Journal of Dental Sciences 17 (2022) 176–183

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Original Article

Nasal obstruction promotes alveolar bone destruction in the juvenile rat model

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Received 21 May 2021; Final revision received 25 May 2021
Available online 11 June 2021

KEYWORDS
Hypoxia;
Inflammation;
Mandible;
Mouth breathing;
Rhinitis

Abstract *Background/purpose:* Nasal obstruction leads to oral breathing and consequently hypoxia. The purpose of this study was to determine the influence of hypoxia on inflammatory response and the effect on alveolar bone development in a rat model in which mouth breathing was induced by nasal obstruction.
Materials and methods: Unilateral nasal obstruction was performed by injecting a Merocel sponge into the nasal cavity of 8-week-old Sprague Dawley (SD) rats. After 3 and 6 weeks of nasal obstruction, rats were sacrificed, the organs were weighed, and the changes in mandibular bone quality were examined by micro-computed tomography (μ-CT). The stereomicroscope was used for the morphological analysis of alveolar bone loss in response to nasal obstruction. Hematoxylin and Eosin (H&E) and immunohistochemical staining were employed to examine inflammation and bone remodeling induced by hypoxia.
Results: Nasal obstruction led to a delay in overall growth and organ development. The bone mineral density (BMD) and bone volume/total volume (BV/TV) of the mandible were reduced due to nasal obstruction, and the loss of the alveolar bone was confirmed morphologically. Our nasal obstruction method was observed to be successful in inducing hypoxia along with an increase in hypoxia-inducible factor 1-alpha (HIF-α). Oral hypoxia induced by nasal obstruction increased inflammatory response, and increased expression of receptor activator of nuclear factor kappa-B ligand (RANKL) led to bone destruction.
Conclusion: This study demonstrated that nasal obstruction induced mouth breathing led to hypoxia in a rat model. Under hypoxic conditions, an increase in osteoclast differentiation induced by activation of the inflammatory pathway causes destructive changes in the alveolar bone.

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https://doi.org/10.1016/j.jds.2021.05.012

Introduction

Mouth breathing refers to the behavior of breathing through the mouth. It has been reported that the main causes of mouth breathing are severe rhinitis and tonsillar hypertrophy.[1,2] In particular, mouth breathing in children may lead to serious clinical consequences such as being pale, apathetic, lack of concentration, and often getting tired.[3] It should be noted that mouth breathing not only affects children but adults as well.

The prolonged mouth breathing is mostly related to the nasal obstruction which influences the change in craniofacial structure and function.[4] Consequently, nasal obstruction in growing children seriously affects the normal development of maxillary complex and causes morphological and functional abnormalities, such as clockwise rotation of the lower jaw, narrowing of the upper and lower dental arch, and a high arched palate.[5–7] Adults with frequent nasal obstruction suffer from dental malocclusions and craniofacial bone abnormalities. Nasal obstruction is speculated to be related to a decrease in bone mineral density and further studies are required to clarify the process of alveolar bone destruction at the molecular level.

Rhinitis is a common heterogeneous chronic disorder that is defined as inflammation of the nasal mucosa and characterized by the presence of one or more nasal symptoms including sneezing, itching, nasal discharge, and nasal blockage.[8] Recently, there has been an increase in the incidence of rhinitis worldwide, and the prevalence has been reported at a rate of more than 30% of the population in the industrialized countries.[9] The atmospheric environment has been changing rapidly especially in East Asia, and air pollution is leading to a series of diseases such as rhinitis, asthma, and conjunctivitis.[10] Since air pollution is closely related to nasal obstruction, a study on the association between nasal obstruction caused by nasal inflammation and oral diseases is necessitated.

To investigate the effect of nasal obstruction on the oral cavity, in the present study, we have utilized a juvenile rat model with unilateral nasal obstruction and examined the changes in the salivary glands and alveolar bone.

Materials and methods

Nasal obstruction

The study protocol was approved by the Ethical Committee of Jeonbuk National University (CBNU 2019–092). In the experiment, an 8-week-old SD rat was used as a juvenile rat model.[11] The animals were randomly assigned to two groups: without nasal obstruction (Control; n = 12), with nasal obstruction (NO; n = 12). To induce nasal obstruction, a method of packing the left nasal cavity of rats with a Merocel sponge (Medtronic Xomed, Jacksonville, FL, USA) was used. All surgical procedures were performed under general anesthesia induced by Zoletil 50 (Virbac Laboratories, Carros, France) and Rompun (Bayer Korea Ltd., Seoul, Korea). First, the Merocel sponge was cut into a length of 1.5 cm and a diameter of 1 mm to facilitate the entry into the nasal cavity of rats. Next, after wiping the left nostril of the rat with an alcohol swab, the Merocel sponge was gently inserted into the nasal cavity. Amikacin (Samu Median Co., Chungnam, Korea) was injected into the nasal cavity to allow sponge expansion and prevent infection. After induction of nasal obstruction, body weight was measured once a week for 6 weeks to examine body changes.

