Immunohistochemical Analysis of Nerve Fibers Distribution in Mandibule of Rats

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ラット下顎骨内における神経分布の免疫組織化学的分析

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Abstract

There is no report about the histological study of nerve fiber distribution with in mandibule. Therefore, this study designed to distribution density of nerve fibers in rat mandibular, using immunohistochemical methods with antibodies against PGP9.5 and CGRP. PGP9.5 antibody to stain every nerve and CGRP antibody to stain only sensory nerve were used for immunohistochemical staining.

Wistar rat was used as the experimental animal. Immunohistochemical staining using PGP9.5 antibody and CGRP antibody was applied to the mandibular microsections.The section of mandibule from the alveolar crest to the mandibular canal was compartmentalized to the several regions. Subsequently, in both of PGP9.5 positive nerve fibers and CGRP, the distribution density of the region was measured microscopically. Furthermore, the ratio of CGRP to PGP9.5 positive nerve fibers were measured in each region. The measurement results in each region were statistically compared.

In both of PGP9.5 positive nerve fibers and CGRP, the distribution density of nerve fibers number significantly increased toward the mandibular canal as vertical direction from alveolar crest, and toward the periodontal ligament side as horizontal direction from

periosteum side. From the ratio of CGRP to PGP9.5 positive nerve fibers, CGRP accounted for over 70% approximately in each region.

It was suggested that the pain is likely to develop when the surgical invasion deepens toward mandibular canal or periodontal ligament. Therefore, when the surgical invasion deepens in mandibule, sufficient infiltration of local anesthetics and combination use of conduction anesthesia or periodontal ligament injection are required.

These results will be available to develop the effective humane technique of the local anesthesia and the surgical operation for the invasion to the mandibule, because the painful site was clarified.

Key word
Mandible, Never Distribution, PGP9.5, CGRP

和文抄録

下顎骨内の神経分布ついての組織学的な研究は認められない。そこで，この研究ではラット下顎骨内の神経分布について，神経線維のマーカーである PGP9.5 と感覚神経のみ染色される CGRP を用い，部位別に神経分布密度を統計学的に解析した。総ての神経が染色される PGP9.5 抗体と感覚神経のみ染色される CGRP 抗体で免疫組織化学染色を行った。

実験動物にはウィスター系ラットを用いた。下顎骨の薄切切片を作成し，PGP9.5 抗体と CGRP 抗体を用いて免疫組織化学染色を行った。歯槽骨頂から下顎管までの下顎骨区域を数か所に区分し，その部位別に光学顕微鏡下に PGP9.5 陽性神経線維ならびに CGRP の分布密度を測定した。また CGRP/PGP9.5 陽性神経線維の割合も部位別に測定した。いずれも部位別の測定結果については統計学的に比較検討した。

PGP9.5 陽性神経線維，CGRP の両方において，垂直的には歯槽骨頂から下顎管に近づくほど，また水平的には骨膜側から歯根膜側に近づくほど，神経分布密度は有意に増加した。CGRP /PGP9.5 陽性神経線維の割合から，どの部位でも約 70%以上は CGRP が含まれていた。

下顎骨では下顎管や歯根膜に近づくほど，神経分布密度が増加するため，外科的侵襲が下顎管や歯根膜に近づくほど痛みも発現しやすいことが示唆された。したがって，外科的侵襲が下顎骨の深部に達する時は局所麻酔薬を十分に浸潤させ，歯根膜内注射や伝達麻酔の併用が求められる。

本結果は痛みの好発部位が明らかになることで，下顎骨に外科的侵襲を与える場合の効果的かつ愛護的な局所麻酔方法や手術術式の対応策のために有用な知見になると考えられる。

