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### Lead Intoxication Under Environmental Hypoxia Impairs Oral Health

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## LEAD INTOXICATION UNDER ENVIRONMENTAL HYPOXIA IMPAIRS ORAL HEALTH

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**We have reported that chronic lead intoxication under hypoxic environment induces alveolar bone loss that can lead to periodontal damage with the subsequent loss of teeth. The aim of the present study was to assess the modification of oral inflammatory parameters involved in the pathogenesis of periodontitis in the same experimental model. In gingival tissue, hypoxia increased inducible nitric oxid synthase (iNOS) activity ( $p < .01$ ) and meanwhile lead decreased prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) content ( $p < .05$ ). In submandibular gland (SMG), iNOS activity was enhanced by lead and PGE<sub>2</sub> content was increased by both lead and hypoxia ( $p < .01$ ) and even more by combined treatments ( $p < .001$ ). In the SMG, hypoxia stimulated angiogenesis ( $p < .01$ ) with blood extravasation. Adrenal glands were 22% bigger in those animals exposed to lead under hypoxic conditions. Results suggest a wide participation of inflammatory markers that mediate alveolar bone loss induced by these environmental conditions. The lack of information regarding oral health in lead-contaminated populations that coexist with hypoxia induced us to evaluate the alteration of inflammatory parameters in rat oral tissues to elucidate the link between periodontal damage and these environmental conditions.**

Air pollution in populations residing in Pb-contaminated high altitude areas is now a major issue (Buchanan et al., 2011). It is estimated that Pb dietary intake is around 35.7  $\mu\text{g}/\text{d}$  after the year 2000 in Europe, being higher in areas where mining and metallurgical activities are predominant (Bierkens et al., 2011). Several systemic alterations such as neurocognitive impairment, low hemoglobin levels, and anemia have been described in Andean children (Counter et al., 2012). During infancy and childhood exposure, lead is stored in bone and its effects continue into adolescence and adulthood, inducing lower peak bone mass, leading to osteoporosis in later life (Campbell et al., 2004). The impairment on axial bone structure produced by lead intoxication is well established, but reports regarding mandibular and

alveolar bone are lacking, especially in those populations that coexist with another stress factor. Previously reported studies from this laboratory suggested that chronic intoxication with Pb in immature rats under hypoxic conditions impaired growth parameters, induced negative effects on mandibular structural properties that predispose to fractures, and produced alveolar and interradicular bone loss (Conti et al., 2012). Additionally, these environmental variables aggravated the pathophysiological alterations produced by periodontal disease when experimental periodontitis was induced (Terrizzi et al., 2013). Chronic exposure to Pb significantly affects oral health among exposed workers, increasing the prevalence of periodontal diseases (gingivitis and periodontitis), caries, missing and filled teeth, and dental

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abrasions (Won et al., 2013; El-Said et al., 2008). Research in rats showed that hypoxia (HX) plays an important role in the pathogenesis of periodontitis (Xiao et al., 2012). In an experimental periodontitis model, a close association between damage to the submandibular gland (SMG) and changes in inflammatory mediators such as inducible nitric oxide synthase (iNOS) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) has been found (Amer et al., 2011). Nitric oxide (NO) produced by iNOS during inflammatory events plays an important role in the host immune system, inducing vasodilatation and cell recruitment during periodontitis (Ossola et al., 2012). PGE<sub>2</sub> is known to be a potent stimulator of bone resorption associated with loss of periodontal attachment tissue (Offenbacher et al., 1993). The lack of information regarding oral health in lead-contaminated populations that coexist with hypoxia induced us to evaluate the alteration of inflammatory parameters in rat oral tissues to elucidate the link between periodontal damage and these environmental conditions.

## MATERIALS AND METHODS

### Animals

Female growing Wistar rats, aged 21 d, were used throughout the experiments. They were maintained at stainless-steel cages 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 diet. Rats were randomly divided into 4 groups of 12 animals each, including a control group (C, sodium acetate in tap water and normal ambient pressure); lead-intoxicated group (Pb, 1000 ppm of lead acetate in drinking water and normal ambient pressure) (Hamilton and O'Flaherty, 1994); hypoxic group (HX, 18 h/d in a simulated high-altitude chamber at 506 mbar) (Conti et al., 2012); and both treatments simultaneously (PbHX). All animals were treated in accordance with the National Institutes of Health (NIH) 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 School of Dentistry, Buenos Aires University. After 3 mo, the animals were euthanized by guillotine, weighed, and measured. Blood samples were obtained to assess hematocrit by micromethod, lead, and PGE<sub>2</sub> content. Pb content in bone ashes was determined using an atomic absorption spectrophotometer, Varian AA 475. Adrenal glands were dissected and weighed as stress indicators. Hemimandibles were resected and gum tissue from around the lower first molar was obtained, as well as both SMG, to evaluate iNOS activity and PGE<sub>2</sub> content. Histological studies were performed in SMG stained with hematoxylin and eosin (H&E).

