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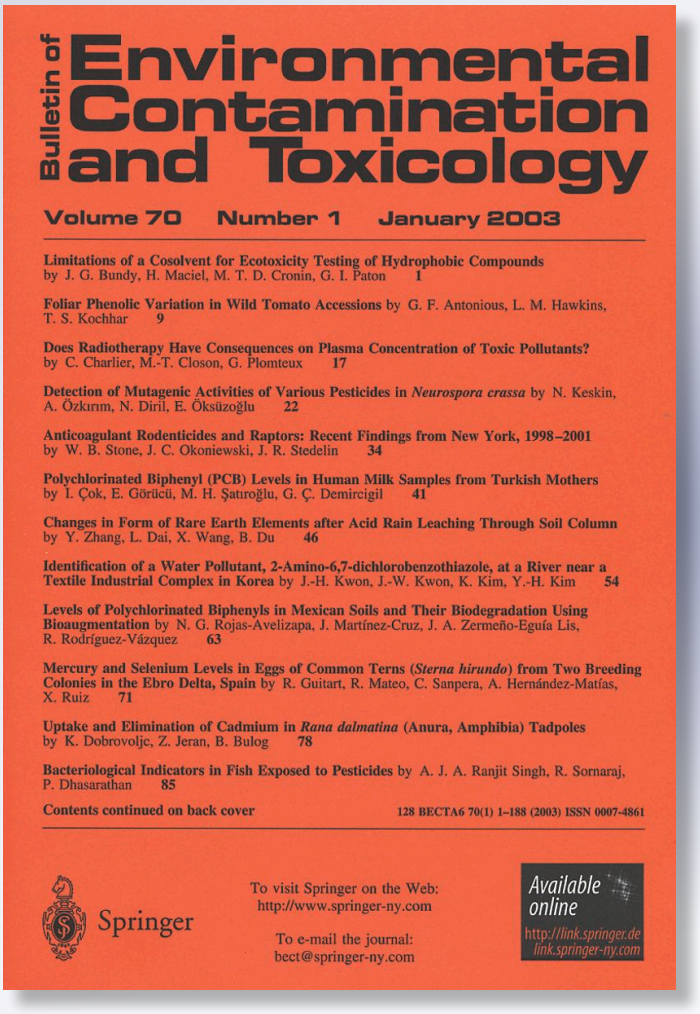

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Abstract The existence of children living at high altitude suffering from lead (Pb) poisoning prompted us to investigate the long term effects of this pollutant on growth and bone biology in growing rats maintained at simulated high altitude (SHA). Pb and hypoxia (HX) significantly reduced body weight (−9.4 % and −24 %; $p < 0.01$) and length (−3 % and −8 %; $p < 0.01$); decreased femoral ultimate load (−16 % and −40 %; $p < 0.01$) and femoral energy absorption capacity (−18 % and −74 %; $p < 0.01$). Oral pathologic alterations were observed in experimental groups. Our findings revealed growth retardation and damages on femoral and mandibular bones that predispose to fractures.

Keywords Lead poisoning · Simulated high altitude · Rats

The increase in mining activities has contributed to the fact that over 100 million people is now living at an altitude of over 2,500 m frequently exposed to environmental pollutants. Among these, lead (Pb) is of particular interest because of its wide distribution in the environment. Some of the highest altitude cities in the world such as La Oroya, located at 3,745 m or Cerro de Pasco at 4,660 m share a significant number of children living in an enclave with widespread environmental Pb contamination (Counter et al. 2007). Drinking water and soil that contains particulate lead has been shown to be significantly hazardous for children, who are more commonly exposed by ingestion of house dust or soil (Lyn Patrick 2006). Lead exposure occurs mainly through the respiratory and gastrointestinal tracts and infants can absorb into the bloodstream up to 50 % of the lead ingested. Once absorbed it is transported by blood to soft tissues and is finally deposited in bones and teeth throughout life. During infancy and childhood, lead is stored in trabecular bone because it is the most active site of remodeling; whereas, in adulthood lead is deposited in both trabecular and cortical bone (Aufderheide and Wittmers 1992). It was demonstrated that long-term effects of lead poisoning in living species induced a reduction in bone mineralization (Gangoso et al. 2009). Previously reported studies from this laboratory (Martínez et al. 2011) suggest that chronic intoxication with an environmental pollutant such as aluminum in immature rats exposed to simulated high altitude (SHA) induced negative effects on femoral material quality. When adult female rats are exposed to SHA, an increase in erythroid precursor cells occurs, leading to hypertrophy of the erythropoietic marrow and negative effects on bone material quality (Bozzini et al. 2009).

