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Deleterious effect of chronic continuous hypoxia on oral health

Antonela R. Terrizzi[a,b], Javier Fernandez-Solari[a,b], Ching M. Lee[a], María Ines Conti[a], María Pilar Martínez[a,*]

[a] Department of Physiology, Faculty of Dentistry, University of Buenos Aires, Argentina
[b] National Council for Scientific and Technical Research (CONICET), Argentina

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ABSTRACT

Objective: To evaluate the effect of chronic continuous hypoxia (CCH) in alveolar bone and its correlation with the inflammatory markers which play a key role in the development of periodontitis.
Material and methods: Wistar rats were exposed to CCH (600 mbar, 3 months). Macroscopic and histological analyses of alveolar bone were performed, together with measurement of oxidative stress and inflammatory parameters in gums and submandibular glands (SMG).
Results: HCC induced cortical alveolar bone loss, decreased interradicular bone volume and increased the periodontal ligament height compared to control rats ($p < 0.05$). CCH enhanced iNOS activity in gums (from $2735{,}04 \pm 662{,}96$ nmol/min/mg proteins to $4289{,}58 \pm 915{,}63$ $p < 0.05$) and in SMG (from $56{,}71 \pm 12{,}05$ nmol/min/mg proteins to $90{,}15 \pm 21{,}78$ $p < 0.05$). PGE_2 did not change in gums or in SMG by means of CCH, while TNFα decreased in gums ($p < 0.05$). Regarding oxidative stress, thiobarbituric acid reactive species concentration in CCH animals was higher both in gums as in SMG, and catalase activity was decreased in SMG.
Conclusion: Higher iNOS activity both in gums and SMG under CCH could be associated with the alveolar bone loss observed. The increase in oxidative stress occurring in SMG and gums, together with a lower antioxidant capacity might indicate a deleterious effect of HX in oral health.

1. Introduction

Hypobaric hypoxia (HX) can be defined as a decrease in barometric pressure with a consequential reduction in the partial pressure of oxygen (pO_2) (Muthuraju & Pati, 2014), which leads to diminished levels of this gas in the body tissues. Many physiological responses to HX in the organism take place due to the activation of transcription factors, mainly at renal, respiratory and cardiovascular levels. Among these, higher renal filtration fraction, increase in the hemoglobin response and right ventricular hypertrophy were found to be the result of a complex regulatory network that tries to adapt the organism to this form of environmental stress (Arestegui et al., 2011). These hypoxia–related events in populations living at high altitude (chronic continuous hypoxia, CCH) are distinct from those who are exposed to intermittent forms of hypoxia (CIH), mainly because of the different signaling pathways activated due to the length and intervals of lower oxygen partial pressure (Siques et al., 2014). Despite the fact that hypoxia related diseases are well characterized, very little is known about the effect of HX in oral tissues.

Periodontitis is one of the most prevalent inflammatory diseases of the oral cavity and when not controlled, it may lead to other systemic alterations, such as metabolic disorders or cardiovascular alterations (El Kholy, Genco, & Van Dyke, 2015). HX seems responsible for the regulation of many inflammatory mediators that play an important role during the development of periodontal disease, such as nitric oxide (NO), tumoral necrosis factor α (TNFα) and prostaglandin E_2 (PGE_2) (Jian et al., 2014). NO is produced by nitric oxide synthases (NOSs) from L-arginine, and during inflammation the role of inducible NOS (iNOS) becomes predominant. The activation of this enzyme by cytokines, interleukins and HX leads to an excess of NO production that correlates with tissue injury (Xie et al., 2014). PGE_2 is another important mediator in the pathogenesis of periodontal disease. Its release from monocytes and fibroblasts is associated with increase activation of osteoclasts and therefore, alveolar bone resorption (Ossola et al., 2012). The interaction between NO and PGE_2 is still not well stablished, meanwhile some studies state that NO increases the content of prostaglandins, other authors claim that higher levels of NO decreases the eicosanoid concentration

* Corresponding author at: Marcelo T. de Alvear 2142 3ªA (1122) Buenos Aires, Argentina.
E-mail addresses: pilarmartinez@fisio.odon.uba.ar, fliapico@hotmail.com (M.P. Martínez).

