

Possible peripheral mechanism for taste disorder in S-1 administered rats

Kumiko Aoki<sup>1)2)3)</sup>, Koji Obata<sup>1)</sup>, Miyako Kurihara<sup>2)3)</sup>, Hiroki Kuniyasu<sup>3)</sup>, Tadaaki Kirita<sup>2)</sup>,  
Miyako Takaki<sup>1)3)</sup>

Departments of <sup>1)</sup>Physiology II, <sup>2)</sup>Oral and Maxillofacial Surgery and <sup>3)</sup>Molecular Pathology, Nara Medical  
University, School of Medicine, 840 Shijo-cho, Kashihara 634-8521, Japan

Correspondence to: Miyako Takaki Ph.D.

Department of <sup>1)</sup>Physiology II and <sup>3)</sup>Molecular Pathology, Nara Medical University,

840 Shijo-cho, Kashihara 634-8521, Japan

TEL +81-744-23-9951, FAX +81-744-25-7308

E-mail: [mtakaki@naramed-u.ac.jp](mailto:mtakaki@naramed-u.ac.jp)

### **Abstract**

**Background:** Taste disorders are frequently observed in cancer patients undergoing chemotherapy and are serious adverse events impairing QOL of the cancer patient. Nevertheless, taste disorder mechanisms in cancer patients undergoing chemotherapy are not fully clarified. The aim of the study was to reveal taste disorder-related peripheral mechanisms using two-bottle preference test (TBPT) and histological examination of tongues by HE staining and immunohistochemistry with PGP 9.5.

**Methods:** One bottle was filled with the 0.01mM quinine hydrochloride (quinine), as a bitter compound, and the other was filled with water in TBPT. S-1 (tegafur/ gimeracil/oteracil potassium: 50, 100 mg kg<sup>-1</sup> day<sup>-1</sup>) was lethal for male Wistar rats and S-1 (2-20 mg kg<sup>-1</sup> day<sup>-1</sup>) was administered for 3 weeks (S-1 rats). Dose of S-1 (2 mg kg<sup>-1</sup> day<sup>-1</sup>) corresponds to a clinically administered dose to cancer patients. The part of the tongue containing the circumvallate papillae was excised following TBPT.

**Results:** Rate of increase in averaged preference rate of the quinine versus all intake (quinine plus water) was significant from the initial S-1 period to the final one, compared with in control rats, suggesting the possibility for worse bitter sensation. In S-1 rats, the area of taste nerve fibers were significantly decreased and the rate of degeneration of intra-tongue ganglionic nerve cells was significantly increased. These changes were significantly correlated with rate of increase in averaged preference rate of the quinine.

**Conclusion:** Neuropathy of the gustatory nerve at the periphery may be involved in taste disorders induced by an anticancer drug.

### **Mini-Abstract**

Taste disorder by an anticancer drug may be generated by neuropathy of the gustatory nerve at the periphery.

**Key words:** taste disorder, anticancer drug administration, neuropathy, S-1

## Introduction

Patients undergoing cancer chemotherapy typically have experienced multiple adverse events. Dysgeusia frequently observed in those patients has been reported as the most distressing one, along with fatigue, nausea, vomiting, and hair loss [1]. It affects the daily quality of life of these patients, leading to malnutrition, weight loss and, in severe cases, difficulty in chemotherapy and finally significant morbidity. Although dysgeusia in patients undergoing cancer chemotherapy has been frequently observed, little is known about its generation mechanisms. The etiology of these disorders seems to be multifactorial. Chemotherapy may cause dysgeusia by the following reasons: (i) alterations of cell structure or receptor surface, (ii) interruption in neural coding [2], (iii) zinc deficiency [3], and (iv) less sensitivity in sensation of taste bud for the taste component due to decreased salivary secretion [4] and (v) due to tongue coating [5]. However, it is still unknown which reasons are decisive.

The gustatory system is functioning by detecting chemicals in the oral cavity, mainly on the tongue, although taste perception also occurs on palate and pharynx. Taste buds on the tongue are the sensory end organs for gustation. In mammals, each taste bud is composed of 0-10 in fungiform and 50-100 elongate taste cells in circumvallate papillae. Ultrastructural studies by EM revealed three different morphological types of elongate taste cells within each taste bud: Type I, Type II and Type III [6]. Each type of taste cells is characterized by DeFazio *et al.* [7]. It is well known that Type II cells detect sweet, umami and bitter taste and that Type III cells detect sour taste [8].

The aim of the current study was to reveal possible peripheral mechanisms related to an anticancer drug, S-1-induced taste disorder. S-1 is orally administered fluorinated pyrimidine that has been reported to be an active agent against solid tumors. In 1999-2007, S-1 was approved for the treatment of the various types of cancers [9]. However, dysgeusia has been routinely reported as one of adverse events [10]. In the present study, as functional studies, we performed two-bottle preference test (TBPT) using 0.01mM quinine hydrochloride solution as a bitter compound [11-14] in S-1 administered rats. Taste disorders may be related to changes in sweet, salty, sour, bitter taste sensations and umami sensation. In anticancer drug administered patients, umami sensation is believed to remain normal. The sweet, salty and sour taste sensations are predicted to show complex changes (better or less sensitivity) in taste-disorders. The bitter taste sensation is predicted to become less sensitive in taste-disorder. Therefore, we selected quinine hydrochloride solution as a bitter compound. The immunohistochemistry with a neuronal marker of the

tongue containing the circumvallate papillae revealed degenerations of taste nerve fibers and intra-tongue ganglionic nerve cells in S-1 administered rats.