The existence of children living in Pb contaminated high altitude areas aimed us to design this study to evaluate the long-term effects of lead poisoning on growth and bone biology in rats maintained at simulated high altitude.

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The studies were performed in two different kinds of bones, femurs and mandibles, as models of axial and appendicular skeleton. The results will try to cover the lack of information regarding the effects of this environmental agent on bone tissue under hypobaric conditions as an aid for potential human health extrapolation.

Materials and Methods

Female Wistar growing rats, aged 21 days, were used throughout the experiments. They were housed in stainless-steel cages and maintained under local vivarium conditions (temperature 22–23°C, 12-h on/off light cycle). All animals were allowed free access to water and a standard pelleted chow diet. Rats were randomly divided into 4 groups of 15 animals each. Pb intoxication was induced in experimental groups through administration of 1,000 ppm of lead acetate in drinking water for 90 days (Hamilton and O'Flaherty 1994). Control animals received equivalent acetate, as sodium acetate, added to tap water. A control group and a Pb-treated group were maintained at normoxic (NX) ambient pressure (CNX and PbNX respectively). Further control and Pb-treated animals were exposed to hypobaric hypoxia (HX), 18 h/day during the whole experimental period (CHX and PbHX respectively) by placing the animals into a SHA chamber in which air pressure was maintained at 506 mbar and allowing the same on/off light cycle than those animals in NX conditions (Bozzini et al. 1997). All animals were treated in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals (NIH 85-23, revised in 1985), and protocols were approved by the Ethical Commission of the Faculty of Dentistry, University of Buenos Aires. Body weight and length were registered at the beginning of the experiment (day 0) and once a week during the experimental period. Body length was determined by measuring the distance between the nose tip and the last hairs of the tail's base with a scaled ruler. At the end of the experimental period, blood samples where obtained by cardiac puncture to asses hematocrit and lead content. Animals were euthanized, weighed and measured. Femurs and mandibles were properly dissected to perform anthropometric and mechanical studies. Body weight and length were registered at the beginning of the experiment (day 0) and once a week during the whole experimental period. Bones were weighed and measured with a digital sliding caliper. The hematocrit values were determined by micromethod. Pb content in blood was determined using an atomic absorption spectrophotometer Varian AA 475 (Roses et al. 1989). Mechanical properties of femurs and hemimandibles of each animal were determined by a three-point bending test on an Instron Universal Testing Machine Model 4442; Canton, MA, USA. The load was applied perpendicularly to the long axis of the bone at a 5 mm/min speed until fracture (Fig. 1 left) so as to obtain the load/deflection (W/d) curves showing the elastic and plastic phases, separated by the yielding point (Fig. 1 right). From these curves, the structural whole-bone properties, maximal load supported at fracture (Wf, ultimate load) and energy absorption capacity by the whole bone (EAC) were measured (Cointry et al. 2005). After mechanical testing, femurs and hemimandibles were dissecated for bone ash determination in a muffle furnace at 600°C for 18 h. Pb content in these ashes was determined by atomic absorption as detailed above. Data were analyzed by one-way analysis of variance (ANOVA), followed by Student–Newman–Keuls Multiple Comparison Test. Analyses were performed using the Software package Instat and Prism V.3 (GraphPad Software Inc., San Diego USA). A p value less than 0.01 was considered statistical significant.