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(Cuzzocrea & Salvemini, 2007). Previously reported studies from our laboratory evidenced an enhanced content of PGE_2 and an increased activity of iNOS in gums of rats exposed to intermittent HX (18 h a day, 506 mbar). The higher levels of these mediators correlated with higher alveolar bone resorption and increased periodontal ligament height suggest the incidence of these environmental conditions in the pathogenesis of periodontal disease (Conti et al., 2012; Terrizzi et al., 2013).

Furthermore, under HX conditions, the reduced levels of oxygen determine less availability of this gas as an electron acceptor in the respiratory chain, enabling the production of reactive oxygen species (ROS) (Maiti et al., 2006). ROS are rapidly generated and diffusible substances, which in high concentrations are associated with tissue damage after initiating free radical chain reactions (Orihuela-Campos et al., 2015). It has been demonstrated that the production of proteolytic enzymes and the respiratory burst of neutrophils lead to generation of ROS and induce oxidative stress. These species could play an important role in the development of periodontal inflammation, as the association between periodontal status and salivary lipid peroxidation markers was confirmed in many clinical studies (Tothova, Kamodyova, Cervenka, & Celec, 2015). Besides the production of ROS, the levels of antioxidant enzymes should be analyzed when studying oxidative stress. Catalase is one of the most important in protecting the cells against the toxic effects of hydrogen peroxide (Kodydková, Vávrová, Kocík, & Žák, 2014). HX is known to decrease the levels of antioxidant enzymes, thus increasing cell damage due to oxidative stress (Maiti et al., 2006).

Salivary glands are key organs in the regulation of hydric, mineral and immunologic balance of the oral environment (Busch, Miozza, Sterin-Borda, & Borda, 2009). Besides its mechanic cleansing effect, saliva also exerts a role in mucosal host defense thanks to the presence of secretory immunoglobulin and many antimicrobial proteins. A decrease in salivary flow and/or an alteration in saliva composition lead to bacterial overgrowth and increased inflammatory response, which may contribute to pathological bone resorption and tissue detachment observed in periodontal disease (Vacas et al., 2008). Regarding glandular function under hypoxic conditions, it has been reported a decrease in submandibular salivary secretion in animals submitted during 3 weeks at 7000 m above sea level (Elverdin, Chiarenza, Frid, & Giglio, 1995). However, no study has approached salivary gland function or any mediator expression under the real HX conditions of those populations that usually inhabit high altitude areas.

Due to the intricate interplay between HX and inflammation in different tissues of the oral cavity and the importance of detecting risk factors for developing periodontal disease, the aim of the present study was to evaluate the effect of chronic continuous hypoxia (CCH) in alveolar bone and its correlation with the inflammatory markers which play a key role in the development of periodontitis. This study could shed some light upon finding whether this environmental condition produces clinical or subclinical manifestations of periodontal inflammation in those populations exposed to high altitude.

2. Materials and methods

2.1. Animal and treatments

Thirty female growing Wistar rats, aged 21 days, were randomly divided into 2 groups of 15 animals per group: control (C; normoxic environment) and chronic continuous hypoxia (CCH; exposed to 23.5 h/d by placing the animals into a chamber at 600 mbar, which equals 4200 m above sea level). All animals were allowed free access to water and a standard pelleted chow diet and were treated in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals (NIH 8th edition, 2011). Protocols were approved by the Ethical Commission of the Faculty of Dentistry, University of Buenos Aires (N° 11/06/2012–23). At the end of the experimental period (3 months) animals were euthanized by cervical dislocation. Immediately after the autopsy, both submandibular glands (SMG) and attached gingival tissue from around the lower first molar were collected and afterwards, right and left hemimandibles were dissected to assess the following parameters:

2.2. Morphometrical analysis of alveolar bone

After autopsy, one hemimandible was resected, defleshed and stained with 1% aqueous methylene blue (Crawford, Taubman, & Smith, 1978) to measure the distance between the cemento-enamel junction (CEJ) and the alveolar crest (AC) of the 3 roots of the first mandibular molar in order to evaluate bone loss in millimeters (Fig. 1). A stereomicroscope (Stemi DV4 Stereomicroscope, Carl Zeiss MicroImaging, Göttingen, Germany) and a digital caliper (Digimess, Geneva, Switzerland) were used. The other hemimandible was resected and fixed in buffered formaldehyde solution for 48 h, decalcified in EDTA pH 7.4 for 25 days and then embedded in paraffin. Seven microns sections were stained with H&E for histological analysis of: I) interradicular bone volume (expressed in% of trabecular bone volume/total interradicular volume, BV/TV%) and II) height of periodontal ligament, expressed in micrometers (μm). To measure the height of the periodontal ligament, ten equidistant points were marked on the alveolar crest of the interradicular bone and a line was drawn from each point to the interradicular surface of the tooth. The length of the lines was measured, and the mean value was calculated to obtain the height of the periodontal ligament (Vacas et al., 2008). Evaluations were performed on digital microphotographs of the sections using Image Pro Plus 4.5 software.

