ONLINE FIRST

ISSN: 0015-5659

e-ISSN: 1644-3284

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DOI: 10.5603/FM.a2022.0074

Article type: Original article

Submitted: 2022-07-04

Accepted: 2022-08-07

Published online: 2022-08-17

Effects of liquid diet intake on nerve growth in salivary glands of growing rats

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Abstract

Background: The growth of parotid glands is inhibited by liquid diet intake during growing period, while that of submandibular glands is not affected. This study examined how liquid diet intake affects nerve growth in the parotid and submandibular glands of growing rats, because nerves are closely involved in the maintenance of salivary gland structure.

Materials and methods: Male Wistar rats were weaned at 21 days of age. Then, rats were fed a pellet diet and a liquid diet in the control group and experimental group, respectively. At 0, 2, 4, or 8 weeks, they were euthanized by isoflurane overdose, and parotid and submandibular glands were removed. The frozen sections were made and immuno-stained with anti-protein gene product 9.5 (PGP 9.5) antibody (general nerve marker), anti-tyrosine hydroxylase (TH) antibody (sympathetic nerve marker), or anti-neuronal nitric oxide synthase (nNOS) antibody (parasympathetic nerve marker).

Results: In control parotid glands, scattered punctate or short linear patterns of PGP 9.5-positive sites were observed at week 0. After 2 weeks, PGP 9.5-positive sites, some of which were arranged in long linear patterns, increased in number. There were some TH-positive sites at week 0. After 2 weeks, there were increasing numbers of TH-positive sites, often in long linear patterns. At week 0, there were very few nNOS-positive sites, and nNOS immunoreactivity increased over time. After week 4, they demonstrated linear patterns. In the experimental parotid glands, there were fewer PGP 9.5- and nNOS-positive sites than in control parotid glands at each time point, although TH immunoreactivity was similar between two groups at each time point. In control submandibular glands, few punctate exhibited PGP 9.5-positive site were observed at week 0. At week 4, PGP 9.5 immunoreaction increased and showed linear patterns. TH-positive sites demonstrated punctate or short linear patterns at week 0, and thereafter TH immunoreactivity increased and were arranged in long linear patterns. There were few nNOS-positive sites at week 0, and they gradually increased after week 4. The immunoreactivities of all antibodies in the experimental submandibular glands were similar to those in the control at each time point.

Conclusions: Parasympathetic nerve growth in rat parotid glands was inhibited by liquid diet intake during the growth period, while liquid diet intake did not affect parasympathetic nerve growth nor sympathetic nerve growth in rat submandibular glands.

Key words: growth, liquid diet, nerve, salivary gland

INTRODUCTION

Salivary glands are exocrine glands that secrete saliva, which helps to maintain the oral environment. Salivary gland function is controlled through innervation by autonomic, sympathetic, and parasympathetic nerves [19]. Generally, sympathetic nerve

stimulation evokes protein-rich secretion, while parasympathetic nerve stimulation evokes large volumes of saliva [20]; however, there are some variations in these effects among salivary glands and species [21]. Because innervation is important for salivary gland function, the effects of nerve damage on salivary glands have been experimentally investigated. Salivary glands with excretory duct ligation including parasympathetic nerves weighed less and exhibited more parenchymal atrophy, compared with salivary glands that were subjected to excretory duct ligation excluding parasympathetic nerves [8, 9]. Furthermore, the weight of parasympathectomized salivary glands decreased [1, 2, 12, 22], and acinar cells became atrophic in parasympathectomized salivary glands [1, 22]. Although sympathectomy had an atrophic effect on salivary glands, it was much weaker than the atrophic effect of parasympathectomy [23, 24, 35]. Thus, autonomic nerves presumably play important roles in maintaining normal salivary gland structure; parasympathetic nerves may have a greater effect, compared with sympathetic nerves [6].

