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Lead Bone Toxicity in Growing Rats Exposed to Chronic Intermittent Hypoxia

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Abstract Lead chronic intoxication under hypoxic conditions revealed growth retardation in growing rats and damages on femoral and mandibular bones that predispose to fractures. These findings aimed us to investigate if bone material and geometric properties, bone mass in terms of histomorphometry or antioxidant capacity are also impaired in such experimental model. Combined treatments significantly reduced hemimandible cross sectional geometry and intrinsic stiffness (−16 % and −34 %); tibia and hemimandible bone volume (−45 % and −40 %) and growth plate cartilage thickness (−19 %). These results show a previously unreported toxic effect of lead on mandible however, longer studies should be necessary to evaluate if an adaptation of bone architecture to maintain structural properties may occur and if the oxidative stress can be identified as the primary contributory agent in the pathogenesis of lead poisoning.

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It has been considered that high-altitude exposure is an extreme physiological stress leading to a wide range of deleterious effects including deterioration of bone biomechanical competition and bone resorption (Shukla et al. 2009). Lead (Pb) is a persistent air pollutant which can be released into the environment via numerous routes principally by industrial and mining activities that arose over the last few years resulting in individuals exposed to chronic intermittent hypobaric hypoxia (HX). The pathogenesis of lead toxicity is multifactorial, as Pb directly interrupts enzyme activation, induces inhibition of bone development and a decrease in bone mass, alters calcium homeostasis, lowers the level of available antioxidant reserves in the body and generates reactive oxygen species (ROS), specifically hydroperoxides and lipoperoxides (Ercal et al. 2001; Lyn Patrick 2006). Previously reported studies from this laboratory suggested that chronic intoxication with Pb in immature rats under hypoxic conditions impaired growth parameters and induced negative effects on femoral and mandibular structural properties decreasing their maximal load supported at fracture and their energy absorption capacity so that plastic deformation and failure in bone tissue structure occurred under lower loads (Conti et al. 2012). These findings aimed us to investigate some of the possible mechanisms by which chronic intoxication with lead in hypoxic conditions impair bone biology by performing three studies: (1) measuring some indicators of bone material properties in relation to bone structural and geometric properties; (2) evaluating bone mass under means of bone histomorphometry and (3) analyzing antioxidant

capacity. The results obtained in the present investigation will be of help to discern whether oxidative stress status and/or changes on bone turnover are responsible for the deterioration of bone strength.

Materials and Methods

Sixty female Wistar growing rats were housed in stainless-steel cages and maintained under local vivarium conditions (temperature 22–23°C, 12-h on/off light cycle, free access to water and a standard pelleted chow diet). Rats were randomly divided into four 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 normal ambient pressure (CNX and PbNX respectively). Further control and Pb-treated animals were exposed to HX, 18 h/day during the whole experimental period (CHX and PbHX respectively) by placing the animals into a simulated high altitude chamber in which air pressure was maintained at 506 mbar using a continuous vacuum pump and an adjustable inflow valve (Wright 1964). 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. At the end of the experimental period, blood samples where obtained by cardiac puncture to evaluate plasmatic antioxidant capacity. After animals were euthanized, femurs, tibiae and mandibles were properly dissected to perform mechanical (Mosier 1969) and histomorphometric studies. Mechanical properties of the left femur and hemimandible 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 bones at a 5 mm/min speed until fracture in order to obtain the load/deflection (W/d) curves showing the elastic and plastic phases, separated by the yielding point (Fig. 1) from which the structural whole-bone properties were measured. Evaluation of geometrical properties (bone architecture) was performed by using an Isomet low-speed diamond saw (Buehler, Lake Bluff, IL, USA). A 2-mm cross-section slide was cut from the regularized fracture section in order to perform micromorphometrical determinations of the vertical and horizontal outer and inner diameters of the elliptic-shaped fracture sections using a stereomicroscope (Stemi DV4 Stereomicroscope, Carl Zeiss Micro Imaging, Göttingen, Germany) and a digital caliper (Digimess, Geneva, Switzerland). This

