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Fluoride increases lead concentrations in whole blood and in calcified tissues from lead-exposed rats

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ABSTRACT

Higher blood lead (BPb) levels have been reported in children living in communities that receive fluoride-treated water. Here, we examined whether fluoride co-administered with lead increases BPb and lead concentrations in calcified tissues in Wistar rats exposed to this metal from the beginning of gestation. We exposed female rats and their offspring to control water (Control Group), 100 mg/L of fluoride (F Group), 30 mg/L of lead (Pb Group), or 100 mg/L of fluoride and 30 mg/L of lead (F + Pb Group) from 1 week prior to mating until offspring was 81 days old. Blood and calcified tissues (enamel, dentine, and bone) were harvested at day 81 for lead and fluoride analyses. Higher BPb concentrations were found in the F + Pb Group compared with the Pb Group (76.7 ± 11.0 μg/dL vs. 22.6 ± 8.5 μg/dL, respectively; $p < 0.001$). Two- to threefold higher lead concentrations were found in the calcified tissues in the F + Pb Group compared with the Pb Group (all $p < 0.001$). Fluoride concentrations were similar in the F and in the F + Pb Groups. These findings show that fluoride consistently increases BPb and calcified tissues Pb concentrations in animals exposed to low levels of lead and suggest that a biological effect not yet recognized may underlie the epidemiological association between increased BPb lead levels in children living in water-fluoridated communities.

1. Introduction

Low-level environmental exposure to lead has been associated with subclinical deficit in neurocognitive function in young children (Needleman et al., 1979) and in adolescents (Needleman et al., 1990), as well as with numerous other conditions prevalent in industrialized societies, such as attention deficit hyperactivity disorder (ADHD) (Braun et al., 2006), delinquent behavior (Dietrich et al., 1991, 2001; Needleman et al., 2002; Wright et al., 2008), hearing impairment (Rothenberg et al., 2000), spontaneous abortions (Borja-Aburto et al., 1999), periodontal disease (Saraiva et al., 2007), decreased renal function (Muntner et al., 2003), and hypertension (Hu et al., 1996). Interestingly, a strong association has been described between preschool blood lead levels and crime rates in many countries (Nevin, 2007). Together, these findings indicate that low-level exposure to lead is still a matter of concern.

Lead enters the human body by inhalation or by gastrointestinal absorption, and a single dose remains in the blood for a short time (35 days) (Rabinowitz, 1976). Thereafter, lead is stored in calcified tissues (Barbosa et al., 2005), thus allowing primary teeth dentine to be used to assess subclinical past exposure to lead (Needleman et al., 1972), as it was done in the seminal studies which showed an association between exposure to lead and adverse effects to children's intelligence (Needleman et al., 1979, 1990). Since then, prospective studies that collected blood at birth and in the first school years showed that early exposure of children to lead results in lower IQ scores (Dietrich et al., 1991, 1993; Bellinger et al., 1992). In this regard, while the U.S. Centers for Disease Control and Prevention (CDC) established in 1991 a blood lead (BPb) concentration of 10 μg/dL as a concentration that should prompt public health actions (U.S. CDC, 1991), there is now clear evidence that BPb concentrations < 10 μg/dL cause cognitive impairment (Lanphear et al., 2000; Canfield et al., 2003; Hu et al., 2006). Indeed, BPb as low as 2.5 μg/dL reduces a child's IQ measurably (Lanphear et al., 2000), and there is no evidence of a threshold under which lead levels appear to be safe in terms of neurobehavioral outcomes (Chiodo et

Abbreviations: BPb, whole blood lead; FSA (H_2SiF_6), fluosilicic acid; ICP-MS, Inductively Coupled Plasma Mass Spectrometry; SiF, silicofluoride; TISAB, total ionic strength adjustment buffer.

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al., 2007). Therefore, any factor that could increase BPb levels must be seriously considered.

