Immunohistochemical Analysis of Nerve Distribution in Mandible of Rats

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After review of the literature, there appears to be no report on the histology of the mandibular nerve fiber distribution. Therefore, using a Wistar rat model, immunohistochemical staining with protein gene product (PGP) and calcitonin gene-related peptide (CGRP) antibody for all nerves and only the pain-sensitive nerves, respectively, was performed. We also statistically compared the nerve distribution density by mandibular region. The section of the mandible from the alveolar crest to the mandibular canal was compartmentalized to several regions. Subsequently, nerve distribution density by region was measured microscopically in both the PGP- and CGRP-positive nerves. Furthermore, the ratio of CGRP- to PGP-positive nerves was measured in each region and statistically compared. In both the PGP- and CGRP-positive nerves, the nerve distribution density significantly increased vertically toward the mandibular canal from the alveolar crest and horizontally toward the periodontal ligament from the periosteum. From the CGRP- to PGP-positive nerve ratio, the pain-sensitive nerve accounted for approximately >70% in each region. Pain would therefore be more likely to develop when surgical invasiveness deepens toward the mandibular canal or periodontal ligament. Therefore, sufficient local anesthetic infiltration and/or combined use of conduction anesthesia or periodontal ligament injection may be required. These results may aid in the development of more effective surgical and anesthetic techniques for mandibular surgery.

Key Words: Mandible; PGP9.5; CGRP; Nerve distribution; Rat.

In dentistry, local anesthetic is required for invasive surgeries, such as wisdom tooth removal or oral implant placement when general anesthesia is not employed. If the effect of the local anesthetic is inadequate, the patient will often complain of pain, which can be accompanied by elevated heart rate and blood pressure.¹ When surgical invasiveness to the mandible increases, both common infiltration and conduction anesthesia are commonly used concomitantly. The combined use of conduction anesthesia significantly suppresses pain compared with the use of infiltration anesthesia alone.^{2,3} Furthermore, 2% lidocaine containing 1:80,000 epinephrine, which is routinely used in dentistry in Japan, significantly inhibits pain via increased lidocaine concentration in the mandible,⁴⁻⁸ suggesting that sufficient infiltration of local anesthetics to the mandible is required for the deep mandibular invasion. Several studies

have reported on the relationship between the effect and concentration of local anesthetic in the mandible.⁴⁻⁸ However, no histological or statistical analyses have been conducted on mandibular nerve fiber distribution.

In the present study, immunohistochemical staining was performed on the mandibular nerves of rats. Moreover, the nerve distribution density was measured for all nerves and for the pain-sensitive nerves and was statistically compared by mandibular region.

MATERIAL AND METHODS

This experiment was conducted with the permission of the Ohu University Animal Experiment Committee (Animal experiment permit number 2016-23, 2017-17). The authors have no conflicts of interest to disclose.

Experimental Animals

Six male Wistar rats (10 weeks old, 300 ± 10 g) were used in the present study (Clea Japan, Tokyo, Japan).

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The rats were housed at room temperature (23°C) and 60% humidity with free access to food (MF, Oriental Yeast, Tokyo, Japan) and water until the day of experimentation.

Sample Preparation Method

The rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 µg/g), thoracotomized, and perfused with saline through the left ventricle. The mandible was excised following perfusion fixation with phosphate buffer containing 4% paraformaldehyde (pH 6.2). The excised mandible was immersed overnight in the same fixative at 4°C and decalcified with 10% EDTA solution (pH 7.0). The specimens were decalcified with 10% EDTA solution (pH 7.0) for 3 to 6 months. The 10% EDTA solution (pH 7.0) was changed once a week during decalcification. The decalcified specimens were embedded in paraffin, and 20-µm sections were obtained using a microtome. Sagittal sections of all specimens were obtained at the right and left first molars of the rats. Furthermore, 24 uninterrupted intact samples from the alveolar crest to the mandibular canal were used.

Immunohistochemical Staining

In the present study, we used the method for immunohistochemical staining of nerve fibers suggested by Iwanaga et al.⁹ and Hsu et al.¹⁰ Paraffin-embedded sections were deparaffinized and treated with 0.3% H₂O₂-containing solution for 15 minutes to inactivate the endogenous peroxidase, blocked with goat serum (VECTASTAINR Elite ABC kit, VECTOR Lab Inc, Burlingame, CA) for 60 minutes, and then reacted with a primary antibody. Rabbit anti-protein gene product (PGP) 9.5 antibody (RA95101; UltraClone Limited, Inc, UK) (1:1000 dilution) was used as the primary antibody for all nerves.¹¹ Goat anti-calcitonin gene-related peptide (CGRP) antibody (LS-C41796; LSBio, Inc, Seattle, WA) (1:500 dilution) was used as the primary antibody for pain-sensitive nerves.¹² The interpretations of PGP-positive and CGRP-positive nerves by classification of nerves¹³ were shown in Table 1. The sections were reacted with the primary antibodies at room temperature for 18 hours. The sections were then reacted with a secondary antibody, a biotin-labeled goat anti-rabbit antibody (VECTASTAINR Elite ABC kit, VECTOR LAB, Inc), at room temperature for 60 minutes, followed by a reaction with peroxidase-conjugated streptavidin (VECTASTAINR Elite ABC

