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**Title:** Recovery of atrophic parotid glands in rats fed a liquid diet by switching to a pellet diet

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**Running Title:** Recovery of atrophic parotid glands

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**Abbreviations:** HE, hematoxylin and eosin; PAS, periodic acid Schiff; BrdU, 5-bromo-2'-deoxyuridine; Casp-3, cleaved caspase-3

## **ABSTRACT**

*Objective:* In this study, we aimed to clarify how parotid glands, made atrophic by a liquid diet, recover after diet change.

*Design:* Seven-week-old male Wistar rats were fed a pellet (control group) or a liquid diet (experimental group) for the first 14 days. Thereafter, all animals were fed a pellet diet for up to 14 days (days 0-14). The parotid glands were removed, weighed and examined histologically and ultrastructurally. Immunohistochemistry was performed for BrdU, a marker of proliferating cells, and Casp-3, a marker of apoptotic cells.

*Results:* Feeding of a liquid diet for 14 days induced atrophy of the parotid glands.

Histologically, acinar cells were small on day 0, compared with the control group. After changing the diet from liquid to pellet form, acinar cells increased in size over time, recovering nearly fully by day 7. Many BrdU-positive acinar cells were observed in the glands in the experimental group on days 1 and 3. Although more acinar cells were Casp-3-positive compared with the control group on day 0, there was no difference between the two groups after the diet change. Ultrastructurally, the cellular organelles did not exhibit a substantial alteration, except for an increase in secretory granules following diet change.

*Conclusions:* Our findings suggest that atrophic parotid glands are able to recover to their normal size by switching the diet from liquid to pellet form and that an increase in both the size and number of acinar cells plays an important role in this recovery process.

**Keywords:** parotid gland, recovery, liquid diet, atrophy

## 1. Introduction

Many experimental studies have shown that continuous intake of soft food, which is currently a popular dietary trend, negatively influences oral maxillofacial regions such as the jaw bones (Shimizu et al., 2013; Hichijo et al., 2014; Bozzini, Picasso, Champin, Bozzini, & Alippi, 2015), masticatory muscles (Kitagawa et al., 2004; Kawai et al., 2010; Fujishita et al., 2015) and temporomandibular joints (Kiliaridis, Thilander, Kjellberg, Topouzelis, & Zafiriadis, 1999; Kato, Takahashi, & Domon, 2015; Uekita, Takahashi, Domon, & Yamaguchi, 2015). The effect of a liquid diet on salivary glands has been investigated in animals in several reports. These studies have revealed differences in the response to a liquid diet among the three major salivary glands. While a liquid diet induces strong atrophy of the parotid glands (Hall & Schneyer, 1964; Wilborn & Schneyer, 1970; Scott & Gunn, 1991; Nakamura, 1997; Kurahashi & Inomata, 1999; Kurahashi, 2002; Takahashi et al., 2012; Takahashi, Uekita, Kato, Inoue, & Domon, 2015), its effect on the submandibular and sublingual glands is negligible or very limited (Scott & Gunn, 1991; Nakamura, 1997; Kurahashi & Inomata, 1999; Kurahashi, 2002; Takahashi et al., 2014; Takahashi, Uekita, Taniwaki, & Domon, 2017). It has been reported that acinar cell shrinkage (Hall & Schneyer, 1964; Wilborn & Schneyer, 1970; Hand & Ho, 1981; Scott, Berry, Gunn, & Woods, 1990; Scott & Gunn, 1991; Takahashi et al., 2012), reduction of proliferative activity of acinar cells (Takahashi et al., 2012) and an increase in apoptotic acinar cells (Takahashi et al., 2012; ElGhamrawy, 2015) play important roles in parotid gland atrophy.

As salivary glands are important for the health of the oral cavity, an important question from the standpoint of clinical dentistry is whether atrophic parotid glands are able to recover following a change from a liquid to a pellet diet. However, there are only a few reports addressing this question (Hall & Schneyer, 1964; Schneyer & Hall, 1975; Nakamura 1997). Of these, Hall & Schneyer (1964) and Nakamura (1997) found that the atrophic parotid glands recovered their weight after diet change. Hall & Schneyer (1964) and Schneyer & Hall (1975) counted acinar cells per a field under a light microscope and found that the number of acinar cells per a field decreased after changing the diet from liquid to pellet form, suggesting that acinar cells become larger following diet change. They also counted the mitotic figures of acinar cells and reported that they were most numerous on day 2 after diet change (Schneyer & Hall, 1975). These observations suggest that acinar cells recover their size and increase in number after diet change.