Two studies have reported an association between the use of silicofluoride (SiF)-treated community water and increased BPb concentrations among children (Masters and Coplan, 1999; Masters et al., 2000). Masters et al. (2000) showed this association after studying data from 151,225 children (0–6 years) that lived in the State of New York, USA. Curiously, they found the highest likelihood of having BPb > 10 (μg/dL) in children exposed to SiF-treated water and to another risk factor associated with high BPb. In line with these findings, Macek et al. (2006) studied 9477 individuals (1–16 years) and showed a statistical interaction between the water fluoridation method and BPb levels when the individuals lived in old houses (built before 1946 or of unknown age), which is a risk factor for increased lead exposure. Taking into consideration that SiF is used to provide fluoride to >90% of fluoridated water in the U.S. (Maas et al., 2007) and in many other countries (Canadian Cancer Society, 2009; Australian Drinking Water Guidelines, 2004), the possible increases in BPb associated with SiF should be better studied.

Lead and fluoride are elements with high affinity to bone, and they might have an effect on each other's absorption, metabolism, and accumulation. Therefore, this study aimed at testing whether the administration of fluosilicic acid (H_2SiF_6) could increase BPb and mineralized tissue lead concentrations in rats exposed to low levels of lead from the beginning of gestation.

2. Materials and methods

2.1. Animals

This study was approved by the Ethical Committee for Animal Research of the University of Sao Paulo/Campus of Ribeirao Preto (Protocol 07.1.346.53.3), and complied with the guidelines established by this Committee. Animals were handled humanely, and in accordance with the guiding principles published by the National Institutes of Health. Twenty-eight Wistar rats (190–210 g, 24 females and 4 males) were obtained from the University's colony. The rats were randomly divided into 4 groups of 6 females and 1 male, according to the amount of fluoride and lead in drinking water. Mating began at the same time that the animals received different water treatments, and lasted for 1–2 weeks maximally.

Food was provided ad libitum, and animals were maintained under 12-h light/dark cycle. Rat chow, as pellets, was purchased from Nuvital (Nuvilab CR-1, Colombo, PR, Brazil). Pb and F concentrations did not exceed 0.05 mg/kg, according to the manufacturer. Animal room temperature and humidity were 24–26 °C, and 40–60%, respectively. Maximally 12 offspring were housed per cage until weaning, and 4 animals were housed per cage after weaning. Plastic cages with fitted stainless steel wire lids, whose dimensions were 41 cm × 34 cm × 16 cm (height), giving 1394 cm^2 floor space. All rats were checked daily for health, feed and water, and clean cages. Rooms were stocked with in-date supplies. Water was provided ad libitum via glass bottle, stopper and sipper tube. Three times a week bottles were cleaned with soap and copious washing with tap water.

Control animals received water containing maximally 0.1 mg/L of F and 0.5 μg/L of lead. Animals in the Fluoride group (F Group) received water containing 100 mg/L of fluoride as fluosilicic acid (H_2SiF_6). Animals in the group exposed to lead (Pb Group) received water containing 30 mg/L of lead as lead acetate ($Pb(CH_3COO)_2{\cdot}3H_2O$). Animals co-exposed to fluoride and lead (F+Pb Group) received water containing both 100 mg/L of fluoride as fluosilicic acid and 30 mg/L of lead as lead acetate.

When the females were pregnant, they were separated from the males, and housed in a separate cage until the end of the weaning period. Offspring was born 4–5 weeks after the beginning of the experiment. After the rats were weaned, they received the same drinking water solution that was given to their mothers. At 81 days of age, the rats were anesthetized with ketamine 100 mg/kg and xylazine 10 mg/kg i.p. and blood samples were collected by cardiac puncture. Thereafter, the rats were euthanized with an anesthetic overdose and both femurs, and both lower and upper incisors from each animal were collected post-mortem for lead and fluoride analysis. Blood and all tissues were harvested in trace element-free tubes and stored at −20 °C until use. Ten female rats from each group were used for the measurement of BPb and mineralized tissue Pb and F concentrations.

