

Original Article

Expression of substance P in rat periodontal alveolar bone after denervation

Xi-Jiao Yu^{1,2*}, Li Zhang^{1,3*}, Shuang Liu^{1*}, Yan-Mei Du², Wei Wang², Chang-Jie Xiao^{1,2}, Yi Du², Shu Li¹

¹Shandong Provincial Key Laboratory of Dental Tissue Regeneration, Department of Periodontology, School and Hospital of Stomatology, Shandong University, Jinan, Shandong, People's Republic of China; ²Department of Endodontics, Jinan Stomatological Hospital, Jinan, Shandong, People's Republic of China; ³Department of Endodontics, Central Hospital of Taian, Taian, Shandong, People's Republic of China. *Equal contributors.

Received October 26, 2015; Accepted December 24, 2015; Epub February 1, 2016; Published February 15, 2016

Abstract: The aim of this study is to investigate substance P (SP) expression in rat periodontal alveolar bone after inferior alveolar nerve transection. 60 male Wistar rats were divided into six groups randomly. Mandibular alveolar bones were removed from the denervated rats according to sacrifice time points (0, 3, 7, 14, 21, and 28 days after operation). Immunohistochemical staining was applied to trace the expression of SP. The mRNA and protein levels of SP in periodontal alveolar bone were quantified by real-time PCR and Western blot techniques. Positive expressions of SP were located in sensory nerve fibres and blood vessels that distributed in periodontal ligaments and alveolar bone. SP-immunopositive expressions changed at different time points as a result of denervation. Expressions of SP decreased in periodontal alveolar bone at day 3, and the lowest expression level was measured at day 7 after operation. In the late stages of the study, SP expressions increased since 14 days and then gradually got back to normal levels at 28 days. To conclude, inferior alveolar nerve transection leads to the down-regulation of SP mRNA and protein in alveolar bone. SP may also play an important role in the compensation or recovery of innervation.

Keywords: Substance P, inferior alveolar nerve, alveolar bone, neuropeptide

Introduction

Substance P is a sensory neuropeptide released from the peripheral ends of sensory nerves including inferior alveolar nerve, and found in the nerve fibers of periodontal ligaments and alveolar bone. It is well known that nervous system as a systemic regulatory factor conducts sensations of pain and pressure in the periodontal tissues [1, 2]. The nerves also secrete a variety of neuropeptides such as substance P (SP), calcitonin gene-related peptide (CGRP) and neuropeptide y (NPY), which are synthesized in the ganglion sensory cells and distributed in the peripheral tissues [3, 4]. The neuropeptides play important parts in homeostasis and repair of periodontal tissues [5, 6]. Studies show substance P is a local regulator for mitosis of bone marrow-derived mesenchymal stem cells, and accelerates osteoblasts proliferation and differentiation in vitro [7, 8]. SP can also regulate microcirculation in bone, and improves

formation of good-quality bone and accelerates bony union during rat mandibular distraction osteogenesis [9].

Rat inferior alveolar nerve transection models have been used as an experimental model to investigate the neuropeptides and inferior alveolar nerve innervation in health and disease [10-12]. Many studies have emphasized the roles of substance P in bone metabolic activities and wound healing [6, 13]. Our previous study shows that the number of osteoclasts increased with the bone density of alveolar bone at root furcation decreased after IAN-T [14]. But the mRNA and protein levels of SP in periodontal alveolar bone after inferior alveolar nerve transection remains unclear. In this study, a rat inferior alveolar nerve transection model was established, and the mRNA and protein levels of SP in periodontal alveolar bone were detected at different time points so as to illustrate its roles.

Materials and methods

Animal care and group setup

Male Wistar rats (7 weeks of age, weight 260-300 g, Laboratory Animal Center, Shandong University) that acclimated for 1 week before the experiments were maintained on a normal hard food diet and water ad libitum. 60 male Wistar rats were randomly assigned to 6 groups (n=10; 5 for tissue staining, the other 5 for molecular study) according to the sacrifice time points (0, 3, 7, 14, 21 and 28 days post operation). Animals in this study were maintained and used in accordance with guidelines established by the Institutional Animal Care and Use Committee of school of stomatology, Shandong University in Jinan, Shandong Province, P. R. China.

