Original Article

Alveolar Bone Density Reduction in Rats Caused by Unilateral Nasal Obstruction

Wang et al. Nasal Obstruction Reduce Bone Density

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Background: Oral breathing can cause morphological changes in oral and maxillofacial regions.

Aims: This study aimed to investigate whether oral breathing affected structural changes of bone tissues.

Study design: Animal Experimentation.

Methods: A total of 48 8-day-old male Sprague–Dawley (SD) rats were divided into two groups: breathing groups and sham control groups. All SD rats were killed at 7 weeks after unilateral nostril obstruction modeling. Then the structural changes of bone tissues were detected by micro-CT, and the expression levels of receptor activator of nuclear factor-κB (RANK), osteoprotegerin (OPG), and receptor activator of nuclear factor-κB ligand RANKL in the signal pathway of bone metabolism within local alveolar bone and serum of rats were detected by RT-qPCR and Western blot.

Results: The results showed RANKL and RANK levels of bone tissues and serum in the oral breathing group were higher than those in the control group (Maxillary alveolar bone (XAB): RANKL (pRNA=0.009, pprotein=0.008), RANK (pRNA=0.008, pprotein=0.009); Mandibular alveolar bone (NAB): RANKL (pRNA=0.047, pprotein=0.042), RANK (pRNA=0.041, pprotein=0.007); Serum: RANKL (pRNA<0.001, pprotein<0.001), RANK (pRNA<0.001, pprotein<0.001)) and decreased OPG expression (XAB: pRNA=0.038, pprotein=0.048; NAB: pRNA<0.001, pprotein<0.001; Serum: pRNA=0.009, pprotein=0.006). Along with decreased OPG expression, RANKL/OPG and elevated RANKL/OPG. The micro-CT analysis indicated that significant difference was found between level of BV/TV, as
well as Tb.Th. in maxillary alveolar bone of experimental group and control group (p= 0.049, p= 0.047). Meanwhile, Tb.Th. and Cort.Th. levels in mandibular alveolar bone of experimental group also showed significance compared with that in control group (p= 0.043, p= 0.024).

**Conclusion:** This study confirmed that structural changes of the respiratory tract affect alveolar bone structure, and the unilateral nostril obstruction may lead to the change of regional specific bone density.

**Keywords:** Nasal obstruction; Bone mineral density; RANK; RANKL; Signal pathway of bone metabolism

Oral breathing has an important impact on the growth and development of craniofacial structures(1-5). Animal experiments demonstrated that changes in breathing patterns from nasal cavity to oral cavity can induce changes in craniofacial muscle activity(6-11). Recent studies have focused on the effect of oral breathing on morphology of maxillofacial adaptation significantly(12,13). However, the adaptive structural component of alveolar bone remains unclear.

In orthodontic patients, many factors contribute to the success of orthodontic microimplants (OMIs), among which, cortical bone thickness and bone density are important factors affecting the success of OMIs(14-24). Oral breathing is speculated to be related to the decrease of bone mineral density in local alveolar bone. Nonetheless, further studies are needed to determine whether the poor bone quality of local alveolar bone can be attributed to oral breathing.

In this study, we hypothesized that after unilateral nostril obstruction, the resistance changes in the nasal cavity to the oral passage may change the cancellous bone of the alveolar bone and the bone mass of the cortical bone due to local and systemic changes, thereby reducing the bone density of the alveolar bone.

**Materials and methods**

2.1 Animals and feeding:
SD female rats (250-300 g) during lactation and their 8-day-old neonatal rats (18–20 g) were purchased from Shanghai Laboratory Animal Center in Chinese Academy of Sciences (Shanghai, China). All rats were fed with standard rodent food and given free access to water in a SPF animal room with constant temperature and 12 h of light–dark cycle. All animal experiments were examined and endorsed by the animal and ethical review committee of XXXXX (XXXXX Policy and Welfare Committee; File ID No.: XXX-2011-AP-0013).