### Determination of iNOS Activity

iNOS activity was determined by a modification of the method of Bredt and Snyder that indirectly measures NO production by the conversion of [<sup>14</sup>C]arginine into equimolar amounts of [<sup>14</sup>C]citrulline and NO (Lomniczi et al., 2001; Bredt and Snyder, 1989). Data were expressed as nanomoles NO per minute per microgram of protein.

### Measurement of PGE<sub>2</sub> Content

To determine PGE<sub>2</sub> content by radioimmunoassay, tissues were homogenized in absolute ethanol, and after centrifugation the supernatant was dried at room temperature. The residues were resuspended with buffer; anti-serum (Sigma–Aldrich) was used as described by Mohn et al. (2011). The results were expressed as picograms per milligram of tissue weight (SMG) or as picograms per gum (Ossola et al., 2012). <sup>3</sup>H-PGE<sub>2</sub> was purchased from New England Nuclear Life Science Products (Boston, MA).

### Histological Analysis of SMG

The SMG removed were fixed with 10% neutral buffered formaldehyde, embedded in paraffin, and 5- $\mu$ m sections were stained with H&E (Amer et al., 2011). The number of blood vessels per square millimeter was evaluated.

SMG morphology and histopathological characteristics were analyzed. Light microscopy was performed on a Bausch & Lomb microscope (New York, NY).

### Statistical Analysis

Statistical analyses were performed by analysis of variance (ANOVA) followed by Tukey tests (GraphPad Software, Inc., San Diego, CA), considering statistically significant a  $p$  value less than .05.

## RESULTS

Results showing the changes in anthropometry measurements, lead content, hematological parameters, and adrenal gland weight are shown in Table 1. As expected, treatments clearly impaired body weight and length. Hematocrit values were increased by HX and decreased by lead intoxication. Additionally, we confirmed that the administered Pb reached significant amounts in bone ashes and blood of Pb and PbHX groups. The higher adrenal gland weight observed in PbHX group suggests that the presence of the metal aggravates the stressing effect of HX.

### iNOS Activity in Gum Tissue and SMG

We observed a significantly increased activity of iNOS in gum tissue in those animals exposed to HX ( $p < .01$ ) versus every other group.

In SMG, Pb was the only experimental group that showed a significantly higher activity of iNOS as compared to controls ( $p < .001$ ) (Figure 1).

### PGE<sub>2</sub> Content in Gum Tissue, SMG, and Plasma

In gum tissue, Pb intoxication produced an unexpected lower content of this inflammatory marker ( $p < .05$ ).

PGE<sub>2</sub> content in SMG was increased in every experimental group versus the control group, observing even higher values when both treatments were applied together (Figure 2).

### Histological Analysis of SMG

The number of blood vessels per square millimeter was greater in those groups exposed to HX. Blood extravasation and vasodilatation were observed only in those animals exposed to HX. SMG of all groups investigated showed normal structural organization of the gland without visible edema or presence of inflammatory cells (Figure 3).