Inferior alveolar nerve model transection (IAN-T)

The protocol of the IAN-T operation was the same as previously described [14]. Briefly, male Wistar rats were anesthetized by intraperitoneal injection (0.35 ml 10 % chloral hydrate per 100 g body weight). After routine disinfection and skin preparation, a skin incision about 1 cm (5 mm under left mandibular angle) was carried out (**Figure 1A**). At this position, muscle was bluntly dissected along mandibular medial wall, and downward separated to a depth of approximately 1 cm. Then, the left inferior alveolar nerve (IAN) was exposed and transected (**Figure 1B**). Finally, the wounds were washed with 0.9 % sodium chloride solution, and the muscle and skin were sutured using 4-0 sutures.

Tissue preparation

Rats were fixed with 4% PFA (paraformaldehyde) by systemic circulation fixation for half an hour after being anesthetized by an intraperitoneal injection (0.35 ml 10% chloral hydrate per 100 g body weight). The left mandibulars were dissected and fixed immediately in 4% PFA for another 12 h at 4°C. Then, the tissues were demineralized in 10% EDTA for 4 months. After dehydrated using gradient ethanols, cleared with xylene and embedded in paraffin. 5 µm serial sections were made in the bucco-lingual direction for immunohistochemical staining. The specimens for real-time PCR and western

blots were not fixed. The molars and incisor of the left mandibles were gently removed using a needle holder, and the mandibular body was taken out using bone cutting forceps and then rapidly frozen in liquid nitrogen and stored at -80°C for real-time PCR and western blots.

Immunohistochemical staining

After deparaffinized using xylene, hydrated in gradient ethanols, the sections were treated with 3% H₂O₂ for 15 min at 37°C to inhibit endogenous peroxidase activities, and reacted with polyclonal antibody SP (1:100, Bioss, China) for 16 h at 4°C. After washing with 0.01 M PBS, the sections were then incubated with polymer auxiliary agent for 15 min at 37°C, washed with 0.01 M PBS 5 min×3 times, and then incubated with Poly-HRP anti-rabbit IgG (Zhongshan, China) for 15 min at 37°C. Afterwards, the sections were visualized with 3,3-diaminobenzidine tetra hydrochloride (DAB, Zhongshan, China) as recommended by the manufacturer. 0.01 M PBS instead of antibody served as negative control. The sections were examined and photographed under a light microscope (OLYMPUS CX-71, Japan). The protocol of immunohistochemical staining was the same as previously described [15].

Western blot analysis

The frozen samples were homogenized with hypotonic lysis buffer (potassium phosphate 50 mM pH 7.0, sucrose 0.3 M, DTT 0.5 mM, EDTA 1 mM pH 8.0, PMSF 0.3 mM, NaF 10 mM and phosphatase inhibitor). Protein concentrations were determined by using a bicinchoninic acid assay (BCA) protein assay kit (Beyotime, China), and the curves in the BSA Protein Standard curves were used. Equal amounts of total proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF Transfer membranes (Invitrogen, Carlsbad, CA, USA). The membranes were washed three times with Tris-buffered saline/Tween-20 (TBST) and blocked with 5% skim milk in Tris-buffered saline at room temperature for 1 hour. Then, the membranes were incubated with antibody against SP (diluted 1:500, Bioss, China) overnight at 4°C. The secondary antibodies were horseradish peroxidase (HRP)-linked goat-anti rabbit IgG (CWBiotech, China). Blots were visualized using ECL chemiluminescence reagents (Milipore, USA), and