Micro-computed tomography (μ-CT) analysis

The mandibles were scanned using μ-CT after anesthesia, and the dynamic changes in the alveolar bone were evaluated at 3 and 6 weeks after nasal obstruction. The μ-CT was performed using a Skyscan 1076 (Bruker, Kontich, Belgium) with an anode electrical current of 100 kV at 18 μm resolution. The region of interest (ROI) of the alveolar bone was manually established in the interradicular septum bone of the left mandibular first molar (M1). The coronal and horizontal planes of M1 were confirmed by two-dimensional (2D) images. First, in the coronal plane passing through the center of the buccal and lingual roots, two horizontal surfaces were selected that individually passed through the alveolar ridge crest and apex of the buccal root. Second, on the horizontal plane of the M1 tooth, the interalveolar septum was selected by drawing a contour from the center of one root canal to another root canal by avoiding the roots and other structures. The selected ROI was analyzed for BMD and BV/TV using the computer tomography analyzer (CTAn) software.

Morphometric analysis of alveolar bone loss

The left mandible of the rat was isolated to remove soft tissue and washed with phosphate-buffered saline (PBS). The left mandible molars and alveolar bone crest images were captured with a stereomicroscope (Leica Microsystems GmbH, Wetzlar, Germany) and evaluated with software Image J (National Institutes of Health, Bethesda, MD, USA) software. Alveolar bone loss was measured based on the distance and area from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC).

Hematoxylin and Eosin (H&E) staining

The mandibles were isolated and fixed in a 10% formalin solution. After decalcification in 15% EDTA and 0.1 M Tris

(pH 7.0), the mandibular tissues were dehydrated. The mandible samples were embedded in paraffin, cut into 8-μm sections, and mounted onto the glass slides. The sectioned mandible sample was dewaxed with xylene, rehydrated with a series of ethanol, and rinsed with distilled water. Next, the samples were stained with H&E and examined using a light microscope.

Immunohistochemistry (IHC) staining

IHC staining was performed using the immunohistochemistry accessory kit (Bethyl Laboratories, Montgomery, TX, USA). Each primary antibody was used at a 1:200 dilution according to the protocol. Antibody HIF-α was purchased from Novus (Novus Biologicals, Centennial, CO, USA). The antibodies to TNF-α, IL-6, RAGE, OPG, BMP-2, and BMP-7 were purchased from Bioworld (Bioworld Technology, Louis Park, MN, USA). RANKL antibody was purchased from Enzo (Enzo Life Sciences, Farmingdale, NY, USA). The levels of antibody expression were measured with software Image J (National Institutes of Health).

Statistical analysis

All results of the control and experimental groups were analyzed independently. An independent two sample *t*-test at a significance level of $p < 0.05$ was used to examine the differences in variables under different experimental conditions.

Results

Physical changes due to nasal obstruction

After induction of nasal obstruction in 8-week-old SD rats, the morphological changes in different organs and mandible at 3 and 6 weeks were analyzed (Fig. 1A). One-