Introduction

In dentistry, for the invasive surgical operation such as wisdom tooth removal or oral implant placement, the infiltration of local anesthesia is required. If the effect of local anesthesia is insufficient, patient often complains of pain along with blood pressure elevation[1]. Especially, when the surgical invasion to the mandibule increases, not only common infiltration anesthesia but also conduction anesthesia is used concomitantly. The combination use of conduction anesthesia demonstrates significant pain suppression than the single use of infiltration anesthesia[2,3]. Furthermore, 1:80,000 epinephrine additive 2% lidocaine which is routinely used in dentistry inhibits the pain significantly, when the lidocaine concentration in mandibule increased sufficiently[4-8]. There are several reports that the intraosseous concentration of local anesthetics changed significantly by the injection point, the injection pressure, and the presence of vasoconstriction, when the local anesthetics was injected to mandibule[5-8]. In other research, it was reported that the intraosseous remaining concentration of lidocaine decreases remarkably if the surgical operation is performed with the periosteum elevation and the irrigation by saline or water[4,5]. Consequently, it was considered that if the local anesthetics success to infiltrate to the deeper

level of mandibule, pain will develop easily by long surgical operation or many irrigations. This suggests that the sufficient infiltration of the local anesthetics to the mandibule is important when the strong surgical invasion to the mandibule is predicted.

Up to present, several research about nerve distribution of oral soft tissue and bone except for the oral area[9-16]. However, there is no histological study and no statistical analysis about nerve distribution in mandibule which local anesthetics response.

In this study, the immunohistochemical staining was applied for the nerve fibers number in mandibule of rats. And, the distribution density of nerve fibers number was measured at both of PGP9.5 positive nerve fibers and CGRP containing nerve fibers by the region of alveolar in mandibule, and it was compared statistically.

There are the serious ethical problems to use the normal human mandible for the tissue specimens, therefore, the mandible of rat was applied for this study and the distribution density of nerve fibers number was analyzed. The mandible of rat as an experimental model that imitates human has been frequently used for the studies of anatomy, histology, and surgery. In addition, it has been demonstrated as a highly reproducible ideal histologic model[17,18].

Materials 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 author has no conflicts of interest to disclose.

1. Experimental animals

Six male Wistar rats (10 weeks, 300±10g) were used in this study (Clea Japan, Tokyo, Japan). Until the day of experimentation, the rats ware housed at a room temperature of 23℃ and humidity of 60% with free access to food (MF, Oriental Yeast, Tokyo, Japan) and water.

2. Sample preparation method

Rats were anesthetized by an intraperitoneal injection of pentobarbital sodium (50μg/g), thoracotomized, and perfused with saline through the left ventricle. After perfusion fixation with phosphate buffer containing 4% paraformaldehyde (pH6.2), the mandibule was excised. The excised mandibular was fixed by immersion in the same fixative at 4℃ for overnight, and decalcified with 10% EDTA solution (pH 7.0). The specimens were decalcified with 10% EDTA solution (pH 7.0) for 3-6 months. During decalcification, exchange 10% EDTA solution (pH 7.0) once a week. The decalcified specimens were embedded in paraffin, and 20μm

sections were obtained using a microtome.

In all specimens, sagittal sections were applied at right and left first molar position of rats. And, uninterrupted intact 24 samples from alveolar crest to mandibular canal were used.

3. Immunohistochemical staining

In this study, the methods of immunohistocheimical staining of nerve fibers number were adopted from the reports of Iwanaga[19] et al. and Hsu[20] et al. Paraffin-embedded sections were deparaffinized and treated with 0.3% H_2O_2-containing method solution for 15 minutes in order to inactivate endogenous peroxidase, blocked with goat serum (VECTASTAINRR Elite ABC kit, VECTOR Lab. Inc., USA) for 60 minutes and then reacted with a primary antibody. Rabbit anti-Protein gene product 9.5 (PGP9.5) antibody (RA95101, UltraClone Limited Inc., PA, UK) (1:1000 dilution) were used as the primary antibody for peripheral nerve marker[21,22]. Goat anti-Calcitonin gene-related peptide (CGRP) antibody (LS-C41796, LSBio Inc. WA, USA) (1:500 dilution) were used as the primary antibody for the marker of trigeminal ganglion transmitter[23,24]. Sections were reacted with the primary antibodies at room temperature for 18 hours. The sections were then reacted with the secondary antibody, a biotin-labeled goat anti-rabbit antibody

(VECTASTAINRR Elite ABC kit, VECTOR Lab. Inc., CA, USA) at room temperature for 60minutues, followed by a reaction with peroxidase-conjugated streptavidin (VECTASTAINRR Elite ABC kit, VECTOR Lab. Inc., CA, USA) for 60minutues. Peroxidase-conjugated streptavidin (VECTASTAINRR Elite ABC kit, VECTOR Lab. Inc., CA, USA) was prepared 30 minutes before dropwise addition.

