.9% and 98.1%±1.6%, respectively.

2.6. Statistical analyses

A SIR computer-database application was developed for the entry of data for all subjects. To obtain approximately Gaussian distributions, we logarithmically transformed all measurements of Hg, renal and oxidative-stress parameters. The urinary concentrations of Hg and all biomarkers were normalized by creatinine. Hg levels in urine were presented as μg/g creatinine to adjust for either highly diluted or concentrated urine samples. There were 9 children with creatinine<30 mg/dL that we included in the analysis. A recent study by Trachtenberg et al. (2010) revealed that the association between creatinine excretion and flow rate was within the recommended “acceptable” range of 3–34 mg/dL. The authors found that despite the increased rate of creatinine and Hg excretion with urinary flow rate, the Hg-adjusted creatinine ratio was unaffected by urinary flow rate. Descriptions of the variables under study have been reported previously (Al-Saleh and Al-Sedairi, 2011). Concentrations of Hg and renal and oxidative stress markers in urine were described as a median within the interquartile range (IQR) (25th and the 75th percentile). Some values in the text are presented as means±SD. Body mass index (BMI) was calculated as body weight (kg)/height (m)². However, since the amount of a child’s body-fat changes with age as well as the fat proportion between boys and girls is different, the BMI percentiles based on the child’s age, and gender were computed using the Center for Disease Control excel spreadsheet (CDC, 2000). The CDC defined-cutoffs for BMI were: underweight (<5th percentile), normal (5th–85th percentile), overweight (≥85th–95th percentile), and obese (≥95th percentile). Due to the limited number of children in the obese group, we combined it with the overweight group and three groups were created: <5th; 5th–85th and ≥85th percentile.

The outcomes of the study were NAG, α1-MG, β2-MG, RBP, ALB, MDA and 8-OHdG. The main predictors used for each outcome were the presence of dental amalgam fillings dichotomized (yes versus no) and UHg-C as a continuous variable. An unpaired Student’s t-test was used to compare the means of NAG, α1-MG, β2-MG, RBP, ALB, Cr, MDA and 8-OHdG between the two groups of children. We also assessed potential confounders extracted from the questionnaire that might be related to the outcome and the levels of UHg-C using bivariate analyses: Pearson’s correlation analysis for continuous variables and one-way analysis of variance (ANOVA) or Student’s t-test (two-tailed) for categorical variables. Subsequently, separate regression models were run only for outcomes (NAG, α1-MG and 8-OHdG) that were found to be significantly associated with dental amalgam fillings (P<0.05). UHg-C was entered twice, either as a continuous or a categorical variable by dividing it into quartiles in the multiple regression analyses. The lowest quartile was used as the reference group. Other confounders were also included that were related to UHg-C levels in the univariate analyses with P less than 0.05. Dunnett’s post hoc test was used to adjust for multiple pairwise comparisons after adjusting for confounding variables in the models. Some variables were excluded due to insufficient cases, such as children living in the central region of the current residence. Statistical parameters presented are β (standardized regression coefficient) and adjusted R² (coefficient of multiple determination). Our findings were similar when we used unadjusted urinary Hg in the regression analyses (data not shown). Analyses were performed using SPSS version 13.0 for Windows (SPSS Inc., Chicago, IL, US). A P-value of less than 0.05 was considered significant.

3. Results

3.1. Descriptive statistics

In this study, 182 children aged 5–15.5 years (mean 9.18 years) were enrolled. There were 106 children with dental amalgam fillings and 76 without. Table 1 presents the median and IQR for the main biological and demographic parameters in all children and for those with and without dental amalgam fillings. Of the studied children, 89 (48.9%) were males and 93 (51.1%) were females. The mean BMI was 16.429±4.372 kg/m² (range, 6.45–38.78 kg/m²). Based on the percentile’s cutoffs of BMI from the CDC (2000), there were 24% underweight (<5th), 56% normal (5th–85th), and 20% overweight or obese (≥85th) children. The median number of dental amalgam fillings was 4 in the range of 2 to 8.