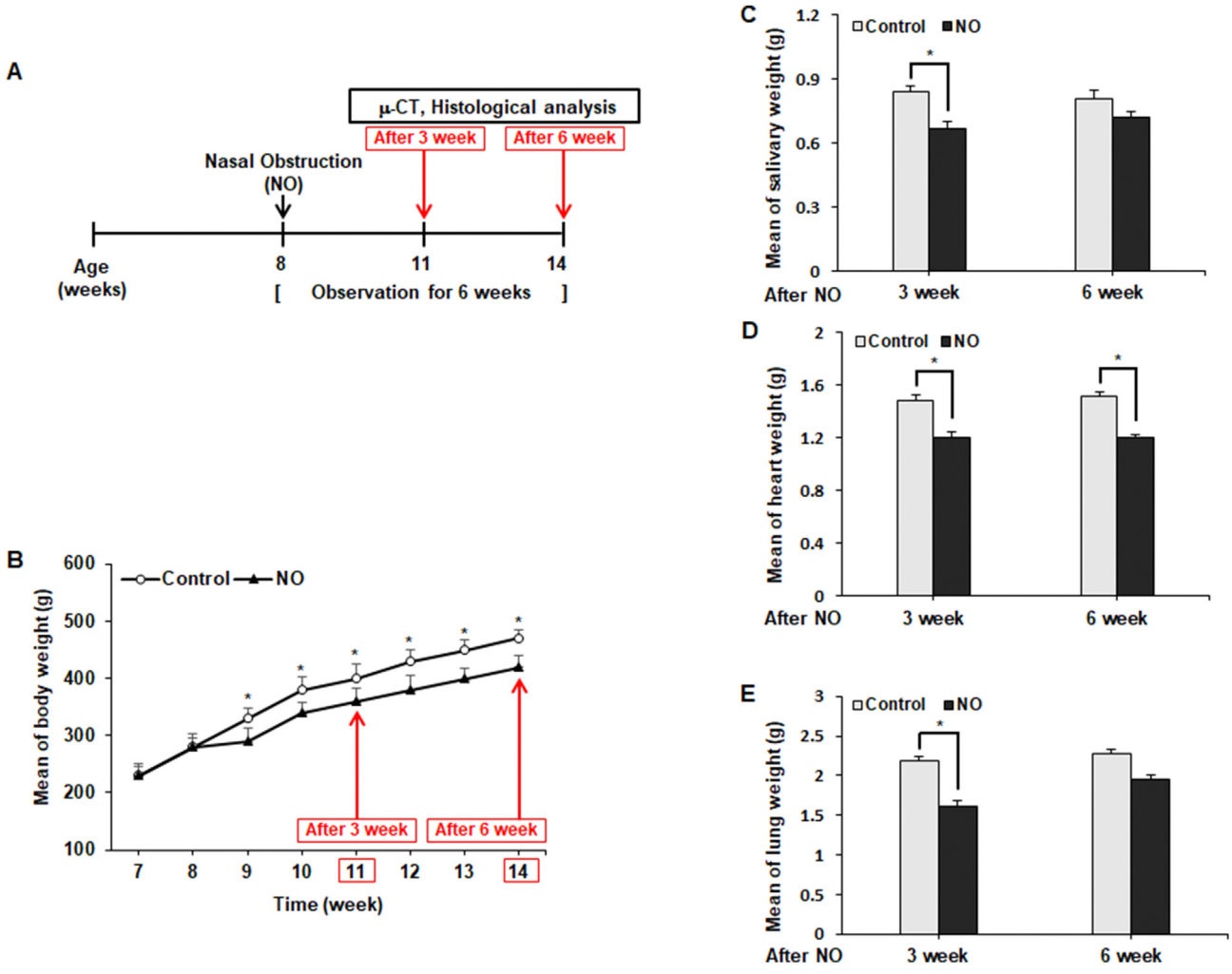

Figure 1 Changes in the physical status of the rats due to nasal obstruction. (A) Schematic procedure of the *in vivo* model. (B) Changes in the body weight of rats due to nasal obstruction. Control, without nasal obstruction; NO, with nasal obstruction. (C) Weight of the salivary glands after 3 and 6 weeks of nasal obstruction. (D) Weight of the heart after 3 and 6 weeks of nasal obstruction. (E) Weight of the lungs after 3 and 6 weeks of nasal obstruction. *: $P < 0.05$.

week after induction of nasal obstruction, body weight in the NO group was identified to be significantly lower compared to the control group, and the difference was significant During entire experimental period (Fig. 1B). In addition, the weight of the submandibular gland in the NO group was significantly lower than the control group at 3 weeks after nasal obstruction (Fig. 1C). Heart weight was significantly lower in the NO group than the control group at both 3 and 6 weeks after nasal obstruction (Fig. 1D). The lung weight was also significantly lower in the NO group at 3 weeks after nasal obstruction, compared to the control group (Fig. 1E).

Mandibular changes due to nasal obstruction

Rats were anesthetized and the changes in the mandible induced by nasal obstruction were observed by μ-CT. Empty space could be observed in the ROI in the NO group at 3 and 6 weeks, while it was scarcely observed in the control group (Fig. 2A). In addition, BMD and BV/TV were significantly decreased at 6 weeks in the NO group compared to the control group (Fig. 2B and C).