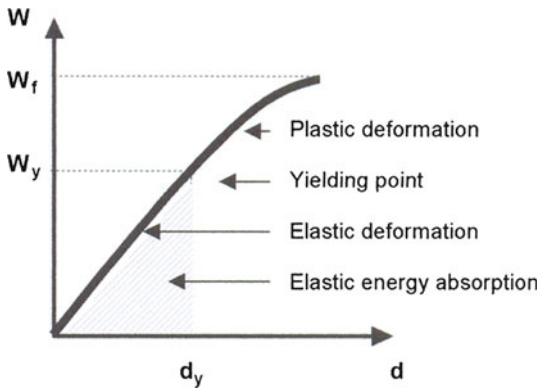

Fig. 1 Diagram of a load (W)/deformation (d) *curve* showing the elastic and plastic phases, separated by the yielding point

procedure enabled calculation of the following cross-sectional (geometric) properties: (a) the entire cross-sectional area (CSA, mm^2); the cross-sectional medullar area (CSMA, mm^2) and the cross-sectional cortical area (CSCA, mm^2), indicators of the amount of bone mass and (b) the second moment of inertia of the cross section in relation to the horizontal axis (CSMI, mm^4) a measure of the architectural efficiency of the cortical design in relation with the distance at which the cortical tissue is distributed from the bending axis in the cross-section, concerning the kind of deformation (Ferretti 1997). Expression of structural properties in relation to geometric properties indirectly allowed calculation of the following material or intrinsic properties of the bone, which are independent of its size and shape: (a) Young's modulus of elasticity of the bone mineralized tissue (E, N/mm^2), an estimator of the intrinsic stiffness of the "solid" bone tissue and (b) Limit elastic stress (stress, N/mm^2) which represents the load supported per unit of cortical bone at the end of the elastic period (bone tissue strength; Ferretti et al. 2001). After mechanical testing, femurs and hemimandibles were dissecated for determination of Pb and calcium content in ashes by atomic absorption (spectrophotometer Varian AA 475).

For histomorphometric studies tibiae and hemimandibles were resected and fixed in buffered formaldehyde solution for 48 h, decalcified in EDTA acid pH 7.4 for 25 days and then embedded in paraffin to perform sections following longitudinal axis. The sections were stained with H&E for histological analysis of: (1) subchondral trabecular bone volume (BV/TV, %) and (2) thickness of growth plate cartilage (GPC.Th, μm) under proximal articular cartilage of tibia; (1) interradicular bone volume (BV/TV, %) and (2) height of periodontal ligament thickness (%) of hemimandibles. Evaluations were performed on digital microphotographs of the sections using Image Pro Plus 4.5 software.

The antioxidant capacity of plasma samples was analyzed by 2,2,5,5-tetramethy-4-piperidin-1-oxyl (TEMPO)

scavenging and electron spin resonance (ESR) (Piehl et al. 2005). This technique evaluates the plasma antioxidant capacity mainly due to ascorbate, based on the oxide-reduction reaction between TEMPO and this antioxidant. Nitroxides were used as models of persistent free radicals to study the antioxidant function of ascorbic acid in human erythrocytes and may be useful as indicators of redox metabolism. These radicals have the advantage that they can be directly detected by ESR spectroscopy due to their paramagnetic properties, being the ESR signal intensity proportional to the nitroxide concentration. Ascorbate is the most effective water-soluble antioxidant in human blood plasma and reacts with most of the oxidizing radicals that could arise in biological systems, protecting biological compounds from oxidative damage. When plasma is subjected to free radical-mediated oxidative stress, there is a decrease of the ascorbate level which was reported as an indicator of the oxidative stress in biological samples (Galleano et al. 2002). TEMPO solution (20 μL) was added to 480 μl of each plasma sample in order to achieve a final concentration in the reaction mixture of 256 μM. Each sample was placed in the ESR cavity and then TEMPO ESR spectra were recorded at 20°C at given acquisition times between 3 and 20 min. Measurements were obtained using an X-band ESR Spectrometer Bruker ECS 106. The standard spectrometer settings for the nitroxide radicals were: center field 3440 G, sweep width 80 G, microwave power 10 mW, microwave frequency 9.62 GHz, conversion time 2.56 ms, time constant 2.56 ms, modulation frequency 50 kHz, modulation amplitude 0.103 G, gain 2.00 × 104, resolution 1,024 points. All spectra were the accumulation of 10 scans. The kinetic of the reaction was studied by analyzing the initial rate (v_0). The constant k is defined as the slope of the curves and can be regarded as the rate constant of the reduction $k = d\ln(h_t/h_0) \cdot d_t^{-1}$, where h_t is the peak height of the ESR spectrum at given time during the reaction and h_0 is the peak height at the beginning of the reaction ($t = 0$).