3.2. Univariate analysis

Initially, the associations between each outcome (NAG, α1-MG, β2-MG, RBP, ALB, MDA and 8-OHdG) and their potential predictors (UHg-C and dental amalgam fillings) as well as other possible confounders were tested by either a Student’s t-test or a Pearson correlation analysis (Table 2). Urinary NAG, α1-MG, β2-MG, 8-OHdG and MDA were positively associated with UHg-C, with P-values of 0 for all. Only urinary NAG levels were significantly higher in children with dental amalgam fillings than in those without fillings (P=0.008). In contrast, both α1-MG and 8-OHdG levels were higher in the non-amalgam group than those with and without dental amalgam fillings. Of the studied children, 89 (48.9%) were males and 93 (51.1%) were females. The mean BMI was 16.429±4.372 kg/m² (range, 6.45–38.78 kg/m²). Based on the percentile’s cutoffs of BMI from the CDC (2000), there were 24% underweight (<5th), 56% normal (5th–85th), and 20% overweight or obese (≥85th) children. The median number of dental amalgam fillings was 4 in the range of 2 to 8.

In Table 3, Pearson’s correlation analysis revealed no correlations between the levels of UHg-C and age or BMI. Children with dental amalgam fillings had significantly higher levels of UHg-C (P=0.019) than those without fillings. On the other hand, no relationship was...
seen between UHg-C and the number of dental amalgam fillings \((P=0.991)\). The levels of UHg-C were lower in males than in females \((P=0.03)\).

### 3.3. Multiple regression analyses

We evaluated further the association between outcomes (NAG, \(\alpha_1\)-MG and 8-OHdG) that were significantly associated with both the presence of dental amalgam fillings \((\text{yes/no})\) and the levels of UHg-C. Potential confounders selected for inclusion in each model were only those that exhibited significant associations \((P<0.05)\) with either the outcome or UHg-C, as shown in Tables 2 and 3.

As illustrated in Table 4 (model I), the excretion of urinary NAG increased significantly with UHg-C levels \((\beta=0.519, P=0)\) and the presence of dental amalgam fillings \((\beta=0.128, P=0.047)\). The overall model was significant \((F=26.559, P=0)\) and explained 30.1% of the variability in urinary NAG levels. When the interaction between UHg-C and the presence of dental amalgam fillings was included in the model, it was statistically significant \((P=0.001)\). Urinary NAG levels were also assessed across the quartiles of UHg-C levels after adjusting for confounding variables. A significant trend was found with a plateau. An increase in urinary NAG levels was observed with the second \((\beta=0.181, P=0.048)\) and third \((\beta=0.262, P=0.005)\) UHg-C quartiles (Table 4, model II). Even though the overall model was significant \((F=4.077, P=0.002)\), it explained only 8% of the variation along UHg-C quartiles. A Dunnett’s post hoc test indicated that urinary NAG excretion was significantly higher in the second \((1.452–2.740 \mu g/\text{ creatinine})\), third \((2.752–4.745 \mu g/\text{ creatinine})\) and fourth \((4.845–19.242 \mu g/\text{ creatinine})\) quartiles than in the first quartile group \((<1.433 \mu g/\text{ creatinine})\), with \(P\)-values of 0.014, 0.001 and 0.036, respectively (Fig. 1a).

In a regression model (Table 4, model I), \(\alpha_1\)-MG was negatively associated with the presence of dental amalgam fillings \((\beta=-0.304, P=0)\) but positively with UHg-C levels \((\beta=0.355, P=0)\). The overall model was significant \((F=11.803, P=0)\) and explained 15.5% of the variance in urinary levels of \(\alpha_1\)-MG. No interaction was seen between the presence of dental amalgam fillings and the levels of UHg-C \((P=0.062)\). When the urinary levels of \(\alpha_1\)-MG were divided by the UHg-C quartiles and adjusted for confounders, the presence of dental amalgam fillings gave the same results (Table 4, model II). However, there was an increasing trend in urinary \(\alpha_1\)-MG excretion among the second, third and fourth quartiles, but the trend was not significant \((P\text{-values: 0.895, 0.865 and 0.092, respectively})\). The same results were obtained after applying Dunnett’s post hoc test; the \(P\)-values were 0.937, 0.842 and 0.226 for the second, third and fourth quartiles, respectively, in comparison to the first quartile. Even though the overall model was significant \((F=2.817, P=0.018)\), the \(R^2\) was very small \((4.9\%)\).