The color development was activated by DAB (Peroxidase Substrate Kit, VECTOR Lab. Inc., USA), and the nuclei were stained with 5% methyl green (Muto Pure Chemicals Co., Tokyo, Japan). Thereafter, the all sections were observed under light microscope, and all images were captured on a personal computer.

4. Measurement of the distribution density of nerve fibers number in mandibule

The specimen of mandibule was vertically divided into five equal regions as A, B, C, D, and E, from the alveolar crest to the mandibular canal (Fig.1). Moreover, the middle region C was horizontally divided into three equal regions (Fig.1). It was divided based on the report of Maeda et al[25]. Namely, periodontal ligament side 1/3 was named $C_{\text{-Ligament side}}$ (C_L), center 1/3 was named $C_{\text{-Center}}$ (C_C), and periosteum side 1/3 was named $C_{\text{-Periosteum side}}$ (C_P).

In each region (A, B, C, D, E, C_L, C_C, C_P), the

bone-area was measured, and the number of nerve fibers number in each bone-area was measured with image analysis software Axio Vision (Carl Zeiss, Tokyo, Japan) (Fig. 2). The distribution density of nerve fibers number was evaluated from the number of nerve fibers number per each bone-area. Finally, distribution density of nerve fibers number per unit area was expressed as nerve fibers number / mm^2.

5. Statistical analysis

The mean distribution density of PGP9.5 positive nerve fibers and CGRP were compared statistically in 5 regions (A, B, C, D, E) and 3 regions (C_L, C_C, C_P). Additionally, the ratio of CGRP to PGP9.5 positive nerve fibers was compared in each region.

A Chi-square test was used for statistics analysis, and a P value of <0.05 was considered to be statistically significant.

Results

1. Statistical analysis of the distribution density of nerve to the vertical direction (Table 1, Fig. 3)

In the mean distribution density of PGP9.5 positive nerve fibers, the values of 34.4±17.9 nerve fibers number/mm^2 at region A, 65.6±24.2 nerve fibers number/mm^2 at region B, 88.4±31.0 nerve fibers number/mm^2 at region C, 88.9±31.3 nerve fibers number/mm^2 at region D, and 125.1 ±32.3 nerve fibers number/mm^2 at region E were obtained to the vertical direction. (Table 1, Fig. 3)

A significant difference ($P=2.511^{-11}$) in the distribution density of PGP9.5 positive nerve fibers were detected by the regions to the vertical direction, and highest value was obtained in region E. (Table 1, Fig. 3)

Moreover, in the mean distribution density of CGRP, the values of 24.7 ± 16.5 nerve fibers number/mm^2 at region A, 65.1±37.1 nerve fibers number/mm^2 at region B, 70.8±35.8 nerve fibers number/mm^2 at region C, 75.1±34.4 nerve fibers number/mm^2 at region D, and 104.2±49.9 nerve fibers number/mm^2 at region E were obtained to the vertical direction. (Table 1, Fig. 3)

A significant difference ($P=1.020^{-9}$) in the distribution density of CGRP was detected by the regions to the vertical direction, and highest value was obtained in region E. (Table 1, Fig. 3)

2. Statistical analysis of the distribution density of nerve to the horizontal direction (Table 1, Fig. 4)

In the mean distribution density of PGP9.5 positive nerve fibers, the values of 72.4±33.0 nerve fibers number/mm^2 at region C_P, 87.1±30.3 nerve fibers number/mm^2 at region C_C, and 105.7 ±81.6 nerve fibers number/mm^2 at region C_L were obtained to the horizontal direction. (Table 1, Fig. 4)

A significant difference ($P=0.0011$) in the distribution density of PGP9.5 positive nerve fibers was detected by the regions to the horizontal direction, and highest value was obtained in region C_L. (Table 1, Fig. 4)

