VIA MEDICA

ORIGINAL ARTICLE

Folia Morphol.
Vol. 83, No. 2, pp. 367–373
DOI: 10.5603/FM.a2023.0053

ISSN 0015–5659
eISSN 1644–3284
journals.viamedica.pl

Immunohistochemical investigation of nerve distribution in mature parotid and submandibular glands of rats with a liquid diet

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[Received: 20 June 2023; Accepted: 29 July 2023; Early publication date: 4 August 2023]

Background: *Although feeding with a liquid diet does not affect the growth of rat submandibular glands, it inhibits the growth of rat parotid glands during growth periods. In these growth-inhibited parotid glands, the growth of parasympathetic nerves is also suppressed. Meanwhile, the mature parotid glands of animals maintained on a liquid diet become morphologically and functionally atrophic, however, there is no effect of a liquid diet on mature submandibular glands. The objective of the present study was to clarify whether the nerve distribution in the mature salivary glands of rats was affected by a liquid diet.*

Materials and methods: *Seven-week-old male Wistar rats were used in this study. Half of the rats were kept on a pellet diet, and half were kept on a liquid diet, for 3, 7, 14, or 21 days. All rats were euthanised by isoflurane at each endpoint. Then, the parotid and submandibular glands were removed, frozen in liquid nitrogen, cryosectioned, and stained with antibodies against protein gene product 9.5 (PGP 9.5; general nerve marker), tyrosine hydroxylase (TH; sympathetic nerve marker), or neuronal nitric oxide synthase (nNOS; parasympathetic nerve marker).*

Results: *In parotid and submandibular glands of the pellet diet group, PGP 9.5- and TH-like immunoreactivity were seen around acini and ducts, and nNOS-like immunoreactivity was lower than PGP 9.5- and TH-like immunoreactivity. In the parotid glands of the liquid diet group, similar immunoreactivities were seen throughout the experimental period. The distribution of antibody labelling in the submandibular glands of the liquid diet group was similar to that of the pellet diet group and remained unchanged during the experimental period.*

Conclusions: *The present study demonstrated no regressive effects of a liquid diet on the distribution of sympathetic or parasympathetic nerves in mature parotid glands and submandibular glands. This differed from inhibitory effects on the growth of parotid glands seen during growth periods.* (Folia Morphol 2024; 83, 2: 367–373)

Keywords: salivary glands, sympathetic nerves, parasympathetic nerves, soft diet

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INTRODUCTION

The opportunity for children and adolescents to consume soft foods such as processed foods and fast food has markedly increased in recent decades [19, 20]. Soft food does not demand extensive mastication [18], and the influence of a continuous intake of soft food during growing periods on salivary glands [30] and other oral tissues [12, 13, 26] have attracted clinical and experimental attention. In growing rats receiving a liquid diet, parotid gland growth is suppressed. The parotid gland weight remains low due to the inhibition of increases in acinar cell size and low proliferative activity of acinar cells [30]. Furthermore, a recent study showed that a liquid diet inhibited the growth of parasympathetic nerves in the parotid gland [28]. On the contrary, gland weight, acinar cell size, acinar cell proliferation [33], and parasympathetic nerve growth [28] were not affected in the submandibular glands of growing rats kept on a liquid diet. Accordingly, there were differences in the responses to liquid diet feeding between parotid glands and submandibular glands in growing rats.