Substance P in alveolar bone

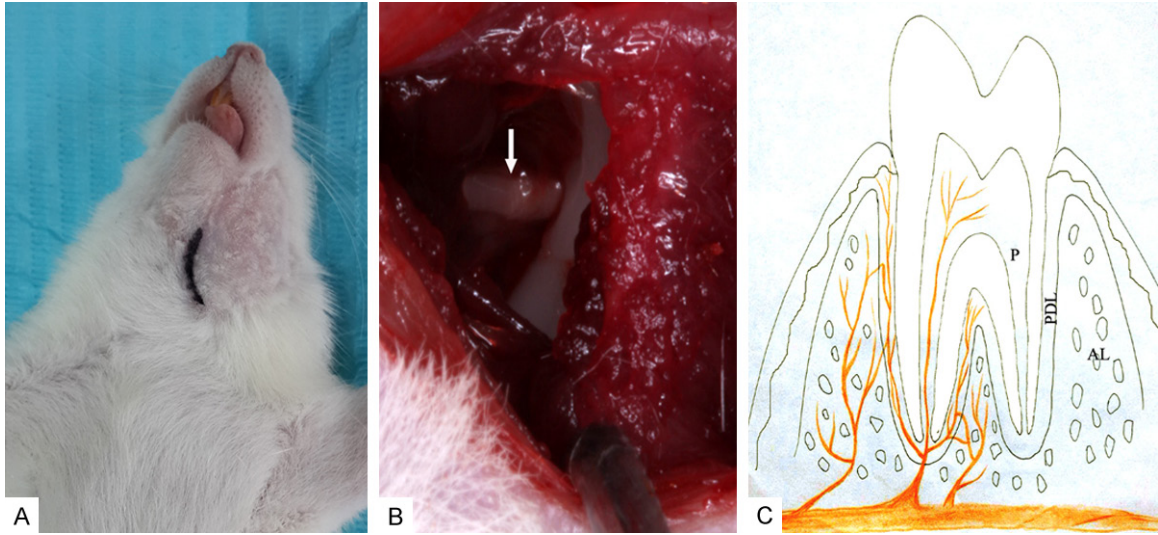
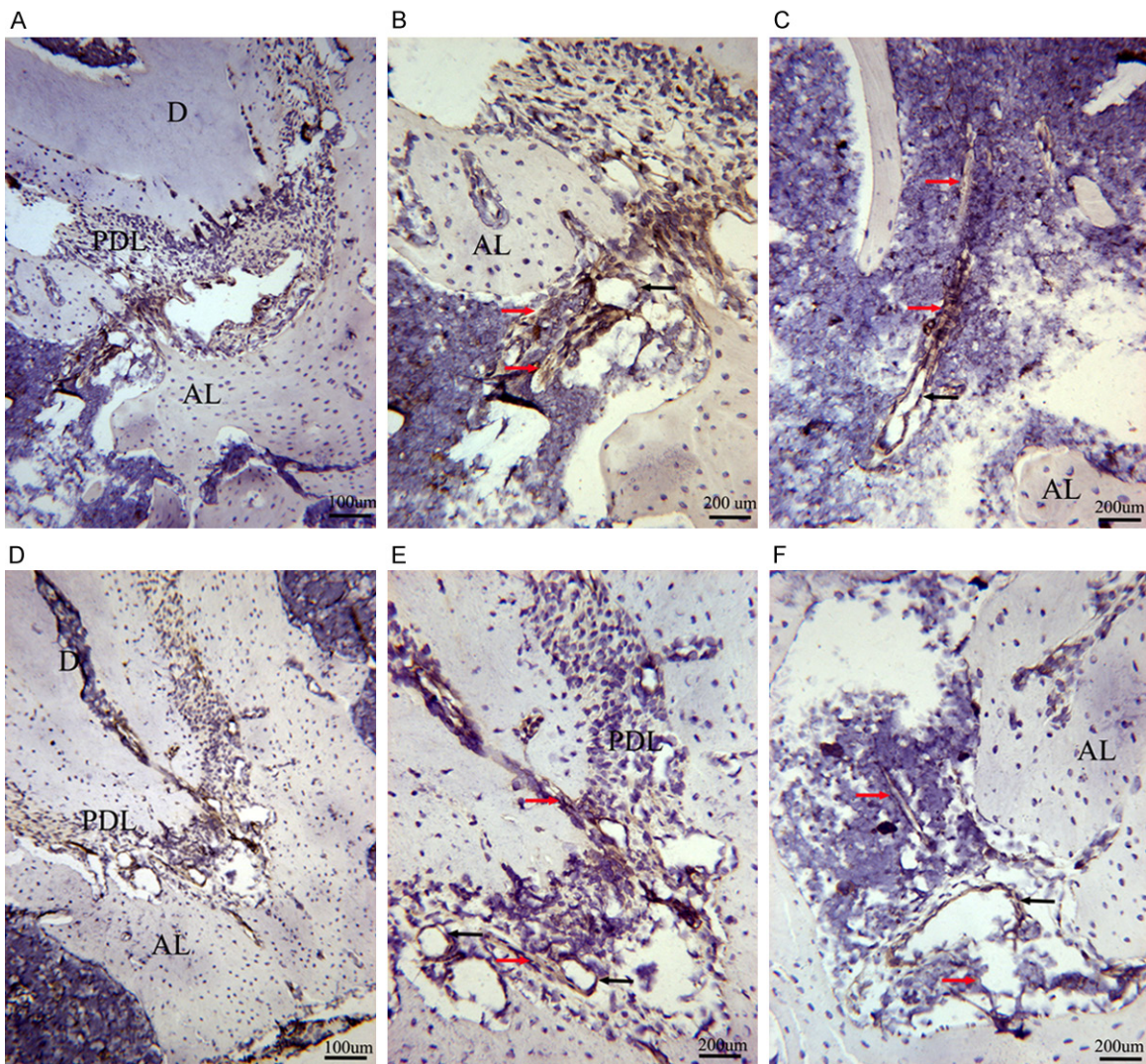