Moreover, in the mean distribution density of CGRP, the values of 49.6 ± 27.6 nerve fibers number/mm^2 at region C_P, 69.4±28.9 nerve fibers number/mm^2 at region C_C, and 93.4±70.8 nerve fibers number/mm^2 at region C_L were obtained to the horizontal direction. (Table 1, Fig. 4)

A significant difference ($P=0.042$) in the distribution density of CGRP was detected by the regions to the horizontal direction, and highest value was obtained in region C_L. (Table 1, Fig. 4)

3. The ratio of CGRP to PGP9.5 positive nerve fibers (Table 1)

In the ratio of CGRP to PGP9.5 positive nerve fibers, the values of 71.8% at region A, 99.2% at

region B, 80.1 % at region C, 84.5% at region D, and 83.3% at region E were obtained to the vertical direction. (Table 1)

No significant difference (P=0.317) in the ratio of CGRP to peripheral nerve fibers was detected by the regions to the vertical direction. (Table 1) Moreover, in the ratio of CGRP to PGP9.5 positive nerve fibers, the values of 68.5% at region C_P, 79.7% at region C_C, and 88.4% at region C_L were obtained to the horizontal direction. (Table 1)

No significant difference (P=0.283) in the ratio of CGRP to peripheral nerve fibers was detected by the regions to the horizontal direction. (Table 1)

However, from the ratio of CGRP to PGP9.5 positive nerve fibers, the ratio of CGRP to peripheral nerve fibers accounted for over 70% approximately in each region. (Table 1)

Discussion

1. Inference of human mandibule from present results

There are the serious ethical problems to use the normal human mandible for the tissue specimens, therefore, the mandible of rat was applied for this study and the distribution density of nerve fibers number was analyzed. However, the mandible of rat as an experimental model that imitates human has been frequently used for the studies of anatomy, histology, and surgery. In addition, it has been demonstrated as a highly reproducible ideal histologic model[17,18]. Therefore, the distribution density of nerve fibers number in human mandible can be analogized from these results to a certain degree.

2. The research significance in the nerve distribution in mandible

Up to the present, several reports about the distribution density of nerve fibers number were found in the oral soft tissues such as temporomandibular joint, periosteum, periodontal ligament, and dental pulp, etc[9-13]. On the one hand, several reports about the distribution density of nerve fibers number in the bone such as thighbone were recognized except for the oral area[14-16]. However, no statistical analysis had been applied for such reports about the nerve distribution, furthermore,

there was no report about the histological study of nerve distribution in mandibule.

By present results, the mean distribution density of the marker of peripheral nerve fibers and the marker of trigeminal ganglion transmitter, ie a substance of pain created in the nerve fibers, and the ratio of those proportions were clarified by the regions of alveolar bone of mandible. In each specimen, the distribution density of nerve fibers number significantly increased down toward mandibular canal from alveolar crest. Additionally, the distribution density of nerve fibers number significantly increased toward periodontal ligament side from periosteal side. Therefore, these results will be available to develop the effective humane technique of the local anesthesia[2,3,6-8] and the surgical operation for the invasion to the mandibule[4,5], because the painful site was clarified.

3. The distribution form of nerve fibers number in mandible

In these results, the distribution density in both of peripheral nerve fibers and CGRP as pain transmitter significantly increased vertically downward toward mandibular canal from alveolar crest. Naturally, inferior alveolar nerve in mandibular canal is a thickest nerve in mandible, which given off from mandibular nerve as third

division of trigeminal nerve. It was speculated that the inferior alveolar nerve fibers numberupplies incalculable microscopic branches around the mandibular canal. Therefore, it was considered that the more distance to the mandibular canal decreased, the more nerve fibers number were recognized.

Moreover, it was reported that the microscopic nerve fibers number accompany with capillary vessels in Haversian canal and Volkmann's canal in bone marrow[14-16,26,27]. Tominaga et al reported that the more cortical bone increases, the more bone marrow decreases[27]. Therefore, it was speculated that the more distance to the surface cortical bone or alveolar crest decreased, the more nerve fibers number decreased conversely.