2.2 Reagents:
Trizol reagents, DNaseI enzyme, M-MLV reverse transcriptase and SYBR Green PCR Master Mix were purchased from Invitrogen (Carlsbad, CA, USA). BCA protein analysis reagent kit was provided by Pierce (Rockford, USA). PVDF was provided by Bio-Rad Laboratories (Hercules, USA). Rabbit anti-human OPG and RANKL were purchased from Novus Biologicals (Littleton, USA), rabbit anti-human RANK, β-Actin as well as goat anti-rabbit IgG HRP were provided by Cell Signaling Technology (Boston, USA). ECL reagent kit was purchased from Amersham Pharmacia Biotech (Piscataway, USA), and all other reagents were of analytical grade.

2.3 Establishment of model rats and sample collection
A total of 48 8-day-old male SD neonatal rats were randomly divided into three groups: group 1 (maxillary alveolar bone of unilateral nostril electrocautery group) vs. group 2 (maxillary alveolar bone of control group (sham group)); group 3 (mandible alveolar bone of unilateral nostril electrocautery group) vs. group 4 (mandible alveolar bone of control group); group 5 (serum of unilateral nostril electrocautery group) vs. group 6 (serum of control group). There were 24 rats in oral breathing group or sham group with 4 rats in each cage, which contained one adult breastfeeding SD female rat. In the 1st to 6th cages of oral breathing group (unilateral nostril electrocautery group), the left nostrils of neonatal rats were blocked with high-frequency electric knife (Figure 1). In the 7th to 12th cages of sham group, the rats received no treatment. Both groups received breastfeeding in the early stage and were provided with standard rodent food and free access to water. All rats were sacrificed after 7 weeks of modeling. The maxillary and mandible alveolar bones were collected immediately and divided into three parts: the first part was soaked in Trizol for subsequent RT-qPCR analysis; the second part was preserved in liquid nitrogen for subsequent western blot analysis; and the third part was soaked in a EP tube with 4% paraformaldehyde for bone density analysis. Meanwhile, venous blood of the neck was collected in each rat, and then centrifuged for 10 min at 4 °C. Afterwards, the serum was obtained through absorbing supernatant and was preserved in liquid nitrogen.

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2.4 Measurement of bone density of SD rats

One-third of the collected maxillary and mandible alveolar bones were immediately soaked in a EP tube with 4% paraformaldehyde for subsequent analysis of bone density (See 2.1). Then, the maxillary and mandible alveolar bones were scanned by micro-CT (PerkinElmer, Model Number: QuantumGX). Micro-CT scanning and 3D reconstruction technology were used to scan the maxillary and mandible bones of rats and standard phantom simultaneously for CT value correction. Quantitative analysis was achieved with system-provided Analyze12.0 software. The scanning pixel size was 18 µm; the scanning voltage was 90 kV; the scanning current was 80 µA; the scanning mode was 360° rotation; the scanning time was 14 min; the scanning field of vision was 9 mm×9 mm×9 mm. Quantitative analysis of bone enrolled the following main measurement parameters: trabecular bone volume fraction (BV/TV), trabecular thickness (Tb.Th.), cortical thickness (Cort.Th.) of maxillary, and mandibular alveolar bone. Bone thickness and density were mainly tested in alveolar bone cortex.

2.5 RT-qPCR detection of genes related to bone mineral density

2.5.1 Design and synthesis of primers

Sequences of OPG, RANK, and RANKL were obtained from NCBI database (http://www.ncbi.nlm.nih.gov), and then Primer premier 6.0 software was used to design the upstream and downstream primers of the three genes by PCR. All primers were synthesized by Beijing Genomics Institute (primer sequences are shown in Table 1).

2.5.2 Total RNA extraction and quality detection

Trizol reagent was used to extract total RNA from the serum of SD rats in the experimental and control groups. Similarly, the total RNA was extracted from the maxillary and mandible alveolar bones of SD rats. Subsequently, the quality of total RNA extracted was detected by 1% agarose electrophoresis and spectrophotometry.

