Chemical Sympathectomy Impairs Peri-implant Osseointegration in Mice: Role of the Sympathetic Nervous System in Osseointegration

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Purpose: The possibility that the sympathetic nervous system (SNS) controls bone remodeling has been raised; however, the actual function of the SNS in osseointegration is still unknown. This study aimed to investigate the effect of chemical sympathectomy on peri-implant osseointegration in adult mice. Materials and Methods: Forty C57BL/6J mice (8-week-old) were divided into two groups: a sympathectomy group and a control group, which were administered 6-hydroxydopamine and saline, respectively, by intraperitoneal injection for 5 days. Then, the mice were exposed to implant surgery. Analyses of serum chemistry, microcomputed tomography, biomechanical test, and bone histomorphometry were employed at 2 and 4 weeks. Results: Compared with the control, the chemical sympathectomy group had a higher serum level of C-terminal collagen I cross-links but lower serum osteocalcin. After 4 weeks, peri-implant trabecular microstructure, including trabecular volume, trabecular thickness, the percentage of osseointegration, and bone-to-implant contact, was lower; however, the trabecular separation was higher in the sympathectomy group mice in comparison with the control group. In addition, the strength of bone-titanium integration measured by the biomechanical resistance test was lower. Furthermore, histomorphologic evidence revealed that the osteoclast counts were higher in the sympathectomy group, while the mineral apposition rate and the bone formation rate per bone surface were significantly lower. Conclusion: Within the limitations of this experimental study, the data showed that chemical sympathectomy has a negative effect in peri-implant bone healing. Int J Oral Maxillofac Implants 2019;34:91–98. doi: 10.11607/jomi.7086

Keywords: chemical sympathectomy, implant, osseointegration, sympathetic nervous system

Osseointegration at the implant-bone interface has been widely studied and regarded as foundational to functional dental implants. However, peripheral nerve fibers and associated Ruffini endings may lead to degeneration or damage during implant surgery. Sprouting of new nerve fibers and gradually increased nerve endings have been observed close to the bone-implant surface, which indicated reinnervation around osseointegrated implants.¹⁻³ Additionally, cortical somatosensory areas can be activated by punctuated mechanical stimulation of osseointegrated maxillary implants⁴ and progressive improvement in the perception,⁵ which indicates a peripheral feedback mechanism of osseoperception. However, the type and location of the mechanoreceptors that mediate osseoperception remain a matter of debate.⁶ The role of nerve fibers in peri-implant osseointegration is open to debate for low-density and less-specialized terminal structures.
Both myelinated and unmyelinated nerve fibers, which are involved in sensory and sympathetic innervation, have been observed along different bone envelopes and in the periosteum, bone marrow space, bone cells, and bone matrix. Various neuropeptides, including vasoactive intestinal peptide (VIP), neuropeptide Y (NPY), substance P (SP), calcitonin gene-related peptide (CGRP), and tyrosine hydroxylase (TH), have been found in these fibers. In vivo and in vitro studies, as well as clinical observations, have established the involvement of the peripheral nervous system in the regulation of bone modeling and remodeling. However, the actual effect of sympathetic innervation in implant osseointegration is still unknown.

The purpose of this study was to investigate the role of the sympathetic nervous system (SNS) in the osseointegration of titanium implants using a sympathetic denervation mouse model.

**MATERIALS AND METHODS**

**Animals**

Forty male C57BL/6J mice (8-week-old) were assigned to two groups. This study was in accordance with international standards and approved by the Animal Research Committee of Sichuan University. The mice were housed under an automatically controlled condition and fed a standard diet ad libitum. Body weights of mice were measured and recorded every other day during the experiment.

**Chemical Sympathectomy**

Chemical sympathectomy was established by serial injections of 6-hydroxydopamine (6-OHDA, Sigma-Aldrich) as described previously. All mice were subjected to daily intraperitoneal injections for 5 days: the control group was given 0.9% saline (1 mL/kg/day), and the experimental group received 6-OHDA (100 μg/g on days 1 and 2, 250 μg/g on days 3 and 5). Two days later, both groups were subjected to implant placement.

**Implant Surgery**

Rod-shaped machined titanium implants, with a length of 3 mm and diameter of 1 mm, were obtained from the National Engineering Research Center of Biomaterials, Sichuan University, China (Fig 1a). Briefly, the distal aspect of the femur was exposed carefully as described in a previous paper. After preparation with sequential 0.7-mm and 1.0-mm drill-holes under saline irrigation, implants were placed on anterior-distal surfaces of both femurs in each mouse (Figs 1b and 1c). The muscles and skin were carefully sutured to protect implants in a biologic environment. Ten animals from each group were harvested at 2 and 4 weeks. Each femur was examined by x-ray radiography (Fig 1d).