The effects of soft food on mature salivary glands have been studied more specifically. The mature parotid glands of rodents kept on a liquid diet became atrophic and their weights decreased [3, 4, 7, 11, 14–17, 24, 25, 27, 31]. A reduction in the levels of amylase [7, 11, 14, 15, 27] and proteins [11] in parotid saliva were biochemically identified. Physiological examination revealed that the flow rate of parotid saliva was reduced [4, 10, 24]. These results suggest that a liquid diet disturbs the function of mature parotid glands in experimental animals. In such atrophied parotid glands, reductions in acinar cell size were histologically identified [22, 24, 25, 27, 31]. In addition, immunohistochemical and ultrastructural investigations reported that acinar cell proliferation [31] was suppressed and acinar cell apoptosis increased [5, 31]. These results suggest that liquid diet also affects the structure of mature parotid glands in experimental animals. In mature submandibular glands of animals maintained on a liquid diet, there is no or little atrophic alterations as recognized in mature parotid glands [3, 14, 16, 17, 25, 32]. Thus, it could be considered that the responses of mature parotid and submandibular glands to a liquid diet are similar to those of these glands during growth periods. Therefore, we hypothesised that liquid diet feeding reduces parasympathetic nerve distribution in mature parotid glands but not in mature submandibular glands.

The objective of the present study was to elucidate whether the nerve distribution in mature salivary glands of rats was affected by a liquid diet. Mature parotid and submandibular glands of rats maintained on a liquid diet were immunohistochemically investigated using autonomic nerve markers.

MATERIALS AND METHODS

Ethics

All animal experiments in this study complied with the Hokkaido University Guide for the Care and Use of Laboratory Animals. The Hokkaido University Laboratory Animal Committee approved the experimental design in advance (Approval No. 09-0009). Animals were kept in rat cages in a humidity and temperature-controlled room (approximately 50% and 22°C) on a 12-h light/dark cycle and free access to drinking water.

Experimental protocol

The present study included 32 seven-week-old male Wistar rats (Japan Laboratory Animals, Inc., Tokyo, Japan). Half of the animals were maintained on a pellet diet (25 g/day; Labo MR Standard; Nosan Corp., Yokohama, Japan; pellet diet group), and half of the animals were maintained on a liquid diet made by mixing 25 g of the pellet diet in powdered form with 50 mL of water (liquid diet group). Animals in both groups were fed their respective diet for 3, 7, 14, or 21 days ($n = 4$ at every time point in each group). At the end of the experimental period, rats were fasted for 12h to synchronise the functional status of the salivary glands, weighed, and then euthanised with overdose of isoflurane. Then, parotid and submandibular glands were collected and embedded in Tissue Tek OCT compound (Miles Scientific, Naperville, IL, USA) and frozen in liquid nitrogen.

Significant differences in body weight between the groups at every tested time point were statistically analysed using Mann-Whitney *U* test (Ystat2008; Igakutosho, Tokyo, Japan), and P-values < 0.05 were considered statistically significant.

Immunohistochemistry

Fresh frozen sections were cut at a thickness of 10 μm with a cryostat and immersed in 4% paraformaldehyde buffered at pH 7.4 with 0.1 M phosphate buffer

Table 1. Primary antibodies

Antibody	PGP 9.5	TH	nNOS
Marker	General nerve	Sympathetic nerve	Parasympathetic nerve
Clonality	Monoclonal	Polyclonal	Polyclonal
Dilution	1:200	1:1000	1:100
Company	Abcam	Merck Millipore	Frontier Institute
City	Cambridge	Darmstadt	Ishikari
Country	UK	Germany	Japan

PGP 9.5 — protein gene product 9.5; TH — tyrosine hydroxylase; nNOS — neuronal nitric oxide synthase

for 5 min. This study used three primary antibodies (Table 1): anti-protein gene product 9.5 (PGP 9.5; rabbit) as a general nerve marker, anti-tyrosine hydroxylase (TH; rabbit) as a sympathetic nerve marker, and anti-neuronal nitric oxide synthase (nNOS; rabbit) as a parasympathetic nerve marker. Sections were immersed in primary antibodies overnight at 4°C. Then, samples were incubated in biotinylated goat anti-rabbit polyclonal secondary antibodies (Histofine; Nichirei Bioscience, Tokyo, Japan) for 1 h at room temperature and finally immersed in peroxidase-conjugated streptavidin (Histofine, Nichirei Bioscience) for 30 min at room temperature. Peroxidase activity was developed using 3, 3'-diaminobenzidine tetrahydrochloride, and the sections were lightly stained with Mayer's haematoxylin. The sections were washed with phosphate buffered saline (PBS) after each incubation step.