2.3. Measurement of TBA-RS and catalase activity

Thiobarbituric acid reactive species (TBA-RS) were evaluated in gingival tissues and SMG quantifying malondialdehyde as the product of lipid peroxidation that reacts with trichloroacetic acid, yielding a pink-stained TBA-RS determined in a spectrophotometer (Hitachi U-2001) at 540 nm. TBA-RS were calculated as nmoles per milligram of tissue. Catalase activity, expressed as U/mg proteins, was measured spectrophotometrically by monitoring the disappearance of H_2O_2 at 240 nm, as it was previously described by Medina et al. (2006).

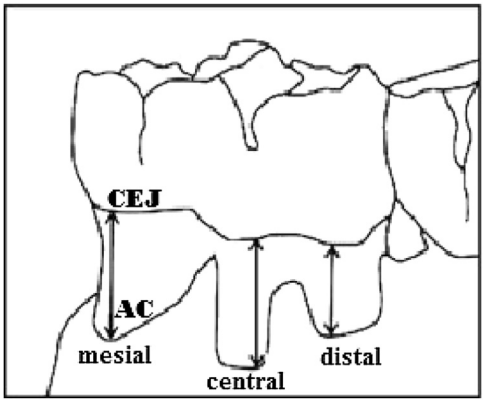

Fig. 1. Distance method: diagram of a section of the lower first molar. Three distances (arrows) were measured from the cement–enamel junction (CEJ) to the most apical area of the alveolar crest (AC).

2.4. Assessment of inflammatory parameters

2.4.1. TNFα concentration

After extraction, gum and SMG were immediately homogenized in PBS buffer containing protease inhibitory cocktail for mammalian tissue extracts (Sigma-Aldrich). The concentration of rat TNFα was determined using a sandwich ELISA according to the manufacturer's instructions (BD Pharmingen, USA).

2.4.2. Radioimmunoassay of PGE_2

To evaluate PGE_2 content, SMG and gum tissue were homogenized separately in 1 ml ice cold ethanol (100%), centrifuged at 10,000*g* for 15 min at 4 °C, and the supernatant was collected and evaporated. The residues were resuspended with radioimmunoassay buffer and Sigma antiserum was used as described in Mohn et al. (2011). The PGE_2 content was expressed as pg/mg of weight tissue.

2.4.3. Measurement of iNOS activity

The activity of inducible iNOS was measured by the method described by Ossola et al. (Ossola et al., 2012). Gingival tissue and SMG were homogenized in 500 ul of 20 mM HEPES (pH 7.4; Sigma-Aldrich) with EGTA (2 mM) and DL-dithiothreitol (DTT, 1 mM; Sigma-Aldrich). After the tissue was homogenized, NADPH and [^{14}C]-arginine monochloride (Perkin–Elmer, Waltham, MA, USA) were added to each tube and incubated for 10 min at 37 °C in a Dubnoff metabolic shaker (50 cycles per min; 95% O_2/5% CO_2). The tubes were then centrifuged at 10,000*g* for 10 min at 4 °C. The supernatants were applied to individual columns containing 1 ml of Dowex AG 50 W-X8 Na^+ form mesh 200–400 (Bio-Rad Laboratories, Hercules, CA, USA), and washed with 2.5 ml of double-distilled water. All collected effluent fluid from each column was counted for activity of [^{14}C]-citrulline in a liquid scintillation analyzer (TriCarb 2800TR, Perkin-Elmer). Since NOS converts arginine into equimolar quantities of NO and citrulline, the data were expressed as pmol of NO produced per min per mg of protein.

2.4.4. Histological analysis of SMG

The SMG removed were fixed with 10% neutral buffered formaldehyde, embedded in paraffin and 5 μm sections were stained with H&E[16]. SMG morphology and histopathological characteristics were analyzed. Light microscopy was performed on a Bausch & Lomb microscope (NY, USA).