The methods used in the above studies were appropriate in those days, and their observations were valuable. However, there seems to be some problems requiring additional examinations from the point of view of the present studies. In these older studies, the area of the individual acinar cells was not measured, and it is difficult to exclude completely the area of other parenchymal cells and stromal components from the observed field. Therefore, the measurement of the individual area of the acinar cells with an image analysis system is necessary to confirm the recovery of acinar cells in size. In addition, mitotic figures are difficult to quantify because the proliferative stage (M-phase) is very short (Yu, Woods, & Levison, 1992). Currently, immunohistochemical methods that identify the cycling cells in

other phases of the cell cycle are generally used to assess proliferation (Hall & Woods, 1990; Yu et al., 1992; Liu & Klein-Szanto, 2000).

It has been reported that apoptosis takes place in progressive (Fan, Kren, & Steer, 1998; Taira, Hiroyasu, Shiraishi, Muto, & Koji, 2001; Takahashi, Nakamura, Domon, Yamamoto, & Wakita, 2005) as well as in regressive (Walker, 1987; Walker, Bennett, & Kerr, 1989; Takahashi et al., 2000) changes of some exocrine glands. Because apoptosis is an important factor in the regulation of cell populations (Kerr, Wyllie, & Currie, 1972), it is desirable to examine apoptosis in acinar cells in atrophic parotid glands undergoing recovery after diet change.

In the present study, we aimed to clarify how atrophic parotid glands in rats fed a liquid diet by switching to a pellet diet recovered. For this purpose, those parotid glands were examined using histological analysis, histomorphometric analysis with an image-analysis system, immunohistochemical analysis with BrdU, a marker of proliferating cells, and Casp-3, a marker of apoptotic cells, and ultrastructural analysis with a transmission electron microscope.

## **2. Materials and methods**

### *2.1. Animals*

This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals of Hokkaido University and was approved by the Laboratory Animal Committee of Hokkaido University (Approval No.13-0206).

Forty male Wistar rats aged 7 weeks (CLEA Japan Inc., Tokyo, Japan) were maintained in a temperature-controlled room (about 22 °C) with a 12 h light/dark cycle, with *ad libitum* access to drinking water. The animals were weighed, and their health status was checked daily by visual observation during the course of the experiments.

## 2.2. Experimental protocol

The animals were divided into control ( $n = 20$ ) and experimental ( $n = 20$ ) groups. For the first 14 days, rats in the control group were given a pellet diet (25 g/day; Labo MR Standard, Nosan Corp., Yokohama, Japan), while those in the experimental group were given a liquid diet prepared daily by mixing 25 g of the powdered form of the pellet diet with 50 mL water. Subsequently, both groups were fed a pellet diet for 0 (day 0), 1, 3, 7 or 14 days ( $n = 4$  for both groups at each time point). For each time point, the rats were fasted for 12 h at night to synchronize the state of storage of acinar secretory granules and then perfused with 4% formaldehyde prepared by dissolving paraformaldehyde in 0.1M sodium phosphate buffer (pH 7.4) under pentobarbital anesthesia, 1 h after administration of BrdU at a dose of 25 mg/kg body weight by intraperitoneal injection (Sigma-Aldrich, St. Louis, MO). Then, the right parotid glands were excised and weighed. A small portion of each gland was set aside for transmission electron microscopy, and the major part of each gland was used for histological and immunohistochemical examination.

## 2.3. Histological analysis

The samples were placed in the 4% formaldehyde prepared by dissolving paraformaldehyde in 0.1M sodium phosphate buffer (pH 7.4) for 24 h, routinely processed, and embedded in paraffin. Then, 4  $\mu$ m sections were cut, stained with HE and PAS, and observed with a light microscope.

Histomorphometric analysis was performed to determine individual acinar cell area. Three HE-stained sections from each animal were used, and approximately 1,000 acinar cells were measured in each section by the image-analysis system (DS-L2, Nikon, Tokyo, Japan). The mean of the data from 3 sections was calculated, and used as the representative value for that animal ( $n = 4$ ).