In Table 4, model I, the same pattern was observed for urinary 8-OHdG, which was negatively associated with the presence of dental amalgam fillings \((\beta=-0.327, P=0)\) and positively associated with UHg-C \((\beta=0.363, P=0)\). The concentrations of urinary 8-OHdG were significantly higher in females than in males \((\beta=-0.174, P=0.009)\). The overall model was significant \((F=19.004, P=0)\) and explained 23.2% of the variability in 8-OHdG. Significant interaction was noted between UHg-C levels and the presence of dental amalgam fillings \((P=0)\). After adjusting urinary 8-OHdG levels for the UHg-C quartiles in the multiple regression model, the results were the same. However, we observed a statistically significant increasing trend in the urinary excretion of 8-OHdG in relation to the second \((\beta=0.261, P=0.001)\), third \((\beta=0.383, P=0)\) and fourth \((\beta=0.538, P=0)\) UHg-C quartiles (Table 4, model II). The overall model was significant \((F=15.935, P=0)\) and explained 29.3% of the variation in urinary 8-OHdG.

### Table 2

<table>
<thead>
<tr>
<th>Predictor variables</th>
<th>NAG (\mu g/\text{ creatinine})</th>
<th>(\alpha_1)-MG (\mu g/\text{ creatinine})</th>
<th>(\beta_2)-MG (\mu g/\text{ creatinine})</th>
<th>RBP (\mu g/\text{ creatinine})</th>
<th>ALB (\mu g/\text{ creatinine})</th>
<th>8-OHdG (\mu g/\text{ creatinine})</th>
<th>MDA (\mu mol/\text{ creatinine})</th>
</tr>
</thead>
<tbody>
<tr>
<td>UHg-C (\mu g/\text{ creatinine})</td>
<td>0.545 (0)*</td>
<td>0.281 (0)*</td>
<td>0.548 (0)*</td>
<td>0.086 (0.253)*</td>
<td>0.039 (0.624)*</td>
<td>0.330 (0)*</td>
<td>0.466 (0)*</td>
</tr>
<tr>
<td>Age (years)</td>
<td>-0.115 (0.125)*</td>
<td>-0.053 (0.479)*</td>
<td>-0.141 (0.066)*</td>
<td>-0.118 (0.116)*</td>
<td>-0.249 (0.001)*</td>
<td>-0.021 (0.781)*</td>
<td>-0.161 (0.032)*</td>
</tr>
<tr>
<td>BMI percentiles (5th/5th–85th; ≥85th)</td>
<td>0.351 (0.704)*</td>
<td>0.450 (0.639)*</td>
<td>0.486 (0.616)*</td>
<td>3.777 (0.025)*</td>
<td>0.303 (0.739)*</td>
<td>0.170 (0.844)*</td>
<td>1.022 (0.362)*</td>
</tr>
<tr>
<td>The presence of dental amalgam fillings (yes/no)</td>
<td>-2.706 (0.008)*</td>
<td>2.919 (0.004)*</td>
<td>-1.125 (0.263)*</td>
<td>0.918 (0.360)*</td>
<td>-0.246 (0.806)*</td>
<td>4.131 (0)*</td>
<td>-1.893 (0.061)*</td>
</tr>
<tr>
<td>Number of dental fillings</td>
<td>0.126 (0.200)*</td>
<td>-0.004 (0.626)*</td>
<td>-0.048 (0.626)*</td>
<td>0.107 (0.278)*</td>
<td>0.115 (0.287)*</td>
<td>0.111 (0.259)*</td>
<td>0.055 (0.576)*</td>
</tr>
<tr>
<td>Gender (female/male)</td>
<td>1.364 (0.175)*</td>
<td>-0.085 (0.933)*</td>
<td>0.601 (0.549)*</td>
<td>2.601 (0.01)*</td>
<td>3.806 (0)*</td>
<td>2.843 (0.005)*</td>
<td>0.109 (0.914)*</td>
</tr>
</tbody>
</table>

*Pearson’s correlation analysis.

ANOVA.