Figure 1. Animal operation processes. A. Operation incision designs. B. Mandibular foramen and inferior alveolar nerve (white arrow). C. Schematic diagram of the inferior alveolar nerve branches innervating mandibular alveolar bone (AL), periodontal ligaments (PDL) and tooth pulp (P).



Substance P in alveolar bone

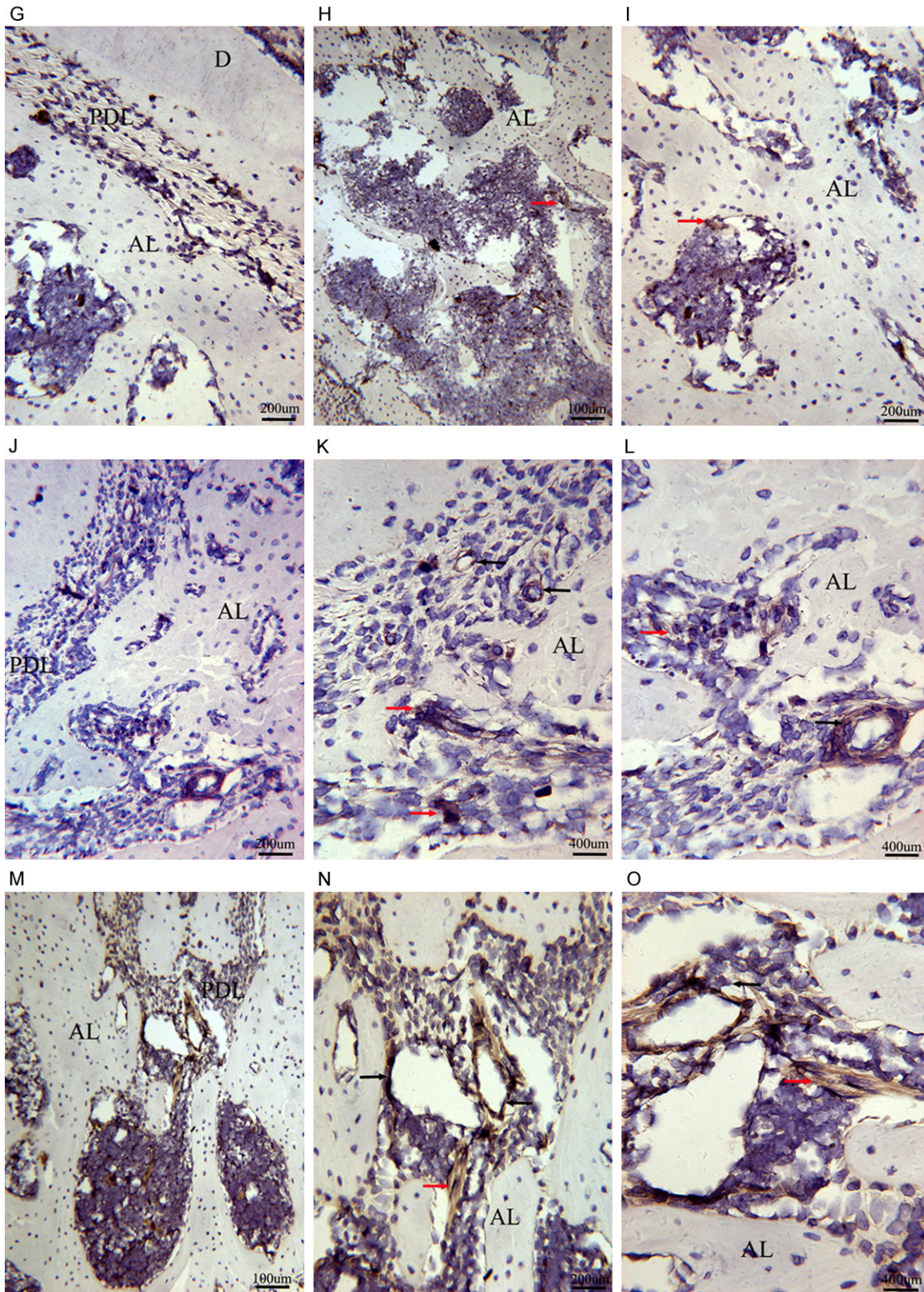


Figure 2. Expressions of substance P in rat periodontal ligaments and alveolar bone at different time points after IAN-T. A-O. showed 0, 3, 7, 14 and 28 days after operation respectively. Substance P-positive expressions were mainly distributed in blood vessels (black arrow) and nerve fibers (red arrow) of the periodontal ligaments and alveolar bone. Immunoreactivities of substance P occurred weakly 3 and 7 days after operation and became stronger gradually in the late stage. The positive expressions of substance P were similar to the normal level at 28 days.

Substance P in alveolar bone

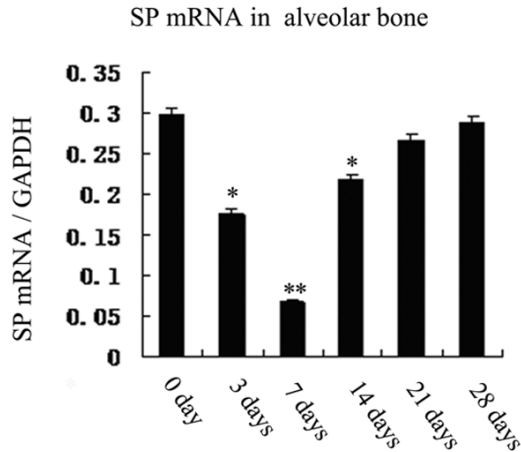


Figure 3. Expressions of substance P mRNA in rat periodontal alveolar bone at 0, 3, 7, 14, 21 and 28 days after IAN-T. IAN-T led to reduction of substance P mRNA at 3-14 days. The levels of substance P mRNA decreased from 3 to 7 days, and then increased gradually, approaching to normal level at 21 and 28 days (* $P < 0.05$, ** $P < 0.01$).

quantified by densitometric analysis. Equal protein loading was shown by stripping and incubation with an anti-GAPDH antibody (diluted 1:5000, CWBiotoch, China).

Real-time PCR analysis

The protocol of real-time PCR analysis was the same as previously described [14]. Total RNA was isolated from frozen tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, reverse transcription was performed in a 200 μ l reaction in the presence of 50 mM Tris-HCl pH 8.3, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM dNTPs, and 50 ng of random primers with 200 units of Moloney murine leukemia virus-reverse transcriptase (Invitrogen). The reaction product was amplified by real-time PCR with the LightCycler 480II/96 (Roche) using the SYBR Green core reaction kit (Thermo, USA). The primers used were the followings: rat SP forward primer 5'-TAATGGGCAAACGGGATGCT-3' and reverse primer 5'-TCGTAGTTCTGCATTGC-GCT-3'; rat GAPDH forward primer 5'-TGATGGGTGTGAACACGAG-3' and reverse primer 5'-CCCTCCACGATGCCAAAGT-3'.

Statistical analysis

Data were analyzed by the SPSS software 13.0 and values were expressed as mean \pm S.D.

Statistical significance was determined by ANOVA followed by the post hoc Fisher's least significant difference test. Significance for statistical analyses was set at $P < 0.05$.

Results

Immunohistochemical staining

Substance P-positive expressions were stained lightly yellow or dark yellow, and were mainly distributed in blood vessels and nerve fibers of the periodontal ligaments and alveolar bone (**Figure 2A-C**). After denervation, substance P-positive staining weakened (**Figure 2D-F**) and the lowest expression level was observed at 7 days (**Figure 2G-I**), then, became stronger gradually in the late stage (**Figure 2J-L**). By 28 days, the expressions of substance P were similar to the level seen in normal rats (**Figure 2M-O**).

Real-time PCR

IAN-T led to down-regulation of SP mRNA in rat periodontal alveolar bone. The levels of SP mRNA decreased at 3-7 days, then increased since 14 days, and approached to the normal level at 28 days. Compared with the 0 day group, SP mRNA was significantly decreased at 3, 7 and 14 days ($P < 0.05$). In addition, the lowest expression of substance P was observed at 7 days ($P < 0.01$). But no significant difference was found between 0 and 21 days or 0 and 28 days (**Figure 3**).