#### *2.4. Immunohistochemical analysis*

Deparaffinized sections were immersed in 0.3% hydrogen peroxide in methanol to inhibit endogenous peroxidase. The sections for BrdU staining, to detect proliferating cells, were incubated with 0.1% trypsin for 20 min at 37 °C and later with 3N HCl for 10 min at 37 °C. After pretreatment, the sections were reacted with anti-BrdU mouse monoclonal antibody (Bu-20a, DakoCytomation, Glostrup, Denmark) at a dilution of 1/50 for 2 h, and then with biotinylated rabbit anti-mouse polyclonal antibody (DakoCytomation) for 1 h at a dilution of 1/100, in turn. The sections for Casp-3 staining, to detect apoptotic cells, were boiled in 10 mM Tris/1 mM EDTA buffer (pH 8.8) for 15 min. After antigen retrieval, the sections were reacted with anti-Casp-3 rabbit polyclonal antibody (Asp 175, Biocare Medical, Concord, CA) at a dilution of 1/20 overnight at 4 °C, and then with biotinylated swine



anti-rabbit polyclonal antibody (DakoCytomation) at a dilution of 1/100 for 1 h. Then, the sections for both BrdU and Casp-3 staining were incubated with peroxidase conjugated streptavidin (Histofine, Nichirei Bioscience, Tokyo, Japan) for 30 min. Peroxidase activity was visualized with 3, 3'-diaminobenzidine, and lightly counterstained with Mayer's hematoxylin. For the respective negative controls, normal mouse serum was used in place of the anti-BrdU primary, while rabbit serum was used in place of the anti-Casp-3 primary.

After immunohistochemical staining, approximately 1,000 acinar cells were observed to calculate the percentages of BrdU and Casp-3-positive acinar cells. The mean of the labeling indices of three sections was taken as the representative value for that animal ( $n = 4$ ).

## 2.5. Statistical analysis

Quantitative data such as body weight, parotid gland weight, individual acinar cell area and labeling indices for BrdU and Casp-3-positive acinar cells, were expressed as medians and ranges in box plots for four control and four experimental animals. Differences between control and experimental groups were assessed with the Mann-Whitney *U*-test (Ystat2008, Igakutosho, Tokyo, Japan).  $P < 0.05$  was considered to indicate a significant difference.

## 2.6. Ultrastructural analysis

The specimens for transmission electron microscopy were immersed in 2% formaldehyde prepared by dissolving paraformaldehyde-1.25% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) for 2 h, and then post-fixed in 1% osmium tetroxide for 2h.

After fixation, the tissues were stained *en bloc* with 4% uranyl acetate, and embedded in Epon 812. Semithin sections were cut and stained with toluidine blue to determine the area for ultrastructural examination. Ultrathin sections stained with both uranyl acetate and lead citrate were observed with a transmission electron microscope (JEM-1400, JEOL, Akishima, Japan).

### **3. Results**

#### *3.1. Body weights and parotid gland weights*

The body weights of the experimental animals were not significantly different from those of the controls at any tested time points (Fig. 1). The parotid gland weights of control animals did not change much from day 0 to day 14. The weights of the parotid glands were significantly less in the experimental group than in the control group on day 0. After changing diet from liquid to pellet form, the weight of the parotid glands in the experimental group increased, and there was no significant difference between the two groups on days 7 and 14 (Fig. 2).

#### *3.2. Histological analysis*

The parotid glands in the control rats consisted of serous acinar cells that were PAS-positive, and were histologically normal at all time points (Fig. 3A, E). In contrast, on day 0, the parotid glands in the experimental rats were atrophic, and acinar cells were smaller than in the controls (Fig. 3B). After the switch from the liquid to pellet diet, acinar cells enlarged, and mitotic figures were sometimes identified in these cells on days 1 and 3 (Fig.

3C). After day 7, parotid acinar cells in the experimental group were histologically similar to those in the control group (Fig. 3D). The intensity of PAS staining in acinar cells in the experimental group remained unchanged throughout the observation period (Fig. 3F, G, and H) and was similar to that in the controls.

The quantitative analysis revealed that individual acinar cell areas in the control parotid glands were constant throughout the observation period, while those in the experimental glands increased from day 0 to day 7. There were significant differences between the two groups on days 0, 1 and 3 (Fig. 4).

### *3.3. Immunohistochemical analysis*

In all control parotid glands at all tested time points, there were small numbers of BrdU-positive acinar cells (Fig. 5A). In the parotid glands from the animals in the experimental group, BrdU-positive acinar cells were very rare on day 0 (Fig. 5B). However, many acinar cells were BrdU-positive on days 1 and 3 after the change in diet (Fig. 5C). Thereafter, BrdU-positive acinar cells decreased in number. The percentage of BrdU-labeled acinar cells are shown in Fig. 6. The percentage of BrdU-labelled acinar cells in the experimental group was significantly lower on day 0, compared with the control group, but significantly higher on days 1 and 3.

In the experimental group, a few acinar cells were Casp-3-positive on day 0 (Fig. 5E), but were very rare at the other time points (Fig. 5F). In the control group, Casp-3-positive acinar cells were very rare at all time points (Fig. 5D). The percentage of Casp-3-positive

acinar cells are shown in Fig. 7. The percentage of Casp-3-positive acinar cells was significantly higher in the experimental group compared with the control group on day 0, although there was no significant difference between the two groups on days 1, 3, 7 and 14.