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 metabolic indicators, bone cross sectional geometry and material (intrinsic) mechanical properties are summarized in Table 1. Significantly high-level lead accumulation was observed in ashes from both kinds of bones in PbNX and PbHX groups. Calcium content did not show statistical differences between groups. The mechanical properties of bone strongly depend on the intrinsic mechanical quality of its constitutive substance (material or intrinsic properties) and the amount and spatial distribution of the mineralized tissue (geometrical properties; Ferretti 1997). To fully elucidate the effect of Pb intoxication under normoxic and hypoxic conditions on bone biomechanics, the differences between control and intoxicated rats with either normal or increased erythropoiesis were examined for both intrinsic mechanical properties and cross-sectional geometry. Diaphyseal bone marrow medullar cavity (CSMA) was decreased by Pb intoxication while it was increased in rats exposed to HX. Lead treatment produced a trend toward an increased cross-sectional diaphyseal cortical area (CSCA); however it was negatively affected under hypoxic conditions. Both treatments significantly enhanced hemimandible CSMA and lowered its CSCA. The CSMI, measure of the architectural efficiency of the cortical design, was impaired by both treatments only in the mandible. Stress, a bone material quality indicator, was significantly reduced either by Pb or by HX the latter being more severe only on the femoral bone. The other intrinsic property, the Young's modulus of elasticity (E), was only significantly reduced on mandibular bone by effect of both treatments. Concerning bone geometric properties, the femoral medullar area of HX treated rats was larger than that of controls, which support findings anticipated of Bozzini et al. (2009), most likely due to periosteal bone formation, which was coupled with increased endosteal bone resorption. Lead intoxication significantly depressed the femoral medullar area with no alterations in the cross sectional moment of inertia. In relation to these findings, when lead-containing matrix is resorted by osteoclasts, they develop structural alterations including lead inclusions that interfere with the osteoclasts ability to resort bone (Haschek et al. 2010). However, both treatments had a different effect on geometrical properties of the mandible showing an increase in the cross sectional medullar area with impairment in the moment of inertia. Treatments impaired stress at the yield point, an indirect indicator of bone tissue strength, of both kinds of bones. The modulus of elasticity, which depends on its constitution but not on its amount of spatial distribution, was not significantly modified in femur by either Pb or HX. This intensive property of bone material was impaired in the mandible, reinforcing our findings that mandibles do not respond to the decline in their material properties with an adaptation of its architecture to maintain structural properties (Martínez et al. 2011).

Histologic sections of the tibiae corresponding to the different groups shown in Fig. 2 evidenced a diminution of trabecular volume and growth plate cartilage thickness. The histomorphometric results shown in Fig. 3 indicate

Table 1 Metabolic indicators, bone cross sectional geometry and material (intrinsic) mechanical properties