Student’s t-test.
mean and median UHg-C found in children with dental amalgam fillings were 3.739 and 2.943 μg/g creatinine, respectively, which were much higher than the median value of 1.4 μg/g creatinine and the mean value of 0.9 μg/g creatinine reported by Ye et al. (2009) and Barregard et al. (2008), respectively. Both groups of authors found no evidence of adverse effects on renal tubular function. Similar results were reported by others (Bellinger et al., 2006; Barregard et al., 2008; Woods et al., 2008). Various biomarkers of renal dysfunction have been recommended over the years as predictive of renal damage due to heavy-metal exposure (Roels et al., 1999). Results, though, still seem inconsistent with respect to their sensitivity. Excretion of NAG is considered an early sensitive biomarker of renal tubular cell injury caused by Hg and other heavy metals (Goyer, 1990; Kawada, 1995). Elevated NAG excretion was seen only at higher urinary Hg in workers of 35 μg/g creatinine (Barregard et al., 1988), 25.4 μg/g creatinine (Langworth et al., 1992), >17.3 μg/g creatinine (Jarosikiska et al., 2008) and 10 μg/g creatinine (Ellingsen et al., 2000). It has been proposed that an increase in urinary NAG is seen usually at higher Hg exposures, close to the levels found in occupationally exposed subjects (Barregard et al., 2008) or in children who had amalgam fillings for only a short time (Ye et al., 2009). In contrast to Mortada et al. (2002) who demonstrated an association between the urinary excretion of NAG and ALB with the number of fillings in adults, none of the tested biomarkers in the present study were related. This might be explained by the fact that children usually have fewer dental amalgam fillings than adults. The majority of children (81%) in the present study had less than 5 amalgam fillings. However, Maserejian et al. (2008) proposed that counting the number of filled surfaces rather than filled teeth can capture the effect of cumulative Hg exposure in children over time. Yet the authors recommended that simple counts of current amalgam fillings can be an adequate predictor of amalgam-related current urinary Hg. Nevertheless, no relationship was detected in the present study between the number of dental amalgam fillings and urinary Hg. Our findings closely match those of Khordi-Mood et al. (2001) and Ye et al. (2009).

Results of multiple regression analysis revealed that both the presence of dental amalgam fillings and the UHg-C levels contributed significantly to higher urinary excretion of NAG. However, they accounted for only 29.6% and 1.7% (adjusted R²), respectively, of the variation in the excretion of urinary NAG. This may suggest that the increase in urinary NAG levels due to Hg exposure does not all come from the presence of dental amalgam fillings, but exposure to other sources might have also contributed to the body’s total Hg burden. By using Dunnett’s post hoc test, the lowest UHg-C concentration for which there was a significant observed effect on urinary NAG was ≥1.452 μg/g creatinine, reaching a plateau at a level higher than 4.745 μg/g creatinine. Although this value
is much lower than Hg levels (10–35 μg/g creatinine) found in occupationally exposed adults with renal tubular damage. De Burbure et al. (2006) reported evidence of tubular dysfunction at much lower levels of UHg (0.06 μg/g creatinine) in children, after adjusting for blood lead and urinary cadmium. The possibility of concurrent exposure to other nephrotoxic metals such as lead and cadmium in our study was not taken into account. Nordberg (2010) found that combined exposure, with increased levels of arsenic and cadmium in urine, caused considerably higher biomarker values of renal tubular damage (NAG and β2-MG).

It seems that children's kidneys could be more sensitive to Hg than those of adults. Despite the low effect of Hg on NAG observed in this study and others (De Burbure et al., 2006), there is a need to establish a threshold taking into consideration children and special-risk populations. A urinary Hg value of 35 μg/g creatinine has been proposed for occupationally exposed adults as a biological threshold limit for effects on kidneys (ACGIH, 2007) and a background level of 5 μg/g creatinine in unexposed populations (WHO, 2003). On the other hand, there is no information on background Hg exposure in children. Evaluating our data based on the adult's reference limit for urinary Hg of 5 μg/g creatinine, there was 22.6% and 19.7% of tested children with and without dental amalgam respectively above this value. It is actually rather disturbing to see high percentages of children in both groups that were exposed to Hg. Children who are exposed to environmental pollutants during their growth and development are more susceptible to permanent impairment (Jarup, 2003). High levels of Hg exposure might cause subtle effects on the children's renal system (Martin and Woods, 2006).

Skálová and Chladek (2004) established a pediatric reference range for urinary NAG. The authors estimated upper reference values (95th percentiles) of 6.11 units/g creatinine and 4.68 units/g creatinine for age groups 6–10 and 10–18 years, respectively. Taking the average of the two values in order to have it within our children's age range, we found 70.8% and 43.4% of children with and without dental amalgam fillings, respectively, had urinary NAG > 5.4 units/g creatinine. This increase in both groups might suggest an early renal tubular dysfunction due to Hg exposure or other nephrotoxic chemicals, but further studies are necessary to confirm this.

