Title: Acinar cell response to liquid diet during rats' growth period differs in submandibular and sublingual glands from that in parotid glands

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ABSTRACT

Continuously feeding a liquid diet to growing rodents strongly inhibits parotid gland growth, due to suppressed growth of acinar cells. This study investigated whether a liquid diet had a similar effect on submandibular and sublingual glands of growing rats. Rats were weaned on day 21 after birth and then fed a pellet diet in the control group and a liquid diet in the experimental group for 0, 1, 2, 4, and 8 weeks. Their submandibular and sublingual glands were excised, weighed, and examined histologically, immunohistochemically (using antibodies to 5'-bromo-2-deoxyuridine and cleaved caspase 3), and ultrastructurally. The submandibular glands did not significantly differ between the control and experimental groups at all tested points. Only at Week 8, acinar cell area and 5'-bromo-2-deoxyuridine-labeling index of acinar cells in sublingual glands were significantly lower in the experimental group than in the control group. These results show that a liquid diet during rats' growth period had no effect on acinar cells in their submandibular glands, and only a slight effect on acinar cells in their sublingual glands of growing rats, in contrast to the marked effect of a liquid diet on parotid glands.

1. Introduction

Soft foods are common in modern diets, but reportedly have unfavorable effects on oral tissues. In experimental rodent models, liquid or soft diets suppressed growth of the craniofacial bone (Watt and Williams, 1951; Ito et al., 1988; Enomoto et al., 2010), masseter muscle (Kiliaridis et al., 1988; Maeda et al., 1990; Miehe et al., 1999), and temporo-mandibular joint (Kiliaridis et al., 1999; Kato et al., 2015; Uekita et al., 2015). Their effects on salivary glands, particularly the parotid glands, have been widely investigated in mature animals. Such studies have concluded that liquid diets cause severe atrophy to parotid glands. Short-term liquid diets have been shown to reduce parotid gland weight (Hall and Schneyer, 1964, Ekstrom and Templeton, 1977, Scott et al., 1990, Scott and Gunn, 1991; Kurahashi and Inomata, 1999; Takahashi et al., 2012), secretion (Ekstrom and Templeton, 1977; Ito et al., 2001), and amylase activity (Hall and Schneyer, 1964; Hand and Ho, 1981; Johnson, 1984; Kurahashi and Inomata, 1999). In such atrophic parotid glands, acinar cell shrinkage (Hall and Schneyer, 1964; Wilborn and Schneyer, 1970; Hand and Ho, 1981; Scott et al., 1990; Scott and Gunn, 1991; Takahashi et al., 2012), decrease in acinar cell numbers (Johnson, 1982), decreased acinar cell replication (Takahashi et al., 2012), and increased acinar cell apoptosis (Takahashi et al., 2012; ElGhamraey, 2015) have been observed.

Submandibular and sublingual glands, as well as parotid glands are major salivary glands, but these three major salivary glands differ in histological structure and physiological function; and the effects of liquid diet on submandibular and sublingual glands have been less studied than those of parotid glands. Therefore, whether submandibular and sublingual glands

would respond in the same way to liquid diets as parotid glands was unclear. Some studies appear to show slight atrophy of the submandibular (Kuntsal et al., 2003) or sublingual glands (Mansson et al., 1990; Kurahashi and Inomata, 1999) whereas others found no effect on these glands by liquid diets (Ekstrom, 1973; Scott and Gunn, 1991; Takahashi et al., 2014). Whether atrophy of submandibular or sublingual glands is induced by the liquid diet is thus controversial, although many investigations consistently report that submandibular and sublingual glands are much less affected by liquid diets than parotid glands (Ekstrom, 1973; Scott and Gunn, 1991; Nakamura, 1997; Kurahashi and Inomata, 1999; Takahashi et al., 2012; Takahashi et al., 2014). These findings could imply that liquid diets affect parotid glands and the other salivary gland types differently.

The aforementioned studies mainly focused on salivary glands of mature animals, but the effects of a liquid diet on growing salivary glands have received little or no attention. Our previous study revealed that the weight of parotid glands of growing rats fed a liquid diet increased much less than solid-fed rats. Histologically, acinar cell size in these parotids did not increase, and acinar cell proliferation was suppressed during the growing period. However, acinar cell apoptosis was not found as a factor in inhibition of parotid gland growth (Takahashi et al., 2015). Our previous study showed that parotid glands of both mature and growing rats were affected negatively by liquid diet and that atrophic induction differs between mature and growing parotid glands. However, the influence of liquid diets on the growth of other major salivary glands is still unclear.

The aim of this report was to clarify whether the growth of submandibular and

sublingual glands was affected by a liquid diet. For this purpose, we examined histologically, histochemically, immunohistochemically, and ultrastructurally glands of growing rats that were fed a liquid diet. In histochemical analysis, we used periodic acid Schiff (PAS) and Alcian blue (AB) staining to demonstrate the full complement of tissue proteoglycans, because the mucous component of the submandibular glands reacts to PAS and the mucous component of the sublingual glands is strongly AB-positive (Cecchini et al., 2009), and they are useful to examine the nature of secretory granules in an acinar cell.

2. Materials and Methods

2.1. Animal model

The animal protocol of this study was approved by the Laboratory Animal Committee of Hokkaido University (Approval No. 14-0108); it complied with the Guide for the Care and Use of Laboratory Animals of Hokkaido University. After weaning at 21 days, 63 male Wistar rats (CLEA Japan Inc., Tokyo, Japan) were divided into control (*n*=35) and experimental groups (*n*=28). In the control group, each rat was given a pellet diet (25 g/day) for 0, 1, 2, 4, and 8 weeks; in the experimental group, each rat was given a liquid diet prepared daily by mixing 25 g powdered form of the pellet diet with 50 mL water for 1, 2, 4, and 8 weeks. At the end of the experimental period, rats were deprived of food for 12 h before perfusion.

2.2. Histological study

At each test point, five control and five experimental rats were perfused with 4% paraformaldehyde buffered with 0.1M phosphate buffer (pH 7.4) under pentobarbital anesthesia at 1 h after administering 5’-bromo-2-deoxyuridine (BrdU) (Sigma-Aldrich, St. Louis, MO; 25 mg/kg body weight; intraperitoneal injection). The right submandibular and sublingual glands were then removed, weighed, immersed in the same fixative for 24 h, and processed for routine paraffin embedding. Sections were cut at 4 μm and stained with Hematoxylin and Eosin (HE), PAS, and AB (pH 2.5).

The image-analysis system (DS-L2, Nikon, Tokyo, Japan) was used to determine

areas of individual acinar cells. Each HE-stained section was roughly divided into 5 parts to avoid uneven distribution of observation fields, and a field was chosen from the central area of each part without intention. In the 5 fields, approximately 1000 acinar cells were measured at an objective magnification of 40x. Three sections were used from each animal (n=5), and the mean of the data from the 3 sections was calculated, as the representative value for that animal.

2.3. Immunohistochemical study

The sections prepared as described above were treated with 0.3% hydrogen peroxide in absolute methanol for 10 min after deparaffinization to quench endogenous peroxidase activity, in readiness for immunohistochemical staining. For BrdU stains to detect proliferating cells, sections were pretreated with 0.1% trypsin for 20 min at 37℃ and later with 3N HCl for 10 min at 37℃ for denaturation of the DNA double strand. The sections were then incubated with anti-BrdU mouse monoclonal antibody (Bu-20a, DakoCytomation, Glostrup, Denmark, 1:50 dilution) for 2 h, biotinylated anti-mouse rabbit polyclonal antibody (DakoCytomation, 1:100 dilution) for 1 h, and peroxidase labeling streptavidin (Histofine, Nichirei Bioscience, Tokyo, Japan) for 30 min. The antigen antibody reaction sites were visualized by 3, 3’-diaminobenzidine; sections were then lightly counterstained with Mayer’s hematoxylin. To quantify acinar cell proliferative activity, we used 3 sections stained with BrdU from each animal. Each section was roughly divided into 5 parts to avoid unevenly distributed observation fields; a field was chosen from the central area of each part without

intention. In the 5 fields, BrdU-positive acinar cells were counted per approximately 1000 acinar cells at an objective magnification of 40x. The labeling index (percentage of labeled acinar cells) of each section was then calculated. The average of the labeling indices of the 3 sections from each animal (n=5) was used as the representative value of that animal.