Results and Discussion

Results showing the changes in anthropometry measurements, lead content, hematological parameters and whole bone structural properties are shown in Table 1. Exposure of rats to SHA produced an expected significant increase in hematocrit values in CHX group at the end of the experimental period showing an enhanced rate of erythropoiesis. The effects of the anemia due to the lead intoxication resulted in less increase in the hematocrit values in PbHX group. Significantly high-level lead accumulation was observed in ashes from both kinds of bones in PbNX and PbHX groups indicating that the administered Pb was deposited in the skeleton in significant amounts. Treatments clearly influenced body weight and length, both serving as indexes of somatic growth (Fig. 2). Like body size, lead administration significantly depressed mandibular and femoral weight and length at the end of the experimental period. Long-term exposure to SHA reduced further more those parameters. Growth retardation associated with exposure to SHA and lead intoxication has been previously reported. Early studies in Peru showed that children born and raised at altitudes above 3,500 m tended to exhibit reduced birth mass, slower growth rate, longer overall period of growth, poorly defined adolescent growth spurt, and delayed onset of certain aspects of psychomotor development (Pawson 1976). Growth rates of body weight and length are significantly depressed in hypoxic well-nourished rats when compared to NX ones (Bozzini et al. 2005). Lead exposure reduced somatic growth, longitudinal bone growth, and bone strength during the pubertal period (Ronis et al. 2001). Studies in children have found a negative association between blood lead level and height,

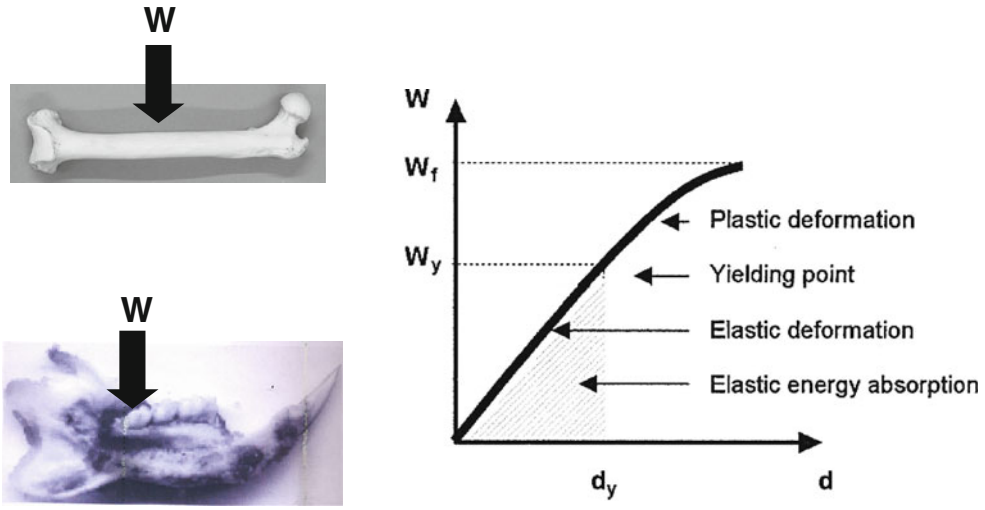

Fig. 1 Schematic representation of femur (*Upper left*) and medial aspect of the right mandible (*Down left*) showing the load (W) applied to perform the three-point bending mechanical test on an Instron Universal Testing Machine Model 4442. *Right*: Diagram of a load (W)/deformation (d) curve showing the elastic and plastic phases, separated by the yielding point

Table 1 Changes in anthropometry measurements lead content, hematological parameters and whole bone structural properties