## **Materials and methods**

### *Animals*

Six-week-old male Wistar rats housed individually in cages and maintained on a 12-h light-dark cycle at about 23°C, were randomly divided into 4 groups, i.e., control (n=4), S-1 (2 mg kg<sup>-1</sup>day<sup>-1</sup>)(n=6), S-1 (10 mg kg<sup>-1</sup>day<sup>-1</sup>)(n=4) and S-1 (20 mg kg<sup>-1</sup>day<sup>-1</sup>)(n=4). All rats were fed on a normal diet and subjected to TBPT for 4 weeks including a custom period of one week. In control group, S-1 was not administered under the same conditions as in other three groups. In other three groups, S-1 was administered according to the protocol described below. All experimental procedures were performed according to the Guide for the Care and Use of Laboratory Animals published by US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and reviewed and approved by the animal care and use committee of Nara Medical University.

### *Administration of S-1*

A 0.5% carboxymethyl cellulose sodium solution was used as the vehicle to suspend S-1 (tegafur/gimeracil/oteracil potassium). S-1 (50, 100 mg kg<sup>-1</sup> day<sup>-1</sup>) was lethal for male Wistar rats and thus the appropriate dose of S-1 (2, 10, 20 mg kg<sup>-1</sup> day<sup>-1</sup>) was administered by a sonde for 3 weeks, during when S-1 administration was performed once in a day in 5 consecutive days and paused on the 6th and 7th day every week. Dose of S-1 (2 mg kg<sup>-1</sup> day<sup>-1</sup>) corresponds to a clinically administered dose in cancer patients.

### *Two-bottle preference test (TBPT)*

The taste preference experiments were performed using the TBPT. Two bottles were set up on each cage, and rats were allowed to make free choice of bottles. The left-right position of the bottles was alternated every day to avoid any influence of side preference or recognition. One bottle was filled with the 0.01mM quinine hydrochloride solution (quinine)[12], and the other with water. After a custom period of one week, during when both two bottles contained water, TBPT was performed for one week before S-1 administration. Then the S-1 administration was performed for three weeks (three courses; initial, mid and final S-1 period). The rate of increase from the initial S-1 period to the final one in averaged preference rate of quinine versus all intake (quinine plus water) or averaged preference volume of quinine was compared between control and S-1 rats.

### *Tissue preparation*

On the next day of the last day of TBPT, rats were anesthetized with pentobarbital and exsanguinated from abdominal aorta. The posterior part of the tongue containing the circumvallate papillae was excised, fixed with 4% paraformaldehyde overnight at 4°C and then embedded in paraffin. Number of taste buds in circumvallate papillae was sufficient and the size was big. Other papillae have no taste buds or small number of taste buds. Therefore, we sampled the tongue including circumvallate papillae. Although taste perception also occurs in taste buds on palate and pharynx, the sampling of taste buds in the tongue is much easier than that of taste buds on palate and pharynx.

### *Immunohistochemistry*

Immunohistochemical staining for a neuronal marker, protein-gene product 9.5 (PGP 9.5), was used to detect nerve cells and fibers in the tongue. Consecutive 5 µm sections were cut from each block. Immunohistochemistry was performed by the immunoperoxidase technique, following antigen retrieval by incubation with pepsin (Sigma Chemical Co., St. Louis, MO) for 20 min, specimens were rinsed with phosphate-buffered saline (PBS). Anti-PGP9.5 antibody (CHEMICON International, Temecula, CA) was diluted at 1:1000. After overnight incubation at room temperature, specimens were rinsed with PBS and incubated at room temperature for 1h with secondary antibody conjugated to LSAB2 Kit (DAKO Corp, Carpinteria, CA). The specimens were then rinsed with PBS and color-developed with diaminobenzidine (DAB) solution (DAKO). Immunostaining of all samples was performed at the same conditions of antibody reaction and DAB exposure.

### *Cell counting*

The number of taste cells was determined by manual counting on hematoxyline and eosin-stained sections including the most taste buds in each one slide selected from each tongue. The number of ganglia in the tongue and nerve cells in each ganglion was determined on PGP9.5 stained sections (x40). We defined degenerated nerve cells in ganglia as nerve cells with degenerated nucleus and cytosol. We counted the degenerated nerve cells in conformity with the definition on PGP9.5 stained sections (x400).

### *Nerve fiber quantification*

To quantify taste nerve fibers, we measured the area of nerve fibers and the total area of the epithelium including taste buds and the subepithelium layer of the taste buds at the area of  $2.6 \pm 0.4 \text{ mm}^2$  including the most taste buds in each section image (x100) and calculated the rate of each area in the same slide as that

used for cell counting.