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

### *3.4. Ultrastructural analysis*

Parotid acinar cells in the control group had developed rough endoplasmic reticulum situated basally around the nucleus, and there were many secretory granules in the luminal cytoplasm. These characteristics were unchanged throughout the observation period (Fig. 8A). In the experimental group, apoptotic acinar cells were rarely identified on day 0. In apoptotic acinar cells, the nuclear fragments, which showed marginated and sharply delineated masses of dense chromatin, and whorled rough endoplasmic reticulum were observed (Fig. 8B). On days 0 and 1, secretory granules in acinar cells were fewer than in the control group. However, other ultrastructural characteristics were similar to those in the control (Fig. 8C). After 7 days, acinar cells in the experimental group were not ultrastructurally different from those in the control group because of an increase in secretory granules (Fig. 8D).

## **4. Discussion**

In the present study, the rats were fed a pellet (control group) or a liquid diet (experimental group) for the first 14 days. Because our previous study showed that feeding of a liquid diet for 14 days induced adequate atrophy of parotid glands to observe the recovery

after diet change (Takahashi et al., 2012). It has been reported that parotid glands, made atrophic by a liquid diet recover their weight 14 days after change of diet (Hall & Schneyer, 1964; Nakamura, 1997). However, the histological changes during this recovery period had not been investigated. In this study, we investigated the recovery process, and found that parotid gland weight increased immediately after change of diet and reached the control level at 7 days. Because there was no difference in body weight between the control and experimental groups, this quick recovery was likely not impacted by changes in general body conditions. It would be an important fact for clinical dentistry that the atrophic parotid glands are able to recover quickly by change of diet consistency.

The histomorphometric analysis using the image analysis system showed that the individual acinar cell area recovered after the change of diet, consistent with previous reports (Hall & Schneyer, 1964; Schneyer & Hall, 1975). Ultrastructurally, secretory granules increased in number, but cellular organelles remained unchanged during parotid gland recovery, suggesting that the increase in acinar cell size is mainly caused by an increase in secretory granules. In addition, we found that acinar cells actively proliferated immediately after diet change, in line with the study by Schneyer & Hall (1975). Furthermore, apoptosis in acinar cells diminished to the same level as in the controls soon after diet change. These findings suggest that acinar cells in the parotid glands increase in number after switching the diet from liquid to pellet form, although it is difficult to explain the relationship between active proliferation and diminished apoptosis. The increase in both the size and number of acinar cells occurred in the first 7 days after the change in diet, temporally coinciding with the

recovery in the weight of the parotid. Therefore, the increase in both acinar cell size and number most likely contribute to the recovery of the parotid glands after the change in diet. The powdered diet also have been shown to result in atrophy of rat parotid acini (Sreebny & Johnson, 1968; Johnson, 1981). Accordingly, the importance of the recovery of parotid acini from a liquid diet might be extended to recovery from a powdered diet as well.

How are the increases in the size and number of acinar cells produced by the change of diet? The parasympathetic nerve may play an important role in the process. Reportedly, the parasympathetic nerve stimulates not only salivary secretion (Garrett, 1987), but also mitotic activity of acinar cells in the parotid glands (Schneyer, Humpreys-Beher, Hall, & Jirakulsomchok, 1993; Burlage, Faber, Kampinga, Langendijk, Vissink, & Coppes, 2009). In addition, Nakamura (1997) found that after changing the diet from liquid to pellet form, the levels of acetylcholine, a neurotransmitter in the parasympathetic nervous system, increased in the parotid glands. Therefore, an increase in masticatory activity caused by the diet change may activate of the parasympathetic nerve to stimulate acinar cells to produce secretory granules and proliferate. However, further physiological studies are needed to test this hypothesis.

In the study by Hall & Schneyer (1964), the concentration of amylase in the parotid glands and the electrophoretic pattern of parotid secretions were altered by a change in diet. In the present study, the intensity of PAS staining and the ultrastructure of secretory granules in the acinar cells in the atrophic parotid glands did not differ from those in the recovered glands. Although the biochemical data in their study and the histological data in the present study

may seem contradictory, the biochemical alterations may be too weak to affect histology during the recovery of parotid acinar cells.

The present study focused on acinar cells in the parotid glands after diet change and did not examine duct cells. In the intercalated duct cells of rat parotid glands, secretory granules are often identified (Carlsoo & Ostberg, 1976). Accordingly, it would be also an interesting theme in further studies to investigate whether the liquid diet affects the intercalated duct cells.