Variable	CNX	PbNX	CHX	PbHX
Metabolic indicators				
Bone ash Pb (mg g^{-1})	1.0 ± 0.6^{a}	630.5 ± 90.2^{b}	1.0 ± 0.5^{a}	701.1 ± 78.5^{b}
Bone ash Ca (mg g^{-1})	427 ± 9^{a}	428 ± 3^{a}	429 ± 4^{a}	428 ± 4^{a}
Geometrical properties				
Femoral cross sectional medullar area, CSMA (mm^2)	4.01 ± 0.29^{a}	3.57 ± 0.33^{b}	4.82 ± 0.42^{c}	3.77 ± 0.45^{ab}
Femoral cross sectional cortical area, CSCA (mm^2)	5.12 ± 0.46^{a}	5.45 ± 0.35^{a}	4.09 ± 0.26^{b}	4.25 ± 0.35^{b}
Femoral cross sectional moment of inertia, CSMI (mm^4)	4.11 ± 0.52^{a}	4.16 ± 0.55^{a}	4.11 ± 0.88^{a}	4.01 ± 0.27^{a}
Hemimandible cross sectional medullar area, CSMA (mm^2)	1.65 ± 0.11^{a}	2.01 ± 0.18^{b}	2.08 ± 0.23^{b}	2.12 ± 0.13^{b}
Hemimandible cross sectional cortical area, CSCA (mm^2)	9.98 ± 0.45^{a}	8.59 ± 0.64^{b}	9.01 ± 0.67^{b}	9.07 ± 0.41^{b}
Hemimandible cross sectional moment of inertia, CSMI (mm^4)	5.02 ± 0.49^{a}	3.78 ± 0.67^{b}	4.13 ± 0.51^{b}	4.23 ± 0.13^{b}
Material properties				
Femoral limit elastic stress (N mm^{-2})	102.08 ± 13.84^{a}	90.36 ± 8.91^{b}	59.95 ± 4.89^{c}	80.61 ± 5.03^{d}
Femoral Young's modulus of elasticity, E (N mm^{-2})	$2{,}398.39 \pm 689.60^{a}$	$2{,}478.06 \pm 730.26^{a}$	$2{,}964.12 \pm 780.15^{a}$	$3{,}055.58 \pm 778.26^{a}$
Hemimandible limit elastic stress (N mm^{-2})	39.25 ± 8.50^{a}	22.96 ± 5.96^{b}	26.39 ± 4.68^{b}	24.69 ± 3.92^{b}
Hemimandible Young's modulus of elasticity, E (N mm^{-2})	796.32 ± 59.36^{a}	565.32 ± 46.31^{b}	555.39 ± 49.31^{b}	526.31 ± 45.31^{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

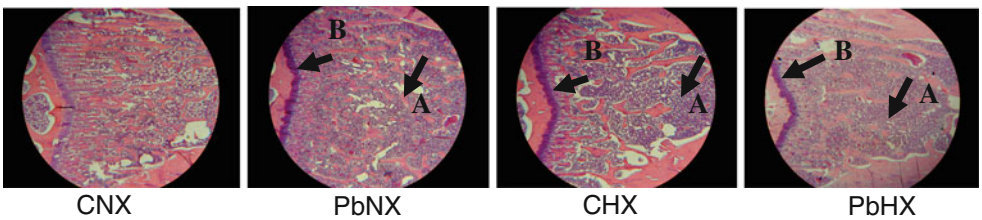

Fig. 2 Photographs of transverse slices of trabecular bone of one animal per group selected randomly. Columns from *left* to *right* represent: *CNX* normoxic control rats, *PbNX* lead-treated normoxic rats, *CHX* hypoxic control rats, *PbHX* lead-treated hypoxic rats. *Arrows* indicate diminution of trabecular volume (**a**) and growth plate cartilage thickness (**b**). Resected tibiae stained with H&E were observed under a stereomicroscope ×5

that Pb decreased bone volume (Fig. 3a) and growth plate cartilage thickness (Fig. 3b). These findings were anticipated from that of Hamilton & O'Flaherty suggesting a decrease in bone mass and an inhibitory effect on the process of endochondral bone formation as measured by histomorphometry. Both histomorphometric parameters were similarly decreased by HX, being the impairment even greater in the PbHX group (Fig. 3a, b) which explain the growth retardation reported in a previous study from our laboratory. On the other hand, the histomorphometric evaluation of hemimandibles showed a trend towards a reduction in the interradicular bone volume by means of Pb and HX being the decrease on bone mass only significant in the PbHX group (Fig. 3c) by expense of an enhancement of the thickness of the periodontal ligament (Fig. 3d). It is thus proposed that even though mandibles are less affected by treatments, combined Pb + HX can impair a previous or simultaneous alteration of bone tissue structure. These changes could be due to the diminution in interradicular bone volume as a consequence of the extended coronary destruction with necrosis and juxtaradicular inflammatory focuses previously reported (Conti et al. 2012).