Table 1. Interpretations of PGP- and CGRP-Positive Nerves

<i>Nerve Classification</i>	<i>Fiber</i>	<i>PGP*</i>	<i>CGRP</i>
Somatic			
Motor (efferent)	Aα	+	
	Aγ	+	
Sensory (afferent)	Aα	+	
	Aβ	+	
	Aδ Fast pain	+	+
	C Slow pain	+	+
Autonomic			
Sympathetic	B	+	
Parasympathetic	C Visceral pain	+	+

* +, positive nerves; PGP, protein gene product; CGRP, calcitonin gene-related peptide; PGP-positive nerves, all nerves; CGRP-positive nerves, pain-sensitive nerves (Aδ and C fibers).

kit, VECTOR LAB, Inc) for 60 minutes. Peroxidase-conjugated streptavidin was prepared by dropwise addition over 30 minutes.

Color development was activated using DAB (Peroxidase Substrate Kit, VECTOR Lab, Inc), and nuclei were stained using 5% methyl green (Muto Pure Chemicals Co, Tokyo, Japan). All sections were subsequently observed using light microscopy, and all images were captured on a personal computer.

Distribution Density Measurement of the Mandibular Nerves

The bone area was divided into 5 main regions, A to E (Figure 1). Moreover, the C region was divided horizontally into equal thirds (Figure 1), namely, the periodontal ligament one-third was named C-Ligament side (C_L), the center one-third was named C-Center (C_C), and the periosteal one-third was named C-Periosteum side (C_P).

The bone area was measured in each region (A, B, C, D, E, C_L, C_C, and C_P), and the number of nerves in each bone area was measured with image analysis software AxioVision (Carl Zeiss, Tokyo, Japan) (Figure 2). The nerve distribution density was determined from the number of nerves per each bone area. Moreover, the nerve distribution density per unit area was expressed as nerve number per mm².

Statistical Analysis

The mean distribution density of PGP- and CGRP-positive nerves was statistically compared in 5 regions (A, B, C, D, and E) and three subregions (C_L, C_C, and

Figure 1. The region was divided vertically into 5 sections (A, B, C, D, and E). In addition, the C region was divided horizontally into equal thirds (C_L , C_C , and C_P). Nerve distribution density was measured in each region, and the number of nerves per bone area was expressed as number of nerve fibers per mm^2 .

C_P). Additionally, the ratio of CGRP- to PGP-positive nerves was compared in each region.

A chi-square test was used for statistical analysis, and a p value of $< .05$ was considered statistically significant.

RESULTS

Statistical Analysis of Vertical Nerve Distribution Density

The mean distribution density of PGP-positive nerves was as follows: 34.4 ± 17.9 nerves/ mm^2 in region A, 65.6 ± 24.2 nerves/ mm^2 in region B, 88.4 ± 31.0 nerves/ mm^2 in region C, 88.9 ± 31.3 nerves/ mm^2 in region D, and 125.1 ± 32.3 nerves/ mm^2 in region E were obtained in the vertical direction (Table 2; Figure 3).

A significant difference ($p < .001$) was detected in the distribution density of PGP-positive nerves in the vertical region, and the highest value was obtained in region E.

The mean distribution density of CGRP-positive nerves was as follows: 24.7 ± 16.5 nerves/ mm^2 in region A, 65.1 ± 37.1 nerves/ mm^2 in region B, 70.8 ± 35.8 nerves/ mm^2 in region C, 75.1 ± 34.4 nerves/ mm^2 in region D, and 104.2 ± 49.9 nerves/ mm^2 in region E were obtained in the vertical direction.

A significant difference ($p < .001$) in the distribution density of CGRP-positive nerves was detected in the vertical region, and the highest value was obtained in region E.

Statistical Analysis of Horizontal Nerve Distribution Density

The mean distribution density of PGP-positive nerves is as follows: 72.4 ± 33.0 nerves/ mm^2 in region C_P , 87.1 ± 30.3 nerves/ mm^2 in region C_C , and 105.7 ± 81.6 nerves/ mm^2 in region C_L were obtained in the horizontal direction (Table 2; Figure 4).