Alveolar bone loss induced by nasal obstruction

A stereomicroscope was used for the morphometric analysis of the alveolar bone loss. In the control group, the alveolar bone crest (ABC) supported the teeth in a serrated shape, but in the NO group, the ABC on the M2 root side demonstrated a blunt serrated shape after 6 weeks (Fig. 3A). Furthermore, it was confirmed that the area of alveolar bone loss at 6 weeks in the NO group was significantly widened when the area between the cementoenamel junction (CEJ) and ABC of the control group and the NO group was measured and compared (Fig. 3B). Histomorphology analysis of the H&E stained mandibular bone in the NO group at 6 weeks showed more empty spaces and inflammatory cell infiltrates in the alveolar bone compared to the control group (Fig. 3C).

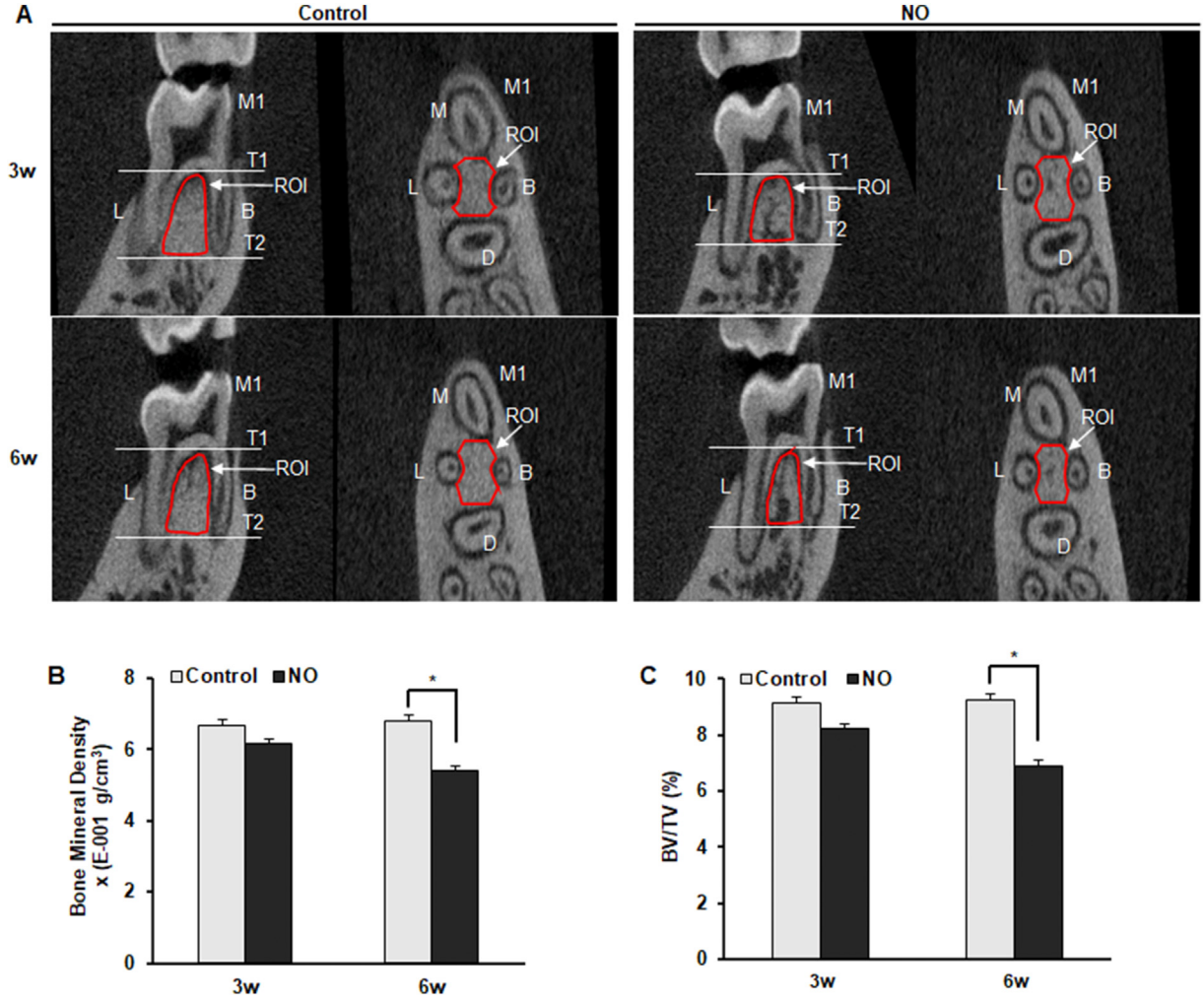

Figure 2 μ-CT analysis of mandibular changes in the rats due to nasal obstruction. (A) μ-CT images of the interradicular septum bone of the left mandibular first molar. M1, the mandibular first molar; M, mesial root; B, buccal root; D, distal root; L, lingual root; and T1 and T2, the two parallel horizontal planes passing through the alveolar ridge and the apex of the buccal muscle separately. (B) Bone mineral density analysis after 3 and 6 weeks of nasal obstruction. (C) BV/TV analysis after 3 and 6 weeks of nasal obstruction. *: $P < 0.05$.

Induction of HIF-1α and inflammatory response in response to nasal obstruction