3. Statistics

Statistical analyses were performed by unpaired t-Student test (GraphPad Inc. software, San Diego, USA). Significant difference were considered when $p < 0,05$. All measurements are mean ± SD of 15 rats.

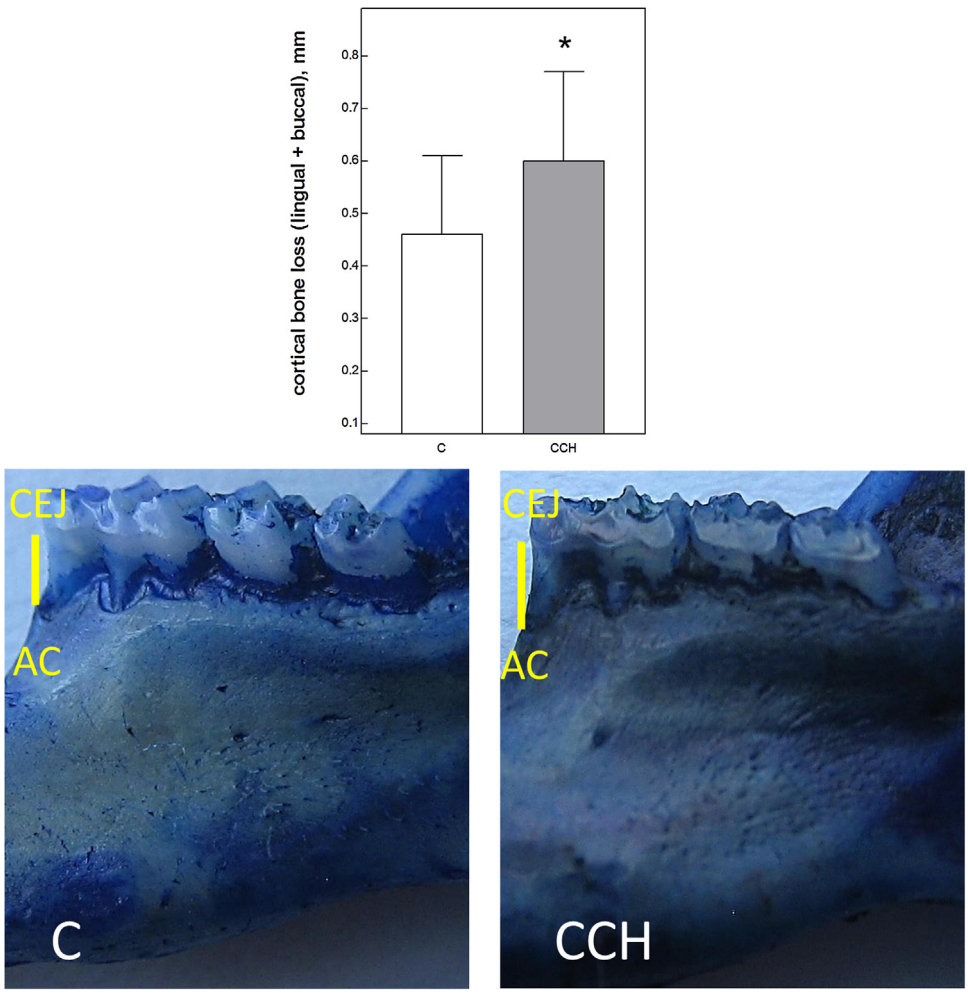

Fig. 2. Upper: Measurements by distance method showing lingual + buccal sections of mandible first molars. A significant difference between groups was chosen as $p < 0.05$ determined by t-test. Lower: photograph of the lingual side of one hemimandible per group selected randomly. CEJ: cement-enamel junction, AC: alveolar crest.

4. Results

4.1. Body weight and length

At the end of the experimental period, body weight and body length were determined. HX clearly influenced body weight in g (C: 279.1 ± 26.89, CCH: 256.77 ± 23.75) and length in cm (C: 22.24 ± 0.71, CCH: 21.57 ± 0.85).