PBS was used instead of primary antibodies for negative control staining.

RESULTS

Body weight

Body weights of rats in the liquid diet group were not significantly different from those in the pellet diet group at any examined time points (Fig. 1).

Immunohistochemical observations of parotid glands

In the parotid glands of the pellet diet group, thick bundles of nerves in interlobular connective tissue showed strong PGP 9.5-like immunoreactivity. In the lobules, PGP 9.5-like immunoreactivity, much of which showed a linear pattern, was commonly identified around acini and ducts (Fig. 2A). In the parotid glands of the liquid diet group, PGP 9.5-like

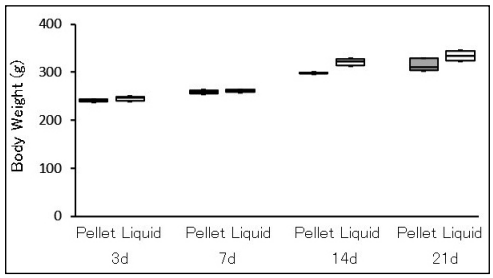

Figure 1. Box-and-whisker plots showing body weights of rats maintained on a pellet diet (Pellet) and a liquid diet (Liquid). No statistically significant differences were identified throughout the experimental period. Top of box, 75th percentile; bottom of box, 25th percentile; horizontal bar within box, median; upper whisker, maximum value; lower whisker, minimum value

immunoreactivity was very similar to that seen in the pellet diet group on Day 3 (Fig. 2B). Although atrophy of the parotid glands progressed over time, PGP 9.5-like immunoreactivity remained unchanged throughout the experimental period (Fig. 2C).

The distribution of TH-like immunoreactivity was similar to that of PGP 9.5 in both the pellet diet group (Fig. 2D) and the liquid diet group at each time point (Fig. 2E, F).

In the pellet diet group, there was less nNOS-like immunoreactivity than PGP 9.5- and TH-like immunoreactivity. The thick nerve bundles in interlobular connective tissues showed nNOS-like immunoreactivity, while some positive nNOS-like puncta were scattered among acini and ducts in the lobules (Fig. 2G). In the liquid diet group, similar nNOS-like immunoreactivity was observed on Day 3 (Fig. 2H), and this immunoreactivity was consistent throughout the liquid diet feeding period (Fig. 2I).

Immunohistochemical observations of submandibular glands

There were PGP 9.5-positive thick nerve bundles in the interlobular connective tissue in the submandibular glands of the pellet diet group. In the glandular lobules, PGP 9.5-like immunoreactivity was identified around acini and ducts, often taking a linear shape and sometimes a dot shape (Fig. 3A). Similar immunoreactivity to PGP 9.5 was found in the submandibular glands of rats in the liquid diet group at every tested time point (Fig. 3B, C).

TH-like immunoreactivity was similar to that of PGP 9.5 in both the pellet diet group (Fig. 3D) and the liquid diet group throughout the experimental period (Fig. 3E, F).