In using cleaved caspase 3 (Casp-3) to detect apoptotic cells, the ready sections were boiled in 10mM tris/1 mM EDTA buffer (pH 8.8) for 15 min as antigen retrieval treatment. After cooling, sections were reacted with anti-Casp-3 rabbit polyclonal antibody (Asp 175, Biocare Medical, Concord, CA, 1:20 dilution) overnight at 4℃, biotinylated anti-rabbit swine polyclonal antibody (DakoCytomation, 1:100 dilution) for 1 h, and peroxidase labeling streptavidin for 30 min in turn. Visualization of immunoreaction and counterstaining were as described above. We did not calculate labeling indices for Casp-3-positive acinar cells, because Casp-3-positive cells were extremely rare in both control and experimental groups at all time intervals.

Negative control sections were incubated with normal mouse or rabbit serum instead of primary antibody for BrdU or Casp-3, respectively, and showed no reaction.

2.4. Statistical analysis

Numerical data such as body weights, gland weights, areas of individual acinar cells, and labeling indices of BrdU are shown as medians and ranges in box plots for five control animals and five experimental ones at each tested time point. To determine significant differences between control and experimental groups, the Mann-Whitney *U*-test was used

(Ystat2008, Igakutosho, Tokyo, Japan). $P<0.05$ was considered significant.

2.5. Ultrastructural study

At each tested time point, we perfused two control and two experimental rats with 2% paraformaldehyde-1.25% glutaraldehyde buffered with 0.1M sodium cacodylate buffer (pH 7.4) under pentobarbital anesthesia. After perfusion, the excised right submandibular and sublingual glands were trimmed into small pieces and immersed in the same fixative for 2 h. The samples were then post-fixed in 1% osmium tetroxide, stained *en bloc* with 4% uranyl acetate, and embedded in Epon 812. Ultrathin sections were cut with a diamond knife on an ultramicrotome, stained with both uranyl acetate and lead citrate, and studied with a transmission electron microscope (JEM-1400, JOEL, Akishima, Japan).

3. Results

3.1. General condition of experimental animals

All animals in both groups appeared healthy, with no symptoms of diarrhea, and their body weights increased during the experiment. The control and experimental groups did not significantly differ in body weight at any of the time points (Fig. 1).

3.2. Submandibular glands

The weights of submandibular glands increased throughout the experiment, and did not significantly differ between two groups at all tested time points (Fig. 2).

At the beginning of the experiment, terminal portions were composed of small seromucous acinar cells (Fig. 3A), which were PAS-positive (Fig. 3D) and weakly AB-positive (Fig. 3G). Thereafter, acinar cells in the experimental group became larger over time (Fig.3C), and their intensity for PAS (Fig. 3F) and AB (Fig. 3I) staining were unchanged until Week 8. These findings were similar to those of the control group at each time point (Fig. 3B, E, and H). The two groups were also similar in histomorphometric data for areas of individual acinar cells in submandibular glands at all time points tested (Fig. 4).

Many acinar cells were BrdU-positive at Week 0 (Fig. 5A), but BrdU-positive acinar cells decreased over time in both the control (Fig. 5B) and experimental groups (Fig. 5C). The BrdU labeling indices for acinar cells in submandibular glands did not significantly differ between the two groups at any time intervals tested (Fig. 6). Only a few Casp-3-positive acinar cells were seen in immunostained submandibular gland sections from either group

throughout the experiment (Fig. 5D), and none in the late phase of the experiment (Fig. 5E and F).

At Week 0, acinar cells already had typical ultrastructures, such as well-developed endoplasmic reticulum around basally-situated, spherical nucleus, electron-lucent secretory granules with relatively fine structures, and Golgi apparatus (Fig. 7A and B). Thereafter, the secretory granules increased; the above ultrastructural characteristics of acinar cells were seen in submandibular glands of both control (Fig. 7C and D) and experimental animals (Fig. 7E and F).

3.3. Sublingual glands

Sublingual glands in both groups grew in weight over time, and did not significantly differ between the control and experimental groups at all time points measured (Fig. 8).

At Week 0, histological observations showed mucous acinar cells mostly composed the terminal portion of sublingual glands. Small acinar cells (Fig. 9A) were PAS-positive (Fig. 9D) and strongly AB-positive (Fig. 9G). In control sublingual glands, acinar cells grew larger over time (Fig. 9B) and their reaction to AB was still strong (Fig. 9H), despite weak reaction to PAS (Fig. 9E). Acinar cell histology and levels of PAS and AB staining were similar in experimental and control sublingual glands (Fig. 9F and I), except that smaller acinar cells were often seen at Week 8 (Fig. 9C). Histomorphometric analysis of areas of individual acinar cells showed no significant differences between the two groups until Week 4, but at Week 8, acinar cells in the experimental group were significantly smaller than in controls ($P<0.05$)

(Fig. 10).

The BrdU-positive acinar cells were often seen at the start of the experiment (Fig. 11A), but thereafter decreased in both experimental and control groups (Fig. 11B and C). Statistical analysis showed no significant differences in BrdU labeling indices between the two groups at 1, 2, and 4 weeks, but the index of the experimental group was significantly lower than that of the control group at Week 8 ($P<0.05$) (Fig. 12). We saw very few Casp-3-positive acinar cells in sublingual glands of either groups in the early phase of the experiment (Fig. 11D), and none after Week 4 (Fig. 11E and F).

Ultrastructurally, acinar cells had low columnar or cuboidal shapes at Week 0, with electron-lucent secretory granules in their cytoplasm and rough endoplasmic reticulum observed to be basally situated around their nuclei (Fig. 13A and B). In control sublingual glands, acinar cells became larger with flat nuclei at Week 8 (Fig. 13C and D). The ultrastructure of acinar cells in the experimental sublingual glands was basically similar to that of controls. Cytoplasm of acinar cells was filled with many electron-lucent secretory granules, which pressed the flat nucleus basally (Fig. 13E and F).

All numerical data (mean and standard deviation) are shown in Table 1.

4. Discussion

We found no differences between the two groups in the weight, the area of individual acinar cells, acinar cell proliferation, and acinar cell apoptosis in submandibular glands. The sublingual glands differed between the two groups only in the areas of individual acinar cells and proliferative activity of acinar cells at Week 8, but showed no significant differences at earlier points. These findings demonstrate that liquid diets exert little or no inhibition of the growth of rats' submandibular and sublingual glands. In addition, both PAS and AB staining results and the ultrastructure of secretory granules in the experimental submandibular and sublingual glands were similar to those in the control glands. These observations indicate that liquid diets have no effect on the nature of secretory granules in both salivary glands during the growing period.

Reportedly, mature parotid glands become strongly atrophic by intake of a liquid diet (Hall and Schneyer, 1964; Ekstrom and Templeton, 1977; Scott et al., 1990; Scott and Gunn, 1991; Kurahashi and Inomata, 1999; Takahashi et al., 2012), whereas mature submandibular and sublingual glands show no or slight atrophy (Ekstrom, 1973; Mansson et al., 1990; Scott and Gunn, 1991; Takahashi et al., 2014), which indicates that mature parotid glands are much more sensitive to liquid diets than the other two salivary glands. Similar results for growing salivary glands were shown in our previous study (Takahashi et al., 2015) and in this study. Therefore, different sensitivities to liquid diet between parotid glands and the other two glands are consistent, regardless of growing or mature phases. Salivary gland response to a liquid diet is very similar to that of surgical removal of parasympathetic innervation (Poat and

Templeton, 1982; Carpenter et al., 2005) or ligation of parasympathetic nerves (Harrison and Garrett, 1976; Harrison et al., 2001). These data suggest that parasympathetic innervation may contribute to the differences in response among these glands (Johnson and Cardenas, 1993). Parotid glands are innervated by the glossopharyngeal nerve, arising from the inferior salivary nucleus; whereas submandibular and sublingual glands are supplied by the facial nerve, arising from the superior salivary nucleus. The difference in sensitivity to liquid diets coincides with the different nerve supplies. In addition, acetylcholine level (Nakamura, 1997), choline acetyltransferase activity (Ekstrom, 1973), and neuropeptide level (Mansson et al., 1990) in parotid glands of liquid-fed rats physiologically decreased but were unchanged in the submandibular and sublingual glands. These physiological facts support the participation of parasympathetic innervation in the different response among the glands. Moreover, some possible effect of nerve growth factor might be associated as well. Further immunohistochemical investigation, using markers of parasympathetic nerve and nerve growth factor will be necessary to clarify this question.

In the present study, slightly different reactions to a liquid diet were seen between growing submandibular glands and sublingual glands. This difference cannot be explained simply through parasympathetic innervations alone, because the parasympathetic innervations of these glands are same. However, their different histological structures could be a factor. In rodents, parotid glands are serous, submandibular glands are seromucous, and sublingual glands are mainly mucous (Sbarbati et al., 1994; Cecchini et al., 2014). It has been reported that different types of acinar cells show different responses to other pathological conditions

such as X-ray irradiation (Abok et al., 1984; Seifert et al., 1986) and excretory duct ligation (Takahashi et al., 2000; Takahashi et al., 2002). Therefore, differences among acinar cell types might be also involved in different reactions to liquid diets.