To examine dynamic mineralization deposition, sequential subcutaneous injections of 1% calcein (10 mg/kg, Sigma-Aldrich) and 1% alizarin red S (20 mg/kg, Sigma-Aldrich) in phosphate buffer solution were performed at 10 and 3 days before the mice were euthanized, respectively.

**Serum Biochemical Assays**

At 2 and 4 weeks postoperatively, approximately 200 μL of blood from each mouse was obtained by tail snipping. Serum was separated from blood via centrifugation (2,500 × g for 30 minutes at 4°C). Osteocalcin and C-terminal collagen I cross-links (CTX-I) in serum were analyzed using a Mouse Osteocalcin ELISA Kit and Mouse CTX-I ELISA Kit.

**Histomorphometric Measurement**

Two or four weeks after surgery, one femur was harvested from each mouse and fixed for 48 hours. For hard tissue histology, specimens were dehydrated with alcohol at first and were embedded in epoxy resin without decalcification (Technovit 7200 VLC, Heraeus Kulzer). At a site 0.5 mm from the implant end, the specimen was sawn perpendicular to its long axis. Embedded specimens of 50-μm thickness were obtained after grinding (Exakt Apparatebau).
Photographs were taken by means of a confocal laser scanning microscope (CLSM, TCS SP2). The mean distance between the first calcein label and the second alizarin red S label was defined as the bone mineral apposition rate (MAR, μm/day). According to the formula, MAR × (single-labeled surface/2BS + double-labeled surface/BS), the bone formation rate per bone surface (BFR/BS, μm²/μm²/day) was calculated. Regions of interest (ROI) were defined as a circle of 200 μm around the implant to the whole area.

Additionally, undecalcified specimens were stained with 1% toluidine blue. Histomorphometric analyses were performed using a Nikon Eclipse E600 microscope. Bone-to-implant contact (BIC) was measured as the linear ratio between the interface in direct contact with bone and the total circle interface of the implant.

The second femur from each mouse was decalcified in 17% ethylenediaminetetraacetic acid (EDTA; pH 7.4). Implants were pushed out gently, and the specimens were dehydrated in ethanol and embedded in paraffin. Then, implants were sectioned perpendicular to the implants with a microtome (Leica RM 2255) and stained for tartrate-resistant acid phosphatase (TRAP, Sigma-Aldrich) according to a standard protocol. The number of osteoclasts (TRAP stained-positive multinucleated cells) per peri-implant surface (Oc.N/BS) was measured on a peri-implant bone area (Nikon Eclipse 80i). Five sequential sections per specimen in each group were examined (ImageJ Software, NIH).

**Microcomputed Tomography (micro-CT) Analysis**

The femurs harvested at 4 weeks were subjected to a micro-CT analysis system (YXLON Y.cheetah), which was set to 90 kV, 35 μA, and 600 ms integration time. The volume of interest (VOI) was determined with the bone tissues beginning 1.5 mm below the implant end and extending to a radius of 200 μm. A total of 50 consecutive slices were analyzed for the bone volume to total volume ratio (BV/TV), bone surface to total bone volume ratio (BS/BV), mean trabecular number (Tb.N), mean trabecular thickness (Tb.Th), and mean trabecular separation (Tb.Sp). The percentage of osseointegration (%OI) was calculated as the percentage of bone in direct contact with the implant to total voxels of the implant.

**Push-in Test**

A push-in test was conducted with a universal material testing machine (Instron 5566, Instron). As described previously, each femur was placed in a custom designed holder, which aligned an implant vertically to maintain downward compression perpendicularly. The testing machine equipped with a rod (diameter of 0.8 mm) pushed the implant vertically downward at a constant speed (1 mm/min). A load-displacement curve was used to determine the maximal force.

**Data Analysis and Statistics**

Results are shown as mean ± SD. An independent Student t test was used to analyze the data with statistical software (SPSS 17.0, SPSS). A P value of less than .05 was considered statistically significant.

**RESULTS**

Three to four days after daily 6-OHDA treatment, the experimental mice developed ptosis, which demonstrated the success of the sympathectomy procedure. The condition persisted throughout the experiment period without any systemic disorders.

**Serum Biochemistry**

As shown in Table 1, serum osteocalcin decreased significantly (P < .05) by 40.9% and 23.8% at weeks 2 and 4, respectively, and the level of CTX-I increased significantly (P < .01) by approximately 2.8-fold and 2-fold after 2 weeks and 4 weeks of healing, respectively, in the sympathectomy group compared with the control.