Western blot analysis

The expressions of SP protein in rat periodontal ligaments and alveolar bone were similar to the expression of its mRNA. SP protein was down-regulated at 3-7 days, and then increased till 28 days, nearly back to the normal level. The lowest level was observed at 7 days ($P < 0.01$). There was significantly statistical difference between 0 and 3, 14, 21 days respectively ($P < 0.05$) (**Figure 4**). But no significant difference was found between 0 and 28 days (**Figure 4**).

Discussion

In recent years much attention has been given to the participation of sensory nerves in inflammatory, immunological responses and healing responses after injury. Growing evidences show that neuropeptide substance P, released from

Substance P in alveolar bone

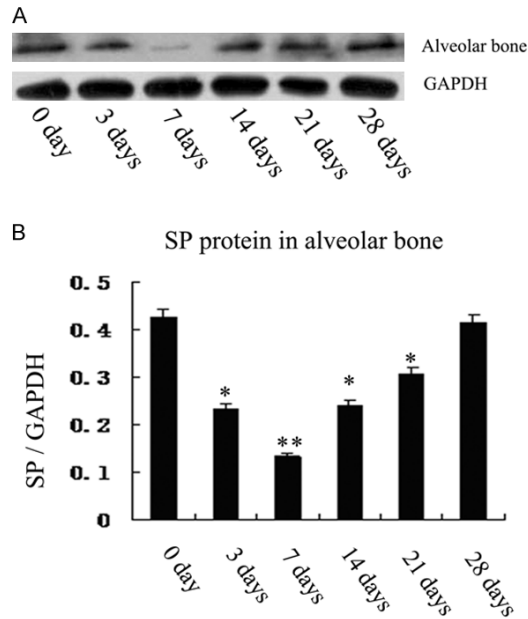


Figure 4. Expressions of substance P protein in rat periodontal alveolar bone at 0, 3, 7, 14, 21 and 28 days after IAN-T. IAN-T led to reduction of substance P protein at 3-14 days. Substance P protein was down-regulated at 3-7 days, and then increased till 28 days, nearly back to the normal level (* $P < 0.05$, ** $P < 0.01$).

peripheral sensory nerve endings, is involved in parts of the inflammatory and healing responses after injury. Substance P is a member of the tachykinin family, and consists of 11 amino acids linked by 10 peptide bonds. Substance P exerts several regulatory functions such as vascular permeability-increasing and vasodilatory effects [16, 17], a chemotactic effect on neutrophils and macrophages [18, 19], improved wound healing potential [20], and stimulation of angiogenesis [21].

Inferior alveolar nerve is a branch of the mandibular nerve. The inferior alveolar nerve enters the mandibular foramen in the ramus of the mandible and occupies the inferior alveolar canal in the body of the mandible. The sensory branches innervate mandibular teeth, periodontal ligaments and alveolar bone. After inferior alveolar nerve transection, Wallerian degeneration occurs in distal neurites, axoplasmic transport is blocked, and sensory corpuscles disappear due to degeneration and denervation. Previous studies show the expressions of neuropeptides in trigeminal ganglion cells are changed after inferior alveolar nerve (IAN)

section, and these changes may also play a part in the persistent sensory abnormalities [22-24]. However, little is known about how neuropeptide substance P changes in periodontal alveolar bone after denervation. In this present study, we quantified the levels of SP in rat periodontal alveolar bone at 3, 7, 14, 21 and 28 days after denervation. Positive expressions of SP were observed in sensory nerve fibers and blood vessels that distributed in periodontal ligaments and alveolar bone. SP expressions in alveolar bone gradually decreased at 3 days, and little positive expressions were observed at 7 days post transection. There was significantly statistical difference between 0, 3 and 7 days respectively, which supported previous studies that inferior alveolar nerve secretes neuropeptide SP. This suggests that substance P significantly decreases in the early stages after injury, and may be useful as a sensitive index to estimate the early stages of inferior alveolar nerve injury.

Studies show that the nervous system is involved in bone repairing and remodeling, and can influence the callus quantity and quality of bone formation by the intermedium of sensory neuropeptides [25-27]. Neuropeptides released from nerve endings distributed in bone is critical to bone metabolism [28, 29]. The present immunohistochemical study provided clear evidence of substance P-positive nerves innervate the periodontal ligaments and alveolar bone, indicating that SP might participate in bone metabolism of the periodontal tissues. In the late stage of the study, SP levels increased since 14 days, and approached to the preoperative level at 28 days. Increased release of substance P provides a microenvironment of innervation compensation and reflects the function of the reinnervation. SP is known to mediate nociception [30], protect nerves against further injury and potentiate normal growth or reconstruction of nerves by transmitting nociceptive information to the central nervous system [31]. We conclude that substance P may play an important role in the compensation or recovery of innervation.