#### *Statistical analysis*

Statistical significance of differences between means was determined by unpaired t-test or Mann-Whitney U test. A P value of  $< 0.05$  was considered statistically significant. Correlations between the rate of the area of nerve fibers and the rate of increase in averaged preference rate as well as between the degeneration rate of nerve cells and the rate of increase in averaged preference rate were tested by Pearson's  $r$ , with significance identified by  $p < 0.05$ .

#### **Results**

##### *Morphological changes in taste bud structure and number of taste buds and taste cells after S-1 administration.*

Morphological changes in mucosal epithelial basal cells and taste buds of the circumvallate papillae after S-1 administration were not observed in comparison with those in control group (Fig. 1, A and B). Table 1 indicated that the averaged number of taste buds and taste cells after S-1 administration in all S-1 (2, 10, 20 mg kg<sup>-1</sup> day<sup>-1</sup>) groups was not significantly different from that in control group.

##### *Morphological changes in nerve fibers in the epithelium and subepithelium of the taste bud.*

PGP 9.5-positive nerve fibers were detected in the epithelium including taste buds and subepithelium of the taste bud. The nerve processes within the taste bud appeared to decrease in an S-1 rat in comparison with those in a control rat (Fig. 1, C and D). The averaged rates of the area of nerve fibers to the total area of the epithelium and subepithelium of the taste bud in all S-1 groups were significantly smaller than that in the control group ( $P < 0.05$  or  $0.01$ ) (Fig. 1, E), suggesting that S-1 administration degenerated taste nerve fibers, i.e., decreased the nerve fibers remained. There were no significant differences in the averaged rates among different doses (2, 10, 20 mg kg<sup>-1</sup> day<sup>-1</sup>) of S-1 groups.

##### *Morphological changes in intra-tongue ganglionic nerve cells and number of degenerated nerve cells in the ganglia after S-1 administration.*

We found PGP 9.5-positive ganglionic nerve cells distributed in the tongue unrelated to salivary gland (Fig. 2, A and C). They distributed in the subepithelium of the taste bud. We defined these nerve cells as intra-tongue ganglionic nerve cells. In the control group, the nucleus and the cytosol were clear and the cytoplasm density appeared uniform (Fig. 2, B). In contrast, in S-1 groups, chromatic agglutination (karyopyknosis), degeneration of the nucleus of the cell (marginal hyperchromatosis), destructive

fragmentation of the nucleus (karyorrhexis), and disintegration and dissolution of the cell nucleus (karyolysis) were observed in the ganglion (Fig. 2, D). These results indicated that the intra-tongue ganglionic nerve cells were degenerated. Furthermore, the averaged numbers of degenerated nerve cells per ganglion in S-1 groups were significantly larger than that in the control group (Table 1). There were no significant differences in the averaged number of degenerated nerve cells among different doses (2, 10, 20 mg kg<sup>-1</sup> day<sup>-1</sup>) of S-1 groups. The averaged degeneration rates of each ganglionic nerve cell in S-1 groups were significantly larger than that in the control group (Fig. 2, E). There were no significant differences in the averaged degeneration rates among different doses (2, 10, 20 mg kg<sup>-1</sup> day<sup>-1</sup>) of S-1 groups.

*Effect of S-1 administration on taste preference in TBPT and correlation between changes in averaged preference rate of quinine and the rate of the area of nerve fibers to the total area as well as between changes in averaged preference rate and degeneration rate of the intra-tongue ganglionic nerve cells*

TBPT was performed with quinine as a bitter taste substance in rats. S-1 administered rats preferred the quinine at the rate of 11.0 - 54.7% at the final S-1 period. The rate of increase in averaged preference rate of quinine from the initial S-1 period to the final one in S-1 rats (n=5) was significant compared with that in control rats (n=4)(Fig. 3, A).

The correlation between the rate of increase in averaged preference rate and the rate of the nerve fibers remained in each rat (n=7) was significant ( $P < 0.05$ )(Fig. 3, B).

The dispersion diagram of the rate of increase in averaged preference rate and the degeneration rate of the intra-tongue ganglionic nerve cells in each rat (n=2 control and 5 S-1 rats) showed that the degeneration rate rather decreased at the maximal rate of increase in averaged preference rate (Fig. 3, C). Other factors than the degeneration rate may increase the rate of increase in averaged preference rate. Excluding these data, the correlation between the rate of increase in averaged preference rate and the degeneration rate of the intra-tongue ganglionic nerve cells in each rat (n=2 control and 3 S-1 rats) was significant ( $P < 0.005$ )(Fig. 3, D).

## **Discussion**

The most important findings of this study were: (i) 0.01mM quinine hydrochloride solution with a bitter taste was dominantly preferred after S-1 administration, (ii) the rate of decrease in remained nerve fibers in the epithelium including taste buds and subepithelium of the taste bud and the rate of increase in degenerated intra-tongue ganglionic nerve cells were significant compared to that in the control by

immunohistochemistry, (iii) no morphological changes in mucosal epithelial basal cells and taste buds of the circumvallate papillae were observed by hematoxyline and eosin staining and (iv) the correlation between (i) and (ii) was significant. These results suggest the possibility that taste disorder observed in rats underwent S-1 administration is related to degenerated taste nerve fibers and intra-tongue ganglionic nerve cells without degeneration of mucosal epithelial basal cells and taste buds, differently from that in zinc-deficient rats [12].