4.2. Morphometrical analyses of alveolar bone

In order to explore the effects of CCH on periodontal tissue, we evaluated the alveolar bone loss, at cortical and interradicular level. CCH enhanced cortical alveolar bone loss (C: 0.46 ± 0.15 mm; HCC: 0.60 ± 0.17 mm) measured by the distance method from the CEJ to the AC, in lingual and buccal side of the three roots of the first mandibular molar (Fig. 2). Photographs of the alveolar bone loss are also shown in Fig. 2. The histological analysis showed a significant decrease of the interradicular bone volume, calculated as a percentage of total bone, in HX exposed animals compared to the control group (C: 42.66 ± 4.22%; CCH: 32.66 ± 4.60%; Fig. 3(a) A). The decrease in the alveolar bone volume is associated with an expansion of the bone marrow, as observed in appendicular bones during hypoxic conditions, but not so frequently in axial bones like the mandible. As expected, the CCH group showed a higher number of blood vessels due to the hypoxic stress. The periodontal ligament height was significantly enhanced in the CCH animals (C: 201.68 ± 12.09 μm; CCH: 269.67 ± 20.01 μm; Fig. 3(b)B). Photographs of transverse slices of mandibular interradicular are shown in Fig. 3(b and c).

4.3. Inflammatory parameters in gum and SMG

Chronic exposure to continuous hypoxia significantly increased iNOS activity in gums and in SMG. PGE_2 content did not significantly differ from the control animals in neither of the oral tissues analyzed. Surprisingly, in gums of CCH exposed animals TNFα content was lower than in control rats. The last inflammatory indicator was not detected in SMG (Table 1).

4.4. TBA-RS content and catalase activity

Since inflammation generates large amounts of ROS that interact with lipids, we measured membrane lipid peroxidation