On the other hand, the distribution density in both of peripheral nerve fibers and CGRP as pain transmitter significantly increased horizontally toward periodontal ligament side from periosteal side. It was reported that there are many microscopic nerve fibers number accompanying with capillary vessels in periodontal ligament[25-29]. Therefore, it was speculated that these microscopic nerve fibers number accompanying with capillary vessels communicate countlessly from periodontal ligament to mandibule, then many nerve fibers number were recognized in the bone of periodontal ligament side.

Mainly, the pain pathway in mandible depends on afferent sensory nerve fibers numberuch as Aδ fiber in thin myelinated nerve and C fiber in thinner unmyelinated nerve[9,10,23,24]. As a staining for this research, CGRP antibody which collaborates with substance P relates to nociception and acute inflammation expression[13,16], and it used widely as a marker to detect the thin sensory nerve (Aδ and C fiber) for the pain pathway[9-11]. The results demonstrate that the ratio of CGRP accounted for over 70% approximately in mandibule. CGRP is the marker of trigeminal ganglion transmitter. And It was thought that the intraosseous nerve fibers number consist of few efferent motor nerve fibers number and many afferent sensory nerve fibers number, because there is no dynamic tissue such as voluntary muscle in the intraosseous tissue.

4. Pain and local anesthesia in mandibule

The results demonstrate many CGRP positive fibers considered to be sensory nerve fibers number distribute around mandibular canal, nonetheless, the mandibular canal is deep away from gingivobuccal fold and interdental papilla that are the common injection point of local anesthetics. Consequently, it is possible that the pain develops easily when the surgical invasion reaches to the deeper level of mandibule. Because,

the infiltration anesthesia is difficult to reach the deeper level of mandibule which consists of peripheral nerve fibers. Conversely, it is possible that the infiltration anesthesia works easily with relatively low dosage of local anesthetics in the case limited to bone surface surgery such as periodontal surgery or orthodontic short screw placing etc. because, the distribution density of nerve fibers number decreases around alveolar crest and bone cortex.

Generally, local anesthetics which was injected around periosteum of mandibule infiltrates through the cortical bone, thereafter, it reaches intraosseous tissue. It was reported that the infiltration of the local anesthetics tends to be difficult if the cortical bone tends to be thick[5-8]. Especially, it was considered that the local anesthetics is difficult to reach to the deeper level of mandibule. There are several reports that the intraosseous concentration of local anesthetics changed significantly by the injection point, the injection pressure, and the presence of vasoconstriction, when the local anesthetics was injected to mandibule[5-8]. It was reported that if 1:80,000 epinephrine additive 2% lidocaine as common dental local anesthetics was injected to mandibule, the intraosseous lidocaine concentration increased significantly when it was injected to interdental papilla than gingivobuccal fold[6]. Other report demonstrated

that the intraosseous lidocaine concentration in mandibule increased significantly by the infiltration anesthesia of 1:80,000 epinephrine additive 2% lidocaine than epinephrine free 2% lidocaine[7]. Furthermore, there is a report that the more injection pressure increases, the more intraosseous concentration of local anesthetics in mandibule increases[8].

From these reports and our results, to infiltrate the sufficient local anesthetics to the surround of mandibular canal in the deep level of mandibule which consists of nerve fibers number of high density, it is thought effective to use the vasoconstrictor additive local anesthetics, and inject high pressure to interdental papilla. In addition, many nerve fibers number were recognized in the bone surround of the periodontal ligament.

Therefore, if the periodontal ligament injection is used in combination, there is a high possibility to suppress the pain, because the local anesthetics infiltrates to the intraosseous tissue via the periodontal ligament space, and the anesthesia effect to the dental pulp is also expected. In other research, it was reported that the intraosseous remaining concentration of lidocaine decreases remarkably if the surgical operation is performed with the periosteum elevation and the irrigation by saline or water[4,5]. Consequently, it was considered that if the local

anesthetics success to infiltrate to the deeper level of mandibule, pain will develop easily by long surgical operation or many irrigations. Accordingly, the combination use of conduction anesthesia should be considered, if the surgical operation of long time with such surgical invasion to the deeper level of mandibule is scheduled.