There is clinical interest in how the daily intake of soft foods, which is a characteristic of modern eating habits, affects the oral maxillofacial region. Therefore, many experimental studies have been conducted in which experimental animals were fed a liquid diet; the salivary glands of liquid diet-fed animals have also been examined. In these studies, biochemical analysis showed decreases in salivary amylase [7, 11, 14, 15, 27] and salivary protein [11] levels, as well as a decreased salivary flow rate [4, 10, 25]; these findings indicated reduced parotid gland function. Histological examinations revealed atrophic changes in parotid glands, such as reduction of gland weight [3, 4, 7, 11, 14-17, 25-27, 29], shrinkage of acinar cells [23, 25-27, 29], reduction of proliferative activity [29], and apoptotic death of acinar cells [5, 29]. However, many studies demonstrated that atrophic changes were minimal or absent in the submandibular glands of liquid diet-fed animals [3, 14, 16, 17, 26, 30].

There is a need to clarify the effects of successive intake of soft food during growth periods on salivary gland growth because children tend to prefer soft food, rather than hard food [37]. In a previous study, increases in parotid gland weight were smaller in rats that were continuously fed a liquid diet immediately after weaning, compared with rats that were fed a pellet diet [28]. In the parotid glands of rats that were continuously fed a liquid diet, acinar cells did not grow larger and proliferative activity was reduced during the growth period, demonstrating that parotid gland growth was strongly inhibited by liquid diet intake during the growth period [28]. In contrast, submandibular gland growth was not inhibited by liquid diet intake during the growth period [31]. These results indicated that the parotid and submandibular glands differed in their response to continuous intake of a liquid diet during the growth period. Because autonomic nerves are closely involved in the maintenance of salivary gland structure, as described above, it was speculated that salivary glands, particularly parotid glands, would be negatively affected in animals that were fed a liquid diet during the growth period.

The purpose of this study was to determine how liquid diet intake influenced nerve growth in salivary glands during the growth period. For this purpose, rats were fed a liquid diet beginning immediately after weaning; their parotid and submandibular glands were examined by immunohistochemical analysis with anti-protein gene product 9.5 (PGP 9.5) antibody (a marker of all nerves), anti-tyrosine hydroxylase (TH) antibody (a sympathetic nerve marker), and anti-neuronal nitric oxide synthase (nNOS) antibody (a parasympathetic nerve marker).

MATERIALS AND METHODS

Ethics and animal welfare

Animal experiments in this study were carried out in accordance with the Hokkaido University Guide for the Care and Use of Laboratory Animals; the

experimental protocol was approved by the Hokkaido University Laboratory Animal Committee (approval no. 14-0108). During the experiment, animals were housed in a temperature-controlled room (approximately 22°C) with a 12-h light/dark cycle and free access to drinking water; they were weighed and visually observed to confirm health status. Statistical comparisons in body weight between the control and experimental groups at each time point were made using Mann-Whitney *U* test (Ystat2008, Igakutosho, Tokyo, Japan). $P<0.05$ was considered significant.

Experimental procedures

Twenty-eight 12-day-old male Wistar rats and their mothers were obtained from CLEA Japan Inc. (Tokyo, Japan); the male rats were weaned at 21 days of age and divided into two groups. Each control rat was fed a pellet diet (25 g/day; Labo MR Standard, Nosan Corp., Yokohama, Japan) for 0, 2, 4, or 8 weeks (n=4 at each time point); each experimental rat was fed a liquid diet (prepared daily by mixing 25 g of the pellet diet in powder form with 50 mL of water) for 2, 4, or 8 weeks (n=4 at each time point). At the end of the experimental period, all animals were subjected to food deprivation for 12 h at night to synchronize the salivary gland status. They were then euthanized by isoflurane overdose; the parotid and submandibular glands were immediately removed. Extracted glands were embedded in Tissue Tek OCT compound (Miles Scientific, Naperville, IL, USA), frozen in liquid nitrogen, and stored at -80°C until preparation.