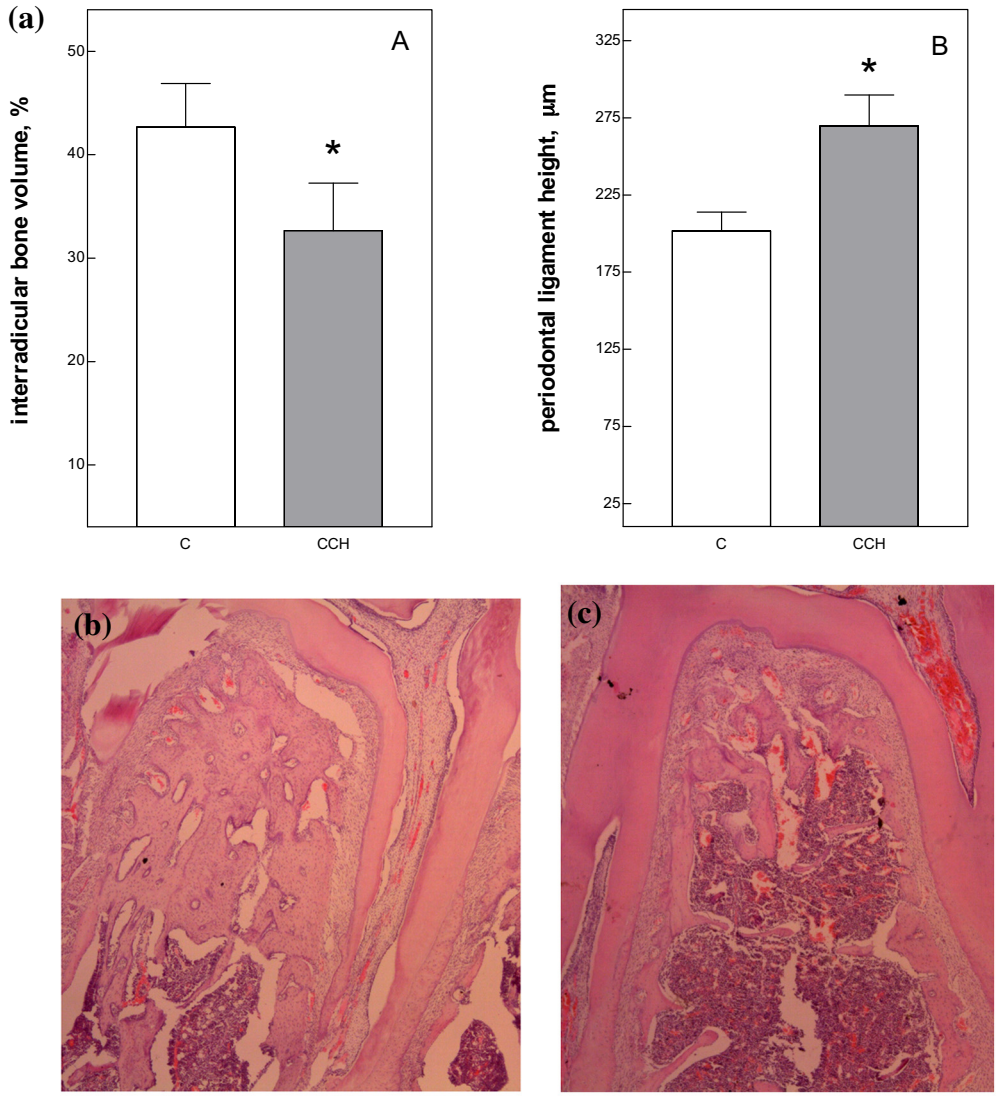

Fig. 3. (a) Interradicular bone volume (A) and periodontal ligament height (B). A significant difference between groups was chosen as $p < 0.05$ determined by t-test. (b and c) Photographs of transverse slides of the longitudinal sections of the mandibular interradicular bone of one animal per C & CCH groups respectively selected randomly. Resected hemimandibles stained with H&E were observed under a stereomicroscope (4X).

Table 1
iNOS activity, PGE_2 content and TNFα concentration in gums and GSM.

Parameter	Gum C	Gum CCH	SMG C	SMG CCH
iNOS activity (nmol/min/mg protein)	2735,04 ± 662,96	4289,58 ± 915,63*	56,71 ± 12,05	90,15 ± 21,78*
PGE_2 content (pg/mg wet tissue)	20,05 ± 2,21	20,40 ± 3,41ns	3,16 ± 0,80	2,79 ± 0,30ns
TNFα concentration (pg/mg wet tissue)	23,24 ± 4,85	13,59 ± 6,23*	non detectable	non detectable

Values are mean ± SD of 15 rats.
* indicate significant difference compare to each control animals ($p < 0.05$ determined by t-Student test). ns indicate no significant differences. C: control, CCH: chronic continuous hypoxia.

as an indicator of oxidative stress in our experimental model. Chronic continuous hypoxia increased the levels of TBA-RS in gums (Fig. 4A) and SMG (Fig. 4B) of the HX exposed animals compared to the control ones. Catalase activity in SMG (Fig. 4C) was significantly depressed in HX animals, suggesting a decrease in the antioxidant capacity of this organ.

4.5. Histological analyses of SMG

No parenchymal or stromal alterations were observed in the SMG of animals exposed to CCH regarding control animals, concluding that this environmental condition is not enough to induce inflammatory infiltration or other structural changes in the gland (Fig. 5A and B).