In order to evaluate if plasma antioxidant capacity was altered in rats subjected to HX and/or Pb treatment, we determined the capacity of plasma to reduce the stable spin label TEMPO. Reduction kinetics of TEMPO was followed by ESR. Results are shown in Fig. 4. Both groups subjected to HX treatment presented a significant higher

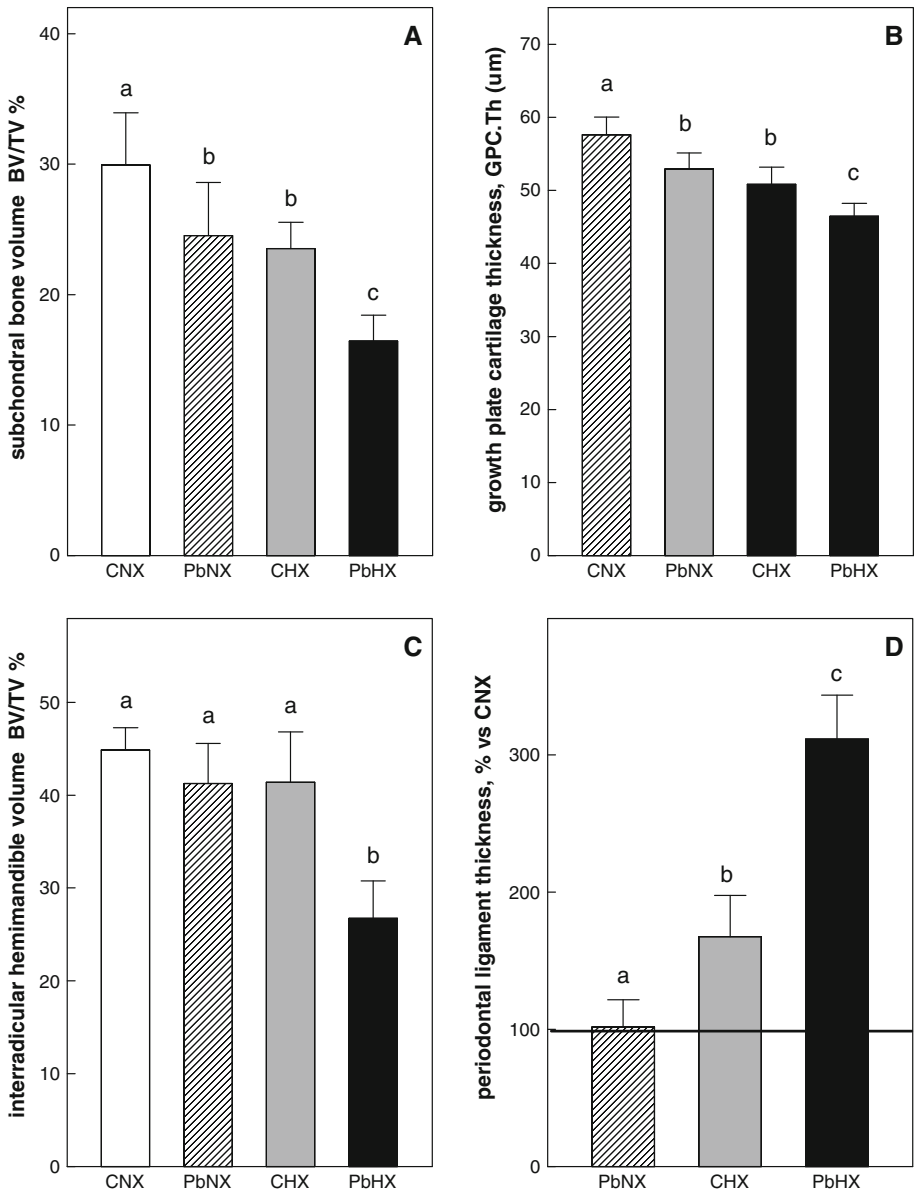

Fig. 3 Histomorphometric evaluations of subchondral bone volume (BV/TV, %) (**a**) and thickness of growth plate cartilage (GPC.Th, μm) (**b**) under proximal articular cartilage of tibia and interradicular bone volume (BV/TV, %) (**c**) and height of periodontal ligament thickness (%) (**d**) of hemimandibles. Evaluations were performed on digital microphotographs of the sections using Image Pro Plus 4.5 software. 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