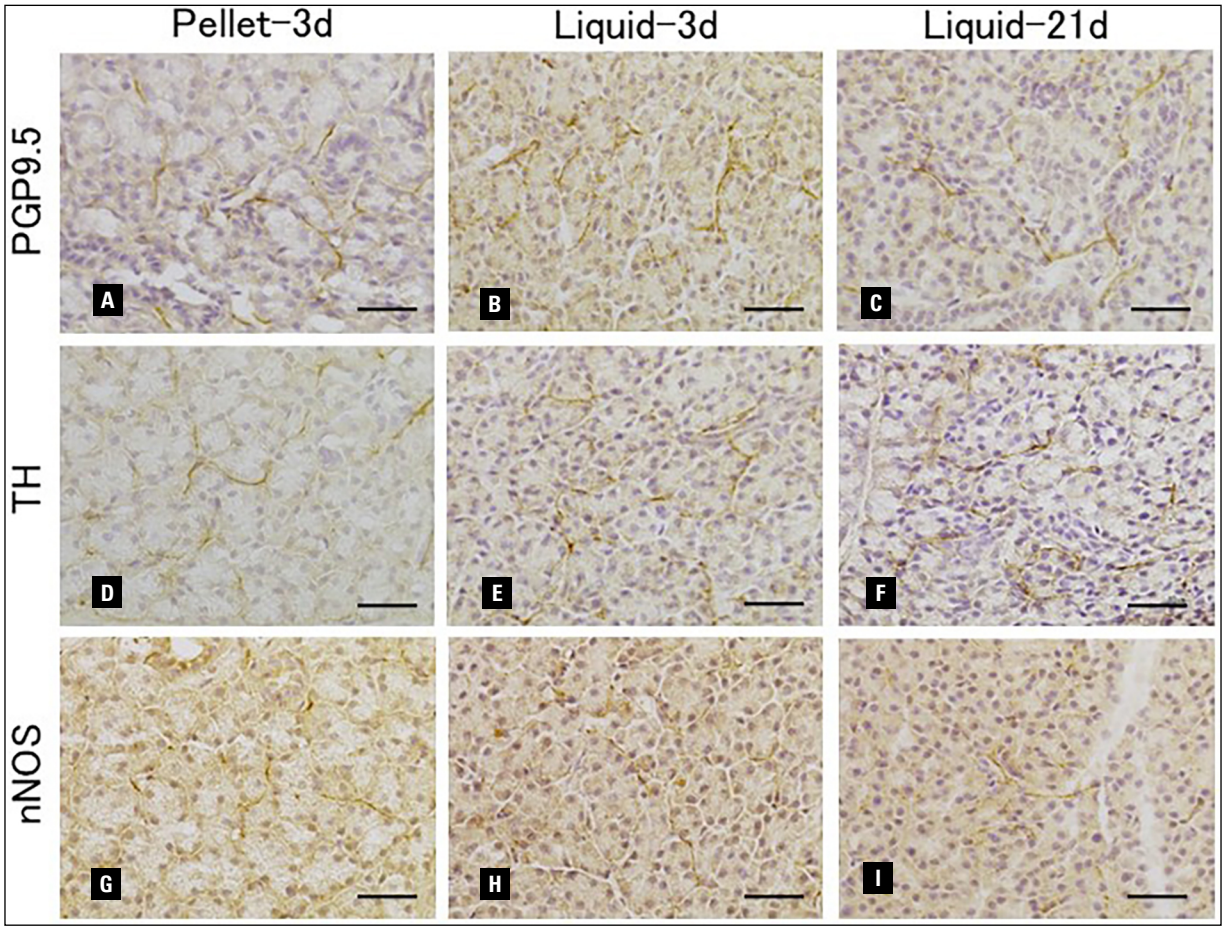

Figure 2. Parotid glands. **A–C.** PGP 9.5; **D–F.** TH; **G–I.** nNOS. **A, D, G.** Pellet diet group on Day 3; **B, E, H.** Liquid diet group on Day 3; **C, F, I.** Liquid diet group on Day 21. Scale bars = 30 μm. There was no difference in PGP 9.5-, TH-, and nNOS-like immunoreactivity between parotid glands of the pellet diet group (A, D, G) and liquid diet group (B, C, E, F, H, I) at all time points.

The distribution of nNOS-like immunoreactivity was similar in the submandibular glands and the parotid glands of the pellet diet group (Fig. 3G). There was no difference in the distribution of nNOS--like immunoreactivity in the submandibular glands between both groups at each time point (Fig. 3H, I).

There was no immunoreactivity in all negative control sections.