2.2. Pre-analytical measures

High purity deionized water (resistivity 18.2 MΩ cm) obtained by a Milli-Q water purification system (Millipore, Bedford, MA, USA) was used throughout. All employed reagents were of high purity analytical grade. HNO_3 (from Synth, Diadema, SP, Brazil) was previously purified in a quartz sub-boiling still (Kürner Analysentechnik, Rosenheim, Germany) before use. All transfer pipette tips, centrifuge tubes, plastic bottles, autosampler cups, and glassware materials were cleaned by soaking in 10% (v/v) HNO_3 for 24 h, rinsing 5 times with Milli-Q water, and drying in a laminar flow hood (class 100).

2.3. Surface bone and whole bone samples

One of the femurs from each animal was used to obtain a surface bone sample. A dry femur was maintained for 1 min (half submerged) in 3 mL of 2% HNO_3 (ultrapure grade). One hundred microlitres of this solution were used for fluoride determination, while 60 μL was further diluted into 2 mL for lead and phosphorus determinations.

The other femur from each animal was digested overnight with 6 mL of 65% HNO_3 (ultrapure grade) at room temperature. One hundred microlitres of this solution were used for fluoride determination, while 60 μL was further diluted into 5 mL for lead and phosphorus determinations.

2.4. Tooth samples

The enamel biopsy was performed in a 0.5-mL Eppendorf tube containing 500 μL of 1.8% HNO_3 (v/v). The labial face of the incisal third of the lower incisor was maintained in contact with the acid for 20 s. One hundred microlitres of this solution were used for fluoride determination, while 60 μL was further diluted into 2 mL for lead and phosphorus determinations.

A dentine fragment was obtained from the cervical lingual third of tooth. This fragment was devoid of enamel, since continually growing rat incisors have only enamel on their labial face (Porto et al., 2009). This dentine fragment was completely digested overnight in 1 mL of HNO_3 at 50% (v/v). One hundred microlitres of this solution were used for fluoride determination, while 60 μL was further diluted into 5 mL for lead and phosphorus determinations.

2.5. Phosphorus determination

The mass of calcified tissues was calculated based on the phosphorus concentration in the solution. Phosphorus was determined according to the Fiske and Subbarow method, as described by Costa de Almeida et al. (2007). In short, each enamel etch sample was assayed in triplicate, and reactions were loaded in ELISA plates for reading. Reaction mixtures consisted of 30 μL sample, 220 μL of ultrapurified water, and 50 μL of molybdic acid solution (ammonium molybdate at 2.5% (w/w) in 4N H_2SO_4), which were thoroughly vortexed. After 10 min, 20 μL of reducing agent was added, and the mixture was vortexed again. After 20 min, absorbance was measured at 660 nm. The plate reader was calibrated with standards containing known concentrations of phosphorus (1, 2, 4, and 8 mg/ml). Variation within triplicates ranged from 0.2% to 6.3%. The phosphate content in the solution was used to determine the total mass of enamel, dentine, and bone, assuming a phosphorus content of 17% for enamel (Halse and Selvig, 2007), 15.97% for dentine (Tjäderhane et al., 1995), and 13.5% for bone (Bezerra de Menezes et al., 2003). Please refer to Bezerra de Menezes et al. (2003) for details.

2.6. Lead determination

The whole blood samples were collected with metal-free syringes containing lyophilized heparin. Blood samples were frozen (−20 °C) until BPb analysis by ICP-MS (Inductively Coupled Plasma Mass Spectrometry) according to the method proposed by Palmer et al. (2006). Briefly, whole blood specimens were diluted 1 + 49 with a diluent solution containing 0.5% (v/v) double-distilled HNO_3, 25 g/L Rh, and 0.005% (v/v) Triton X-100. Calibration was performed against matrix-matching. The detection limit (DL) for lead was 0.05 μg/L. Quality control for lead determination was assured by analyzing Standard Reference Materials from the U.S. National Institute of Standards and Technologies (NIST 955c). In addition, various secondary reference materials provided either by the New York State Department of Health (NYSDOH Proficiency Testing Program for trace elements in whole blood) or by the Institut National de Santé Publique du Québec, Canada (INSP-external quality assessment scheme (EQAS) for trace elements in whole blood) were analyzed. Reference samples were analyzed before and after ten ordinary samples. Experimental values were always in good agreement with the provided reference or certified ranges. The results were expressed as μg Pb/dL of whole blood or as μg Pb/g of calcified tissue.