In conclusion, inferior alveolar nerve transection leads to the down-regulation of SP mRNA and protein in alveolar bone in the early stages after injury. In the late stages, SP may also play an important role in the compensation or recovery

ery of innervation. However, the mechanisms are still unclear, further studies are required to elucidate the potential mechanisms.

Acknowledgements

This work was supported by National Natural Science Foundation of China (81271138) awarded to Shu Li and Open Foundation of Shandong Provincial Key Laboratory of Oral Biomedicine (SDKQ201403) and Shandong Province Natural Science Foundation (ZR2015-PH017) awarded to Xijiao Yu.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Shu Li, Shandong Provincial Key Laboratory of Dental Tissue Regeneration, Department of Periodontology, School of Stomatology, Shandong University, 44-1 West Wenhua Road, Jinan 250012, Shandong, PR China. Tel: +86 531 8838 2769; Fax: +86 531-8838 2923; E-mail: lishu@sdu.edu.cn

References

- [1] Atsumi Y, Imai T, Matsumoto K, Sakuda M, Kurisu K, Wakisaka S. Effects of neonatal injury of the inferior alveolar nerve on the development and regeneration of periodontal nerve fibers in the rat incisor. *Brain Res* 2000; 871: 201-209.
- [2] Wang L, Shi X, Zhao R, Halloran BP, Clark DJ, Jacobs CR, Kingery WS. Calcitonin-gene-related peptide stimulates stromal cell osteogenic differentiation and inhibits RANKL induced NF-kappaB activation, osteoclastogenesis and bone resorption. *Bone* 2010; 46: 1369-1379.
- [3] Bjurholm A, Kreicbergs A, Brodin E, Schultzberg M. Substance P and CGRP-immunoreactive nerves in bone. *Peptides* 1988; 9: 165-171.
- [4] Lerner UH, Persson E. Osteotropic effects by the neuropeptides calcitonin gene-related peptide, substance P and vasoactive intestinal peptide. *J Musculoskelet Neuronal Interact* 2008; 8: 154-165.
- [5] Jung H, Horiuchi M, Soma K. Changes in the distribution of nerve fibers immunoreactive to calcitonin gene-related peptide according to growth and aging in rat molar periodontal ligament. *Angle Orthod* 2010; 80: 309-315.
- [6] Imai S, Matsusue Y. Neuronal regulation of bone metabolism and anabolism: calcitonin gene-related peptide-, substance P-, and tyrosine hydroxylase-containing nerves and the bone. *Microsc Res Tech* 2002; 58: 61-69.
- [7] Wang L, Zhao R, Shi X, Wei T, Halloran BP, Clark DJ, Jacobs CR, Kingery WS. Substance P stimulates bone marrow stromal cell osteogenic activity, osteoclast differentiation, and resorption activity in vitro. *Bone* 2009; 45: 309-320.
- [8] Rameshwar P, Zhu G, Donnelly RJ, Qian J, Ge H, Goldstein KR, Denny TN, Gascón P. The dynamics of bone marrow stromal cells in the proliferation of multipotent hematopoietic progenitors by substance P: an understanding of the effects of a neurotransmitter on the differentiating hematopoietic stem cell. *J Neuroimmunol* 2001; 121: 22-31.
- [9] He H, Tan Y, Yang M. Effect of substance P in mandibular osteotomies after amputation of the inferior alveolar nerve. *J Oral Maxillofac Surg* 2010; 68: 2047-2052.
- [10] Berggreen E, Sae-Lim V, Bletsa A, Heyeraas KJ. Effect of denervation on healing after tooth replantation in the ferret. *Acta Odontol Scand* 2001; 59: 379-385.
- [11] Elcock C, Boissonade FM, Robinson PP. Changes in neuropeptide expression in the trigeminal ganglion following inferior alveolar nerve section in the ferret. *Neuroscience* 2001; 102: 655-667.
- [12] Tsuzuki K, Fukuoka T, Sakagami M, Noguchi K. Increase of preprotachykinin mRNA in the injured mandibular neurons after rat infra-orbital nerve transection. *Neurosci Lett* 2003; 345: 57-60.
- [13] Hong HS, Lee J, Lee E, Kwon YS, Lee E, Ahn W, Jiang MH, Kim JC, Son Y. A new role of substance P as an injury-inducible messenger for mobilization of CD29(+) stromal-like cells. *Nat Med* 2009; 15: 425-35.
- [14] Yu X, Lv L, Zhang J, Zhang T, Xiao C, Li S. Expression of neuropeptides and bone remodeling-related factors during periodontal tissue regeneration in denervated rats. *J Mol Histol* 2015; 46: 195-203.
- [15] Wang Y, Lv L, Yu X, Zhang T, Li S. The characteristics of epithelial cell rests of Malassez during tooth eruption of development mice. *J Mol Histol* 2014; 45: 1-10.
- [16] Karabucak B, Walsch H, Jou YT, Simchon S, Kim S. The role of endothelial nitric oxide in the Substance P induced vasodilation in bovine dental pulp. *J Endod* 2005; 31: 733-736.
- [17] Rodd HD, Boissonade FM. Substance P expression in human tooth pulp in relation to caries and pain experience. *Eur J Oral Sci* 2000; 108: 467-474.
- [18] Lotz M, Vaughan JH, Carson DA. Effect of neuropeptides on production of inflammatory cytokines by human monocytes. *Science* 1988; 241: 1218-21.