Immunohistochemical analysis

Fresh frozen sections were prepared using a cryostat and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 5 min. After the sections had been washed with phosphate-buffered saline (PBS), they were incubated with the following primary antibodies overnight at 4°C: anti-PGP 9.5 rabbit monoclonal

antibody (1:200; EPR4118, Abcam, Cambridge, UK) to detect all nerves, anti-TH rabbit polyclonal antibody (1:1000; AB152, Merck Millipore, Darmstadt, Germany) to detect sympathetic nerves, and anti-nNOS rabbit polyclonal antibody (1:100; Af480, Frontier Institute, Ishikari, Japan) to detect parasympathetic nerves. The sections were then incubated with biotinylated anti-rabbit goat polyclonal antibody (Histofine, Nichirei Bioscience, Tokyo, Japan) for 60 min at room temperature; subsequently, they were incubated with peroxidase-conjugated streptavidin (Histofine, Nichirei Bioscience) for 30 min at room temperature. Peroxidase activity was visualized using 3, 3'-diaminobenzidine; sections were lightly counterstained with Mayer's hematoxylin. After each incubation step above, sections were thoroughly washed with PBS.

Negative control sections were incubated in PBS without primary antibodies, then subjected to all other treatments as above.

RESULTS

Body weights of animals

Animals in both groups appeared healthy during the experimental period, and their body weights increased as normal. There was no significant difference in body weights between the control and experimental groups at every time point (Fig. 1).

Parotid glands

At week 0, thick nerve bundles in interlobular connective tissue exhibited PGP 9.5-positive sites. In glandular lobules, scattered punctate or short linear patterns of PGP 9.5-positive sites were observed (Fig. 2A). After 2 weeks of pellet diet intake, there were increasing numbers of PGP 9.5-positive sites around acini and ducts; some of these sites were arranged in long linear patterns (Fig. 2B). However, in the parotid glands of liquid diet-fed rats, there remained few PGP 9.5-positive sites at week 8 (Fig. 2C).

Furthermore, there were some TH-positive sites around acini in glandular lobules, as well as in thick nerve bundles in interlobular connective tissue, at week 0 (Fig. 2D). After 2 weeks of pellet diet intake, there were increasing numbers of TH-positive sites at the peripheries of acini and ducts, often in long linear patterns (Fig. 2E). TH immunoreactivity was similar between experimental and control groups at each time point (Fig. 2F).

At week 0, there were very few nNOS-positive sites in glandular lobules, most of which were punctate, although thick nerve bundles in interlobular connective tissues demonstrated extensive nNOS immunoreactivity (Fig. 2G). In control glands, nNOS immunoreactivity gradually increased. nNOS-positive sites were generally punctate at week 2, while they demonstrated linear patterns after week 4 (Fig. 2H). In experimental glands, nNOS immunoreactivity gradually increased. However, there were fewer nNOS-positive sites in experimental glands than in control glands at each time point; these sites mainly exhibited a punctate appearance (Fig. 2I).

Submandibular glands

At week 0, thick bundles of nerve fibers in interlobular connective tissue exhibited PGP 9.5-positive sites; few punctate exhibited PGP 9.5-positive sites were distributed around acini and ducts in glandular lobules (Fig. 3A). In control submandibular glands, PGP 9.5 immunoreactivity gradually increased around parenchymal tissue beginning at week 4; some PGP 9.5-positive sites demonstrated linear patterns (Fig. 3B). Immunoreactivity was similar between experimental and control groups at each time point (Fig. 3C).

Also at week 0, TH-positive sites were observed at the peripheries of acini and ducts, with a punctate or short linear appearance; they were also observed in thick nerve bundles in interlobular connective tissue (Fig. 3D). Thereafter, TH immunoreactivity gradually increased and TH-positive sites were present in long linear patterns (Fig. 3E).

TH immunoreactivity was similar between experimental and control groups at each time point (Fig. 3F).

Finally, some thick nerve bundles in interlobular connective tissue exhibited nNOS immunoreactivity at week 0, while few nNOS-positive sites were present in glandular lobules (Fig. 3G). At week 2, nNOS immunoreactivity remained limited overall, although scattered punctate nNOS-positive sites were observed around acini and ducts in control glands. After week 4, nNOS immunoreactivity in glandular lobules gradually increased; some nNOS-positive sites demonstrated linear patterns (Fig. 3H). nNOS immunoreactivity was similar between experimental and control groups at each time point (Fig. 3I).