Zinc-deficiency causes the decrease in taste bud cell proliferation [12]. Yamazaki et al. [15] proposed a different conclusion from ours; X-ray irradiation-induced taste dysfunction is caused by a decrease of the number of taste cells like in zinc-deficient rats [12].

Taste disorder frequently loses the appetite of the patient undergoing chemotherapy, leads to malnutrition and, in severe cases, difficulty in continuation of chemotherapy per se [16,17]. Although multiple biochemical abnormalities, trace metal and vitamin deficiencies, metabolic and endocrine abnormalities of growth factors supporting taste buds [18-22] have been reported as taste disorder-related mechanisms, taste disorder-generating mechanisms in cancer patients undergoing chemotherapy have not yet been clarified. There is agreement, however, that damage to sensory receptor cells and abnormal neuronal activities are possible causes of the taste abnormalities in these patients [2].

In S-1 (50, 100 mg kg<sup>-1</sup> day<sup>-1</sup>) administered rats, the severe morphological changes in mucosal epithelium were observed after death. In contrast, in S-1 (2, 10, 20 mg kg<sup>-1</sup> day<sup>-1</sup>) administered rats, the nerve fibers in the epithelium including taste buds and subepithelium of the taste bud, and the intra-tongue ganglionic nerve cells were degenerated. These morphological changes were not dose-dependent within 2-20 mg kg<sup>-1</sup> day<sup>-1</sup>. These may be associated with “on-off” effect induced by S-1, or the effect has reached to plateau in 2 mg kg<sup>-1</sup> day<sup>-1</sup>. Anyway, no dose-dependency of S-1 in the present study was similar to that observed in clinical application of S-1 [10].

In S-1 (2, 10, 20 mg kg<sup>-1</sup> day<sup>-1</sup>) administered rats, the increase rates of body weights after 3 weeks were 116 ± 13% (n=6), 123 ± 28% (n=4) and 118 ± 16% (n=4), respectively, compared to that in control (140 ± 19%, n=5). There were no significant differences between each S-1 group and control group, suggesting no relationship at least between weight loss and taste-related peripheral changes.

TBPT was performed with quinine as a bitter compound in rats [12, 13]. At first we prepared 0.3 mM quinine, but all rats did not drink such high concentration of quinine after S-1 administration for 3 weeks.



Then, the solution was diluted to a final concentration of 0.01 mM. This concentration is the same as that used in studies of taste disorder in zinc-deficient rats [12, 13].

In the present study, a bitter taste compound, quinine was preferred in performance of TBPT. To further investigate taste disorder generation mechanisms, TBPT with a sweet, salty or sour taste substance should be expected.

Since the correlation between the degeneration rate of the intra-tongue ganglionic nerve cells and the rate of increase in preference rate of a bitter compound in TBPT was significant, it seems likely that the degeneration of these nerve cells is related to S-1 induced taste disorder at least as a peripheral mechanism.

The ganglionic nerve cells are associated with tongue branch of glossopharyngeal nerves [23]. The glossopharyngeal nerve innervates the circumvallate papillae and the posterior foliate papillae on the posterior tongue. The cell bodies of the glossopharyngeal nerve are located in the petrosal ganglion, located on the medial edge of the tympani bulla. Glossopharyngeal, chorda tympani and greater superficial petrosal nerves form terminal fields in the ipsilateral nucleus of the solitary tract and contribute to the rat gustatory system [24]. However, as a peripheral mechanism, intra-tongue ganglionic nerve cells could play some roles on taste sensation transmission to the nucleus of the solitary tract. Further studies are needed to clarify the role of these ganglionic nerve cells.

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#### Conflict of interest

The authors have no conflicts of interest and there are no potential competing interests.

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## Legends for Figures

Figure 1. Hematoxylin and eosin staining of the taste buds.

A: control rat. B: S-1 ( $2 \text{ mg kg}^{-1} \text{ day}^{-1}$ ) administered (S-1) rat. In B, no morphological changes in mucosal epithelial basal cells and taste buds were observed. C: PGP 9.5-positive nerve fibers distributed in the epithelium including the taste bud and subepithelium of the taste buds in a control rat. D: PGP 9.5-positive nerve fibers distributed in the epithelium and subepithelium of the taste bud in an S-1 ( $2 \text{ mg kg}^{-1} \text{ day}^{-1}$ ) administered (S-1) rat. The nerve processes appeared to decrease in D. Scale bars:  $100 \mu\text{m}$  in A-D. E: The averaged rates of the area of nerve fibers to the total area of the epithelium and subepithelium of the taste bud in each section image ( $\times 100$ ) in control ( $n=5$ ) and S-1 ( $2 \text{ mg kg}^{-1} \text{ day}^{-1}$ )( $n=17$ ), ( $10 \text{ mg kg}^{-1} \text{ day}^{-1}$ )( $n=4$ ) and ( $20 \text{ mg kg}^{-1} \text{ day}^{-1}$ )( $n=4$ ) administered rats. \* $P < 0.05$  vs. control. \*\* $P < 0.01$  vs. control. There were no significant differences in the averaged rates among S-1 ( $2$ - $20 \text{ mg kg}^{-1} \text{ day}^{-1}$ ) groups.