**Micro-CT Evaluation**

The trabecular topography and bone-implant interface of each femur were examined using high-resolution three-dimensional (3D) micro-CT evaluation (Fig 2, Table 2). Sympathectomy treatment significantly decreased BV/TV (−40.0%, P = .005), Tb.Th (−14.5%, P = .033), Tb.N (−20.6%, P = .001), and %OI

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Results of Osteocalcin and CTX-I for Control and Sympathectomy Groups 2 and 4 Weeks After Implantation</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Osteocalcin (pg/mL)</td>
</tr>
<tr>
<td></td>
<td>2 wk</td>
</tr>
<tr>
<td>Control</td>
<td>318.18 ± 69.15</td>
</tr>
<tr>
<td>Sympathectomy</td>
<td>187.91 ± 56.64</td>
</tr>
<tr>
<td>P values</td>
<td>.012</td>
</tr>
</tbody>
</table>

Values are represented as mean ± SD. CTX-I = C-terminal collagen I cross-links.
However, BS/BV (1.2-fold) and Tb.Sp (1.6-fold) exhibited a moderate increase in the sympathectomy group (\(P < .01\)).

**Histologic and Histomorphometric Evaluation**

Observations of undecalcified histologic sections provided detailed information about implant osseointegration (Fig 3). The BIC ratio in the sympathectomy group was 32.25% ± 5.86% at 2 weeks, which was close to that found in the control (\(P = .16\), Fig 3c). After 4 weeks, however, the BIC ratio of the sympathectomy group increased to 43.60% ± 6.84%, which was significantly lower than that of the control group (70.87% ± 4.88%, \(P = .001\)) (Fig 3f).

The interlabel width between calcein and alizarin red S labeling lines on peri-implant fluorescence micrographs was measured, and the MAR and BFR/BS were calculated (Fig 4). This showed an obvious narrowing in the sympathectomy group compared with the control group. The values of MAR and BFR/BS decreased by 23.6% and 35.9% (\(P < .01\), both, Table 3), respectively, in the sympathectomy group compared with the control group.

Additionally, TRAP-positive stained osteoclasts (Oc.N/BS) were detected during the implant osseointegration period (Fig 5). Compared with the control group, Oc.N/BS was significantly increased at week 2 (1.4-fold, \(P < .01\)) and week 4 (1.2-fold, \(P < .05\)), respectively, in the sympathectomy group (Table 4).
Biomechanical Analysis
The maximal push-in force was calculated on the push-in test, which represents the biomechanical property parameters of osseointegration. Over a 4-week period, bone-implant integration strength significantly declined in the sympathectomy group (5.54 ± 1.01 N) in comparison with the control group (8.80 ± 1.01 N) (–37.0%, \( P < .01 \)).

### DISCUSSION
In this study, lower expression of osteogenic markers (osteocalcin) and higher expression of osteoclast markers (CTX-I) were found in serum, suggesting that treatment with 6-hydroxydopamine (6-OHDA) strongly impairs bone remodeling in adult mice. Trabecular volume and trabecular thickness were lower while trabecular separation was higher in the sympathectomy group.
The percentage of osseointegration (%OI), BIC, and the biomechanical resistance tests were lower in the experiment group, which shows the detrimental effect of chemical sympathectomy on osseointegration around implants. Histomorphologic evidence of higher numbers of osteoclasts, decreased MAR, and diminished BFR/BS supported the above conclusion, indicating that the SNS is an important factor in osseointegration.

This study used serial injections of 6-OHDA to disrupt catecholaminergic neurons, which severely reduced catecholamine content in tissues. Since 6-OHDA does not penetrate the blood-brain barrier of adult mice, it could be used to evaluate the role of peripheral sympathetic nerves on implant osseointegration. Although the systemic application of 6-OHDA could cause damage to the SNS, significant recovery of NA fibers was observed a few days following cessation of administration, and the sympathetic nerve was demonstrated to disappear after 28 days or even 5 weeks. Furthermore, a decrease in the density of NPY-immunoreactive fibers was revealed in chemical and surgical sympathectomies.