HIF-1α expression was increased in the NO group at 3 and 6 weeks compared to the control group, thereby suggesting that hypoxia was induced by nasal obstruction (Fig. 4A). The levels of inflammatory cytokines such as TNF-α and IL-6 were increased at 3 and 6 weeks in the NO group (Fig. 4B and C). Also, the RAGE level was increased at 3 and 6 weeks in the NO group (Fig. 4D).

Bone destruction due to nasal obstruction

To investigate the mechanism by which nasal obstruction induces alveolar bone destruction, the expression of molecules related to bone remodeling was examined. The expression of RANKL, an osteoclast differentiation marker, was increased at 3 and 6 weeks in the NO group compared to the control group (Fig. 5A). On the contrary, OPG, an osteoblast differentiation marker, was suppressed in the NO group at 3 and 6 weeks compared to the control group (Fig. 5B). Similarly, the level of bone formation molecules such as BMP-2 and BMP-7 was decreased in the NO group at 3 and 6 weeks compared to the control group (Fig. 5C and D).

Discussion

In this study, we have examined the effect of nasal obstruction on the growth and alveolar bone using a juvenile rat model. Nasal obstruction in the rats resulted in delayed normal growth of body and organs, loss of alveolar bone, and a decline in alveolar bone quality. It has been reported that nasal obstruction negatively affects alveolar bone.[12,13] However, considering that previous studies were conducted using neonatal rats, our study provides meaningful data on the effect of nasal obstruction in adolescents.

Nasal obstruction can lead to hypoxia.[14,15] Under a hypoxic state, the amount of tissue HIF-1α increases, which