Variable	CNX	PbNX	CHX	PbHX
Body weight (g)	308.35 ± 16.44^{a}	279.45 ± 17.06^{b}	232.79 ± 14.31^{c}	236.05 ± 6.09^{c}
Body length (cm)	23.17 ± 0.51^{a}	22.45 ± 0.34^{b}	21.26 ± 0.27^{c}	21.54 ± 0.60^{c}
Femoral weight (g)	0.95 ± 0.08^{a}	0.89 ± 0.08^{b}	0.75 ± 0.07^{c}	0.75 ± 0.06^{c}
Femoral length (mm)	32.88 ± 0.61^{a}	32.14 ± 0.67^{b}	30.94 ± 0.98^{c}	30.77 ± 0.77^{c}
Hemimandible weight (g)	0.54 ± 0.02^{a}	0.50 ± 0.04^{b}	0.45 ± 0.03^{c}	0.43 ± 0.03^{c}
Hemimandible length (mm)	26.42 ± 1.11^{a}	25.07 ± 0.46^{b}	23.97 ± 0.10^{c}	23.86 ± 0.28^{c}
Blood lead level (ug/dl)	3.17 ± 1.42^{a}	48.28 ± 8.54^{b}	4.22 ± 0.69^{a}	43.78 ± 7.20^{b}
Bone ash Pb (mg g^{-1})	0.90 ± 0.62^{a}	632.29 ± 94.23^{b}	1.06 ± 0.60^{a}	700.03 ± 78.52^{b}
Hematocrit (%)	43.50 ± 3.11^{a}	36.00 ± 5.10^{b}	73.13 ± 6.54^{c}	63.21 ± 7.49^{d}
Femoral ultimate load, Wf (N)	174.04 ± 18.31^{a}	146.33 ± 17.92^{b}	103.11 ± 15.10^{c}	107.81 ± 14.17^{c}
Femoral energy absorption capacity, EAC (N mm^{-1})	20.18 ± 1.80^{a}	16.50 ± 1.22^{b}	5.54 ± 1.54^{c}	4.85 ± 1.96^{c}
Hemimandible ultimate load, Wf (N)	58.42 ± 2.39^{a}	48.95 ± 5.26^{b}	53.33 ± 2.58^{b}	52.93 ± 3.28^{b}
Hemimandible energy absorption capacity, EAC (N mm^{-1})	9.02 ± 080^{a}	7.28 ± 0.70^{b}	6.53 ± 0.75^{b}	7.51 ± 0.82^{b}

Values are Mean ± SD of 15 rats. Equal letters indicate no significant differences. A significant difference between groups was chosen as $p < 0.01$ determined by ANOVA followed by Student–Newman–Keuls Multiple Comparison Test

CNX normoxic control rats, *PbNX* lead-treated normoxic rats, *CHX* hypoxic control rats, *PbHX* lead-treated hypoxic rats

this inhibition of stature when young results in a lower peak bone mass being achieved, thus predisposing to osteoporosis in later life (Campbell et al. 2004). Although our results are similar to those previously reported it was reasonable to propose that combined treatments would have decreased further more the anthropometric parameters; however they showed no such behavior as the results in the PbHX group were similar to those induced by HX alone.

It was of our interest to investigate if the impairment found in bone growth had compromised the stiffness affecting bone strength. The analysis of the structural properties obtained after performing the biomechanical tests of both kinds of bones demonstrated that treatments decreased their maximal load supported at fracture and their energy absorption capacity, being the effects of SHA more severe only on femoral bone. We have noticed that Pb intoxicated bones showed a decreased yielding point compared to control ones, so that plastic deformation and failure in bone tissue structure occurred under lower loads. This could be due to detrimental effects of lead on bone mineral density, bone mineral and matrix composition and bone turnover markers resulting in a higher risk of fractures