At Week 8, the areas of individual acinar cells and BrdU-labeling index for sublingual glands of liquid-fed rats were significantly less than those of pellet-fed rats, but the weight of these sublingual glands did not significantly differ between the two groups at that time. In parotid glands, the gland weight of liquid-fed rats was significantly less than that of pellet-fed rats, and acinar cell size and acinar cell proliferation of the experimental group were remarkably different from those of the control group (Takahashi et al., 2015). In sublingual glands in this study, differences in acinar cell size and proliferation between the two groups were unremarkable, in contrast to the parotid glands. Possibly the suppressive effect of liquid diet feeding on acinar cell growth (for both cell size and proliferative activity) might be too weak to affect the entire sublingual gland weight enough to be detected as numerical data.

In conclusion, we found that liquid diets in growing rats had no effect on acinar cells in submandibular glands and only slight effects on acinar cells in sublingual glands. This differs from the strongly inhibitory effect of liquid diets on the growth of parotid glands.

Conflict of interest

The authors declare no conflict of interest.

References

Abok K., Brunk U., Jung B., Ericsson J., 1984. Morphologic and histochemical studies on the differing radiosensitivity of ductular and acinar cells of the rat submandibular gland. Virchows Arch. Cell Pathol. 45, 443-460.

Carpenter G. H., Proctor G. B., Garrett J. R., 2005. Preganglionic parasympathectomy decreases salivary SIgA secretion rates from the rat submandibular gland. J. Neuroimmunol. 160, 4-11.

Cecchini M. P., Merigo F., Cristofoletti M., Osculati F., Sbarbati A., 2009. Immunohistochemical localization of Clara cell secretory proteins (CC10-CC26) and Annexin-1 protein in rat major salivary glands. J. Anat. 214, 752-758.

Cecchini M. P., Parnigotto M., Merigo F., Marzola P., Daducci A., Tambalo S., Boschi F., Colombo L., Sbarbati A., 2014. 3D printing of rat salivary glands: the submandibular-sublingual complex. Anat. Histol. Embryol. 43, 239-244.

Ekstrom J., 1973. Choline acethyltransferase and secretory responses of the rat's salivary glands after liquid diet. Q. J. Exp. Physiol. 58, 171-179.

Ekstrom J., Templeton D., 1977. Difference in sensitivity of parotid glands brought about by disuse and overuse. Acta Physiol. Scand. 101, 329-335.

ElGhamrawy T. A., 2015. The effect of liquid diet on the parotid gland and the protective role of L-carnitine: immunohistochemical and ultrastructural study. Folia Morphol. 74, 42-49.

Enomoto A., Watahiki J., Yamaguchi T., Irie T., Tachikawa T., Maki K., 2010. Effects of

mastication on mandibular growth evaluated by microcomputed tomography. Eur. J. Orthod. 32, 66-70.

Hall H. D., Schneyer C. A., 1964. Salivary gland atrophy in rat induced by liquid diet. Proc. Soc. Exp. Biol. Med. 117, 789-793.

Hand A. R., Ho B., 1981. Liquid-diet-induced alterations of rat parotid acinar cells studied by electron microscopy. Arch. Oral Biol. 26, 369-380.

Harrison J. D., Foud H. M. A., Garrett J. R., 2001. Variation in the response to ductal obstruction of feline submandibular and sublingual salivary glands and the importance of the innervation. J. Oral Pathol. Med. 30, 29-34.

Harrison J. D., Garrett J. R., 1976. Histological effects of duct ligation of salivary glands of the cat. J. Pathol. 118, 245-254.

Ito G., Mitani S., Kim J. H., 1988. Effect of soft diet on craniofacial growth in mice. Anat. Anz. 165, 151-166.

Ito K., Morikawa M., Inenaga K., 2001. The effect of food consistency and dehydration on reflex parotid and submandibular salivary secretion in conscious rats. Arch. Oral Biol. 46, 353-363.

Johnson D. A., 1982. Effect of a liquid diet on the protein composition of rat parotid saliva. J. Nutr. 112, 175-181.

Johnson D. A., 1984. Changes in rat parotid salivary proteins associated with liquid diet-induced gland atrophy and isoproterenol-induced gland enlargement. Arch. Oral Biol. 29, 215-221.

Johnson D. A., Cardenas H. L., 1993. Effects of food mastication on rat parotid gland adrenergic and cholinergic cell surface receptors. Crit. Rev. Oral Biol. Med. 4, 591-597.

Kato T., Takahashi S., Domon T., 2015. Effects of liquid diet on the temporomandibular joint of growing rats. Med. Prin. Pract. 24, 257-262.

Kiliaridis S., Engstrom C., Thilander B., 1988. Histochemical analysis of masticatory muscle in the growing rat after prolonged alteration in the consistency of the diet. Arch. Oral Biol. 33, 187-193.

Kiliaridis S., Thilander B., Kjellberg H., Topouzelis N., Zafiriadis A., 1999. Effect of low masticatory function on condylar growth: a morphometric study in rat. Am. J. Orthod. Dentofacial. Orthop. 116, 121-125.

Kuntsal L., Firat D., Sirin Y., 2003. Prevention of liquid-diet-induced damages on submandibular glands by selenium supplementation in rats. Tohoku J. Exp. Med. 201, 191-199.

Kurahashi M., Inomata K., 1999. Effects of dietary consistency and water content on parotid amylase secretion and gastric starch digestion in rats. Arch. Oral Biol. 44, 1013-1019.

Maeda N., Suwa T., Ichikawa M., Masuda T., Kumegawa M., 1990. Effects of easily chewable diet and unilateral extraction of upper molars on the masseter muscle in developing mice. Acta Anat. 137, 19-24.

Mansson B., Ekman R., Hakanson R., Ekstrom J., 1990. Neuropeptides and disuse of the rat parotid gland. Exp. Physiol. 75, 597-599.

Miehe B., Fraghanel J., Kubein-Meesenburg D., Nagerl H., Schwestka-Polly R., 1999. Masticatory musculature under altered occlusal relationships -a model study with experimental animals. Ann. Anat. 181, 37-40.

Nakamura K., 1997. Effects of short-term bulk or liquid diet feeding on the neurotransmitters in the salivary glands and various sialogogue-induced salivation in mice. Jpn. J. Oral Biol. 39, 655-664.

Poat J. A., Templeton D., 1982. Non-specific supersensitivity in rat parotid salivary glands following parasympathectomy. J. Auton. Pharmacol. 2, 79-85.

Sbarbati A., Baldassarri A., Leclercq F., Merigo F., Antonakis K., Boicelli A., 1994. Magnetic resonance imaging of the submandibular-sublingual complex. Acta Anat. 149, 63-69.

Seifert G., Miehlke A., Haubrick J., Chilla R., 1986. Sialadenitis, 12th Chap. Diseases of the Salivary Glands, 1st ed. Georg Thieme Verlag, Stuttgart, pp.110-170.

Scott J., Berry M. R., Gunn D. L., Woods K., 1990. The effects of a liquid diet on initial and sustained, stimulated parotid salivary secretion and on parotid structure in the rat. Arch. Oral Biol. 35, 509-514.

Scott J., Gunn D. L., 1991. A comparative quantitative histological investigation of atrophic changes in the major salivary glands of liquid-fed rats. Arch. Oral Biol. 36, 855-857.

Takahashi S., Nakamura S., Suzuki R., Islam N., Domon T., Yamamoto T., Wakita M., 2000. Apoptosis and mitosis of parenchymal cells in duct-ligated rat submandibular gland. Tissue Cell 32, 457-463.

Takahashi S., Shinzato K., Nakamura S., Domon T., Yamamoto T., Wakita M., 2002. The roles

of apoptosis and mitosis in atrophy of the rat sublingual gland. Tissue Cell 34, 297-304.

Takahashi S., Uekita H., Kato T., Yuge F., Ushijima N., Inoue K., Domon T., 2012. Involvement of apoptosis and proliferation of acinar cells in atrophy of rat parotid glands induced by liquid diet. J. Mol. Histol. 43, 761-766.

Takahashi S., Uekita H., Kato T., Yuge F., Ushijima N., Inoue K., Domon T., 2014. Immunohistochemical and ultrastructural investigation of acinar cells in submandibular and sublingual glands of rats fed a liquid diet. Tissue Cell 46, 136-143.

Takahashi S., Uekita H., Kato T., Inoue K., Domon T., 2015. Growth of rat parotid glands is inhibited by liquid diet feeding. Tissue Cell 47, 336-341.

Uekita H., Takahashi S., Domon T., Yamaguchi T., 2015. Changes in collagens and chondrocytes in the temporomandibular joint cartilage in growing rats fed a liquid diet. Ann. Anat. 202, 78-87.