The present study revealed an inverse link between urinary α1-MG and the presence of dental amalgam fillings. Children without amalgam fillings had significantly higher urinary α1-MG than those with fillings (P = 0). On the other hand, an increase in urinary α1-MG was associated with levels of UHg-C that could be indicative of early tubular damage. It is unclear whether exposure to other Hg sources might be the reason for elevated urinary α1-MG excretion in children without dental amalgam. However, excreted urinary α1-MG levels were tested among UHg-C quartiles, no dose–effect relationship was seen. This might be the case if the extent of exposure is low, as usually expected in the general population. Induced renal tubular dysfunction usually develops in a dose-dependent pattern based on the extent of exposure to heavy metals (Thomas et al., 2009). Bernard (2004) reported that the critical effect of cadmium that leads to a decline in renal function is around 10 μg/g creatinine in both occupationally exposed and general populations. There is no clear cut-off value for urinary α1-MG. Jarositsiksa et al. (2008) reported an increase in urinary α1-MG in highly exposed workers with UHg-C > 35 μg/g creatinine. Yu et al. (1983) suggested proximal tubular dysfunction in children with α1-MG > 15 mg/g creatinine. None of the studied children had urinary α1-MG equal to or above this value. Hjorth et al. (2000) established an upper reference limit of 7 mg/g creatinine for children between the ages of 1 month and 15 years. Eight children (4.4%) had urinary α1-MG levels above this reference value that might disturb proximal tubular function. Only one of these children had dental amalgam fillings. As it is known, α1-MG has been used increasingly as a biomarker for the diagnosis, monitoring and early detection of tubular disorders such as heavy-metal intoxications, diabetic nephropathy, urinary outflow disorders and pyelonephritis (Penders and Delanghe, 2004). It has also been proven to be a promising biomarker of cadmium-induced tubular dysfunction, with a cut-off value of 5 mg/g creatinine (Moriguchi et al., 2004). The same researchers, however, pointed out in a recent paper that NAG is more sensitive than α1-MG or β2-MG for monitoring cadmium exposure-related tubular effects in the general population (Moriguchi et al., 2009) due to the difference in their excretion mechanisms. Pless-Mulloli, et al. (1998) found that urinary α1-MG is an unsuitable biomarker for renal dysfunction induced by cadmium in epidemiological studies because other environmental pollutants can have an influence on its excretion. Moreover, some researchers have reported other factors that should be taken into consideration, such as age, gender, and diurnal variation, in the interpretation of urinary markers (Trachtenberg and Barregard, 2007; Andersson et al., 2008). Lehrnbecher et al. (1998) found a drop in urinary α1-MG excretion with age that might be related to the maturity of the renal system. In this study, there was a marginally negative correlation between age and urinary α1-MG (r = −0.141, P = 0.066), indicating that it might not have an impact on the results because of the relatively narrow age range.

Chen et al. (2005) reported that high exposure to Hg can induce oxidative damage to DNA, measured as urinary 8-OhdG. In the present study, urinary 8-OhdG levels found in children without dental amalgam fillings (median value: 20.189 μg/g creatinine) was significantly higher than those with fillings (15.420 μg/g creatinine). These values were much higher than the median value of 4.45 μg/g creatinine reported by Mori et al. (2011) in children (aged 3–6 years). Of course, we should consider that Mori et al. used HPLC analysis. Breton et al. (2003) reported that results are usually overestimated.
using ELISAs due to the lack of specificity of the antibodies. Shimoi et al. (2002) found 2-fold increases in ELISA results in comparison to those from HPLC, but for an unknown reason, 10% of the urine samples had more than a 4-fold increase in values using ELISAs, with a correlation of 0.460. Some authors, though, found a correlation of 0.88 (Yoshida et al., 2002) between the two methods. Despite the reliability of the HPLC method, many researchers find ELISAs simple and cost-effective, especially in human monitoring studies (Chiou et al., 2003; Wu et al., 2004). On the other hand, our results are still higher than those of Wong et al. (2005) who used the ELISA method and reported a median value of 11.7 μg/g creatinine in children between the ages of 10 and 12 years.