2.7. Fluoride determination

For the determination of fluoride, 100 μL of the dissolved samples were mixed with 900 μL of deionized water, and 1 mL of TISAB II (1.0 M of acetate buffer, pH 5.0 with 1.0 M NaCl, and 0.4% cyclohexanediaminotetraacetic acid). The samples were agitated at room temperature, and fluoride was determined in an ion analyzer (Orion EA-940) previously calibrated with a standard fluoride curve (0.5–5.0 μg/ml) prepared under the same conditions as the sample. The results were expressed as μg F/g of calcified tissue.

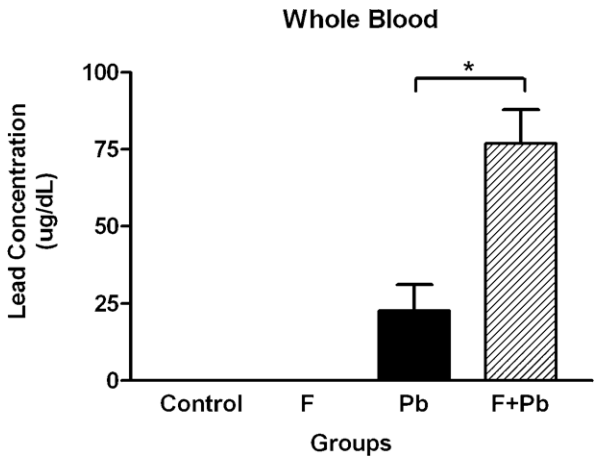

Fig. 1. Whole blood lead concentrations (μg/dL) in 81-day-old female rats exposed to lead and/or fluoride in the drinking water from the beginning of gestation. Control Group received control water, F Group received 100 mg/L of F, Pb Group received 30 mg/L of Pb, and F + Pb Group received 100 mg/L of F and 30 mg/L of Pb. *$p < 0.001$.

2.8. Statistical analysis

The results were analyzed for normality, and the data were normally distributed. Comparison between groups was performed using ANOVA, and a p value < 0.05 was accepted as significant. Differences between groups were analyzed using the Bonferroni Test, and differences were considered significant when a p value of 0.008 was reached, since 6 comparisons were performed.

3. Results

We found no bodyweight differences at the end of the study (data not shown). Fig. 1 shows 3.4-fold higher BPb concentrations in the F + Pb Group (76.7 ± 11.0 μg/dL) compared with the Pb Group (22.6 ± 8.5 μg/dL) ($p < 0.001$). BPb concentrations were below 0.5 μg/dL both in the Control and in the Fluoride groups (Fig. 1).

Fig. 2a–d displays lead concentrations in the calcified tissues, and shows higher lead levels in the F + Pb Group vs. the Pb Group for all tissues examined in this study. Higher superficial enamel lead concentrations were found in the (F + Pb) group compared to the Pb Group (4369 ± 1353 μg/g vs. 1768 ± 1892 μg/g, respectively) ($p < 0.001$), thus indicating a 2.5-fold increase in the amounts of lead in the F + Pb Group. Similarly, higher lead concentrations were found in the F + Pb Group compared with the Pb Group in dentine samples (8.5 ± 2.0 μg/g vs. 4.9 ± 1.7 μg/g, respectively; $p < 0.001$; Fig. 2b). Whole bone lead concentrations doubled in the F + Pb Group (14.2 ± 2.6 μg/g) compared with those found in the Pb Group (6.8 ± 1.7 μg/g) ($p < 0.001$). Lead concentrations in the bone surface were also 3 times higher in the F + Pb Group compared with those found in the Pb Group (28.0 ± 10.6 μg/g vs. 9.0 ± 3.7 μg/g, respectively; $p < 0.001$). The amount of bone dissolved by etching ranged from 1.5 to 10 mg, indicating that the superficial dissolution of bone (on average) ranged from 0.01 to 0.14 μm of superficial bone, with no difference among groups in the etching depth (not shown).