Conclusion

It was clearly that the distribution density of sensory nerve fibers number in mandibule increased toward the mandibular canal and periodontal ligament side. Therefore, when the surgical invasion deepens in mandibule, sufficient infiltration of local anesthetics and combination use of conduction anesthesia are required. In addition, since there are many distributions on the periodontal ligament side, periodontal ligament injection is effective

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Legend for Figure and Table

Fig. 1 The resions to measure the distribution density of nerves in mandibular bone of rats.

The region was divided in fifth (A,B,C,D,E) in a vertical direction. And, the region C was divided in third (CL, CC,CP) in a horizontal direction. The distribution density of nerves was measured in each region. The number of nerves per each bone-area was expressed as nerve fibers number / mm^2..

Fig. 2 PGP positive nerves and CGRP positive nerves in imaging software Axio Vision

In each region, the bone-area was measured, and the number of nerves in each bone-area was measured with image analysis software Axio Vision.

Table 1 The distribution density of nerves and ratio of CGRP/PGP in each region

Fig. 3 The distribution density of nerves in vertical regions

Fig. 4 The distribution density of nerves in horizontal regions

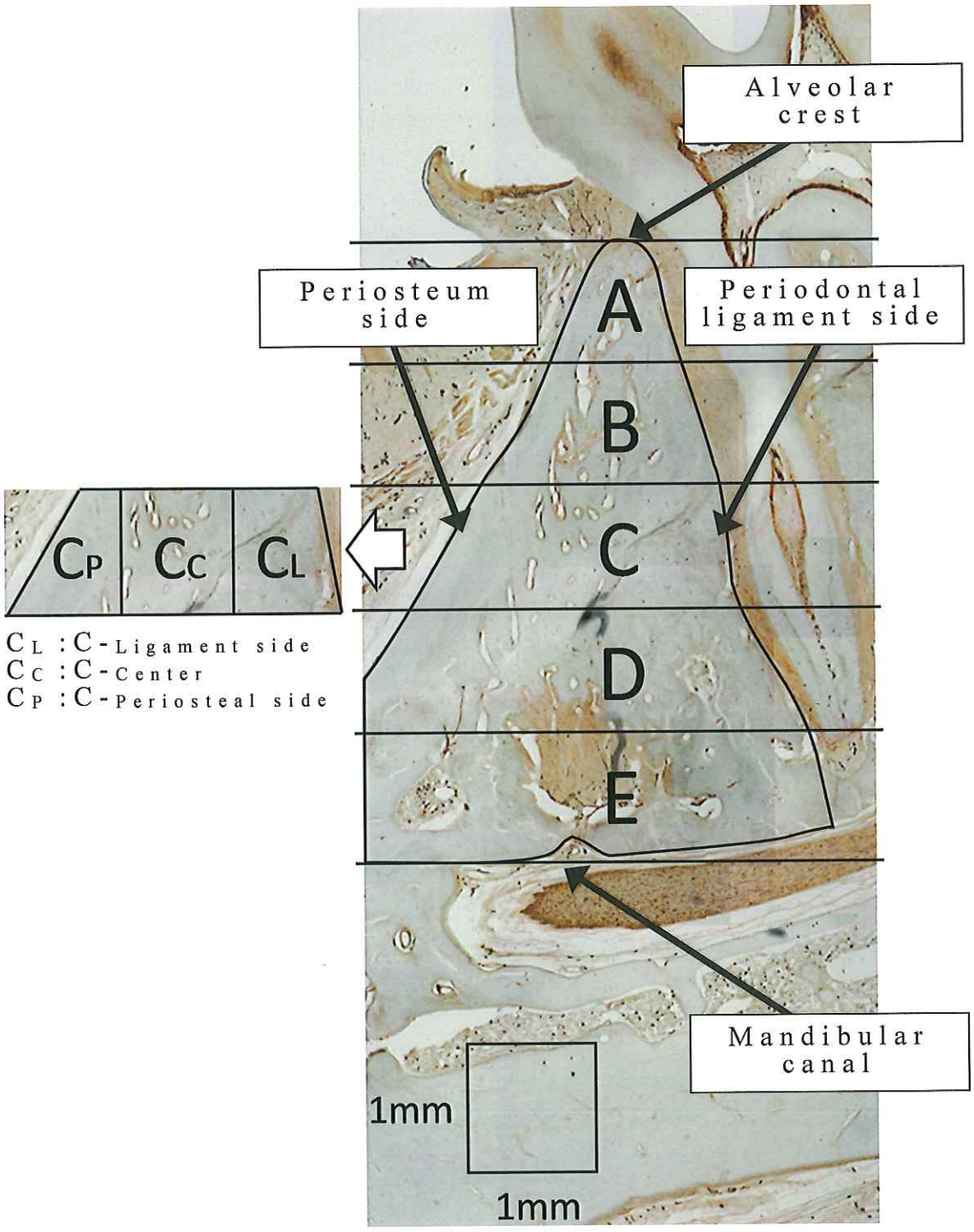
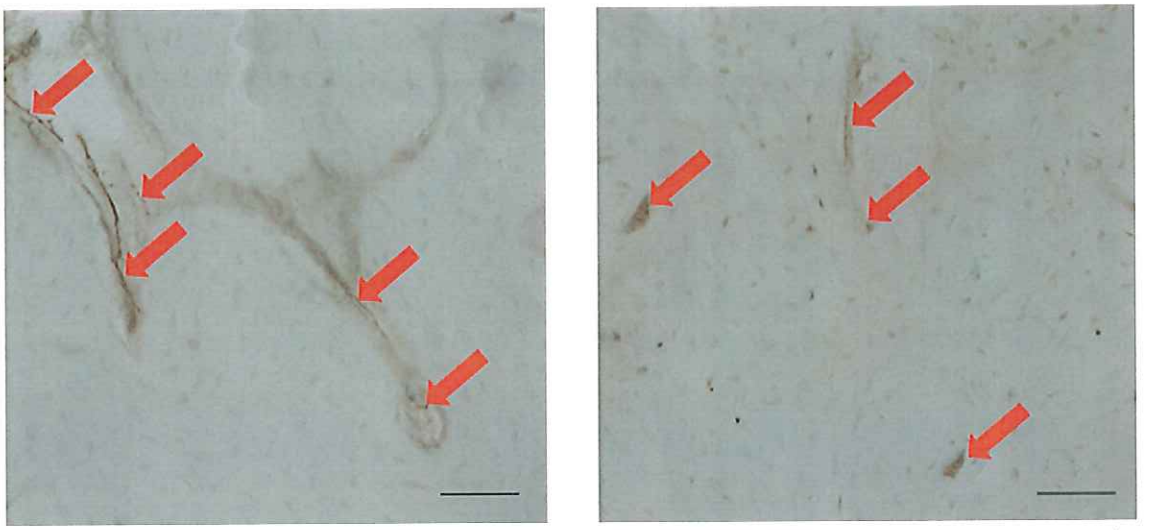