Watt D. G., Williams C. H. M., 1951. The effects of the physical consistency of food on the growth and development of the mandible and the maxilla of the rat. Am. J. Orthod. 37, 895-928.

Wilborn W. H., Schneyer C. A., 1970. Ultrastructural changes of rat parotid glands induced by a diet of liquid metrecal. Z. Zellforsch 103, 1-11.

Figure legends

Fig. 1. Box plot illustrating body weights of control (white box, *n*=5) and experimental rats (gray box, *n*=5). The two groups do not significantly differ at any tested points. Tops of boxes: 75th percentile; bottoms: 25th percentile; horizontal bar: median; upper whisker: maximum value; lower whisker: minimum value.

Fig. 2. Box plot showing submandibular gland weights of control (white box, *n*=5) and experimental rats (gray box, *n*=5). The two groups do not significantly differ at any tested points. (Explanation of box plot: see Fig. 1.)

Fig. 3. Histological observations of submandibular glands. Stains: A-C, HE; D-F, PAS; G-I, AB. Tissues are from control submandibular glands at Week 0 (A, D, and G) and Week 8 (B, E, and H), and experimental ones at Week 8 (C, F, and I). Bars=30μm. Acinar cells are small in size Week 0 (A, D, and G). Acinar cell morphology (B and C) and staining intensity of PAS (E and F) and AB (H and I) are similar in experimental and control submandibular glands.

Fig. 4. Box plot shows areas of individual acinar cells in submandibular glands of control (white box; *n*=5) and experimental animals (gray box; *n*=5). No significant differences are observed between control and experimental groups at any examined points. (Explanation of box plot: see Fig. 1.)

Fig. 5. Immunohistochemical observations of submandibular glands for BrdU (A-C) and Casp-3 (D-F). Tissues are shown from control submandibular glands at Week 0 (A and D) and Week 8 (B and E), and experimental ones at Week 8 (C and F). Bars=30μm. Many BrdU-positive acinar cells are seen at Week 0 (A), but decrease in number in both groups by Week 8 (arrows; B and C). A Casp-3-positive acinar cell (arrow) is rarely observed at Week 0 (D), but not at Week 8 in either group (E and F).

Fig. 6. Box plot of BrdU labeling indices for acinar cells in submandibular glands in control (white box, *n*=5) and experimental animals (gray box, *n*=5). The two groups do not significantly differ at any tested points. (Explanation of box plot: see Fig. 1.)

Fig. 7. Ultrastructural observations of submandibular glands in the control group at Week 0 (A and B) and Week 8 (C and D), and in the experimental group at Week 8 (E and F). The typical ultrastructure of an acinar cell is seen in the experiment's early phase (A and B). Ultrastructural characteristics of acinar cells are similar in control (C and D) and experimental (E and F) submandibular glands. ER: rough endoplasmic reticulum; G: Golgi apparatus; SG: secretory granule. Bars=5μm (A, C, E) and 2μm (B, D, F)

Fig. 8. Box plot for weights of sublingual glands (control rats: white box, *n*=5; experimental rats: gray box, *n*=5). No significant difference is identified between control and experimental groups at any tested points. (Explanation of box plot: see Fig. 1.)

Fig. 9. Histology of sublingual glands, including control glands at Week 0 (A, D, and G) and Week 8 (B, E, and H) and experimental ones at Week 8 (C, F, and I). Stains: HE: A-C; PAS: D-F; and AB: G-I. Bars=30μm. At the start of the experiment, acinar cells are small in size (A, D, and G). At Week 8, acinar cells in controls (B) are slightly larger than in experimental glands (C), but their staining intensity for PAS (E and F) and AB (H and I) are similar in both groups.

Fig. 10. Areas of individual acinar cells in sublingual glands of control (white box; n=5) and experimental animals (gray box; n=5). Areas of individual acinar cells in sublingual glands of rats fed a pellet diet are significantly larger than in those fed a liquid diet at Week 8. *P<0.05. (Explanation of box plot: see Fig. 1.)

Fig. 11. Immunohistochemical observations of sublingual glands for BrdU (A-C) and Casp-3 (D-F). Control sublingual glands at Week 0 (A and D) and Week 8 (B and E), and experimental ones at Week 8 (C and F) are shown. Bars=30μm. Many acinar cells are BrdU-positive at Week 0 (A), but at Week 8, few BrdU-positive acinar cells (arrows) are seen in either group (B and C). Casp-3-positive acinar cells (arrow) are rare throughout the experimental period (D); many sections of either group show none (E and F).

Fig. 12. Box plot shows BrdU labeling indices for acinar cells in sublingual glands in control

(white box; *n*=5) and experimental animals (gray box; *n*=5). The labeling index of control glands is significantly higher than that of experimental glands at Week 8. *$P<0.05$.

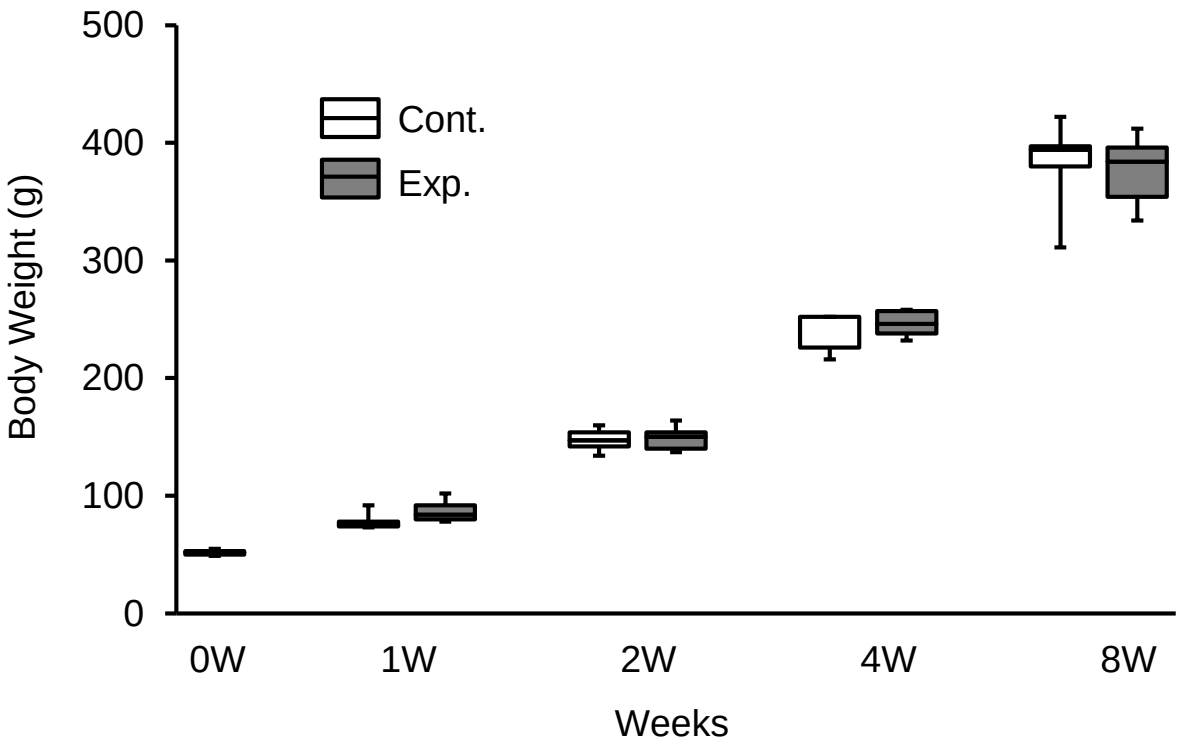
(Explanation of box plot: see Fig. 1.)