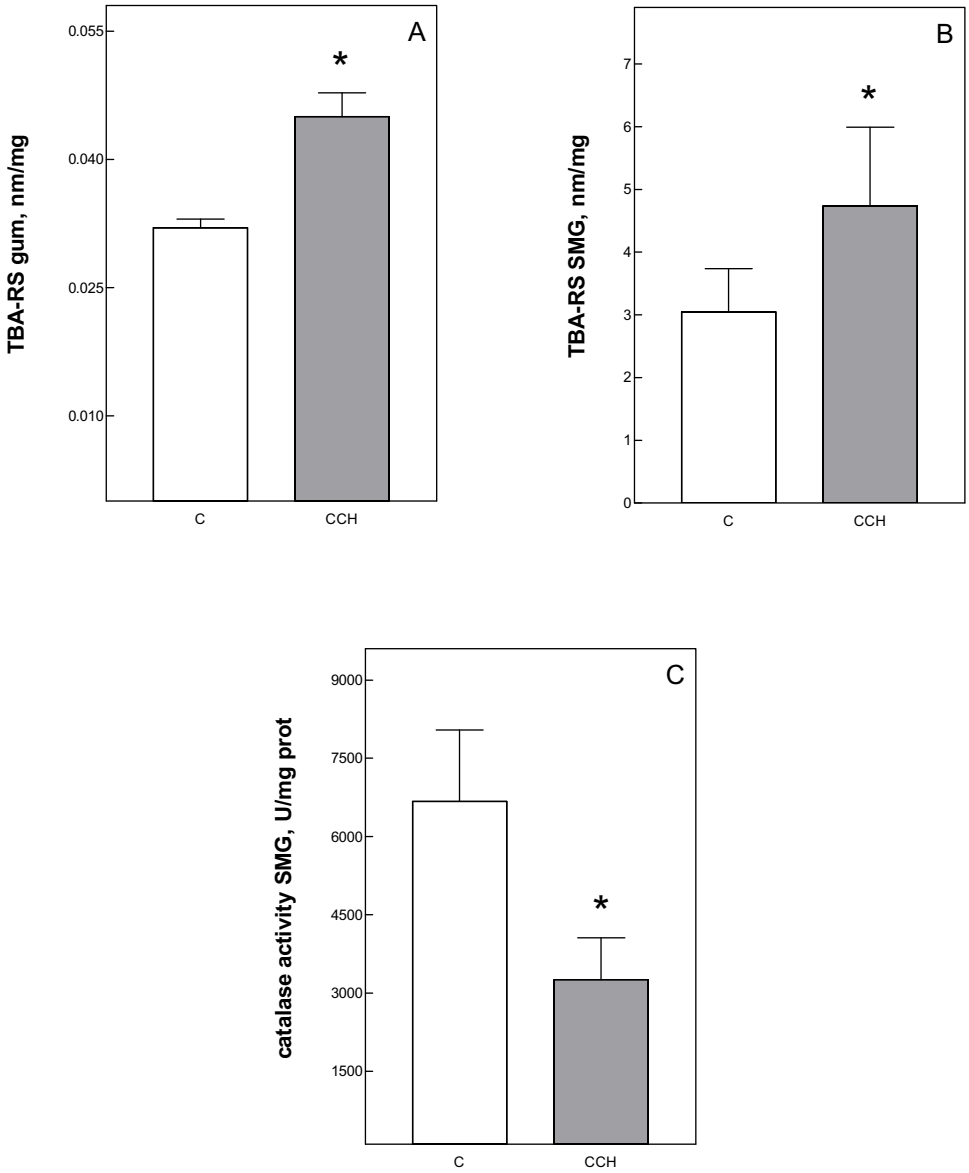

Fig. 4. UPPER: Effect of CCH on TBA-RS content in gum (A) and SMG (B). DOWN: catalase activity in SMG (C). A significant difference between groups was chosen as $p < 0.05$ determined by t-test.

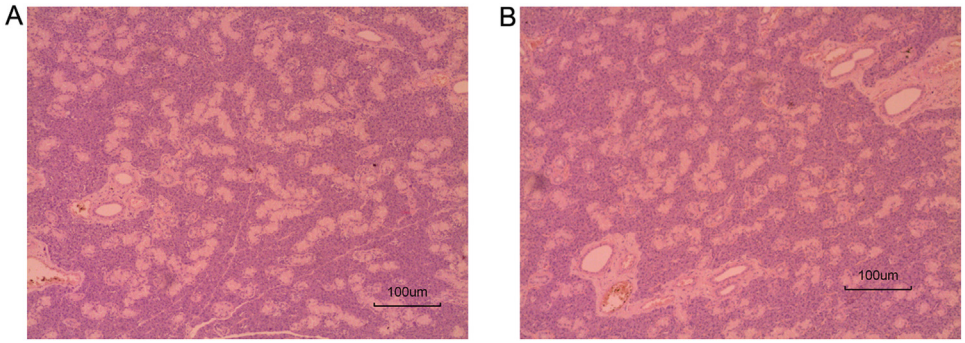

Fig. 5. (A and B) Histological appearance of SMG of one animal per C & CCH groups respectively selected randomly, showing normal structural organization of the gland. H&E staining. Magnification 10X. Scale bar = 100 μm.

5. Discussion

Periodontitis is an inflammatory disease in the teeth-supporting tissues and the main cause of tooth loss. It has been extensively associated with other systemic diseases and therefore the importance of detecting the possible risk factors that might contribute to the progression of periodontal disease (Tothova et al., 2015). Periodontitis is related with the release of many inflammatory mediators, such as nitric oxide, prostaglandins and metalloproteinases (Queiroz-Junior et al., 2013) which, among others, lead to bone and collagen destruction. On the other hand, the effects of low oxygen levels in the oral environment are of particular relevance due to its relationship with bacteria survival and growth and the consequent maintenance of the inflammatory response (Xiao et al., 2012). These findings highlight the importance of studying the effect of chronic continuous hypoxia on periodontal health.

In this study we demonstrated that the exposure to CCH in rats induces alveolar bone loss, increases periodontal ligament height and modifies some inflammatory mediators that play an important role in the development of periodontal disease. In concordance with these results, we have previously demonstrated that chronic intermittent hypoxic condition (506 mbars, 18 h/day during 3 month) increases alveolar bone loss in rats subjected to experimental periodontitis (EP), causing also an incipient damage on alveolar bone in rats not subjected to EP (Terrizzi et al., 2013).