Dentine was the calcified tissue that exhibited the lowest lead concentrations found in this work. Dentine was followed by whole bone, and then by surface bone. The calcified tissues with similar amounts of minerals and with a collagenous organic matrix (dentin and bone) showed 5–9 μg/g of lead in the Pb Group, whereas the same tissues showed 8.5–28 μg/g of lead in the F + Pb Group. Enamel, on the other hand, which was sampled by a superficial etch technique, contained very high concentrations of lead, which reached approximately 500 times as much as those found in dentine in the F + Pb Group. Superficial enamel is known to accumulate

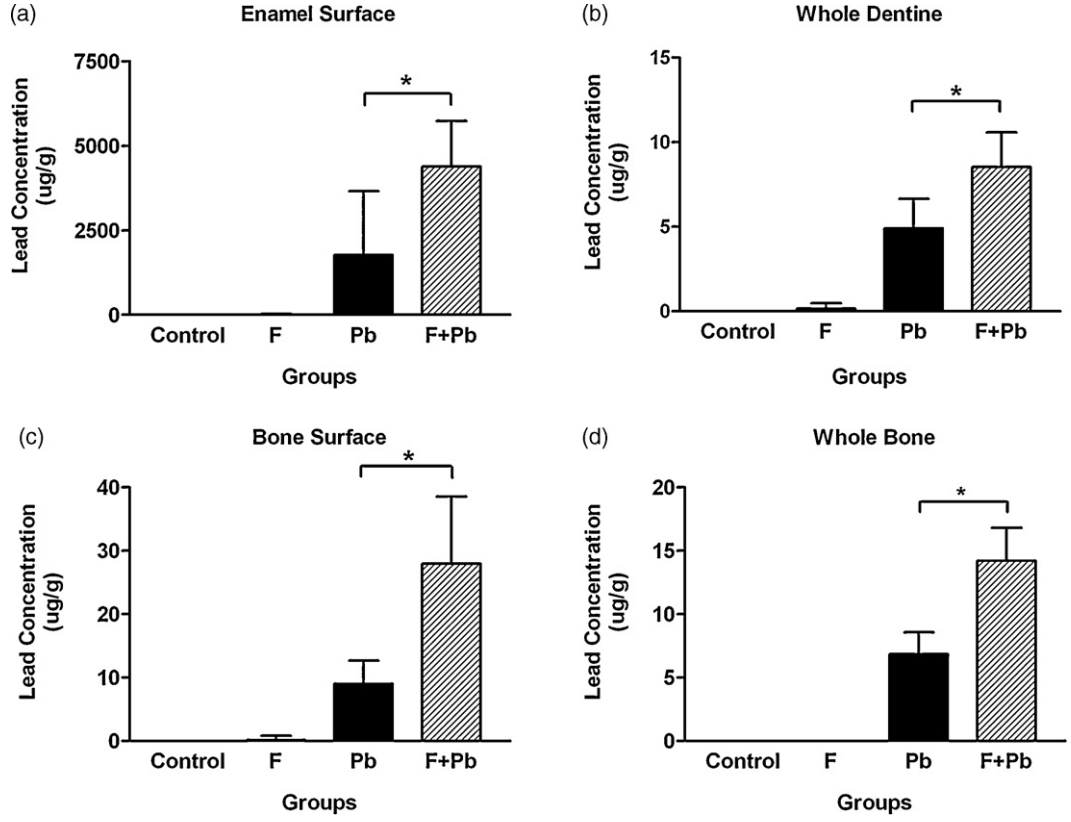

Fig. 2. Lead concentrations (μg/g) in (a) enamel surface, (b) dentine, (c) bone surface, and (d) whole bone from 81-day-old female rats exposed to lead and/or fluoride in the drinking water from the beginning of gestation. *$p < 0.001$.