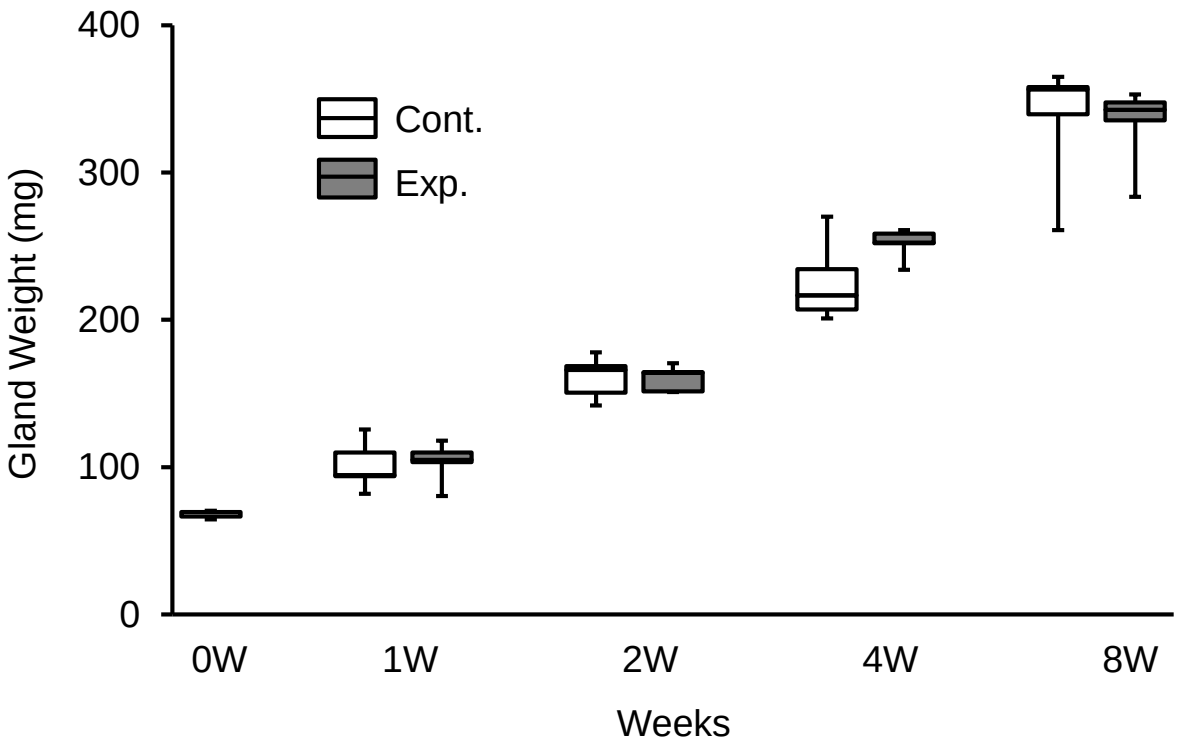
Fig. 13. Ultrastructures of control sublingual glands at Week 0 (A and B) and Week 8 (C and D), and of experimental glands at Week 8 (E and F). At Week 0, acinar cells contain electron-lucent secretory granules and flat, basally situated nuclei (A and B). At Week 8, acinar cell ultrastructural characteristics are similar in control (C and D) and experimental (E and F) sublingual glands. MEC: myoepithelial cell; ER: endoplasmic reticulum; SG: secretory granule. Bars=5μm (A, C, E) and 2μm (B, D, F).

Table 1. Mean and standard deviation values for body weight, gland weight, individual acinar cell area, and BrdU labeling indices.

			0W	1W	2W	4W	8W
Animal	Body Weight (g)	Cont	51.8±2.1	78.4±7.8	147.4±10.1	239.6±17.3	380.8±41.9
		Exp		87.2±9.9	149.0±10.9	246.2±11.5	376.0±31.6
SMG	Gland Weight (mg)	Cont	68.1±2.2	101.2±16.8	161.0±14.5	225.8±27.8	336.0±43.0
		Exp		103.4±14.0	160.3±8.7	251.6±10.6	332.4±28.1
	Cell Area (μm^2)	Cont	100.5±4.4	134.1±5.1	157.9±2.4	172.9±3.6	172.1±4.1
		Exp		136.0±9.3	155.4±1.8	173.1±4.0	174.4±4.0
	BrdU Index (%)	Cont	4.60±1.65	4.68±0.50	3.04±0.91	1.87±0.53	0.91±0.16
		Exp		4.94±0.84	2.71±0.35	1.86±0.28	0.71±0.18
SLG	Gland Weight (mg)	Cont	14.2±2.1	20.9±2.8	31.8±2.7	39.6±4.3	47.7±2.7
		Exp		21.1±2.0	29.4±2.9	39.1±4.8	42.7±5.4
	Cell Area (μm^2)	Cont	176.4±2.3	188.4±8.0	215.5±4.2	217.0±3.1	217.5±2.3
		Exp		189.1±4.5	213.7±3.4	217.0±3.0	212.3±2.1
	BrdU Index (%)	Cont	3.72±1.06	2.77±0.73	1.52±0.35	1.70±0.48	0.89±0.17
		Exp		2.53±0.53	1.48±0.38	1.59±0.15	0.53±0.22

W: week; Cont: control group; Exp: experimental group; SMG: submandibular gland; SLG: sublingual gland.