Fig. 1 The resions to measure the distribution density of nerves in mandibular bone of rats.

The region was divided in fifth (A,B,C,D,E) in a vertical direction. And, the region C was divided in third (C_L, C_C,C_P) in a horizontal direction. The distribution density of nerves was measured in each region. The number of nerves per each bone-area was expressed as nerve fibers number / mm^2.



Stained by PGP antibody　　Stained by CGRP antibody

A red arrow indicates a nerve
A scale bar shows 50μm

Fig. 2 PGP positive nerves and CGRP positive nerves in imaging software Axio Vision®

In each region, the bone-area was measured, and the number of nerves in each bone-area was measured with image analysis software Axio Vision®.

Table 1 The distribution density of nerves and ratio of CGRP/PGP in each region

Region		PGP(/mm^2)	Statistics	CGRP(/mm^2)	Statistics	Ratio of CGRP/PGP	Statistics
Vertical direction	A	34.4±17.9	** P=2.511^{-11}	24.7±16.5	** P=1.020^{-9}	71.8%	ns P=0.317
	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	** P=0.0011	49.6±27.6	* P=0.042	68.5%	ns P=0.283
	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 were shown in mean plus minus standard deviation

PGP: Every nerve that were stained by Protein gene product 9.5 antibody

CGRP: Sensory nerves that were stained by Calcitonin gene-related Peptide antibody