Analysis of negative control sections for all primary antibodies revealed no reactions.

DISCUSSION

PGP 9.5 is a ubiquitin carboxyl-terminal hydrolase [36]. It was originally identified as a new brain-specific protein; it has since been used as a marker of all nerves [32]. TH is an enzyme involved in amino acid and neurotransmitter metabolism; it is the rate-limiting enzyme in the synthesis of catecholamines (e.g., dopamine and noradrenaline) from tyrosine [18]. Therefore, TH can be used as a marker of sympathetic nerves [34]. Nitric oxide is formed from its precursor, L-arginine, by a family of nitric oxide synthases. nNOS is one of three nitric oxide synthase isoforms [33] and has been used as a parasympathetic nerve marker in various tissues [13]. For these reasons, PGP 9.5, TH, and nNOS for immunohistochemical analyses of nerve distribution were used in this study. While comparisons can be performed between control and experimental glands that have been stained with the same antibody, it is difficult to compare glands that have been stained with different antibodies because the degree of immunoreactivity depends on each antibody’s sensitivity.

In this study, PGP 9.5 and nNOS immunoreactivities were lower in experimental parotid glands than in control parotid glands, suggesting that liquid diet intake during the growth period inhibits parasympathetic nerve growth in parotid glands. A previous study [28] showed that liquid diet intake in the growth period suppressed the proliferative activity of the parotid gland; it also suppressed increases in parotid gland weight and acinar cell size. Therefore, present results are consistent with the previous finding that liquid diet intake adversely affects parotid gland growth.

In contrast, nerve growth in submandibular glands was not affected by liquid diet intake during the growth period, as demonstrated by the absence of a difference between control and experimental submandibular glands in all immunohistochemical staining assays in this study. This is consistent with a previous report [31] that investigated gland weight, acinar cell size, and acinar cell proliferation in the submandibular glands of liquid diet-fed growing rats, confirming that submandibular gland growth is not affected by liquid diet intake. Thus, parotid glands are much more sensitive to liquid diet intake, compared with submandibular glands. This discrepancy might be related to differences in parasympathetic nerve system innervation of the two glands [30, 31]. The parasympathetic fibers of the parotid glands arise from the inferior salivary nucleus and accompany the glossopharyngeal nerve, while the parasympathetic fibers of the submandibular glands arise from the superior salivary nucleus and accompany the facial nerve [38]. The expression of choline acetyltransferase, which is necessary for acetylcholine synthesis, is downregulated in the parotid glands of liquid diet-fed rats [3], suggesting suppression of the parasympathetic nerve system that is needed to maintain parotid gland structure. Thus, the parotid glands may exhibit degeneration. In contrast, the parasympathetic nerve system of the submandibular glands is unaffected by liquid diet intake. Our finding of a difference in parasympathetic nerve growth between parotid and submandibular glands might support the above

theory that the different reactions to liquid diet intake are related to differences in parasympathetic nerve innervation between parotid and submandibular glands.

The previous study showed that liquid diet intake inhibited parotid gland growth by suppressing acinar cell enlargement and impeding acinar cell proliferation [28]; the present study showed that liquid diet intake inhibited parasympathetic nerve growth in parotid glands. The next question to address is whether the inhibition of parotid growth affects parasympathetic nerve growth in parotid glands, or whether the inhibition of parasympathetic nerve growth in parotid glands affects parotid growth. Although the present findings do not enable confirmation of either scenario, the inhibition of parasympathetic nerve growth might have a causative effect because parasympathetic nerves contribute to the maintenance of salivary gland morphology. Further studies are needed to investigate this sequence of events.