500
400
300
200
100
0
Body Weight (g)
Cont.
Exp.
0W
1W
2W
4W
8W
Weeks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300
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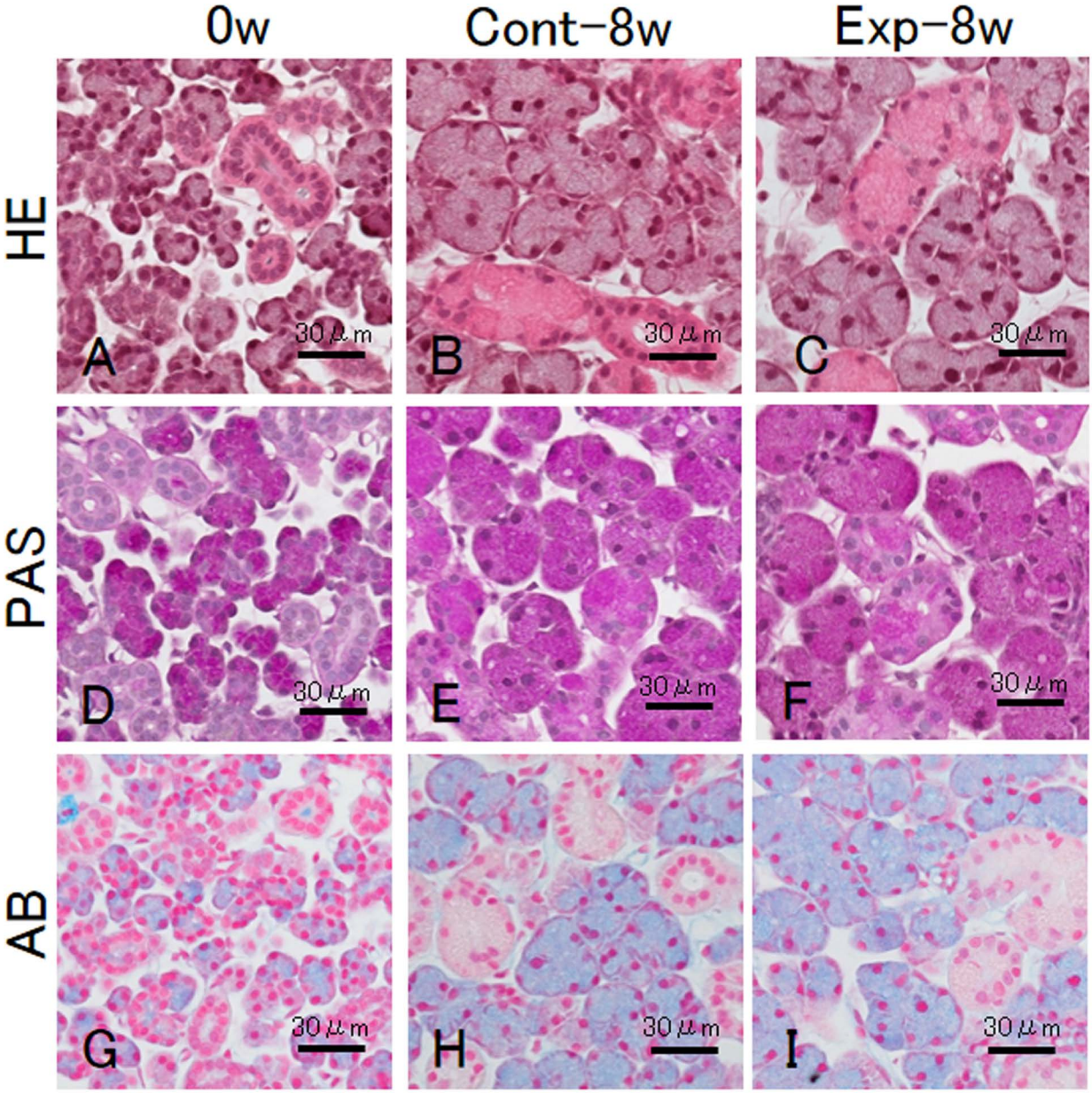
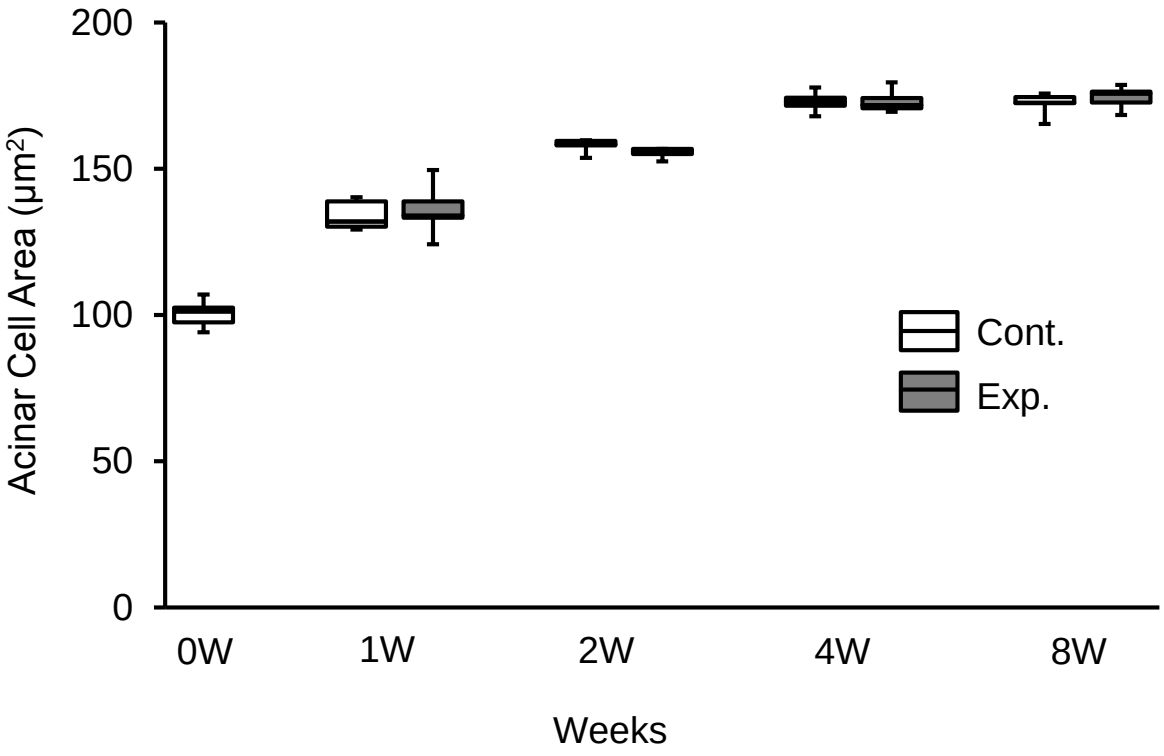
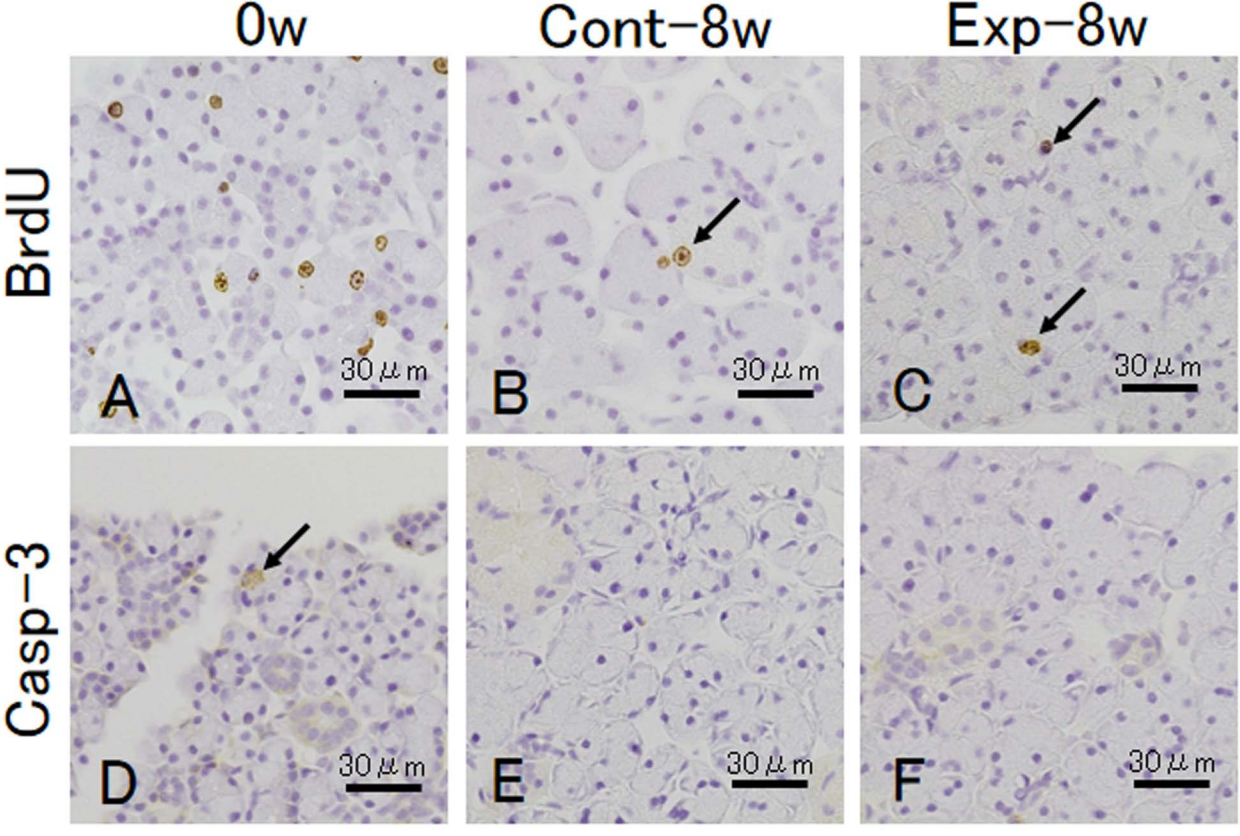
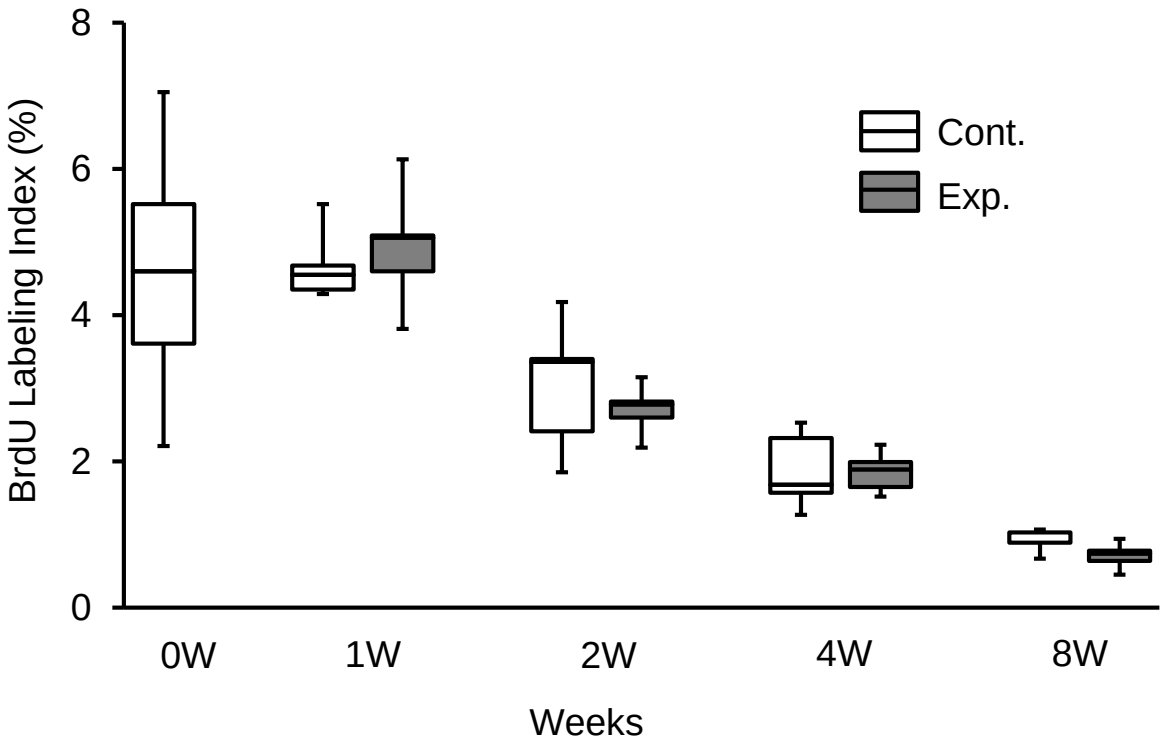
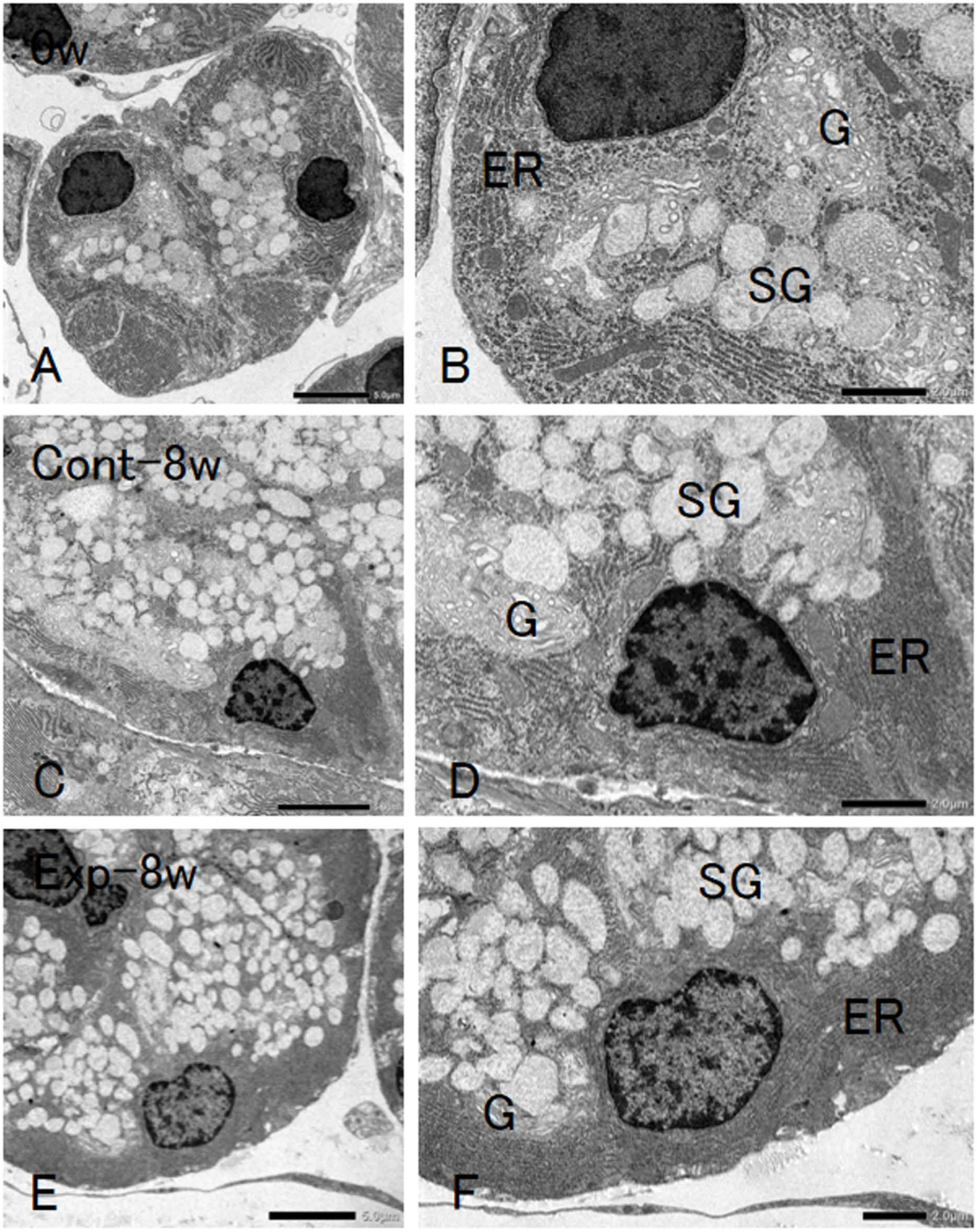
Gland Weight (mg)
Cont.
Exp.
0W
1W
2W
4W
8W
Weeks