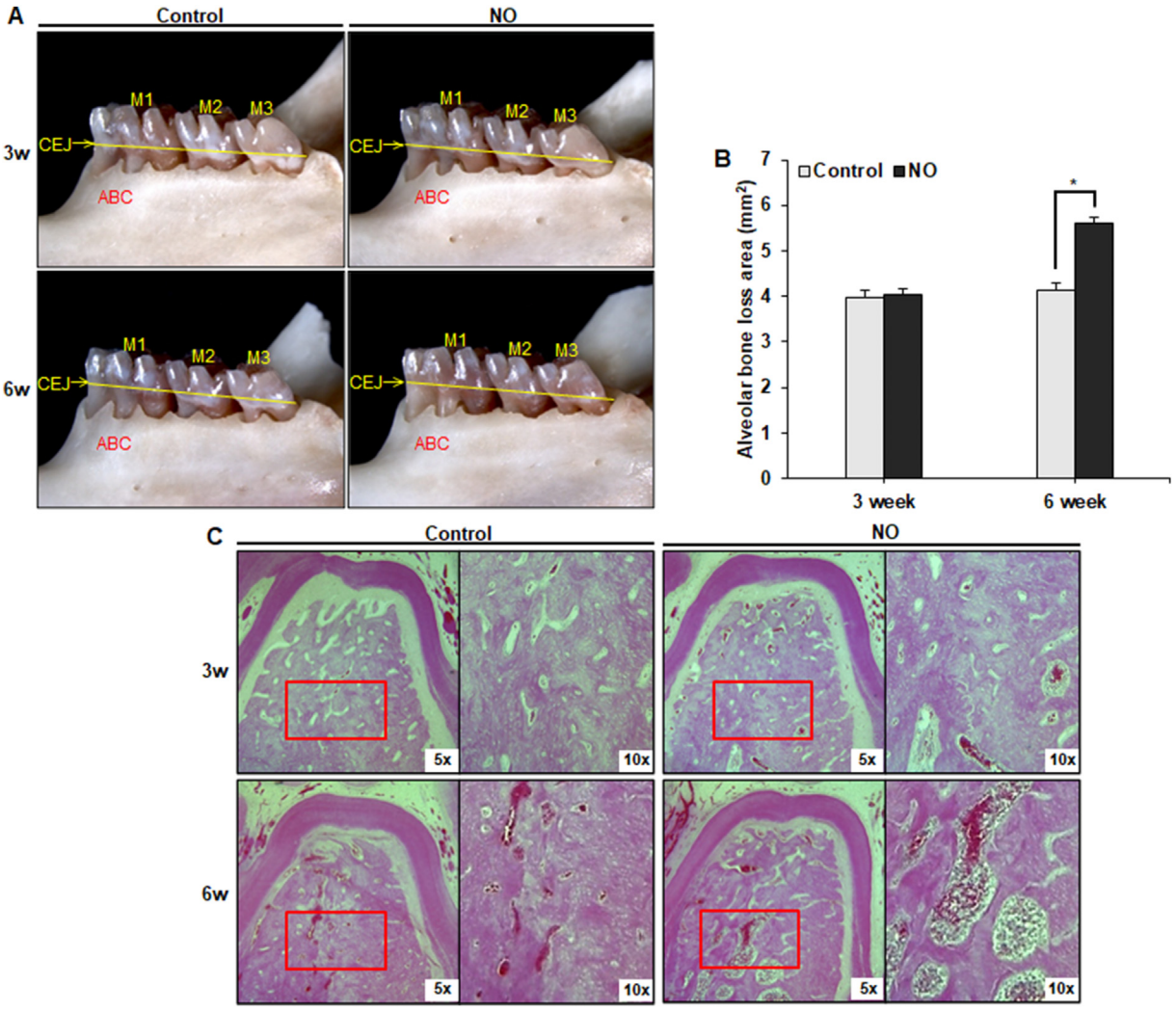

Figure 3 Morphological and histological analysis of mandibular changes in the rats due to nasal obstruction. (A) Stereomicroscopic images of the mandible of rats. CEJ, cementoenamel junction; and ABC, alveolar bone crest. (B) Alveolar bone loss area was measured by ImageJ software. (C) H&E stained tissue section of the rat mandible. *: $P < 0.05$.