Figure 2. PGP9.5-immunohistochemical staining of intra-tongue ganglionic nerve cells in control and S-1 administered rats. A: The epithelium including the taste bud and subepithelium of the taste bud are enclosed by a blue-line square. Five ganglia circled by black-line distributed in the underlayer of the subepithelium unrelated to salivary glands (indicated by red arrows). The circled area **a** shows a single ganglion. B: The enlarged ganglion **a** (**a** in A). The arrows show ganglionic nerve cells. The margin of the nucleus and the cytosol was clear and the cytoplasm density appeared uniform. These nerve cells in the ganglion were defined normal. Scale bars:  $200 \mu\text{m}$  (A),  $20 \mu\text{m}$  (B). C: The epithelium including the taste bud and subepithelium of the taste bud are enclosed by a blue-line square. Four ganglia circled by black-line distributed in the underlayer of the subepithelium unrelated to salivary glands (indicated by red arrows). The circled area **a** shows a single ganglion. D: The enlarged ganglion **a** (**a** in C). The arrows show ganglionic nerve cells. Chromatic agglutination (i: karyopyknosis), degeneration of the nucleus of the cell (ii: marginal hyperchromatosis), destructive fragmentation of the cell nucleus (iii: karyorrhexis) and disintegration and dissolution of the cell nucleus (iv: karyolysis) was observed in these ganglionic nerve cells. Scale bars:  $200 \mu\text{m}$  (C),  $20 \mu\text{m}$  (D). E: The averaged degeneration rate of the intra-tongue ganglionic nerve cells. The degeneration rates of S-1 ( $2 \text{ mg kg}^{-1} \text{ day}^{-1}$ )( $n=17$ ), ( $10 \text{ mg kg}^{-1} \text{ day}^{-1}$ )( $n=4$ ) and ( $20 \text{ mg kg}^{-1} \text{ day}^{-1}$ )( $n=4$ ) administered rats were significantly larger (\* $P < 0.05$ , \*\* $P < 0.01$ ) than that in control rats. But

there were no significant differences in averaged degeneration rate among S-1 ( $2\text{-}20\text{ mg kg}^{-1}\text{ day}^{-1}$ ) groups.

Figure 3. Effect of S-1 ( $2\text{ mg kg}^{-1}\text{ day}^{-1}$ ) administration on taste preference in two-bottle preference test (TBPT)(A) and correlations between changes in the averaged preference rate of  $0.01\text{ mM}$  quinine hydrochloride solution (quinine) and the rate of the area of nerve fibers to the total area (B) as well as between changes in the averaged preference rate of quinine and the rate of the degenerated intra-tongue ganglionic nerve cells (C, D)

A: Comparison of the rate of increase in the averaged preference rate or volume of quinine from the initial S-1 period to the final one between S-1 rats ( $n=5$ ) and control rats ( $n=4$ ). \*,  $P < 0.05$  vs. control. A vertical line indicates standard error. B: The correlation between the rate of increase in averaged preference rate of quinine and the rate of the area of nerve fibers to the total area in each rat ( $n=2$  control indicated by open circles and  $n=5$  S-1 indicated by solid circles) was significant ( $P < 0.05$ ). C: The dispersion diagram of the rate of increase in averaged preference rate of quinine and the degeneration rate of the intra-tongue ganglionic nerve cells in each rat ( $n=2$  control indicated by open circles and  $n=5$  S-1 by solid circles). D: Excluding maximal rate points of increase in averaged preference rate (shown by an arrow in C), the correlation between the rate of increase in averaged preference rate and the degeneration rate of the intra-tongue ganglionic nerve cells in each rat ( $n=2$  control indicated by open circles and  $n=3$  S-1 by solid circles) was significant ( $P < 0.005$ ). In B-D, both results from TBPT and morphology were attained in 2 control rats.