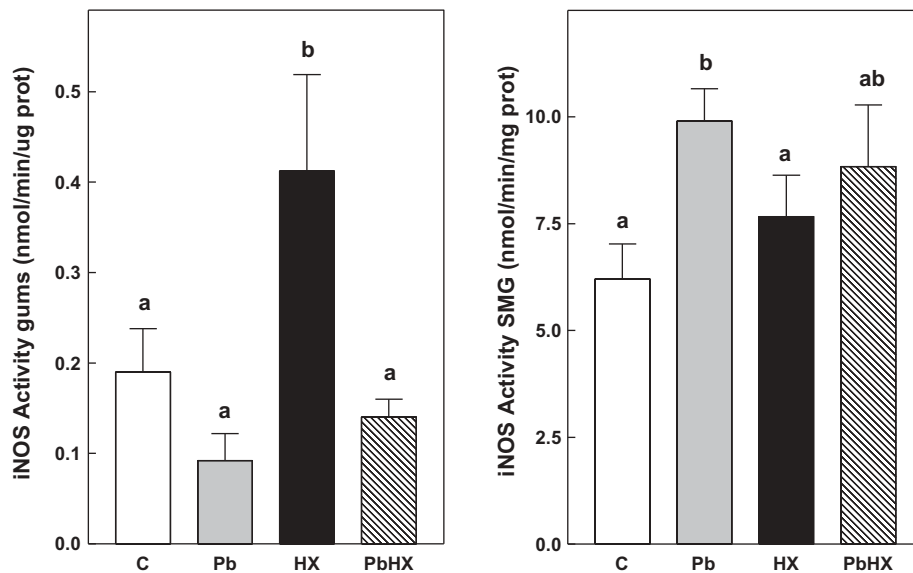
## DISCUSSION

Previously reported studies from this laboratory demonstrated that lead intoxication under a hypoxic environment produced interdicular bone resorption, leaving the teeth without the correct periodontal support and predisposing to their loss. Only hypoxia induced alveolar bone resorption of the buccal side of the mandible (Terrizzi et al.,

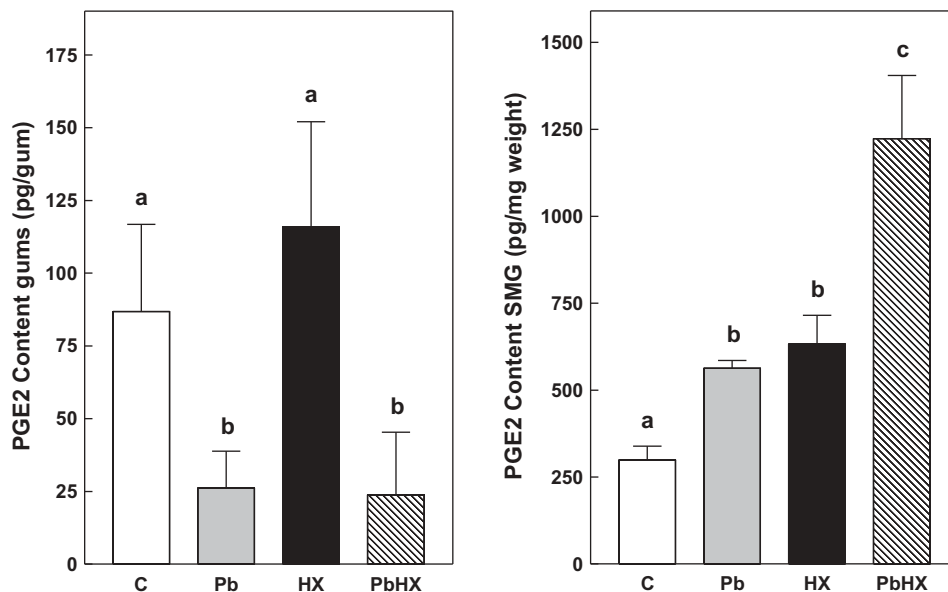
**TABLE 1.** Effects on Lead Levels, Hematological Parameters, and Adrenal Gland Weight

Variable	C	Pb	HX	PbHX
Body weight (g)	243.86 ± 15.39 <sup>a</sup>	225.5 ± 22.37 <sup>b</sup>	211 ± 17.63 <sup>b</sup>	185.93 ± 15.15 <sup>c</sup>
Body length (cm)	21.39 ± 0.4 <sup>a</sup>	20.24 ± 0.67 <sup>b</sup>	20.83 ± 0.4 <sup>b</sup>	20.31 ± 0.43 <sup>b</sup>
Blood lead level (μg/dl)	3.29 ± 1.51 <sup>a</sup>	47.37 ± 8.54 <sup>b</sup>	4.13 ± 0.56 <sup>a</sup>	44.51 ± 6.32 <sup>b</sup>
Bone ash Pb (mg g <sup>-1</sup> )	0.85 ± 0.49 <sup>a</sup>	594.29 ± 87.32 <sup>b</sup>	1.12 ± 0.67 <sup>a</sup>	689.15 ± 69.34 <sup>b</sup>
Hematocrit (%)	38.57 ± 0.97 <sup>a</sup>	34.00 ± 1.29 <sup>b</sup>	47.62 ± 3.96 <sup>c</sup>	48.14 ± 2.27 <sup>c</sup>
Adrenal gland (g/100 g)	12.75 ± 1.34 <sup>a</sup>	13.29 ± 1.47 <sup>a</sup>	14.72 ± 0.93 <sup>b</sup>	16.49 ± 1.17 <sup>c</sup>