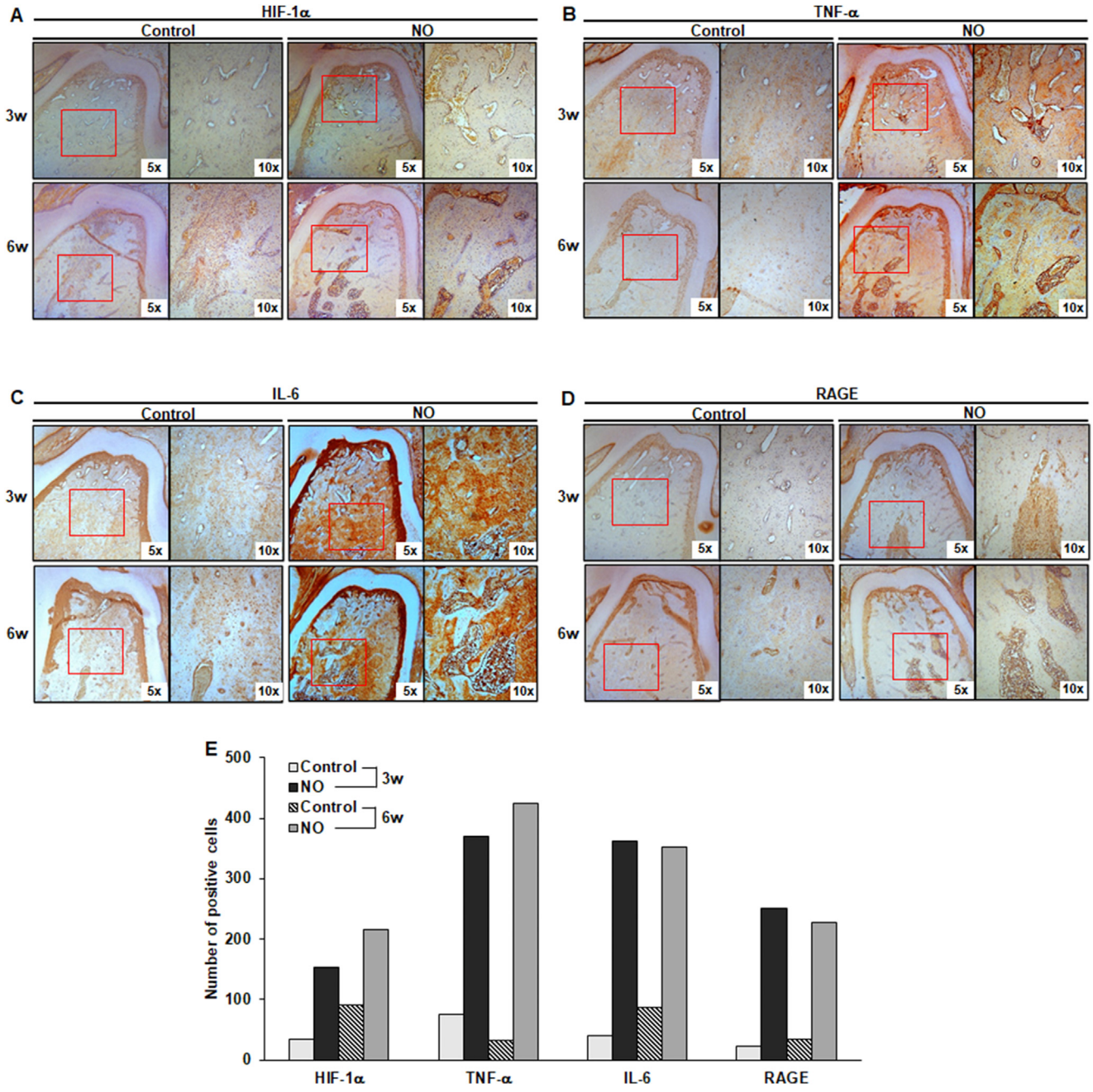

Figure 4 Immunohistochemical analysis of the inflammatory molecules in the rat alveolar bone. (A) HIF-1α, (B) TNF-α, (C) IL-6, and (D) RAGE. (E) The levels of HIF-1α, TNF-α, IL-6, and RAGE expression were measured.

in turn activates inflammatory responses.[16] It has been reported that HIF-1α activates macrophage, dendritic cells, T and B lymphocytes, and induces the expression of inflammatory cytokines such as TNF-α and IL-6.[17—19] Also, an increase in HIF-1α due to hypoxia upregulates RAGE, thereby resulting in the expression of inflammatory cytokines.[20] On the contrary, there have been studies reporting that activating HIF-1α in a normoxic state suppresses inflammation and improves tissue healing.[21,22] Overall, the results in the present study suggest that upregulation of HIF-1α and inflammatory cytokines in tissue was caused by hypoxia induced by nasal obstruction though oxygen saturation was not measured during the experiments.

Chronic inflammation causes bone resorption by the activation of osteoclasts.[23,24] Inflammatory cytokines such as TNF-α and IL-6 and increased expression of RAGE enhances osteoclast differentiation.[25—27] As expected, expression of RANKL, a marker of osteoclast was increased by nasal obstruction. On the contrary, levels of OPG, an osteoclastogenesis inhibitor, and BMP-2 and BMP-7, inducers of bone formation, were decreased due to nasal obstruction. The changes in gene expression are hypothesized to be associated with the observed alveolar bone loss.

Moreover, nasal obstruction can lead to mouth breathing. It has been reported that mouth breathing causes dryness of the oral cavity and decreased salivary flow