CONCLUSIONS

In conclusion, parasympathetic nerve growth in rat parotid glands was inhibited by liquid diet intake during the growth period, while liquid diet intake did not affect parasympathetic nerve growth nor sympathetic nerve growth in rat submandibular glands. Prudence is necessary to apply the conclusions obtained from rats to humans. However, they are considered to be a piece of evidence showing that the dietary habit requiring adequate mastication is important for healthy growth of the oral maxillofacial region. The conclusions of this study are significant in clinical dentistry to encourage patients to have the good dietary habit.

Acknowledgements

We thank Ryan Chastain-Gross, Ph.D., from Edanz (https://jp.edanz.com/ac) for editing a draft of this manuscript.

Conflict of interest: The authors declare no conflict of interest.

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Figure 1. Box plot of body weights of the control (Cont) and experimental rats (Exp). There was no significant difference between two groups at each time point. Explanation of box plot: top of box, 75th percentile; bottom of box, 25th percentile; horizontal bar within box, median; upper whisker, maximum value; lower whisker, minimum value.

Figure 2. Immunohistochemical analyses of parotid glands to determine distributions of PGP9.5 (A–C), TH (D–F), and nNOS (G–I). Normal parotid glands at week 0 (A, D, and G); control parotid glands at week 8 (B and H) and week 4 (E); experimental parotid glands at week 8 (C and I) and week 4 (F). Scale bars=30 μm. PGP9.5 (A) and nNOS (G) immunoreactivities are minimal at week 0. Although PGP9.5 and nNOS immunoreactivities both gradually increased in control glands (B and H), they remained minimal in experimental glands (C and I). TH immunoreactivity gradually increased in both control (E) and experimental glands (F), compared with parotid glands at week 0 (D).

Figure 3. Immunohistochemical analyses of submandibular glands to determine distributions of PGP9.5 (A–C), TH (D–F), and nNOS (G–I). Normal submandibular glands at week 0 (A, D, and G); control submandibular glands at week 4 (B and H) and week 2 (E); experimental submandibular glands at week 4 (C and I) and week 2 (F). Scale bars=30 μm. At week 0, all immunoreactivities were minimal (A, D, and G). Immunoreactivities were similar between experimental glands (B, E, and H) and control glands (C, F, and I).

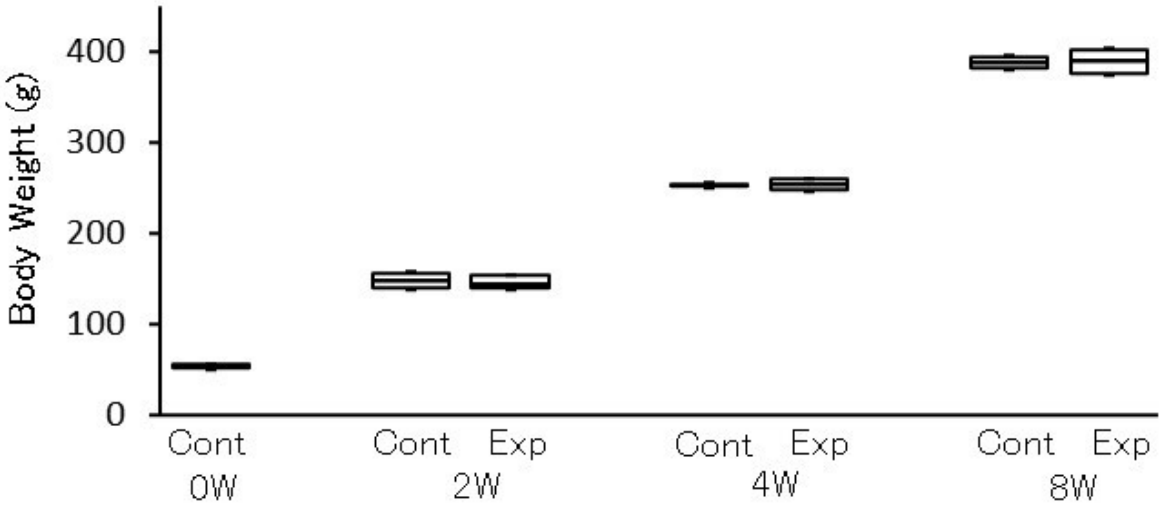
Body Weight (g)
400
300
200
100
0
Cont
0W
Cont
Exp
2W
Cont
Exp
4W
Cont
Exp
8W