Staining by PGP antibody

Staining by CGRP antibody

Figure 2. Protein gene product (PGP)- and calcitonin gene-related peptide (CGRP)-positive nerve to measure the nerve distribution. The bone area in each region was measured, and the number of nerves in each bone area was measured using AxioVision image analysis software. Red arrows indicate nerves. The scale bar shows 50 μm .

Table 2. Nerve Distribution Density and CGRP/PGP Ratio in Each Region

Region	PGP (/mm ²)†	Statistics	CGRP (/mm ²)	Statistics	CGRP/PGP Ratio	Statistics
Vertical direction						
A	34.4 ± 17.9	** <i>p</i> = 2.511 ^{−11}	24.7 ± 16.5	** <i>p</i> = 1.020 ^{−9}	71.8%	ns <i>p</i> = .317
B	65.6 ± 24.2		65.1 ± 37.1		99.2%	
C	88.4 ± 31.0		70.8 ± 35.8		80.1%	
D	88.9 ± 31.3		75.1 ± 34.4		84.5%	
E	125.1 ± 32.3		104.2 ± 49.9		83.3%	
Horizontal direction						
C _P	72.4 ± 33.0	** <i>p</i> = .0011	49.6 ± 27.6	* <i>p</i> = .042	68.5%	ns <i>p</i> = .283
C _C	87.1 ± 30.3		69.4 ± 28.9		79.7%	
C _L	105.7 ± 81.6		93.4 ± 70.8		88.4%	

† All graphs are shown as mean ± standard deviation. PGP, all nerves were stained by protein gene product 9.5 antibody; CGRP, pain-sensitive nerves were stained by calcitonin gene-related peptide antibody.

* $p < .05$; ** $p < .01$; and ns, no significant difference on chi-square test.

A significant difference ($p = .001$) in the distribution density of PGP-positive nerves was detected in the horizontal region, and the highest value was obtained in region C_L.

Moreover, the mean distribution density of CGRP-positive nerves is as follows: 49.6 ± 27.6 nerves/mm² in region C_P, 69.4 ± 28.9 nerves/mm² in region C_C, and 93.4 ± 70.8 nerves/mm² in region C_L were obtained in the horizontal direction. A significant difference ($p = .042$) in the distribution density of CGRP-positive nerves was detected in the horizontal region, and the highest value was obtained in region C_L.

The Ratio of CGRP- to PGP-Positive Nerves

The ratio of CGRP- to PGP-positive nerves is as follows: 71.8% in region A, 99.2% in region B, 80.1% in region C, 84.5% in region D, and 83.3% in region E were obtained in the vertical direction (Table 2).

No significant difference ($p = .32$) was detected in the ratio of the pain-sensitive nerve to every nerve in the vertical region.

Moreover, the ratio of CGRP- to PGP-positive nerves in the horizontal direction is as follows: 68.5% in region C_P, 79.7% in region C_C, and 88.4% in region C_L. No significant difference ($p = .29$) was detected in the ratio of the pain-sensitive nerve to every nerve in the horizontal region.

However, from the CGRP- to PGP-positive nerve ratio, the ratio of the pain-sensitive nerve to all nerves accounted for >70% in each region.

DISCUSSION

Inference of the Human Mandible Based on the Present Results

Serious ethical problems exist with the use of the normal human mandible for tissue specimens; therefore, the rat mandible was used in the present study, and the nerve

Figure 3. Statistical analysis of vertical nerve distribution density. All graphs are shown in mean ± standard deviation. PGP: All nerves were stained by protein gene product 9.5 antibody. CGRP: Pain-sensitive nerves were stained by calcitonin gene-related peptide antibody. * $p < .05$; ** $p < .01$; ns, no significant difference on chi-square test.

Figure 4. Statistical analysis of horizontal nerve distribution density. All graphs are shown as mean $\pm$ standard deviation. PGP: All nerves were stained by protein gene product 9.5 antibody. CGRP: Pain-sensitive nerves were stained by calcitonin gene-related peptide antibody. * $p < .05$; ** $p < .01$; ns, no significant difference by chi-square test.

distribution density was analyzed. The rat mandible has been frequently used as an experimental model imitating the human mandible for anatomical, histological, and surgical studies. In addition, it has been demonstrated as a highly reproducible ideal histologic model.^{14,15} Therefore, the nerve distribution density in the human mandible can be analogized from these results to a certain degree.

Findings of Mandibular Nerve Distribution

There have been several reports on nerve distribution density in oral soft tissues,^{16–20} including the temporomandibular joint, periosteum, periodontal ligament, and dental pulp. Several reports on nerve distribution density in bone, such as the femur, have been recognized.^{21–23} However, no statistical analysis was performed for those reports. Furthermore, there are no histological studies on the mandibular nerve distribution.