2.5.3 RT-PCR, gel extraction and cloning sequencing

The synthesis of cDNA was completed with 2 μg of total RNA, and the 25 μL reverse transcriptional system was composed of the following components: 2 μL of RT primers (10 μmol/L), 5 μL 5×M-MLV buffer, 1.25 ml dNTPs (10 mmol/L), 1 μL of reverse transcriptase (200 U/μL), and 0.6 μL of RNA enzyme inhibitor (40 U/μL); RNase-free water was added to reach 25 μL. Reaction conditions were set at 16 ℃ for 20 min, at 42 ℃ for 30 min, and at 85 ℃ for 10 min to inactivate the reverse transcriptase, which was then preserved at 4 ℃. QPCR amplification system (50 μL) was made up of the following components: 5 μL of cDNA template, 0.2 μL of upstream and downstream primers (20 μM), 25 μL of SYBR Green PCR Master Mix, and ddH2O. PCR amplification was carried out according to the following setting parameters: pre-denaturation at 95 ℃ for 3 min, followed by 40 cycles (denaturation at 95 ℃ for 12 s, annealing at 62 ℃ for 40 s, prolongation at 72 ℃ for 45 s), and then at 72 ℃ for 10 min. The mRNA levels of OPG, RANK, and RANKL were quantified relatively, and GAPDH was used as an internal reference.

Electrophoreses of quantitative PCR products were conducted with 4% agarose gel, and gel extraction was performed according to instructions of general Tiangen agarose DNA recovery kit. Extracted fragments were connected with PMD-19T carrier in accordance with the instruction. Then, the fragments were converted into DH5α competent cells of *Escherichia coli*. Furthermore, eight bacterial colonies were selected for liquid culture by using LA agar plate culture medium. Positive clones identified by PCR were delivered to Beijing Genomics Institute for sequencing.

2.6 Western blot analysis

Total protein in maxillary and mandible alveolar bones or serum of rats was extracted from experimental and control groups. The protein concentration was tested by means of BCA protein analysis kit (Pierce, Chemical Co., USA). Furthermore, 2 μl of 5×SDS-PAGE loading buffer was added to the 8 μl sample, with heat treatment at 100 ℃ for 5 min and centrifugation at 10000 rpm for 1 min to remove insoluble precipitation. The sample was separated with 8% and 10% SDS-PAGE. The amount of sample per hole was 10 μl. After the electrophoresis, PVDF membrane was soaked in methanol for 10 s. Gel and the PVDF membrane soaked in methanol were soaked in the rapid electrophoretic buffer for 10 min, and then the transferring sandwich structure was prepared, followed by transferring with wet phase-inversion method (transferring condition: 200 mA for 25 min). After the completion of transmembrane, PVDF membrane was soaked in methanol for 10 s, and then incubated at 4 ℃ overnight with the primary antibody at an appropriate dilution. The sample was cleaned 5 times with 1×TBST(10 min each time). Transmembrane protein was incubated through enhanced chemiluminescent buffer and then developed with X-ray film. Density of immunooreactive bands was analyzed by version 1.61 NIH image analysis software (National Institutes of Health, Bethesda, USA). OPG (or RANK or RANKL) protein was quantified with β-actin as the internal reference.
2.7 Statistical analysis
SPSS 22.0 software package was used for statistical analysis. The data were expressed as mean ± standard deviation. t test was used for comparison between the two groups (The details are as follows: group 1 vs. group 2, group 3 vs. group 4, group 5 vs. group 6). P<0.05 meant that the difference was statistically significant.

Results
3.1 Bone density analysis
As shown in Figure. 2, A, B, and C display the sagittal view, horizontal view, and cortical thickness. Our study detected BV/TV (trabecular bone volume fraction), Tb.Th. (trabecular thickness), Cort.Th. (cortical thickness) of maxillary and mandibular alveolar bone. As shown in Table 2, significant difference was found between level of BV/TV, as well as Tb.Th. in maxillary alveolar bone of experimental group and control group (p= 0.049, p= 0.047). Meanwhile, Tb.Th. and Cort.Th. levels in mandibular alveolar bone of experimental group also showed significance compared with that in control group (p= 0.043, p= 0.024). However, no significance was found between level of Cort.Th in maxillary alveolar bone of experimental group and that in control group (p=0.072).