TEMPO reduction rate ($k = 0.0074 \pm 0.0013$ min^{-1} and $k = 0.0074 \pm 0.0030$ min^{-1} for CHX and PbHX, respectively) than normoxic groups ($k = 0.0059 \pm 0.0012$ min^{-1} and $k = 0.0057 \pm 0.0032$ min^{-1} for CNX and PbNX respectively). A higher TEMPO reduction rate represents a higher antioxidant status. On the other hand, there was no difference in the groups treated with Pb, regardless if they were under hypoxic or normoxic condition. Like other toxic metals, lead damages cellular material and alters cellular genetics through a mechanism which involves increase production of free radicals and decrease availability of antioxidant reserves (Lawton and Donaldson 1991). In normal conditions, there is a balance between oxidative stress (ROS production) and the antioxidant systems present in the plasma. HX could result in oxidative/reductive stress, enhanced generation of ROS and related oxidative damage to lipids, proteins, and DNA (Dosek et al. 2007). Besides, recent studies have shown that Pb could disrupt tissue prooxidant/antioxidant balance which lead to physiological dysfunction (Hamed et al. 2010). Most researches about lead exposure on various antioxidant enzymes and tissues were mainly experimental and had often divergent results. Using the scavenging of a nitroxide radical TEMPO (Piehl et al. 2005) we evaluated the antioxidant status due to ascorbic acid, the main plasma hydrophilic antioxidant. No differences were observed as a consequence of Pb treatment. Thus, the oxidative stress could not be identified as the primary contributory agent in the pathogenesis of lead poisoning. Unexpectedly, an increase in plasma antioxidant activity regard to the

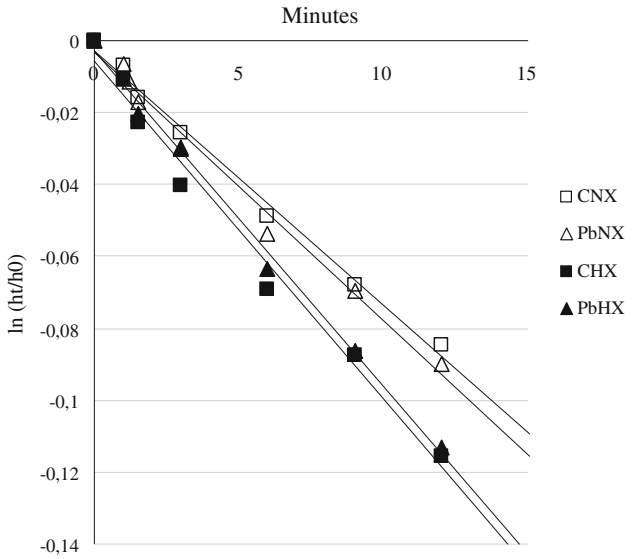

Fig. 4 Plasma antioxidant capacity evaluation determined by the reduction rate of TEMPO radical. Figure represents the kinetics obtained for the four groups evaluated: CNX, PbNX, CHX, and PbHX. The reduction rate constants (k) were obtained from the slope of respective curve: $k = d\ln(h_t/h_o) \cdot d_t^{-1}$, where h_t is the peak height of the ESR spectrum at a given time during the reaction and h_o is the peak height at the beginning of the reaction ($t = 0$). Significant difference between NX and HX groups was chosen as $p < 0.01$ determined by ANOVA followed by Student–Newman–Keuls Multiple Comparison Test

controls was observed in animals subjected to hypoxia. We suggest that the observed increase could be the consequence of an adaptive process to the oxidative challenge, which enhances antioxidant defenses in order to affront oxidative damage. Abundant information has been accumulated on the oxidative stress generated in response to acute hypoxia but less to intermittent hypoxia, although after a program of intermittent hypobaric hypoxia in rats similar to ours, blood and plasma oxidative stress related markers offered no evidence of a significant imbalance in redox status (Esteva et al. 2010).