DISCUSSION

In studies investigating the influence of a liquid diet on oral tissues, it is important that there are no differences in the body weights of animals between pellet and liquid diet groups. If the body weights of animals had differed between the pellet diet group and the liquid diet group, it would have become impossible to judge whether tissue alterations were caused directly by intake of liquid diet or by deterioration of general conditions. In previous studies, liquid diet was prepared by mixing one part of a powdered form of the pellet diet with two parts of water, and no differences in rat body weight were identified between liquid- and pellet-fed groups [12, 28–33]. Therefore, this liquid diet was prepared and used in the present study.

In this study, no immunohistochemical alterations were seen in nerve distribution in mature parotid and submandibular glands in rats maintained on a liquid diet was immunohistochemically identified. As mature submandibular glands do not show atrophic changes histologically [3, 14, 16, 17, 25, 32], this result was as expected. Meanwhile, this result contradicted the hypothesis that a liquid diet induces regressive alterations of parasympathetic nerves in mature rat parotid glands, which did show atrophic alterations histologically. Schultz et al. [23] reported that parasympathetic denervation via bisection of the auriculotemporal nerve reduced the number of neuropeptide Y-immunoreactive fibres around the secretory acini in the parotid glands remarkably.

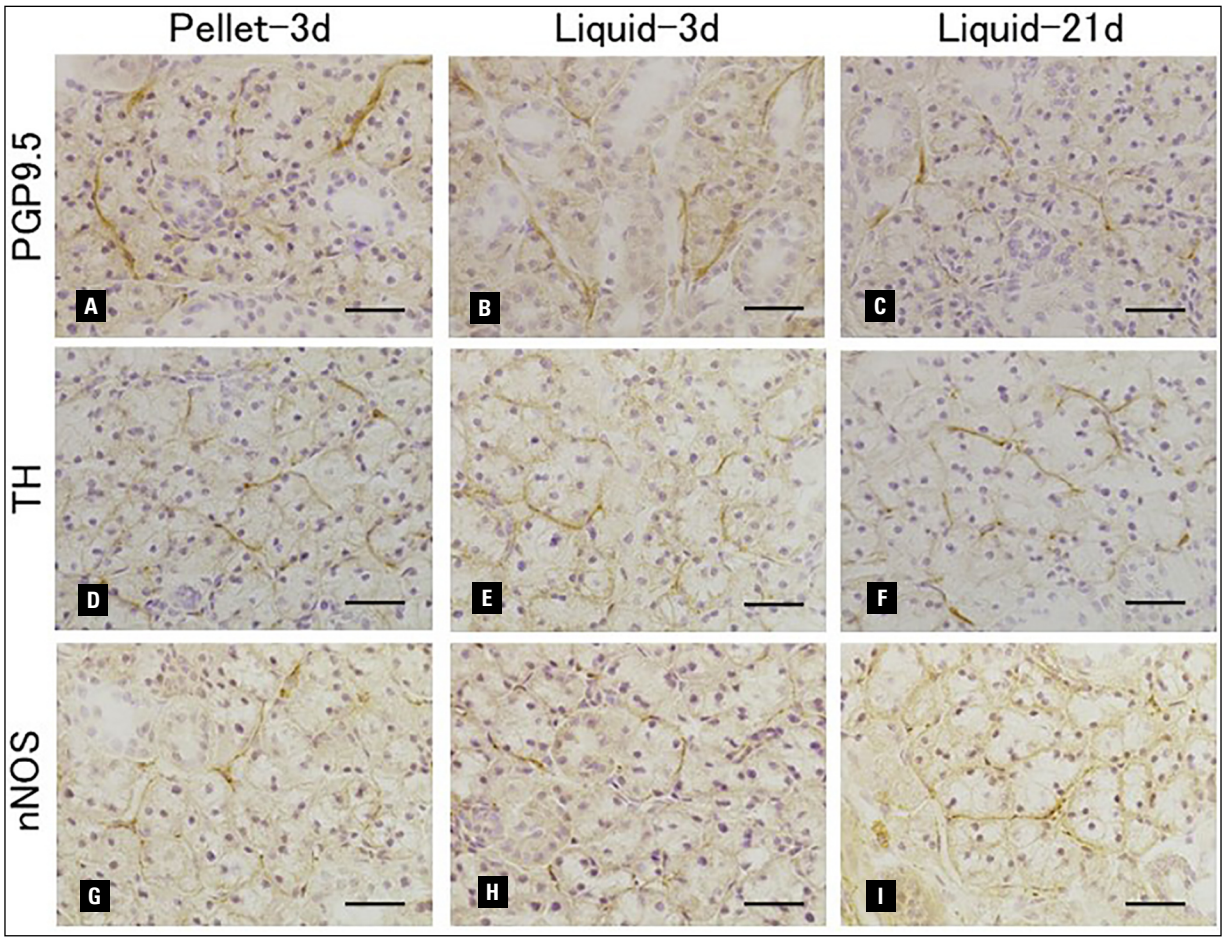

Figure 3. Submandibular glands. **A–C**. PGP 9.5; **D–F.** TH; **G–I.** nNOS. **A, D, G.** Pellet diet group on Day 3; **B, E, H.** Liquid diet group on Day 3; **C, F, I.** Liquid diet group on Day 21. Scale bars = 30 μm. The distribution of PGP 9.5-, TH-, and nNOS-like immunoreactivity in submandibular glands of the liquid diet group at every time point (B, C, E, F, H, and I) were very similar to those of the pellet diet group (A, D, and G).