No significance was found between level of BV/TV in mandibular alveolar bone between experimental and control groups (p=0.076). Thus, oral breathing caused by nasal obstruction did not lead to the decrease of alveolar bone density and bone strength in SD mice.

3.2 Expression of RNA level of bone density-related genes
The sequencing results provided by the Beijing Genomics Institute were considered completely correct. RT-qPCR results showed that the expression levels of RANKL and RANK in maxillary alveolar bone, mandible alveolar bone, and serum were higher in unilateral nostril electrocautery group than in the control group: 1 (maxillary alveolar bone of unilateral nostril electrocautery group) vs. 2 (maxillary alveolar bone of the control group), RANKL (**p=0.009), RANK (**p=0.008); 3 (mandible alveolar bone of unilateral nostril electrocautery group) vs. 4 (mandible alveolar bone of the control group), RANKL (*p=0.047), RANK (**p=0.041); and 5 (serum of unilateral nostril electrocautery group) vs. 6 (serum of the control group), ***p<0.001) (Figure 3 A, B). On the contrary, the expression levels of OPG in the maxillary alveolar bone, mandible alveolar bone, and serum were lower in the experimental group than that in the control group: 1 (maxillary alveolar bone of unilateral nostril electrocautery group) vs. 2 (maxillary alveolar bone of the control group), *p=0.038; 3 (mandible alveolar bone of unilateral nostril electrocautery group) vs. 4 (mandible alveolar bone of the control group), ***p<0.001; 5 (serum of unilateral nostril electrocautery group) vs. 6 (serum of the control group), **p=0.009) (Figure 3 C).

3.3 Expression of protein level of bone density related genes
Western blot results indicated that RANKL and RANK levels in maxillary alveolar bone, mandible alveolar bone, and serum were higher in the experimental group than in the control group: 1 (maxillary alveolar bone of unilateral nostril electrocautery group) vs. 2 (maxillary alveolar bone of the control group), RANKL (**p=0.008), RANK (**p=0.009); 3 (mandible alveolar bone of unilateral nostril electrocautery group) vs. 4 (mandible alveolar bone of the control group), RANKL (*p=0.042), RANK (**p=0.007); and 5 (serum of unilateral nostril electrocautery group) vs. 6 (serum of the control group), ***p<0.001) (Figure 4 A, B, D). In contrast, the expression levels of OPG in the maxillary alveolar bone, mandible alveolar bone, and serum were lower in the experimental group than that in the control group (Namely, 1 (maxillary alveolar bone of unilateral nostril electrocautery group) vs. 2 (maxillary alveolar bone of the control group), *p=0.048; 3 (mandible alveolar bone of unilateral nostril electrocautery group) vs. 4 (mandible alveolar bone of the control group), **p<0.001; and 5 (serum of unilateral nostril electrocautery group) vs. 6 (serum of the control group), **p=0.006) (Figure 4C, D).

3.4 Possible mechanism of alveolar bone osteoporosis
As shown in Figure 5, when the number of RANKL produced in the microenvironment around the osteoclastic precursor is significantly higher than that of OPG (natural antagonist of RANKL), RANKL combine with the RANK expressed in the osteoclastic precursor, thereby breaking the balance of bone remodeling, which was beneficial to the formation of osteoclasts and the activation of bone absorption. On the contrary, when the ratio of RANKL/OPG was reduced, OPG bonded with RANKL, which prevented the binding of RANKL and RANK, thus inhibiting the osteoclast formation. Meanwhile, inhibition of OPG binding to RANKL and RANK also promoted the apoptosis of activated mature osteoclasts. Therefore, the relative ratio of RANKL/OPG determines the speed and intensity of osteoclast-mediated bone resorption.
Discussion

Oral breathing may affect facial and occlusal development in early childhood development. Oral-breathing children are more likely to present reduced posterior facial height and a narrow maxillary (23). Further studies are necessary to explore the changes caused by oral breathing.

It is difficult to recruit clinical patients as oral breathing models. In this study, an oral breathing model of SD neonatal rats was constructed by means of electrocoagulation for unilateral nostril obstruction, with which, bone density and Rank, RANKL, and OPG levels can be detected easily.