Fig.1

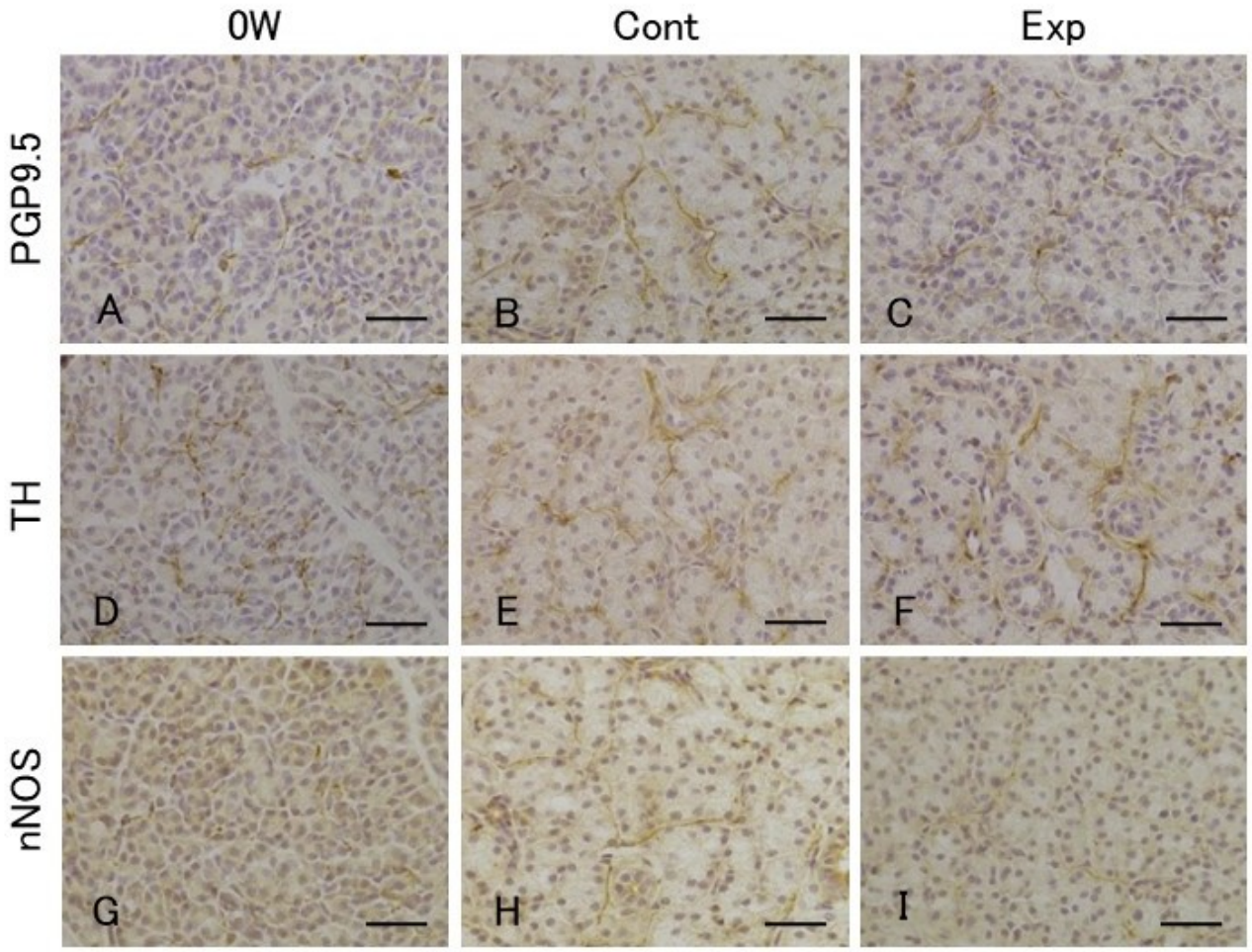