This study clarified the mean distribution density of all nerves and of the pain-sensitive nerves and the ratio of the pain-sensitive nerves to all nerves in the mandibular region. The nerve distribution density significantly increased in each specimen downward toward the mandibular canal from the alveolar crest.

In addition, it significantly increased toward the periodontal ligament from the periosteum. These results could aid in the development of more effective surgical and anesthetic techniques for mandibular surgery.

Pain-Sensitive Versus All Nerves in the Mandibular Nerve Distribution

The results revealed that the distribution density of all nerves and pain-sensitive nerves significantly increased in the vertical direction downward toward the mandibular canal from the alveolar crest. The inferior alveolar nerve in the mandibular canal is the thickest nerve in the mandible and branches off from the mandibular nerve as the third branch of the trigeminal nerve. We speculate that numerous microscopic branches of nerves around the mandibular canal are supplied by the inferior alveolar nerve. Therefore, we found that the shorter the distance from the mandibular canal, the greater was the number of nerves that were recognized.

In general, in most bones, except for the jawbone, the distribution density of nerves generally decreases toward the center of the bone from cortical bone.²² Conversely, it is the special morphology of the jawbone that a thick nerve runs in the center of the bone. Therefore, our result that the distribution density of nerves that increased toward the mandibular canal was a morphologic distinction of the mandible and expected based on anatomy.

In contrast, the distribution density of all nerves and in the pain-sensitive nerves significantly increased in the horizontal direction toward the periodontal ligament from the periosteum. It has been reported that the periodontal ligament membrane is a dense connective tissue having a thickness of about 150 to 400 μm , which is interposed between the teeth and the alveolar bone, and the function of periodontal ligament is not only the support-fixing device of the tooth, but also an important oral sensory device with a rich sensory nerve supply.²⁴ Other reports suggest there are many Ruffini endings as the sensory receptors in the periodontal ligament.^{25–27} It has also been reported that many microscopic nerves are accompanied by capillary vessels in the periodontal ligament.^{19,28,29} Therefore, we speculate that these microscopic nerves can communicate extensively with the mandibular periodontal ligament.

The pain pathway in the mandible primarily depends on afferent pain-sensitive nerves, such as thin myelinated A δ fibers and thinner unmyelinated C fibers.¹⁶ As a stain for this research, CGRP antibody, which correlates with substance P, is associated with nociception and acute inflammation expression²³ and is widely used as a

marker for detecting thin pain-sensitive nerves (A δ and C fibers) in the pain pathway.¹⁶ The results show that the ratio of pain-sensitive nerves accounted for >70% in the mandible. Intraosseous nerves comprise few efferent motor nerves and many afferent pain-sensitive nerves because there is no dynamic tissue, such as voluntary muscle, in the intraosseous tissue.

Pain and Local Anesthesia in the Mandible

The results demonstrate that many pain-sensitive nerves are distributed around the mandibular canal; however, the mandibular canal is deep and away from the gingivobuccal fold and interdental papilla, which are common injection points of local anesthetics. Therefore, it is likely that pain transmission occurs when surgical invasion reaches the deeper mandibular level despite infiltration anesthesia. This correlates with common clinical observations. Conversely, it is likely that infiltration anesthesia works well with a relatively low dose of local anesthesia in surgery limited to the superficial bone, such as periodontal surgery or orthodontic mini-screw placement, etc, because nerve distribution density decreases around the alveolar crest and cortical bone.

It has been reported that local anesthetic infiltration tends to be difficult in cases of thick cortical bone, and the intraosseous concentration of the local anesthetic changes significantly depending on the injection point, injection pressure, and presence of vasoconstrictor.^{5–8} The intraosseous lidocaine concentration is increased significantly when injected into the interdental papilla compared with the gingivobuccal fold.⁶ Other reports have demonstrated that the intraosseous lidocaine concentration in the mandible is increased significantly by the infiltration of 2% lidocaine containing 1:80,000 epinephrine compared with epinephrine free 2% lidocaine.⁷ Furthermore, it has been reported that the more the injection pressure increases, the more the intraosseous concentration of the local anesthetic in the mandible increases.⁸

Other research has reported that the remaining intraosseous concentration of lidocaine decreases markedly if the surgical operation is performed with the periosteum elevated and with saline or water irrigation.^{4,5} Consequently, it would appear that even if local anesthetic successfully infiltrates to the deeper mandibular level, pain can easily develop with longer surgical procedure or copious irrigation. Accordingly, the combined use of infiltration anesthesia and conduction anesthesia should be used, as is commonly provided, when performing a lengthy surgery with deep mandibular invasion.

CONCLUSION

The distribution density of nerves in the mandible increases toward the mandibular canal. Interestingly, the distribution density of nerves also increases toward the periodontal ligament from periosteum. This may have implications for the use of local anesthesia in dentistry.

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