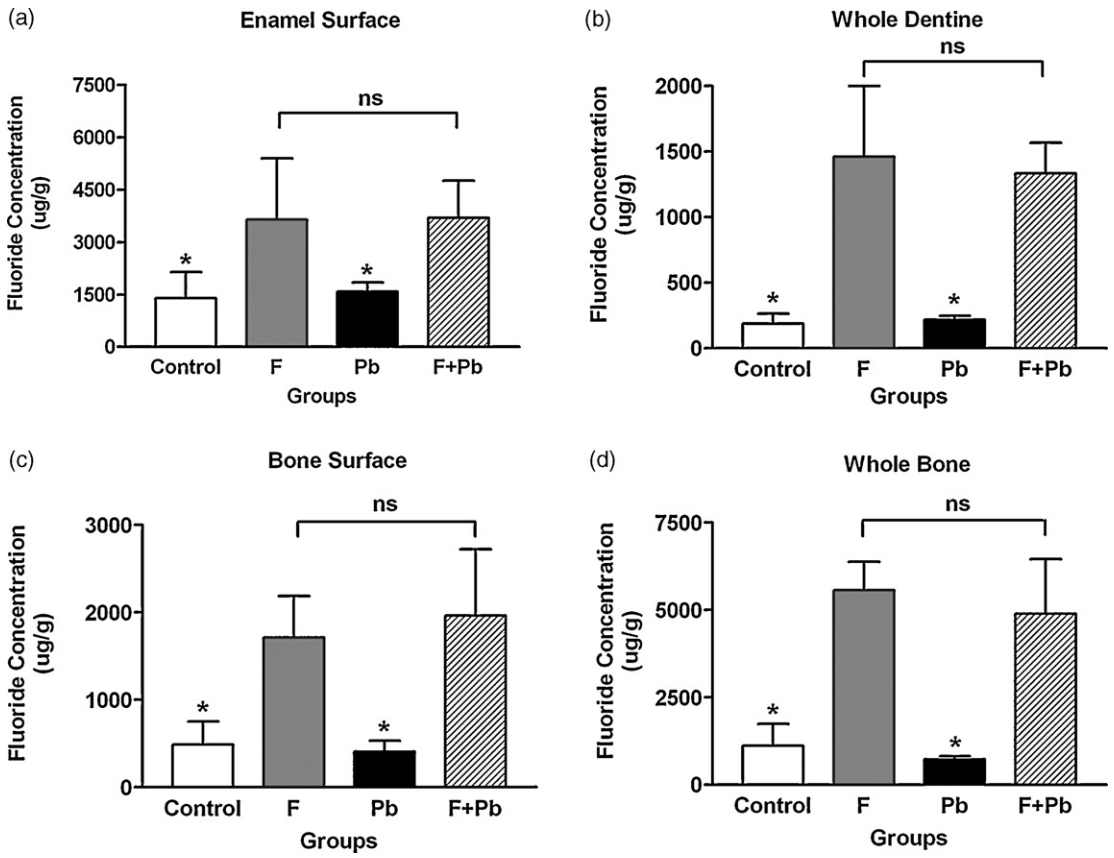

Fig. 3. Fluoride concentrations (μg/g) in (a) enamel surface, (b) dentine, (c) bone surface, and (d) whole bone from 81-day-old female rats exposed to lead and/or fluoride in the drinking water from the beginning of gestation. ns, non-significant. *$p < 0.001$ for comparisons between the F and the F + Pb Groups vs. Control or the Pb Group.

lead in the range of hundreds to thousands of μg/g of lead (Robinson et al., 1995; Gomes et al., 2004; Costa de Almeida et al., 2007).