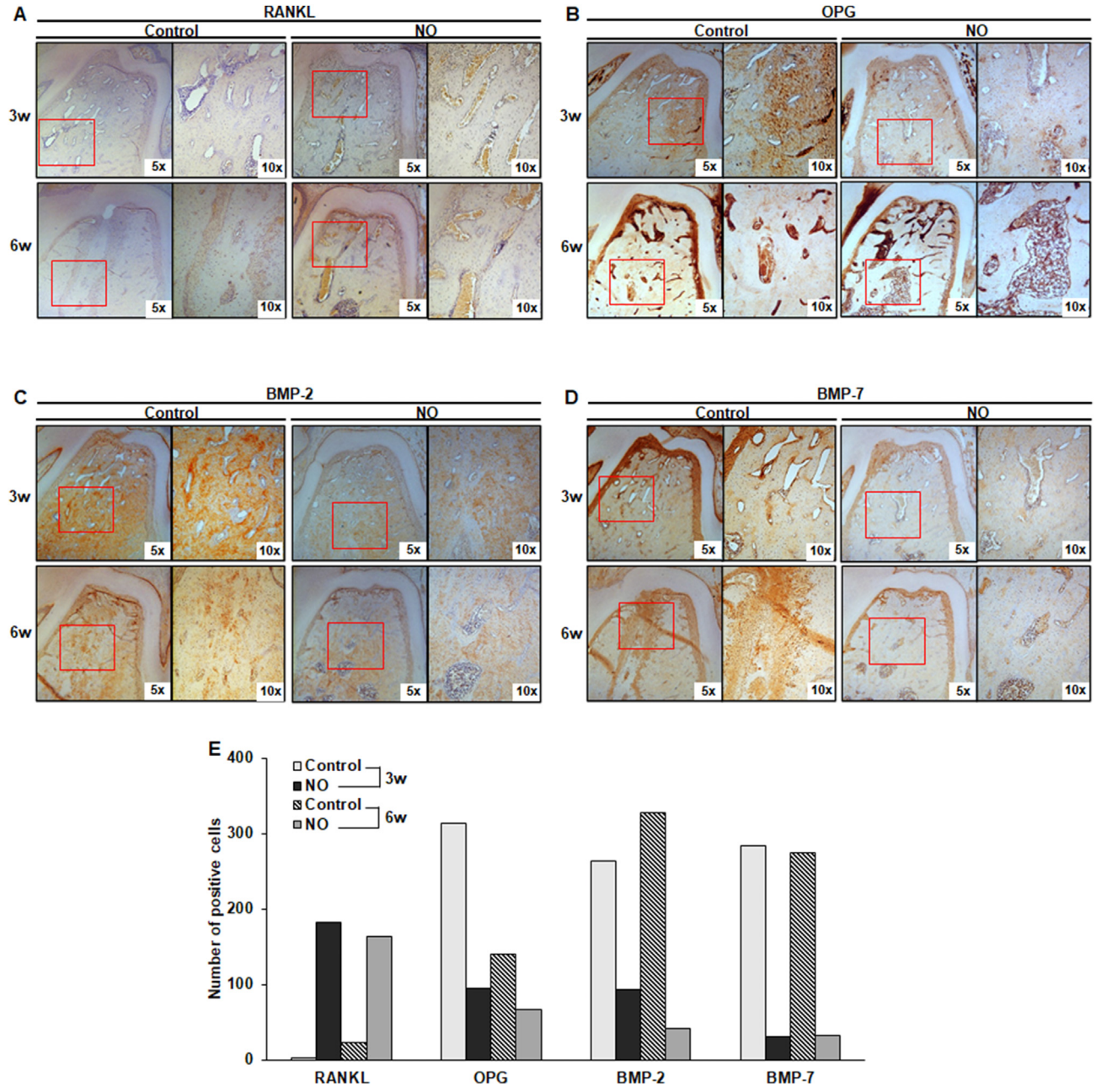

Figure 5 Immunohistochemical analysis of the molecules related to bone remodeling in rat alveolar bone. (A) RANKL, (B) OPG, (C) BMP-2, and (D) BMP-7. (E) The levels of RANKL, OPG, BMP-2, and BMP-7 expression were measured.

induces alveolar bone loss.[28] Thus, it is hypothesized that alveolar bone loss in rats might be a consequence of an inflammatory response that is provoked by hypoxia due to nasal obstruction and dryness of the oral cavity due to mouth breathing.

Though the reason remains unclear, mouth breathing causes growth retardation.[3] In addition, a study reports the negative effect of hypoxia on the growth and development in an intermittent hypoxia rat model.[29] These findings coincide with our results which showed growth inhibition in rats that underwent nasal obstruction. Overall, it can be concluded that nasal obstruction effectively induced mouth breathing and consequently hypoxia in our model. Destructive changes in the alveolar bone might be a consequence of increased osteoclast differentiation, induced by activation of inflammatory pathways under hypoxic circumstances.

Rhinitis is one of the major causes of nasal obstruction. The prevalence of allergic rhinitis is continuously increasing.[30,31] It can be assumed that the occurrence of problems associated with nasal obstruction is becoming more frequent. Our study demands attention on the effect of nasal obstruction and hypoxia on juvenile growth and oral health. Further studies on the effect of nasal obstruction on oral and systemic health are necessitated in the future.

In conclusion, hypoxic conditions caused by nasal obstruction promote destruction in the alveolar bone with

an increase in osteoclast differentiation induced by activation of the inflammatory response.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgment

This work was supported by a grant from the National Research Foundation (NRF) of Korea, funded by the Korean government (MSIP) [(NRF-2018R1A2B6004744).

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