Note. Values are mean ± SD of 12 rats. Statistical analyses were performed by ANOVA followed by Tukey tests (GraphPad Software, Inc., San Diego, CA). Same superscripted letters indicate no significant differences. Different superscripted letters indicate significant at  $p < .05$ . C = control rats, Pb = lead-intoxicated rats, HX = hypoxic rats, PbHX = lead-intoxicated hypoxic rats.



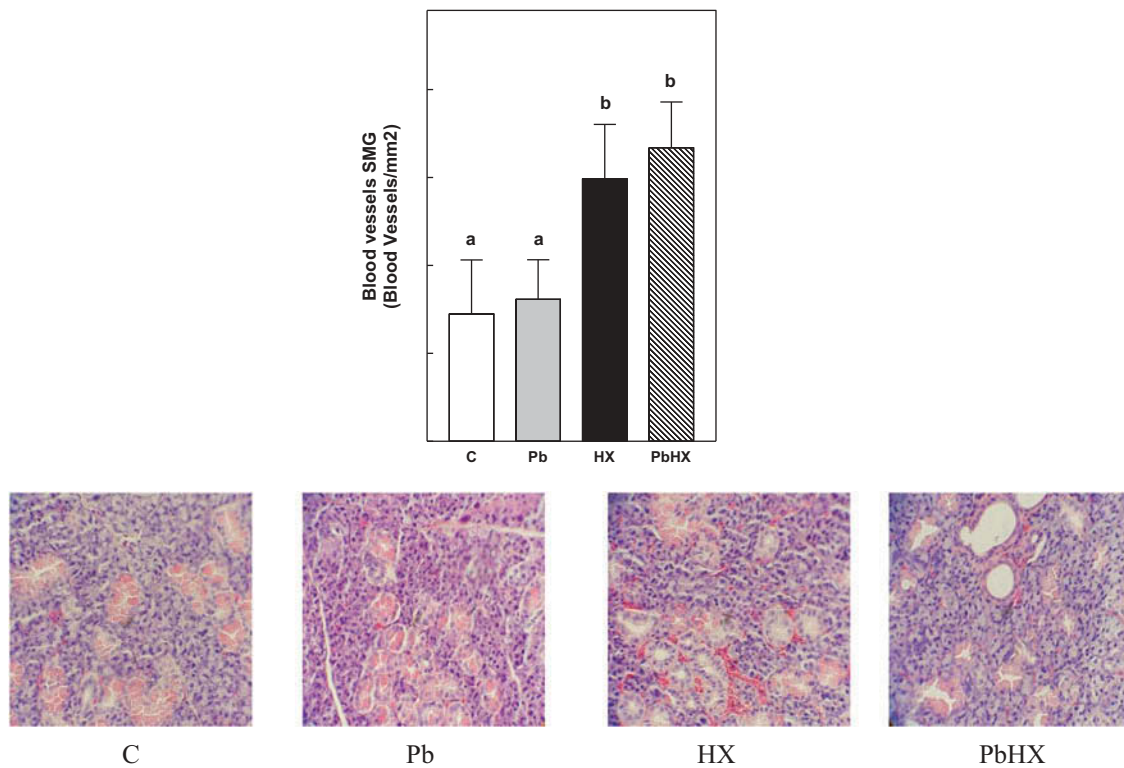
**FIGURE 1.** Effect of Pb (1000 ppm lead acetate in drinking water for 3 mo): HX (18 h/d at 506 mbar for 3 mo) and PbHX (both treatments simultaneously) on iNOS activity in gum tissue (left) and SMG (right). Mean  $\pm$  SD of 12 rats. Equal letters indicate no significant differences. A significant difference between groups is taken as  $p < .05$  determined by two-way ANOVA followed by Tukey test.