Substance P in alveolar bone

- [19] Park SH, Hsiao GY, Huang GT. Role of substance P and calcitonin gene-related peptide in the regulation of interleukin-8 and monocyte chemoattractant protein-1 expression in human dental pulp. *Int Endod J* 2004; 37: 185-92.
- [20] Westerman RA, Carr RW, Delaney CA, Morris MJ, Roberts RG. The role of skin nociceptive afferent nerves in blister healing. *Clin Exp Neurol* 1993; 30: 39-60.
- [21] Alhelal MA, Palaska I, Panagiotidou S, Letourneau R, Theoharides TC. Trigeminal nerve stimulation triggers oral mast cell activation and vascular permeability. *Ann Allergy Asthma Immunol* 2014; 112: 40-45.
- [22] Hernández CJ, Berríos A, Chinapen S. Localization of substance P-like immunoreactivity in the palate and trigeminal ganglion of *Rana pipiens*. *Comp Biochem Physiol C Toxicol Pharmacol* 2003; 134: 465-472.
- [23] Bird EV, Long A, Boissonade FM, Fried K, Robinson PP. Neuropeptide expression following constriction or section of the inferior alveolar nerve in the ferret. *J Peripher Nerv Syst* 2002; 7: 168-180.
- [24] Jacobsen EB, Fristad I, Heyeraas KJ. Nerve fibers immunoreactive to calcitonin gene-related peptide, substance P, neuropeptide Y, and dopamine beta-hydroxylase in innervated and denervated oral tissues in ferrets. *Acta Odontol Scand* 1998; 56: 220-228.
- [25] Hukkanen M, Konttinen YT, Santavirta S, Nord-sletten L, Madsen JE, Almaas R, Oestreicher AB, Rootwelt T, Polak JM. Effect of sciatic nerve section on neural ingrowth into the rat tibial fracture callus. *Clin Orthop Relat Res* 1995; 311: 247-257.
- [26] Adam C, Llorens A, Baroukh B, Cherruau M, Saffar JL. Effects of capsaicin-induced sensory denervation on osteoclastic resorption in adult rats. *Exp Physiol* 2000; 85: 62-66.
- [27] Onuoha GN. Circulating sensory peptide levels within 24 h of human bone fracture. *Peptides* 2001; 22: 1107-1110.
- [28] Chole RA, Tinling SP. Adaptive bone modeling in the middle ear is substance-P dependent. *Am J Otol* 1998; 19: 521-524.
- [29] Elefteriou F. Regulation of bone remodeling by the central and peripheral nervous system. *Arch Biochem Biophys* 2008; 473: 231-236.
- [30] Wu XN, Zhang T, Qian NS, Guo XD, Yang HJ, Huang KB, Luo GQ, Xiang W, Deng WT, Dai GH, Peng KR, Pan SY. Antinociceptive effects of endomorphin-2: suppression of substance P release in the inflammatory pain model rat. *Neurochem Int* 2015; 82: 1-9.
- [31] Keast JR, Forrest SL, Osborne PB. Sciatic nerve injury in adult rats causes distinct changes in the central projections of sensory neurons expressing different glial cell line-derived neurotrophic factor family receptors. *J Comp Neurol* 2010; 518: 3024-3045.