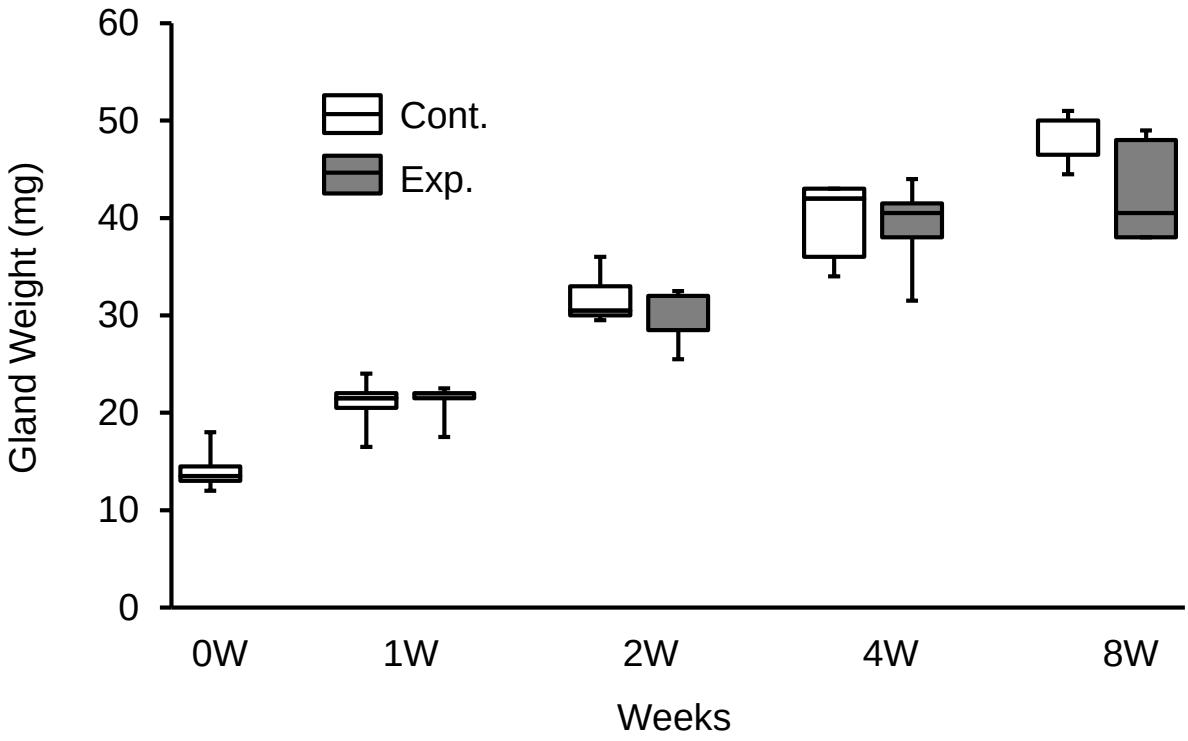

0w
Cont-8w
Exp-8w
HE
PAS
AB
A
B
C
D
E
F
G
H
I
30μm


200
150
100
50
0
Acinar Cell Area (μm²)
0W
1W
2W
4W
8W
Weeks
Cont.
Exp.