## **5. Conclusion**

Here, we show that atrophic parotid glands are able to recover by changing the liquid diet to a pellet diet, and that increases in both the size and number of acinar cells contribute to the recovery. Our findings are clinically significant as they suggest that a simple change of diet may alleviate parotid gland dysfunction caused by a liquid diet.

## **Conflict of interest**

The authors declare no conflict of interest.

## **Acknowledgement**

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## Figure legends

**Fig. 1.** Box plot showing body weights of control (white box,  $n = 4$ ) and experimental (gray box,  $n = 4$ ) rats. There is no significant difference between the two groups at any tested time point. Top of box: 75th percentile; bottom of box: 25th percentile; horizontal bar: median; upper whisker: maximum value; lower whisker: minimum value.

**Fig. 2.** Box plot showing parotid gland weights in control (white box,  $n = 4$ ) and experimental (gray box,  $n = 4$ ) rats. The parotid glands were significantly lighter in the experimental group than in the control group on days 0, 1 and 3.  $*P < 0.05$  (for a description of the box plot, see Fig. 1).

**Fig. 3.** Histology of the parotid glands. Stains: A-D, HE; E-H, PAS. Samples: A and E, control parotids on day 0; B and F, experimental parotids on day 0; C and G, experimental parotids on day 3; D and H, experimental parotids on day 14. Scale bars: A-H, = 30  $\mu\text{m}$ ; Inset of C, = 10  $\mu\text{m}$ . Acinar cells in the experimental parotid glands (B) are smaller in size than those in the controls (A). A mitotic figure in an acinar cell (inset) is observed in the experimental glands on day 3 (C). Acinar cell morphology in the experimental glands on day 14 (D) is similar to that in the control (A). There is no difference in the staining intensity of PAS between the control (E) and experimental glands at any tested time point (F-H).

**Fig. 4.** Box plot of areas of individual acinar cells in the control parotid glands (white box;  $n$

= 4) and experimental parotid glands (gray box;  $n = 4$ ). Parotid acinar cells in the experimental group are significantly smaller than those in the control group on days 0, 1 and 3.  $*P < 0.05$  (for a description of the box plot, see Fig. 1).

**Fig. 5.** Immunohistochemistry of the parotid glands. A-C: BrdU; D-F: Casp-3. A and D: parotid glands in the control group on day 0; B and E: parotid glands in the experimental group on day 0; C: experimental parotids on day 1; F: experimental parotids on day 7. Scale bars: A-C, = 30  $\mu\text{m}$ ; D-F = 10  $\mu\text{m}$ . BrdU-positive acinar cells (arrowhead) are sometimes observed in the control parotid glands (A). In the experimental glands, BrdU-positive acinar cells are rare on day 0 (B), but many acinar cells are positive for BrdU (arrowheads) on day 1 (C). More Casp-3-positive acinar cells (arrowheads) are observed in the experimental parotid glands on day 0 (E), but are few in the control glands at all time points (D) and in the experimental glands at the other time points (F).

**Fig. 6.** Box plot showing the percentage of BrdU-positive acinar cells in the control (white box,  $n = 4$ ) and experimental (gray box,  $n = 4$ ) parotid glands. On day 0, the percentage of BrdU-positive acinar cells is significantly higher in the control group compared with the experimental group. In contrast, the percentage of BrdU-positive acinar cells is higher in the experimental group than in the control group on days 1 and 3.  $*P < 0.05$  (for a description of the box plot, see Fig. 1).

**Fig. 7.** Box plot showing the percentage of Casp-3-positive acinar cells in the control (white box,  $n = 4$ ) and experimental (gray box,  $n = 4$ ) parotids. On day 0, the percentage of Casp-3-positive acinar cells was significantly higher in the experimental group than in the control. At the other tested time points, the two groups did not significantly differ.  $*P < 0.05$  (for a description of the box plot, see Fig. 1).

**Fig. 8.** Transmission electron photomicrographs of parotid glands in the control group on day 7 (A) and in the experimental group on days 0 (B), 1 (C) and 7 (D). ER, rough endoplasmic reticulum; SG, secretory granule; N, nuclear fragment. Scale bar: 2  $\mu\text{m}$ . An acinar cell in the control glands contains many secretory granules and developed rough endoplasmic reticulum (A). Nuclear fragments and whorled rough endoplasmic reticulum (arrow) are observed in an apoptotic acinar cell in the experimental parotid glands on day 0 (B). There are a few secretory granules in acinar cells in the experimental parotid glands on day 1 (C). The secretory granules are comparatively more numerous in the experimental glands on day 7 (D).

Fig. 1



