As shown in Fig. 3, significantly higher fluoride concentrations were found in the F and F + Pb Groups as compared with those found in the Control or in the Pb Groups ($p < 0.001$ for all comparisons). However, no significant differences in the fluoride concentrations in the tested calcified tissues were found when the F and F + Pb Groups were compared ($p > 0.05$ for all comparisons).

4. Discussion

This study shows that co-exposure to fluoride and lead from the beginning of gestation consistently increases the concentrations of lead in whole blood and in calcified tissues of 81-day-old animals, with no changes in the concentrations of fluoride. Lead concentrations were found to be 2.5 times higher in the superficial enamel, 3 times higher in surface bone, 2 times higher in whole bone, and 1.7 times higher in the dentine when the animals were co-exposed to fluoride, thus indicating a consistent rise in the amounts of lead found in whole blood and calcified tissues in the F + Pb Group. This is the first study to show that fluoride affects lead concentrations during lead exposure, and our findings may have serious implications for populations exposed to increased amounts of both lead and fluoride, particularly young children.

Decreased learning ability and low hippocampus glutamate has been recently shown in offspring rats exposed to fluoride and lead (Niu et al., 2009)

Essential and toxic metal concentrations in the body are regulated by gastrointestinal absorption together with urinary excretion (Barbier et al., 2005). Lead (Pb^{2+}) and many other non-essential divalent metals are transported by a protein transporter known as divalent metal transporter 1 (DMT-1) (Garrick et al., 2003), which is expressed both at the intestinal brush border, and at the renal tissue (Barbier et al., 2005). Fluoride is known to inhibit enzymes (Marquis et al., 2003 discuss some examples of inhibition), and sodium fluoride (NaF) is a potent, rapid, and reversible activator of the regulatory heterotrimeric GTP-binding proteins in virtually all in vitro systems (Chabre, 1990). Thus, we speculate that the unknown mechanism that explains the increased lead levels found in the blood and in the calcified tissues may involve the effect of fluoride on the control of lead absorption in the intestine or excretion in the kidney. This effect may be direct, by a direct effect of fluoride on the DMT-1 protein, or indirect, since changes in iron metabolism, for example, will increase DMT-1 expression, thus increasing the absorption of toxic metals. It is of note that changes in the structure of DMT-1 induced by mutations have been shown to completely change the capability of this transporter to recognize metals, changing it from a major transporter of Fe into a Ca transporter (Xu et al., 2004). Therefore, further multidisciplinary physiological, biochemical, and molecular studies are needed to support our findings and provide mechanistic insight.

Lead and fluoride share a common distribution, at least in some calcified tissues such as dental enamel (Robinson et al., 1995). Lead shows a high degree of accumulation in the very first micrometers of superficial (outer) enamel, particularly in the first 6 μm in humans (Brudevold et al., 1975; Purchase and Fergusson, 1986; Cleymaet et al., 1991; Gomes et al., 2004; Costa de Almeida et al., 2007; De Almeida et al., 2008), which correspond to the same accumulation site for fluoride (Robinson et al., 1995). Studies using unerupted molars from rats clearly show that accumulation of lead in the superficial enamel is a pre-eruptive event (Arora et al., 2005, 2007). Therefore, this particular overlap in the distribution of both elements in dental enamel suggests a biological interaction between lead and fluoride, possibly involving the precipitation

chemistry of hydroxyapatite, which is known to be altered by fluoride (Aoba, 1997), and this is the basis for the use of fluoride in caries prevention. Precipitation of hydroxyapatite may be also altered by lead, which can replace calcium in the hydroxyapatite structure (Verbeeck et al., 1981). Finally, this much higher accumulation in superficial enamel is certainly linked to the high degree of calcification of this tissue and the distinct way enamel mineralizes. Mature enamel is the most calcified tissue in vertebrates, comprising 95% of mineral in weight and it mineralizes by acquiring ions across its surface for long periods of time (Smith, 1998). The way lead and fluoride accumulate in surface enamel suggests that they may interact directly or indirectly, possibly changing the amounts of calcium that are available. Such interactions may also account for the increased concentrations of lead found in the animals co-exposed to lead and fluoride.