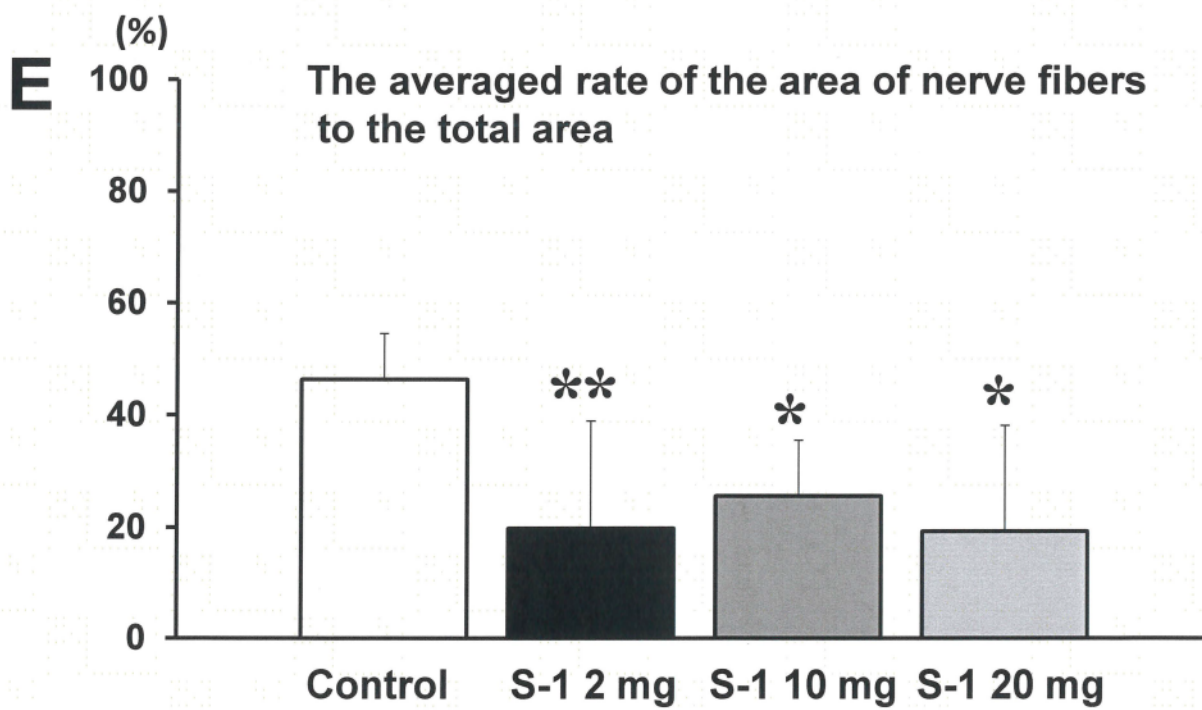
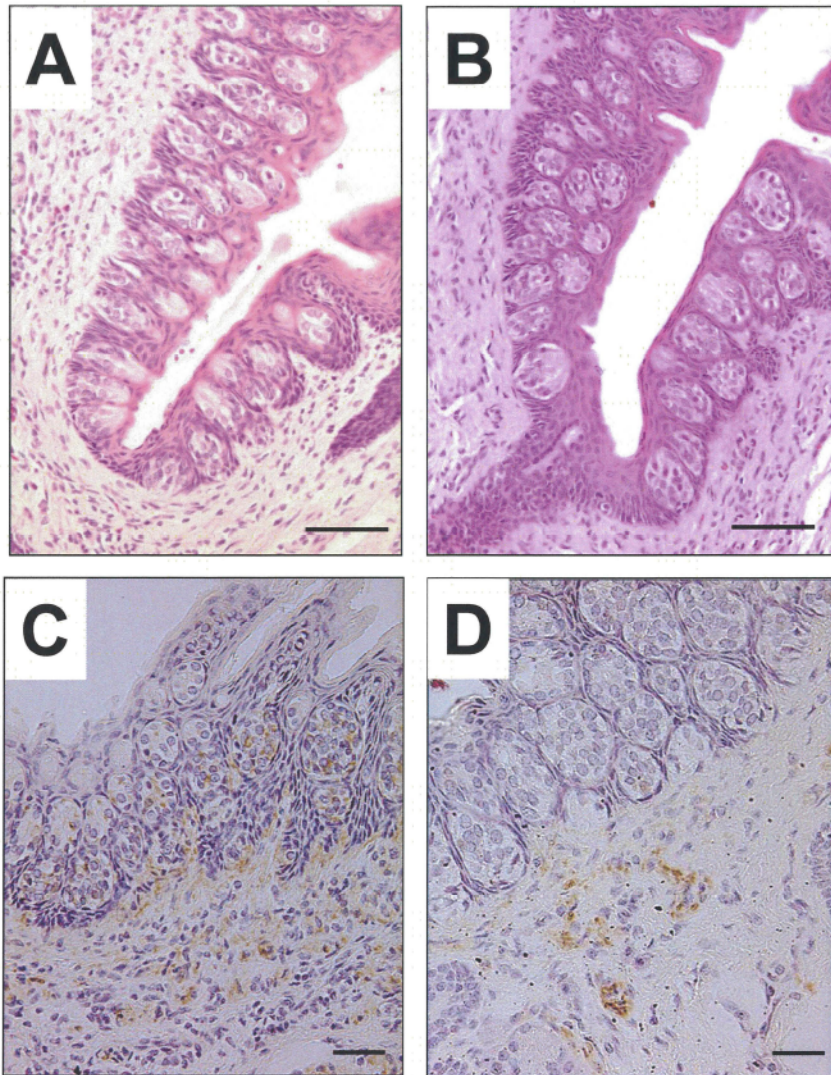


