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**Title:** Growth of rat parotid glands is inhibited by liquid diet feeding

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2nd REVISED VERSION

## **ABSTRACT**

This study investigated how liquid diet feeding affects the growth of parotid glands. We weaned 21-day-old rats and thereafter fed them a pellet diet (control group) or a liquid diet (experimental group) for 0, 1, 2, 4, or 8 weeks. Their parotid glands were excised, weighed, examined, and tested for 5-bromo-2'-deoxyuridine (BrdU) and cleaved caspase-3 (Casp-3) as markers of proliferation and apoptosis, respectively. Parotid gland weights were consistently smaller in experimental animals than in controls. Morphometrical analysis showed that control group acinar cells increased in area during the experiment, but experimental group acinar cells were almost unchanged. Labeling indices of BrdU in acinar cells in both groups declined during the experiment, but were consistently lower in the experimental group than in controls. Casp-3-positive acinar cells were rare in both groups, which consistently express significantly similar Casp-3 levels. Ultrastructurally, terminal portions of the experimental parotid glands consisted of a few acinar cells that were smaller than those in controls. Control acinar cells showed mitotic figures within short experimental periods, but not in experimental glands. These observations indicate that liquid diet feeding inhibits growth of parotid glands in growing rats through suppression of growth and proliferation of individual acinar cells, but not through apoptosis.

## **1. Introduction**

Modern diets often feature relatively soft foods that do not demand extensive mastication (Maki et al., 2001; Suzuki et al., 2005; Murakami et al., 2007). As children in particular tend to prefer soft foods to hard foods (Yamanaka et al., 2009; Ohira et al., 2012), the effect of this dietary habit on functional and structural growth of the oral maxillofacial regions is a clinical concern (Tsuchiya et al., 2014). Many investigations with animal models have addressed this matter, and have shown that the maxillary and mandibular bones (Watt and Williams, 1951; Ito et al., 1988), masticatory muscles (Kiliaridis et al., 1988; Mieke et al., 1999), and temporomandibular joint (Kiliaridis et al., 1999; Kato et al., 2015) can be underdeveloped in animals that are fed soft diets.

Parotid glands are the largest salivary glands, and are important in maintaining the health of oral cavity. Clarifying the influence of soft foods on the parotid glands is therefore clinically significant. Earlier investigations of the effects of soft diet on parotid glands commonly used mature animal models, in which mature parotid glands become macroscopically atrophic by short-term liquid diet feeding (Hall and Schneyer, 1964; Johnson, 1982; Scott and Gunn, 1991; Kurahashi and Inomata, 1999; Leal et al., 2003; Takahashi et al., 2012). This atrophy has been interpreted as a result of shrinking of acinar cells despite no change in duct cells (Hall and Schneyer, 1964; Wilborn and Schneyer, 1970; Hand and Ho, 1981; Scott et al., 1990; Scott and Gunn, 1991; Takahashi et al., 2012). A recent report of reduced acinar cell proliferation and increased acinar cell apoptosis implied that liquid diet-induced parotid gland atrophy is caused by smaller and fewer acinar cells (Takahashi et

al., 2012), but this result was found in mature parotid glands. The influence of soft foods on parotid gland growth has received little attention.

The purpose of the present study was to clarify whether a liquid diet affected the growth of the parotid glands and, if so, whether acinar cells were changed as observed in mature parotid glands. For this purpose, growing rats were fed a liquid diet, and their parotid glands were examined using histological analysis, immunohistochemistry for 5-bromo-2'-deoxyuridine (BrdU) and cleaved caspase 3 (Casp-3), and transmission electron microscopy.

## **2. Materials and Methods**

### *2.1. Experimental procedures*

We purchased 63 male Wistar rats, aged 12 days, with their mothers from CLEA Japan Inc. (Tokyo, Japan). On day 21 after birth, the infant rats were weaned and divided into control (n=35) and experimental groups (n=28). Control rats were fed a pellet diet and experimental ones were fed a liquid diet for 0 (control group only), 1, 2, 4, or 8 weeks, as described previously (Takahashi et al. 2012, 2014). All food was removed for a 12 h-fasting period before each perfusion. BrdU (Sigma-Aldrich, St. Louis, MO) was administered at 25 mg/kg (body weight) to rats for histological and immunohistochemical examination (n=5 for control and experimental groups at each tested point), and they were perfused with 4% buffered paraformaldehyde at 1 h after the BrdU-injections. For ultrastructural examination, rats (n=2 for the control and experimental groups at each tested point) were perfused with 2% buffered paraformaldehyde-1.25% glutaraldehyde. After perfusion, the parotid glands were excised and immersed in each fixative.

All animal experimentation in this study was approved by the Laboratory Animal Committee of Hokkaido University (Approval No. 10-0126), and complied with the Guide for the Care and Use of Laboratory Animals of Hokkaido University.

### *2.2. Histological examination*

Fixed parotid glands were weighed and routinely embedded in paraffin. They were then cut into 4- $\mu$ m sections, stained with hematoxylin and eosin (HE), and observed with a

light microscope.