In this study, the OPG/RANKL ratio was suggested to regulate normal bone metabolism. Accordingly, the relative ratio of RANKL/OPG determines the speed and intensity of bone resorption.

Three proteins (RANK, RANKL, and OPG) and their RNA expression levels were examined. RT-qPCR results revealed that the expression levels of RANKL and RANK in maxillary alveolar bone, mandible alveolar bone, and serum were all higher in the experimental group than those in the control group. While the opposite was found regarding the expression level of OPG. These results were consistent with the western blot analysis. Thus, the increase in RANK and RANKL expression increases the activity of osteoclast, but the decrease of OPG expression inhibits the full reflection of the effect of osteoclast, resulting in excessive bone absorption and probably the decrease of bone mineral density in the alveolar bone.

As shown in Table 2, micro-CT analysis indicated that significant difference was found between level of BV/TV, as well as Tb.Th. in maxillary alveolar bone of experimental group and control group (p=0.049, p=0.047). Meanwhile, Tb.Th. and Cort.Th. levels in mandibular alveolar bone of experimental group also showed significance compared with that in control group (p=0.043, p=0.024). However, no significance was found between level of Cort.Th in maxillary alveolar bones (p=0.072). In addition, no significance was found between level of BV/TV in mandibular alveolar bones (p=0.076). During our previous modeling by electrocoagulation, we found that 8-day-old SD rats with bilateral nasal obstruction had a much higher mortality rate than those with unilateral nasal obstruction. Therefore, 8-day-old SD rats with unilateral nasal obstruction were used in this study.

Our results indicated that oral breathing leads to the decrease of bone density of compact bone and cancellous bone in maxillary and mandible alveolar bones of SD rats. However, the mechanism needs a more intensive study. Previous research demonstrated that oral breathing induces chew activity (24), but whether the decrease of chew activity will reduce bone density of alveolar bones remains unclear. Oral breathing is known to affect craniofacial muscle activity (6,8,9,10). Nonetheless, further studies are needed to explore the relationship between craniofacial muscle activity and bone density of alveolar bones.

Prior experiments have shown that intermittent hypoxia can cause a reduction in bone mass, and the mechanism of OPG/RANKL/RANK system will contribute to the exploration of the mechanism of bone mass decline in alveolar bone (12).

Previous studies documented that breathing can affect occlusion (25). The impact of breathing on the maxillofacial muscles has also been demonstrated, but the impact of breathing patterns on the jaw is not clear (6,7). Western blot analysis for RANK, RANKL, OPG, and their corresponding RNA levels by RT-qPCR also provided insight into the possible mechanism of decline in bone mineral density. The change in nasal passage to oral passage after unilateral nasal obstruction might lead to the change of bone structure of the cancellous bone and the cancellous bone in alveolar bones. In addition, alveolar bone remodeling in patients involving oral breathing, such as the stability of implant anchorage, may require consideration of changes in bone strength of the alveolar bone. Our current research could provide guidance for the selection of implant anchorage in clinical practice. For oral breathing patients, positions with higher bone density are better for implanting anchorage.

The current experimental results cannot be directly applied to humans. However, our experiment has shown that changes in airway structure may affect alveolar bone structure, which may be related to local and systemic factors.

This study confirmed that structural changes of the respiratory tract affect alveolar bone structure, and the unilateral nostril obstruction may lead to the change of regional specific bone density during the growth and
development of the maxillary and the mandible. In addition, changes in alveolar bone structure may be related to local factors and systemic factors.

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References


<table>
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<th>Table 1: Sequences of primers used in PCR assay</th>
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<td>Gene</td>
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<tr>
<td>RANKL</td>
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<td>RANK</td>
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<td>GAPDH</td>
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Notes. Sequences of OPG, RANK and RANKL were obtained in NCBI database (http://www.ncbi.nlm.nih.gov).
Table 2. The effect of experimental groups and control groups on the microstructural data obtained through micro-computed tomography (micro-CT)