In order to correlate alveolar bone damage observed in CCH rats with the most studied periodontal inflammatory mediators, iNOS activity, TNFα concentration and PGE_2 content in gums and SMG were determined. CCH rats showed increased activity of iNOS in the gums closely associated to the damaged alveolar bone. It is known that iNOS is over expressed during inflammation leading to excessive levels of NO that may promote osteoclast maturation and therefore, induces bone destruction. Our results are in agreement with previously reported data in which gums of patients with chronic periodontitis showed higher content of iNOS, suggesting an important role of this enzyme in the pathogenesis of the disease (Uğar-Cankal & Ozmeric, 2006). iNOS activity was also enhanced in SMG by means of CCH. NO is known to participate in the blood flow and salivary secretion regulation, and higher levels of this mediator produced by iNOS have been associated to several oral diseases, such as Sjogren syndrome and tumors (Uğar-Cankal & Ozmeric, 2006). We believe that the higher activity of NO by means of increased levels of iNOS together with increased values of ROS observed in CCH rats, could lead to salivary dysfunction as was previously reported by different authors (Vacas et al., 2008; Lomniczi et al., 2001; Tai et al., 2009) further contributing to higher periodontal damage due to lower saliva volume. TNFα is another biological mediator associated with periodontitis, as it enables the ingress of inflammatory cells into the sites of infection, promoting bone resorption and prostaglandin release (Ossola et al., 2012). In our experimental model, TNFα was decreased in gums of CCH animals compared to the control group. This could be explained by the fact that HX alone not only triggers small amounts of TNFα but also enhances TNFα lysosomal degradation in mouse macrophages (Lahat et al., 2008).

Oxidative stress can be defined as the imbalance between the production of ROS and the ability to scavenge them by endogenous antioxidant systems. In the present study, we found higher gingival TBA-RS levels in CCH exposed animals when compared to control group. It has been shown that oxidative stress leads to a decline in gingival fibroblasts functioning, resulting in impaired collagen synthesis, activation of metalloproteinases and fibroblasts apoptosis (Orihuela-Campos et al., 2015). In addition, our result correlates with clinical data reported in the literature, where high levels of this lipid peroxidation marker have been found in patients with altered periodontal status (Borges et al., 2007). In SMG we observed not only higher levels of TBA-RS but also lower levels of catalase activity due to CCH. Even though no microscopic alterations were observed in this organ, the previous results could indicate a biochemical alteration of the gland, supporting a possible mechanism of salivary secretion impairment due to HX (Elverdin et al., 1995). However, further studies are needed to corroborate salivary hypofunction within the conditions of our experimental model.

Regarding the content of PGE_2, we did not observe an increase in this marker either in gums or in SMG of the animals exposed to CCH. Some authors have reported decreased levels of this inflammatory parameter during long term exposure to HX meanwhile short periods increased them (Zhao et al., 2012). High levels of NO produced by iNOS might block the cyclooxygenase 2 activity, enzyme responsible of PGE synthesis modulating the prostaglandin biosynthetic pathway (Mollace, Muscoli, Masini, Cuzzocrea, & Salvemini, 2005). These findings partly support our results. Additionally, as previously mentioned, eicosanoid production is highly related with TNFα release, which was decreased in our experimental model.

Based on the present evidence, we can conclude that the higher activity of iNOS and the excess in ROS production that occur under CCH in gingival tissue may be involved with the periodontal destruction observed under this experimental condition. Moreover, the alteration in its redox status and the increased iNOS activity in the SMG could contribute to a decline in its secretory function. This could lead to an immunological, hydric and mineral misbalance within the oral environment, worsening the bone resorption. Therefore, we conclude that HX might induce deleterious effects in the periodontium and in the SMG of people continuously exposed to this environmental condition. However, further studies that prove the connection between CCH, periodontitis development and salivary gland impairment are still required.

Conflict of interests

Drs. Fernandez-Solari, Conti & Martinez and dentists Terrizzi and Lee report no conflicts of interest related to this study and agree with the decisions about it, approved the final manuscript and referred to it as original manuscript, with text, figures and photographs that have not appeared in any other publication, This material has been submitted only to Archives of Oral Biology.

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