**FIGURE 2.** Effect of Pb (1000 ppm lead acetate in drinking water for 3 mo): HX (18 h/d at 506 mbar for 3 mo) and PbHX (both treatments simultaneously) on PGE<sub>2</sub> content in gum tissue (left) and SMG (right). Data are reported as means  $\pm$  SD (12 rats per group). Equal letters indicate no significant differences. A significant difference between groups is given as  $p < .05$  determined by two-way ANOVA followed by Tukey test.

2013). Alveolar bone loss is a common consequence of periodontitis, an infectious disease characterized by inflammation of tooth-supporting tissues and by periodontal pocket formation, which also results in loss of

periodontal attachment tissue with evidence that indicates the role of SMG in the regulation of immune/inflammatory reactions (Vacas et al., 2008). Periodontal disease is associated with inflammatory markers in gingival



**FIGURE 3.** Upper: Effect of Pb (1000 ppm of lead acetate in drinking water for 3 mo); HX (18 h/d at 506 mbar for 3 mo) and PbHX (both treatments simultaneously) on the number of GSM blood vessels/mm<sup>2</sup>. Data are reported as means  $\pm$  SD (12 rats per group). Equal letters indicate no significant differences. A significant difference between groups is given as  $p < .05$  determined by two-way ANOVA followed by Tukey test. Lower: Photographs of one animal per group selected randomly.

tissue, as well as in SMG, such as proinflammatory cytokines, prostaglandins, metalloproteinases, and nitric oxide from iNOS enzyme.

In this study, we analyzed some representative inflammatory parameters at both gingival and glandular level to fully elucidate the link between these environmental factors and alveolar bone resorption. Environmental hypoxia increased iNOS activity in gingival tissue of rats, in agreement with the alveolar bone loss we observed under such condition. On the other hand, chronic lead intoxication did not significantly enhance iNOS activity in gingival tissue, according to the absence of bone damage previously observed (Terrizzi et al., 2013). It has been suggested that the increased expression of iNOS leads to the production of NO, a free radical that might be involved in bone destruction by modulating metalloproteinase synthesis (Jung et al., 2013). Our results concerning the SMG showed that Pb

was the variable responsible for enhancing NO production, evidencing a distant effect of this pollutant on periodontal health. These findings are related to previously reported data that demonstrated an association between NO levels in saliva and chronic periodontitis, serving as a biological marker of this disease (Jung et al., 2013).

Regarding PGE<sub>2</sub> content in gingival tissue, we found that Pb intoxication decreased this bone loss marker. Lead seems to alter the balance between vasoconstrictive and vasodilatory prostaglandins, enhancing the first ones and decreasing the latter (Vaziri, 2008). This also correlates with the absence of bone loss mentioned earlier. On the other hand, we evidenced a higher content of PGE<sub>2</sub> in the SMG in all of our experimental groups, and this was even higher when both treatments were applied simultaneously. This would explain the interradicular bone loss observed in PbHX

rats, clearly evidencing a cross-talk between periodontal health and the SMG function. This finding was anticipated by Amer et al. (2011) when reporting an association between high levels of PGE<sub>2</sub> in the SMG and periodontitis, correlated with salivary hypofunction observed in periodontal disease.

As expected, both HX groups showed greater number of blood vessels per square millimeter in SMG as compared to controls, consistent with reports by Scott and Gradwell (1989) that attributed these changes to vascular responses to protect salivary parenchymal elements against hypoxic degeneration. Interestingly, only the hypoxic group evidenced blood extravasation and vasodilatation, perhaps due to angiogenesis and polycythemia induced by HX, which might lead to endothelial fragility of the newly formed blood vessels. This effect could be countered by the anemia induced by Pb, allowing a normal development of blood vessels. SMG alterations would be due to changes on signaling pathways, probably involving cytokines and prostaglandins, since significant anatomical changes were not observed.

In summary, our results showed that chronic lead intoxication and hypoxic environment modified some oral inflammatory parameters, which could contribute to explaining the periodontal damage observed in this experimental model. Lead and lower levels of oxygen would alter the concentration of biologic markers of periodontal disease at both gingival and glandular level, suggesting not only in situ bone damage but also distant effects by means of SMG and systemic alterations. This study lays the foundation for evaluating the periodontal risk of those populations exposed to Pb pollution under hypoxic conditions.

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Department of Physiology, School of Dentistry, University of Buenos Aires. Drs. Fernandez-Solari, Conti, and Martinez and dentists Terrizzi and Lee report no conflicts of interest related to this study.

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