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<tr>
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<th>Experimental groups</th>
<th>Control groups</th>
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<tr>
<td></td>
<td>XAB (n=3)</td>
<td>NAB (n=3)</td>
</tr>
<tr>
<td>BV/TV (%)</td>
<td>16.250±9.092(^{a1})</td>
<td>24.867±4.215(^{b1})</td>
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<td>34.367±6.608</td>
<td>31.967±2.991</td>
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<tr>
<td>Tb.Th (um)</td>
<td>0.133±0.023(^{a2})</td>
<td>0.150±0.016(^{b2})</td>
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<tr>
<td></td>
<td>0.193±0.029</td>
<td>0.178±0.005</td>
</tr>
<tr>
<td>Cort.Th.</td>
<td>0.146±0.013(^{a3})</td>
<td>0.129±0.029(^{b3})</td>
</tr>
<tr>
<td></td>
<td>0.164±0.002</td>
<td>0.193±0.012</td>
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Notes. All data represent mean ± SD. n indicates number of rats in each group; XAB, Maxillary alveolar bone; NAB, Mandibular alveolar bone; BV/TV, trabecular bone volume fraction; Tb.Th., trabecular thickness; Cort.Th., cortical thickness.

\(^{a1}\) Significant difference (p = 0.049) from the XAB control group in BV/TV;

\(^{a2}\) Significant difference (p = 0.047) from the XAB control group in Tb.Th;

\(^{a3}\) No significant difference (p=0.072) from the XAB control group in Cort.Th.

\(^{b1}\) No significant difference (p=0.076) from the NAB control group in BV/TV;

\(^{b2}\) Significant difference (p=0.043) from the NAB control group in Tb.Th.

\(^{b3}\) Significant difference (p=0.024) from the NAB control group in Cort.Th.

Figure 1. Establishment of unilateral nasal obstruction in SD model rats: Rats were raised in an SPF animal house (breastfeeding in the early stage; standard rodent food and free access to water in the later stage) and then sacrificed at 7 weeks after modeling. The alveolar bone of each rat was quickly collected and immersed in an EP tube containing 4% paraformaldehyde for subsequent bone mineral density analysis.
Figure 2. Bone density analysis of maxillary and mandibular alveolar bones: (A) Sagittal view, (B) horizontal view, and (C) axial cross-section images of selected regions of interest in the alveolar bone adjacent to the apical third of the mesial root of the maxillary left first molar. Red and green represent trabecular bone and root of tooth, respectively, in (A) and (B); red represents cortical thickness in (C).

Figure 3. Expression of RNA level of bone density related genes. (A, B, C) RT-qPCR was used to detect the expression of RANKL (*$p=0.047$, **$p=0.009$, and ***$p<0.001$), RANK (*$p=0.041$, **$p=0.008$, and ***$p<0.001$), and OPG (*$p=0.038$, **$p=0.009$, and ***$p<0.001$) in maxillary and mandible alveolar bones as well as in the serum. The values are expressed as mean ± SD from three independent experiments. Note: 1 (maxillary alveolar bone of unilateral nostril electrocautery group) vs. 2 (maxillary alveolar bone of the control group), 3 (mandible alveolar bone of unilateral nostril electrocautery group) vs. 4 (mandible alveolar bone of the control group), and 5 (serum of unilateral nostril electrocautery group) vs. 6 (serum of the control group)).
Figure 4. Expression of protein level of bone density-related genes. (A, B, C, D) Western blot was used to detect the expression of RANKL (*p=0.042, **p=0.008, and ***p<0.001), RANK (*p_{12}=0.009, **p_{14}=0.007, and ***p<0.001), and OPG (*p=0.048, **p=0.006, and ***p<0.001) in maxillary and mandible alveolar bones as well as in the serum. The values are expressed as mean ± SD from three independent experiments. Note: 1 (maxillary alveolar bone of unilateral nostril electrocautery group) vs. 2 (maxillary alveolar bone of the control group), 3 (mandible alveolar bone of unilateral nostril electrocautery group) vs. 4 (mandible alveolar bone of the control group), and 5 (serum of unilateral nostril electrocautery group) vs. 6 (serum of the control group)).

Figure 5. Possible mechanism of alveolar bone osteoporosis induced by nasal obstruction in SD rats.