Alm et al. [1] also showed almost total disappearance of NOS-immunoreactivity after parasympathetic denervation. Considering these results, severe manipulations such as complete denervation might be necessary to induce regressive alterations in parasympathetic nerves in mature salivary glands. The effects of a liquid diet might be too weak to induce of regressive changes in parotid gland parasympathetic nerves, while they might be enough to block further parasympathetic nerve development. Because no regressive alterations in parasympathetic nerves were identified in the parotid glands of growing rats kept on a liquid diet, but the initial distribution of parasympathetic nerves was maintained [30], as in the present study.

Ligation of excretory duct causes salivary gland to atrophy and acinar cells in glandular tissue to decrease in number [8, 9]. Salivary glands become more atrophic after the ligation of both excretory ducts and nerves [8, 9]. Furthermore, salivary gland atrophy, in which acinar cells remarkably decrease or disappear, can be induced by bisection of its nerves, especially parasympathetic nerves [2, 21]. Thus, autonomic nerves, especially parasympathetic nerves, play important roles for the maintenance of salivary glands [6]. Although liquid diet intake induces atrophy in parotid glands with a decrease in size and the number of acinar cells [22, 24, 25, 27, 31], the distribution of parasympathetic nerves was maintained in such atrophic parotid glands in the present study. Taken together, atrophic changes in acinar cells in the parotid glands of liquid-fed rats may be caused by functional rather than morphological changes in parasympathetic nerves.

Although the present study showed that no influence of liquid diet intake on nerve distribution in mature parotid and submandibular glands, good eating habits involving adequate mastication are important

for the maintenance of healthy salivary glands. Dentistry researchers and clinicians should inform people of the importance of adequate mastication.

CONCLUSIONS

This study demonstrated that the distribution of sympathetic and parasympathetic nerves in mature parotid and submandibular glands in rats were not changed after feeding a liquid diet, suggesting that nerve tissue in mature salivary glands was not negatively affected. This differed from suppressive effects seen by a liquid diet in the growth of parotid glands during animal growth periods.

ARTICLE INFORMATION AND DECLARATIONS

Acknowledgments

We thank Alexander S. Chockley from Edanz (https://jp.edanz.com/ac) for editing a draft of this manuscript.

Conflict of interest: None declared.

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