In conclusion, although these results show a previously unreported toxic effect of heavy metals especially on mandible bone, longer studies should be necessary to evaluate if at any time adaptation of its architecture to maintain structural properties may occur and if a possible association between the impairment of bone material quality and the degree of oxidative stress exists.

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References

Bozzini C, Olivera MI, Huygens P, Alippi RM, Bozzini CE (2009) Long-term exposure to hypobaric hypoxia in rat affects femur cross-sectional geometry and bone tissue material properties. Ann Anat 191:212–217

Conti MI, Terrizzi AR, Lee CM, Mandalunis PM, Bozzini C, Piñeiro AE, Martínez MP (2012) Effects of lead exposure on growth and bone biology in growing rats exposed to simulated high altitude. Bull Environ Contam Toxicol. doi:10.1007/s00128-012-0602-2

Dosek A, Ohno H, Acs Z, Taylor AW, Radak Z (2007) High altitude and oxidative stress. Respir Physiol Neurobiol 158(2–3):128–131

Ercal N, Gurer-Orhan H, Aykin-Burns N (2001) Toxic metals and oxidative stress part 1 mechanisms involved in metal-induced oxidative damage. Curr Top Med Chem 1:529–539

Esteva S, Pedret R, Fort N, Torrella JR, Pagès T, Viscor G (2010) Oxidative stress status in rats after intermittent exposure to hypobaric hypoxia. Wilderness Environ Med 21(4):325–331

Ferretti JL (1997) Biomechanical properties of bone. From osteoporosis and bone densitometry. Springer, Berlin, pp 143–161

Ferretti JL, Cointry GR, Capozza RF, Capiglioni R, Chiappe MA (2001) Analysis of biomechanical effects on bone and on the bone muscle interactions in small animal models. J Musculoskel Neuron Interact 1:263–274

Galleano M, Aimo L, Puntarulo S (2002) Ascorbyl radical/ascorbate ratio in plasma from iron overloaded rats as oxidative stress indicator. Toxicol Lett 133:193–201

Hamed EA, Meki AR, Abd El-Mottaleb NA (2010) Protective effect of green tea on lead-induced oxidative damage in rat's blood and brain tissue homogenates. J Physiol Biochem 66(2):143–151

Hamilton JD, O'Flaherty EJ (1994) Effects of lead exposure on skeletal development in rats. Fundam Appl Toxicol 22(4): 594–604

Haschek WM, Rousseaux CG, Wallig MA (2010) Bone toxicity. In: Fundamentals of toxicologic pathology, 2nd edn. Academic Press, imprint of Elsevier Inc., Typeset by MacmillanPublishing Solutions

Lawton LJ, Donaldson WE (1991) Lead-induced tissue fatty acid alterations and lipid peroxidation. Biol Trace Elem Res 28:83–97

Lyn Patrick ND (2006) Lead toxicity part II: the role of free radical damage and the use of antioxidants in the pathology and treatment of lead toxicity. Altern Med Rev 11(2):114–127

Martínez MP, Bozzini C, Olivera MI, Dmytrenko G, Conti MI (2011) Aluminum bone toxicity in immature rats exposed to simulated high altitude. J Bone Miner Metab 29(5):526–534

Mosier HD (1969) Allometry of body weight and tail length in studies of catch-up growth in rats. Growth 33:319–330

Piehl LL, Facorro GB, Huarte MG, Desimone MF, Copello GJ, Díaz LE, Rubín de Celis E (2005) Plasmatic antioxidant capacity due to ascorbate using TEMPO scavenging and electron spin resonance. Clin Chim Acta 359:78–88

Shukla D, Saxena S, Jayamurthy P, Sairam M, Singh M, Jain SK, Bansal A, Ilavazaghan G (2009) Hypoxic preconditioning with cobalt attenuates hypobaric hypoxia-induced oxidative damage in rat lungs. High Alt Med Biol 10(1):57–69

Wright BM (1964) Apparatus for exposing animals to reduced atmospheric pressure for long periods. Brit J Haemat 10:75