For histomorphometric analysis of acinar cells, 3 HE-stained sections from each animal (n=5) were used. In each section, an area was measured of approximately 500 acinar cells in randomly chosen fields, using the image-analysis system (DS-L2, Nikon, Tokyo, Japan). The mean of the data from 3 sections was taken as representative of that animal.

### *2.3. Immunohistochemical examination*

Deparaffinized sections were immersed for 10 min in methanol with 0.3% hydrogen peroxide to block endogenous peroxidase. Sections for BrdU were pretreated with 0.1% trypsin for 20 min at 37°C, then 3N HCl for 10 min at 37°C, incubated with anti-BrdU mouse monoclonal antibody (Bu-20a, DakoCytomation, Glostrup, Denmark, 1:50 dilution) for 2 h, and finally treated with anti-mouse rabbit polyclonal antibody (DakoCytomation, 1:100 dilution) for 1 h at room temperature. Sections for Casp-3 were boiled in 10 mM tris/1 mM EDTA buffer (pH 8.8) for 15 min, incubated with anti-cleaved caspase 3 rabbit polyclonal antibody (Asp 175, Biocare Medical, Concord, CA, 1:50 dilution) overnight at 4°C, and treated with anti-rabbit swine polyclonal antibody (DakoCytomation, 1:100 dilution) for 1 h at room temperature. After reacting to the secondary antibody, both types of immunohistochemistry sections were incubated with streptavidin-biotin horseradish peroxidase complex (DakoCytomation) for 30 min, placed in 3,3'-diaminobenzidine tetrahydrochloride for visualization, and lightly counterstained with Mayer's hematoxylin in turn. Normal mouse or rabbit serum was substituted for the primary antibodies in the negative

controls.

Immuno-positive acinar cells were counted per approximately 1000 acinar cells in randomly selected fields, and calculated as percentages. The average of percentages of 3 immunostained sections from each animal (n=5) was used as the labeling index for that animal.

#### *2.4. Statistical analysis*

The data were expressed as medians and ranges for 5 control animals and for 5 experimental ones at each tested time for parotid gland weights, acinar cell areas, and the labeling indices for BrdU and Casp-3; and were analyzed statistically using the Mann-Whitney *U*-test (Ystat2008, Igakutosho, Tokyo, Japan).  $P < 0.05$  was considered significant.

#### *2.5. Ultrastructural examination*

Tissues for ultrastructural examination were placed in 1% osmium tetroxide, stained *en bloc* with 4% uranyl acetate, and routinely embedded in Epon 812. Ultrathin sections were made with an ultramicrotome, doubly stained with 4% uranyl acetate and lead citrate, and observed with a transmission electron microscope (JEM-1400, JOEL, Akishima, Japan).



### **3. Results**

#### *3.1. Weights of bodies and parotid glands*

Body weights of the control and experimental rats increased throughout the experimental period, and did not significantly differ between the two groups at any tested point (Fig. 1). Weights of parotid glands of both groups also increased, but were significantly greater in the control group at all tested points (Fig. 2).

#### *3.2. Histological observations*

Histological structures of parotid glands such as acini and ducts were normal at week 0 (3 week-old rats), with small acinar cells (Fig. 3A) that grew larger over time in the controls (Fig. 3B). However, in the experimental group, parotid acinar cells were still small at 8 weeks (Fig. 3C). Histomorphometric analysis shows that the area of parotid acinar cells in experimental rats was significantly smaller than those of controls at all tested points (Fig. 4).

#### *3.3. Immunohistochemical observations*

The labeling index of BrdU of acinar cells was the highest at week 0 (Fig.3D), and thereafter declined in both groups (Fig.3E, F), but was significantly higher in the control group at 1, 2, 4, and 8 weeks due to its sharp decrease in the experimental group (Fig. 5). Casp-3-positive acinar cells were very rarely identified in all animals (Fig. 3G, H, I). Labeling indices of Casp-3 in acinar cells were low in both groups, and did not significantly differ at all tested points (Fig. 6).

Negative control sections for BrdU and Casp-3 showed no reaction.

### *3.4. Ultrastructural observations*

Experimental acinar cells were ultrastructurally different from those in the control glands, especially after 4 weeks. Terminal portions of control glands were large with many acinar cells, and their acinar cells were larger, contained many electron-dense secretory granules, and had developed rough endoplasmic reticulum at their basal cytoplasm (Fig. 7A). Experimental glands had smaller terminal portions with fewer acinar cells, and their acinar cells had fewer secretory granules (Fig. 7B). Control acinar cells showed mitotic figures by 1 week, but the experimental acinar cells did not (Fig. 7C). Apoptosis of acinar cells was very rare in both groups (Fig. 7D).