Fig.2

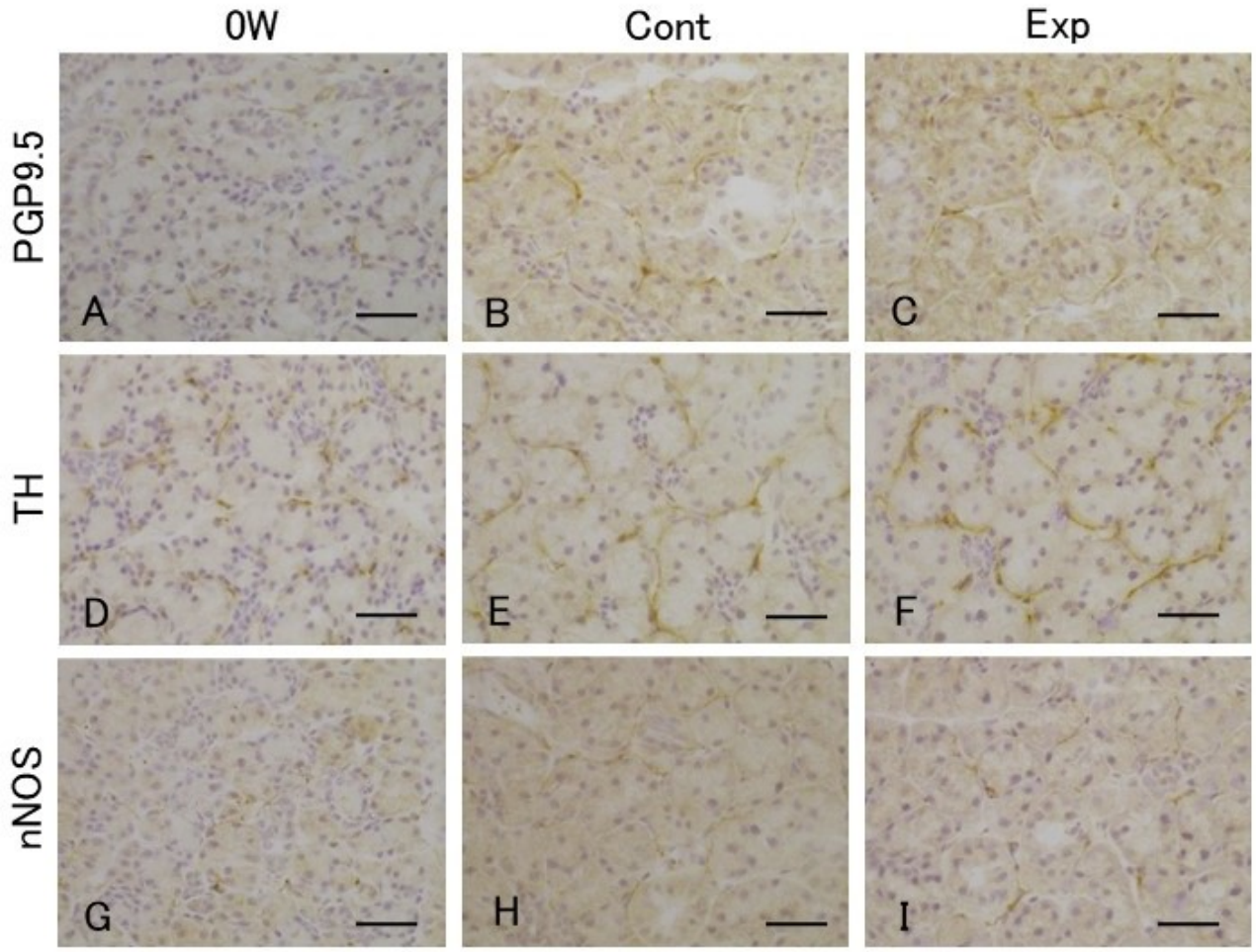

Fig.3