Figure 1

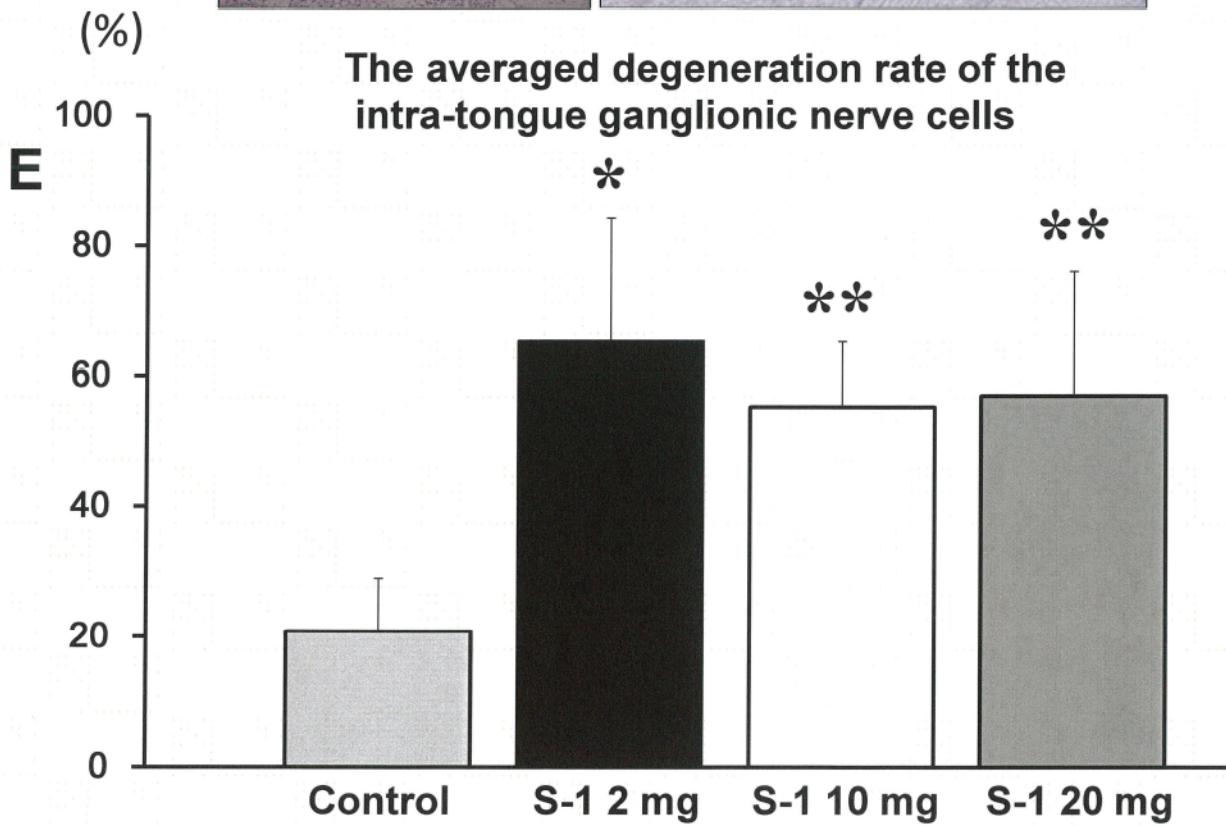
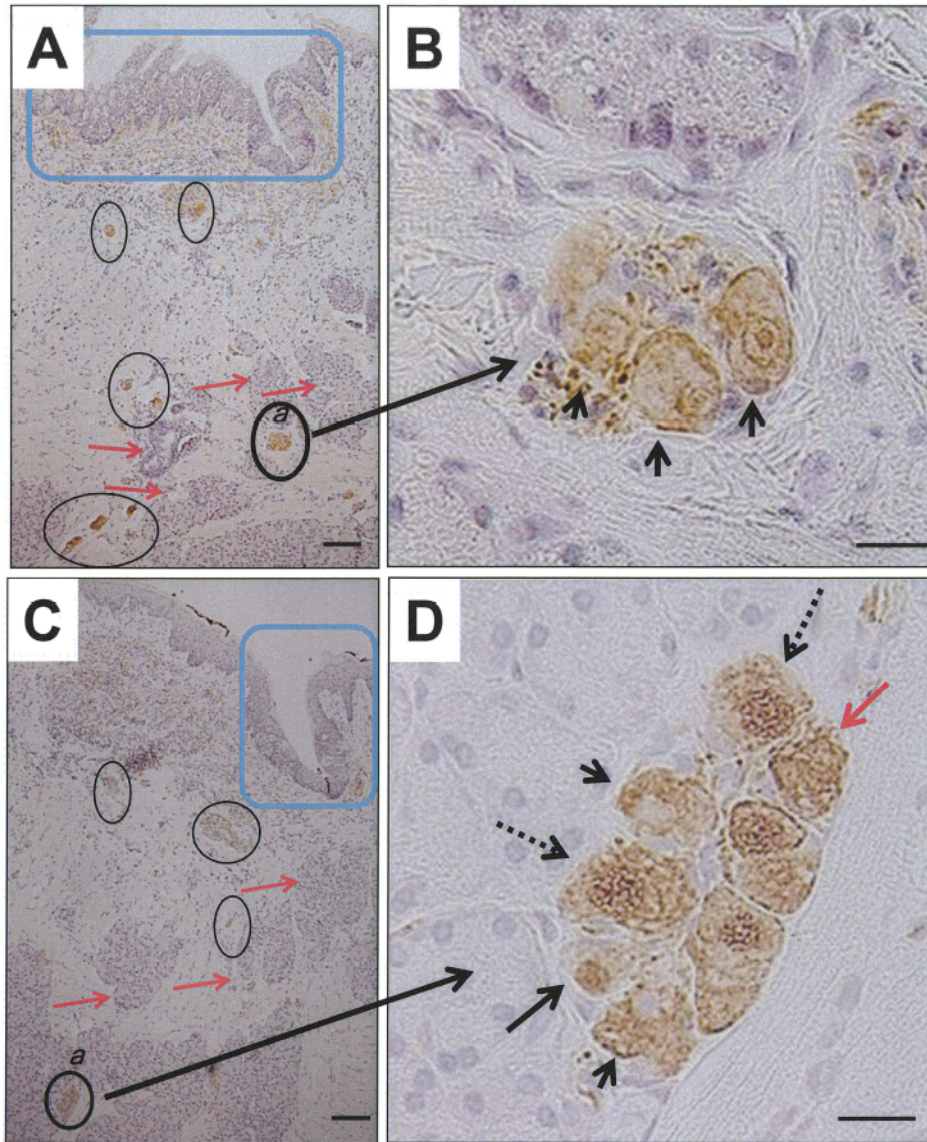