Multiple regression analysis revealed that urinary 8-OHdG did not increase in children with dental amalgam fillings but was significantly influenced by UHg-C levels. Evidence for an interaction between UHg-C and dental amalgam filling, though, was also seen. This may suggest that the presence of dental amalgam fillings had partly contributed to elevated urinary 8-OHdG levels. UHg-C showed a strong association with 8-OHdG, with a clear dose-effect relationship that mostly associated with long-term exposure to low Hg levels. A Dunnett’s post hoc test was used to further analyze mean changes in urinary 8-OHdG for each UHg-C quartile compared to the first. There were significant differences of 0.371, 0.692 and 0.983 μg/g creatinine in urinary 8-OHdG levels between the second, third and fourth UHg-C quartiles and the first, respectively. Our findings support the notion that oxidative DNA damage in children might be due to Hg exposure that comes partly from dental amalgam and other sources in a complex manner. However, the inverse relationship between urinary 8-OHdG and the presence of dental amalgam remains persistent. A possible hypothesis for such an inverse relationship is that the presence of dental amalgam fillings does not increase DNA damage but rather reduces its capacity for repair, leading to a decrease in DNA-repair products excreted in urine compared to those without dental amalgam. Although urinary 8-OHdG is often considered a marker of oxidative damage, it can also reflect repair mechanisms (Wu et al., 2004; Kim et al., 2004; Chen et al., 2005). We also should not rule out the possibility that urinary 8-OHdG levels may be confounded by a variety of factors, such as pollutants or physiological, nutritional, lifestyle or socioeconomic status (Chen et al., 2005; Wong et al., 2005; Mori et al., 2011) that were not taken into account in this work. Concern has also been raised regarding the validity of single assessment of 8-OHdG levels. Although many studies suggest that urinary 8-OHdG level is stable over time (Miwa et al., 2004), it exhibits intra-individual variations (Breton et al., 2007) and multiple assessments are highly recommended.

MDA is another biomarker of oxidative stress that usually complements 8-OHdG. In the present study, we found a significant correlation only between MDA and 8-OHdG (r = 0.301, P = 0.01) in children without dental amalgam fillings. This suggests an effect of lipid peroxidation and DNA damage that were both likely induced by Hg exposure, as evidenced by the significant relationship between UHg-C and MDA (r = 0.661, P = 0.001) and 8-OHdG (r = 0.367, P = 0.001). On the other hand, in children with dental amalgam fillings, UHg-C was correlated with 8-OHdG (r = 0.523, P = 0.012). Besides, MDA and 8-OHdG were not correlated (r = 0.074, P = 0.45). Accordingly, it is reasonable again to speculate that the capacity for repair of oxidized DNA between the two groups is different. None of these associations have been previously assessed, and therefore, no data are available for comparison. Furthermore, the validity of MDA has been criticized for the lack of specificity and its role in the pathogenesis of many health and physiological conditions (Del Rio et al., 2005; Lykkjesfeldt 2007).

Oxidative stress has been suggested to play a role in the early process of tubular damage in the kidney induced by heavy metals (Huang et al., 2009). The role of oxidative-stress indices (MDA and 8-OHdG) in increasing urinary NAG excretion was tested in this study. Upon the simultaneous addition of MDA and 8-OHdG to the multiple regression model, both dental amalgam fillings (β = 0.161, P = 0.027) and UHg-C (β = 0.233, P = 0) remained predictors of the urinary NAG levels. Urinary MDA levels were significantly associated with urinary NAG (β = 0.516, P = 0), and 8-OHdG was correlated with urinary NAG, but with marginal significance (β = 0.134, P = 0.078). The two biomarkers seem to behave quite differently with respect to their excretion in urine, and perhaps we can again assume the involvement of DNA repair mechanisms. In support of this point, no correlation was found between urinary MDA and 8-OHdG (P = 0.168). More importantly, an interaction between UHg-C and MDA was evident (P = 0.003). This observation suggests that exposure to Hg from dental amalgam fillings may play a role in inducing renal damage through oxidative-stress mechanisms.

Finally, this study has a number of limitations that should be acknowledged. First, limited dental records were collected. Second, some children had amalgam fillings for only a short period (17% less than 12 months), thus nephrotoxic effect due to low Hg might not have been noticeable yet. Third, concern should be also raised regarding the specificity of ELISA method used to measure 8-OHdG in comparison to HPLC. Fourth, data on socioeconomic status was not collected, which might significantly affect the utilization of dental care. Bates (2006) suggested that people of higher socio-economic status usually have better dental treatment access and, therefore, may have more amalgam fillings. Fifth, exposure to other nephrotoxic heavy metals such as lead and cadmium that can be potential confounders for our study was not taken into account.

5. Conclusion

Our data provide evidence that low exposure to Hg from dental amalgam fillings exerts an effect on kidney tubular functions in children. Oxidative stress may have played a role in this mechanism. Although the oxidative properties of Hg have been studied, the actual mechanism of inducing oxidative stress to renal tubules is still unclear. The results of this study would also suggest that urinary NAG is the most sensitive of all the investigated renal biomarkers. These results should be confirmed with further investigation.

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