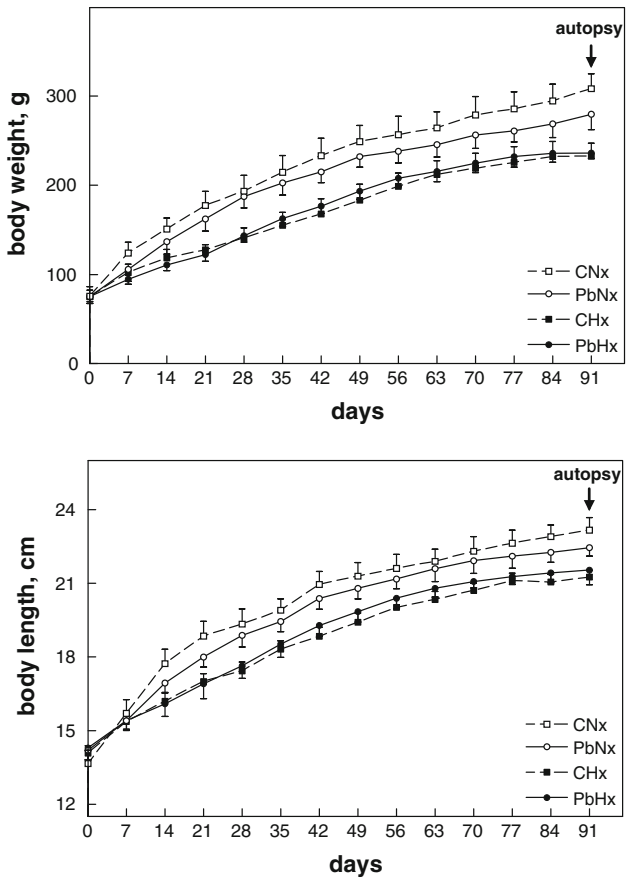

Fig. 2 Changes in body weight (*Upper*) and body length (*Lower*) as a function of time after lead intoxication under normoxic and hypoxic conditions. Each point represents the mean ± SD of 15 animals

(Monir et al. 2010). On the other hand, it was demonstrated that increased densities of erythroid elements in the marrow must result in the expansion of bone marrow cavities and a consequent displacement of the mineralize tissue leading to an impairment in the structural strength of femur diaphyses as well as the bone material quality in growing rats under stress erythropoiesis (Bozzini et al. 2008). These observations, taken together, should indicate that as bone fracture means a sum of microfractures produced by the fatigue of mineral crystals, newly formed bone after the effect of these exogenous deleterious agents would offer a lower resistance to withstand load and would spread these microfractures more than a normal bone. From the analysis of our previous results (Martínez et al. 2011) and the ones presented here, it seems evident that the severity of the impairment in bone biology after SHA probably masks the effects induced by the pollutant itself.

Mandibular bone was significantly less affected by hypoxia than femoral bone. We suggest that the observed effects could have been due to the different embryological origin of the two skeletal bones or to the distinct function that they perform, because femurs are bones designed to support loads meanwhile mandibles are subjected to forces produced during mastication. Resected hemimandibles stained with H&E observed under a stereomicroscope (×5) showed extended coronary destruction with necrosis in all experimental rats and juxtaradicular inflammatory focuses in both hypoxic groups. These oral pathologies were enhanced with the combination of both treatments assayed together where a diminution in interradicular bone volume was also observed (Fig. 3). There is no evidence in the literature reporting these findings and we hypothesize that they could be due to a greater vulnerability of dental tissues to inflammatory processes; however, further studies would be helpful to clarify this point.

In conclusion, we have described a number of changes that suggest that chronic intoxication with Pb under hypoxic conditions impaired growth parameters and induced negative effects on bone structural properties. As the findings obtained in experimental models in rats can be extrapolated to human beings (Frost and Jee 1992) we hope that the results shown in the present study could be taken in consideration for the consequences on dental health in children from populations living in Pb-contaminated high altitude areas.

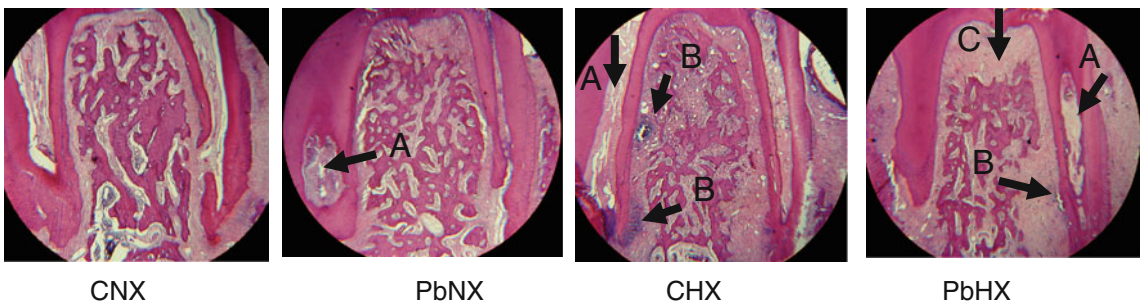

Fig. 3 Photographs of transverse slices of the longitudinal sections of the mandibular interradicular bone of one animal per group selected randomly. Resected hemimandibles stained with H&E were observed under a stereomicroscope (×5). *Arrows* indicate pathologic alterations such as extended coronary destruction with necrosis (*A*), juxtaradicular inflammatory focuses (*B*) and diminution in interradicular bone volume (*C*)

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