The concentration of lead in drinking water used in the present study is considered a low concentration for rodents (Leasure et al., 2008). However, while the fluoride concentration used in the present could be considered relatively high for rodents (100 mg/L or ppm), this concentration was chosen because it produces plasma fluoride levels that are comparable with those commonly found in humans chronically exposed to 8 mg/L of fluoride in the drinking water, which is a concentration known to cause severe fluorosis. Since this study was based on a hypothesis derived from epidemiological evidence from thousands of children (that fluoride from the water might increase BPb levels), we felt that we had to maximize fluoride concentrations to observe its influence on lead levels in this proof-of-concept animal study. Although children are not chronically exposed to high concentrations of fluoride (100 ppm) by means of drinking/cooking water, children are frequently exposed to high levels of fluoride during their first years because of the many sources of fluoride available to them. Since fluoride is not considered a toxic agent, it is widely available through mouth rinses, toothpastes, tablets, besides the fluoride present in drinking water, beverages, and food. Indeed, the widespread presence of fluoride increased the prevalence of fluorosis in the USA (Pendrys, 2000) and in other countries (Leverett, 1986; Jackson et al., 1999; Tabari et al., 2000; Tsutsui et al., 2000; Pereira et al., 2000). Therefore, it is likely that young children may experience episodes of exposure to high levels of fluoride, which may cause their BPb levels to increase and produce more lead toxicity.

A reason for major concern is the fact that exposure to increased amounts of lead and fluoride occurs at about the same age (1–3 years). Some studies of fluorosis prevalence point to a higher degree of fluorosis in front teeth and first molars (Ismail et al., 1990), which is an indirect measure of dose that indicates that the children receive the highest fluoride doses when their front teeth and first molars mineralize (at ages 1–5 years). This is about the same time when BPb levels are the highest in children. In fact, the exposure of children to lead apparently peaks at 12–36 months of age, which is the time when toddlers experience prominent hand-to-mouth behavior (Binns et al., 2007). Therefore, this is a critical time when systemic exposure to fluoride should be minimized, since fluoride may increase lead accumulation, and any preventable exposure to lead should be avoided (Binns et al., 2007).

While the benefits of water fluoridation for caries prevention are unquestionable (Kumar and Moss, 2008), concerns have recently been raised regarding the association of fluoride in the drinking water with increased BPb lead in large populations (Masters and Coplan, 1999; Masters et al., 2000). Children living in communities using H_2SiF_6 (fluosilicic acid) to fluoridate the drinking water have the highest BPb levels (Masters et al., 2000). Consistent with these epidemiological findings, our results showed that fluoride (as H_2SiF_6) increases BPb levels and lead concentrations in calcified tissues. These findings suggest a possible biological effect of the co-exposure to lead and fluoride that deserves further studies using different doses and forms of fluoride to better characterize this effect. Furthermore, such studies are needed to established safety guidelines for the use of topical, mouth rinse of toothpaste fluoride for children under 5 years, since so far the fluoride doses of concern are based on the "probable toxic dose" (Shulman and Wells, 1997).

The concerns about the effects of fluoride may also apply to fluoride-polluted areas, where the exposure of the populations and animals to other toxic metals, such as lead, may be increased. In addition, it is possible that fluoride may affect the concentrations of other metals that are found in calcified tissues and share a similar distribution with fluoride and lead, such as zinc and cadmium (Cleymaet et al., 1991; Robinson et al., 1995). As pointed out by Bellinger (2004) "Co-exposure to other toxicants is another candidate explanation for individual differences in susceptibility (to lead), although greater attention has been paid to the potential of co-exposures to be confounders than to be effect modifiers".

In conclusion, this study showed that co-exposure to fluoride increases lead concentrations in the blood and in calcified tissues in animals exposed to lead from the beginning of gestation. These findings suggest that a biological effect not recognized so far may underlie the epidemiological association between increased BPb levels in children and water fluoridation.

Conflict of interest

None.

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