* ($p<0.05$), ** ($p<0.01$), ns (no significant difference) by Chi-squire test

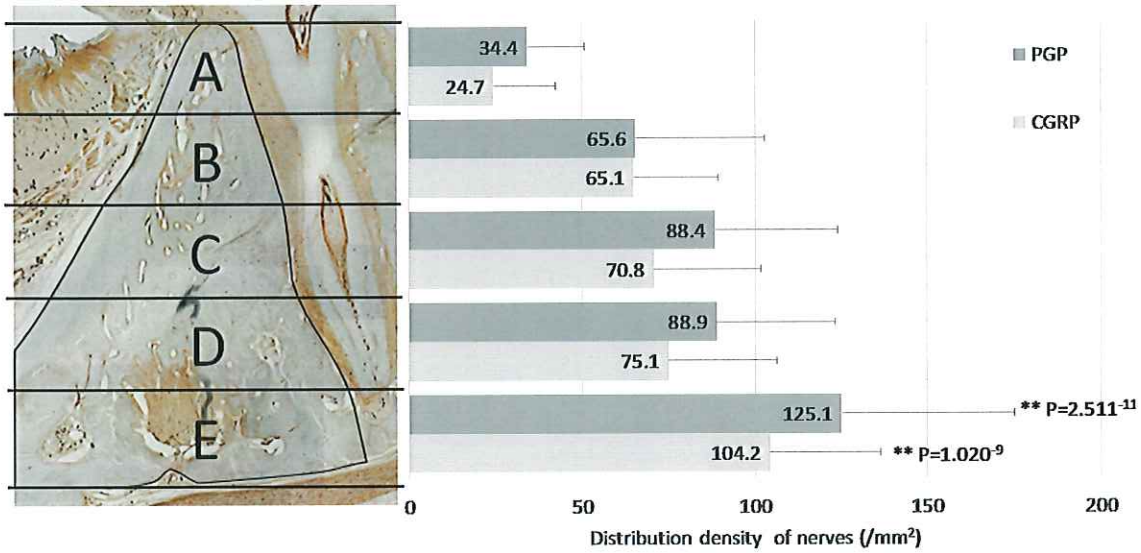


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Fig. 3 The distribution density of nerves in vertical regions

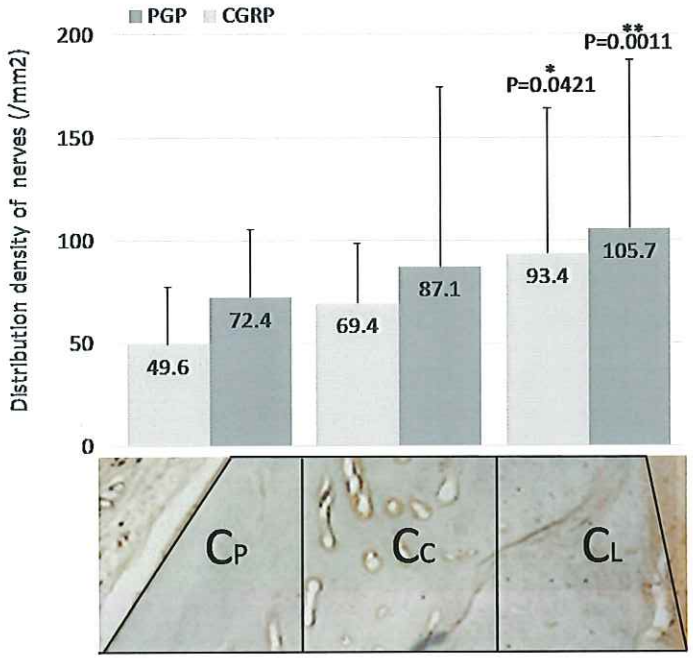

Fig. 4 The distribution density of nerves in horizontal regions

All graphs were shown in mean plus standard deviation

PGP: Every nerve that were stained by Protein gene product 9.5 antibody

CGRP: Sensory nerves that were stained by Calcitonin gene-related Peptide antibody

* ($p<0.05$), ** ($p<0.01$), ns (no significant difference) by Chi-squire test