#### **4. Discussion**

This is the first study to show that the growth of parotid glands in growing rats is inhibited by liquid diet feeding. This did not reflect altered general health, because body weights of rats fed a liquid diet did not differ significantly from those of rats on a pellet diet. In underdeveloped parotid glands, acinar cell proliferative activity decreased, which suggests that the number of acinar cells were insufficient for normal growth, and therefore, suppression of acinar cell growth in both number and size (as observed histologically) inhibited parotid gland growth. These findings may have clinical implications for the diets of children, who tend to prefer soft foods (Yamanaka et al., 2009; Ohira et al., 2012). Parasympathetic nerve stimulation is important for salivary glands, because resection (Poat and Templeton, 1982; Carpenter et al., 2012) and ligation (Harrison and Garrett, 1976; Harrison et al., 2001) of parasympathetic nerve induce regressive changes in salivary glands. In the liquid-fed rats, the parotid gland acetylcholine level an index of parasympathetic nerve stimulation decreases (Nakamura, 1997). Therefore, the unfavorable effects on parotid gland growth in the present study might be caused by decreased parasympathetic nerve stimulation due to a lack of mastication.

Comparison of the effects of this regimen on growing parotid glands with those on mature glands is interesting. Common findings at both stages are small acinar cells (Hall and Schneyer, 1964; Wilborn and Schneyer, 1970; Hand and Ho, 1981; Scott et al., 1990; Scott and Gunn, 1991; Takahashi et al., 2012) and low proliferative activity of acinar cells (Takahashi et al., 2012). However their apoptosis rates differ; acinar cell apoptosis hardly

occurs in the growing parotid glands, but is common in mature ones (Takahashi et al., 2012).

This suggests that acinar cell apoptosis has an important function in shrinkage of parotid glands. This possibility is supported by the fact that acinar cell apoptosis is observed under other conditions, such as duct ligation (Walker and Gobe, 1987; Scott et al., 1999) and withdrawal of isoprenaline (Chisholm et al., 1995), which reduce the size of parotid glands.

As similar phenomena were also reported in involution of the lactating mammary glands after weaning (Walker et al., 1989) and duct-ligated pancreas (Walker, 1987; Abe and Watanabe, 1995), acinar cell apoptosis would be necessary in reducing the size of exocrine glands, including parotid glands. In the present study, why acinar cell apoptosis hardly occurs in the growing parotid glands of the liquid-fed rats is unclear. However, considering that the growing and mature parotid glands have similar reactions to liquid diets, such as smaller and less proliferative acinar cells (Takahashi et al., 2012) but differ in acinar cell apoptosis, some anti-apoptotic factors may be influential during the growing period.

In conclusion, we have shown that a liquid diet inhibits growth of parotid glands in growing rats by suppressing both individual cell growth and proliferation of acinar cells, but does not delete acinar cells through apoptosis.

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## Figure legends

**Fig. 1.** Box plot showing body weights of control rats fed a pellet diet (white box plot, n=5) and experimental rats fed a liquid diet (gray box plot, n=5). The two groups did not significantly differ in weight at any tested 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.

**Fig. 2.** Box plot showing parotid gland weights of control (white box plot, n=5) and experimental rats (gray box plot, n=5). The significant differences are identified between two groups at 1, 2, 4, and 8 weeks. \* $P < 0.05$ . Explanation of box plot is the same as in Fig. 1.

**Fig. 3.** Histology and immunohistochemistry. Panels A-C show hematoxylin-eosin staining; D-F, BrdU labeling; and G-I, Casp-3 labeling. Control samples are shown at 0 weeks (A, D, G); 1 week (E) and 8 weeks (B, H). Experimental samples are shown at 1 week (F) and 8 weeks (C, I). Bars=30 $\mu$ m. At the start of experiment, acinar cells are small in size (A), but by 8 weeks are larger in controls (B) than in experimental gland (C). BrdU-positive acinar cells are common in control samples (D, E), but are relatively few in experimental glands (F). Casp-3-positive acinar cells are rare in both groups (G-I).

**Fig. 4.** Box plot showing areas of individual acinar cells in parotid glands of control (white box plot, n=5) and experimental rats (gray box plot, n=5). Acinar cells in parotid glands of

rats fed a liquid diet are smaller than that in those fed a solid diet at each interval. \* $P < 0.05$ .

Explanation of box plot is the same as in Fig.1.

**Fig. 5.** Box plot showing BrdU labeling indices of acinar cells in parotid glands of control (white box plot,  $n=5$ ) and experimental rats (gray box plot,  $n=5$ ), which were lower in the experimental group than in the control group at weeks 1, 2, 4, and 8. \* $P < 0.05$ . Explanation of box plot is the same as in Fig.1.

**Fig. 6.** Box plot showing Casp-3 labeling indices of acinar cells in parotid glands of control (white box plot,  $n=5$ ) and experimental rats (gray box plot,  $n=5$ ). The two groups did not significantly differ at any tested point. Explanation of box plot is the same as in Fig.1.

**Fig. 7.** Ultrastructure. (A) Control glands at 8 weeks. The terminal portion (TP) consists of many acinar cells with many secretory granules. Scale bar=10  $\mu\text{m}$ . (B) Experimental glands at 8 weeks. Acinar cells are few and small in the terminal portion (TP). Scale bar=10  $\mu\text{m}$ . (C) Mitotic figure of an acinar cell (AC) from control group at 1 week. Scale bar=5 $\mu\text{m}$ . (D) Apoptosis of an acinar cell (AC) from control group at 1 week. Scale bar=5 $\mu\text{m}$ .