The results showing that the peri-implant osseointegration was inhibited by chemical sympathectomy are in agreement with the results of studies of chemical sympathectomy by guanethidine or by 6-OHDA in rats and with the results of a study of surgical removal of the superior cervical ganglion. Both genetic and pharmacologic studies have demonstrated that the activity of the SNS can influence bone formation and bone resorption. Mice deficient in dopamine β-hydroxylase and leptin-deficient Ob/Ob mice had higher osteoblast numbers and activity, as well as greater bone volume. The enhanced SNS activity produced with treatment by β-agonist isoproterenol or clenbuterol upregulated bone resorption by stimulating osteoclastic differentiation, while lowering sympathetic tone in mice via treatment with the β-blocker propranolol, had a positive regulatory role in bone formation.

As described earlier, the results of this study suggest that sympathetic innervation contributes to peri-implant osseointegration. It is notable that the results of other studies have been conflicting. Osteoclastic resorption was impaired when guanethidine

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Table 3  MAR and BFR/BS Calculated Between the Calcein Label and Alizarin Red S Label

<table>
<thead>
<tr>
<th>Group</th>
<th>MAR (μm/day)</th>
<th>BFR/BS (μm²/μm²/day)</th>
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<tbody>
<tr>
<td>Control</td>
<td>1.23 ± 0.10</td>
<td>0.64 ± 0.04</td>
</tr>
<tr>
<td>Sympathectomy</td>
<td>0.94 ± 0.10</td>
<td>0.41 ± 0.04</td>
</tr>
</tbody>
</table>

P values .001 < .001

Values are represented as mean ± SD. MAR = mineral apposition rate; BFR/BS = bone formation rate per bone surface.

Table 4  Quantitative Results of Number of Osteoclasts on Peri-implant Trabecular Surface (Oc.N/BS) After Healing for 2 and 4 Weeks

<table>
<thead>
<tr>
<th>Group</th>
<th>Oc.N/BS(/mm)</th>
<th>2 wk</th>
<th>4 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.13 ± 0.97</td>
<td>7.58</td>
<td>0.71</td>
</tr>
<tr>
<td>Sympathectomy</td>
<td>14.23 ± 0.79</td>
<td>6.19</td>
<td>0.79</td>
</tr>
</tbody>
</table>

P values .001 .039

Values are represented as mean ± SD.
was administered in adult rats, which was assumed to be an acute effect of sympathectomy. Histologic evidence found that catecholamine-containing nerve endings contact osteoblasts directly. Additionally, several functional neuro-osteogenic factors, including adrenergic and neuropeptide receptors, have been identified in bone cells, which suggests that bone remodeling is under the control of a neuroendocrine mechanism. A tightly regulated balance between bone formation and bone resorption is vital for bone mineral homeostasis. Therefore, the release of sympathetic neuropeptides to osteoblasts and osteoclasts is responsible for the effect of chemical sympathectomy on local bone healing processes.

In previous studies, the present authors have demonstrated that α-CGRP has an anabolic role in the osseointegration of titanium implants. On the other hand, the SNS represents one of the main links between the CNS and the skeleton. Patients with reflex sympathetic dystrophy displayed localized sympathetically mediated pain. The release of sympathetic neuropeptides to osteoblasts and osteoclasts was thought to exacerbate bone loss. Sympathetic nerves are often associated with blood vessels that enter the bone through Volkmann's canals, which are predominantly involved in modulating blood-flow through bone. Vascular tone in bone is under the control of sympathetic neurotransmitters, such as NPY and noradrenaline (NA). Many studies have suggested that depletion of sympathetic mediators may be responsible for the balance between bone formation and bone resorption. The depletion of NPY-IR and catecholaminergic fibers by chemical sympathectomy may counteract the positive regulation of blood-flow, which might disrupt or alter the trophic effect of sympathetic fibers and activate bone resorption.

Additionally, the SNS plays a role in upregulation and downregulation of homeostatic mechanisms. Circulating catecholamines, as well as locally released NA and NPY, could modulate cytokine production and the functional activity of different lymphoid cells. It has been demonstrated that the SNS can shift the balance of Th1/Th2 from a pro-inflammatory Th1 response to an anti-inflammatory Th2 response. For example, NA is demonstrated to shift the balance of Th1/Th2 from a pro-inflammatory Th1 response to an anti-inflammatory Th2 response. Furthermore, epinephrine has been shown to enhance DNA synthesis and alkaline phosphatase activity in differentiating MC3T3-E1 cells. In the present study, decreased epinephrine caused by chemical sympathectomy and the anti-inflammation imbalance induced by depletion of catecholamines may have negatively influenced bone formation.

CONCLUSIONS

The data of this study revealed that chemical sympathectomy has a negative effect on peri-implant osseointegration, suggesting that SNS regulation is important in peri-implant bone healing.

ACKNOWLEDGMENTS

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REFERENCES