Figure 2

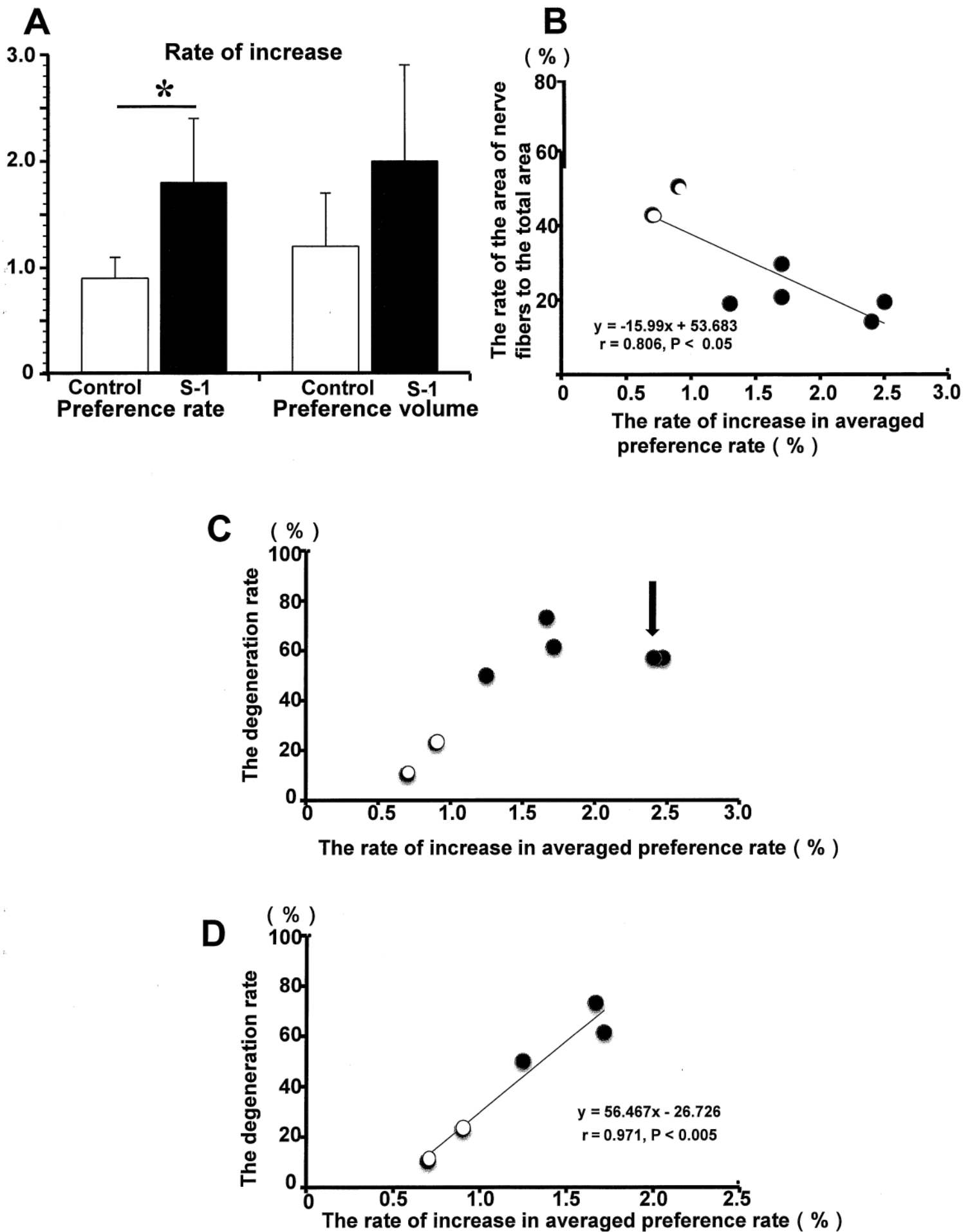


Figure 3