0w
Cont−8w
Exp−8w
BrdU
Casp−3
A
B
C
D
E
F
30μm


8
6
4
2
0
BrdU Labeling Index (%)
Cont.
Exp.
0W
1W
2W
4W
8W
Weeks


0w
A
ER
G
SG
B
Cont-8w
C
SG
G
ER
D
Exp-8w
E
SG
ER
G
F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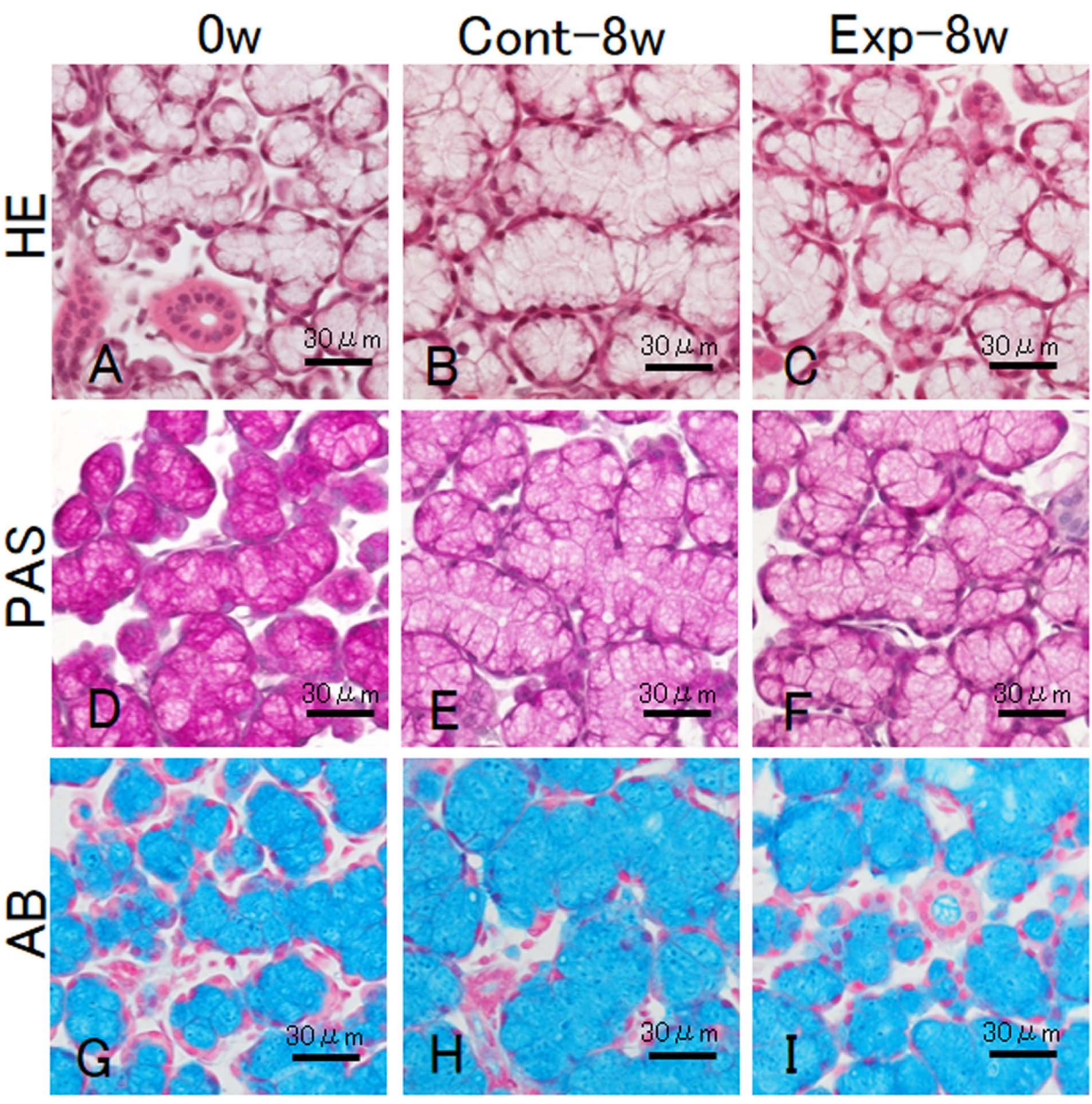
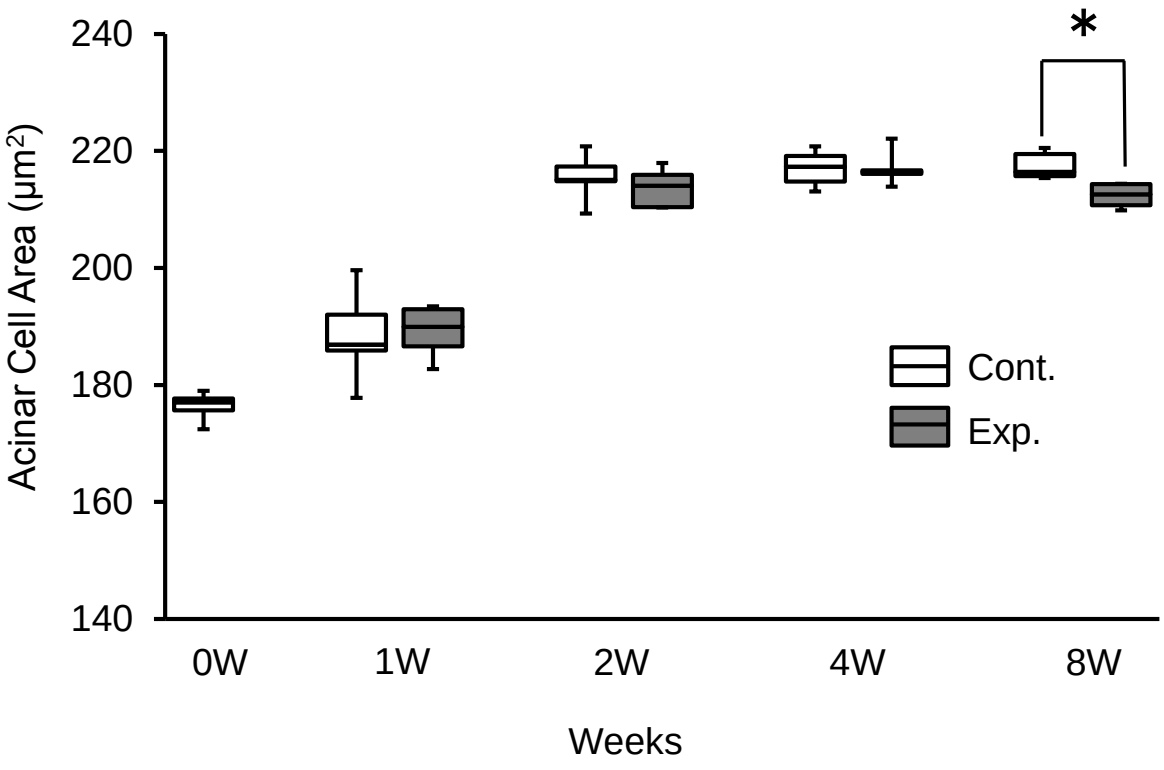
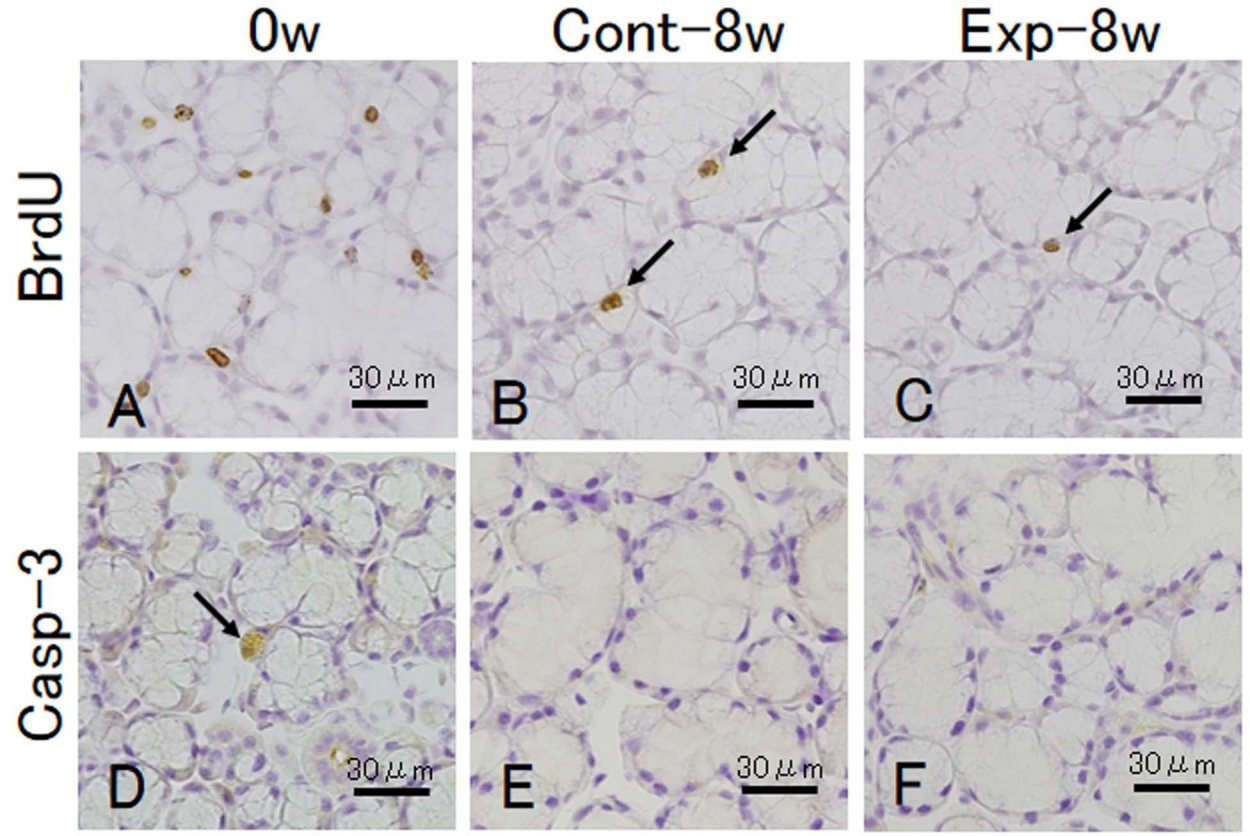
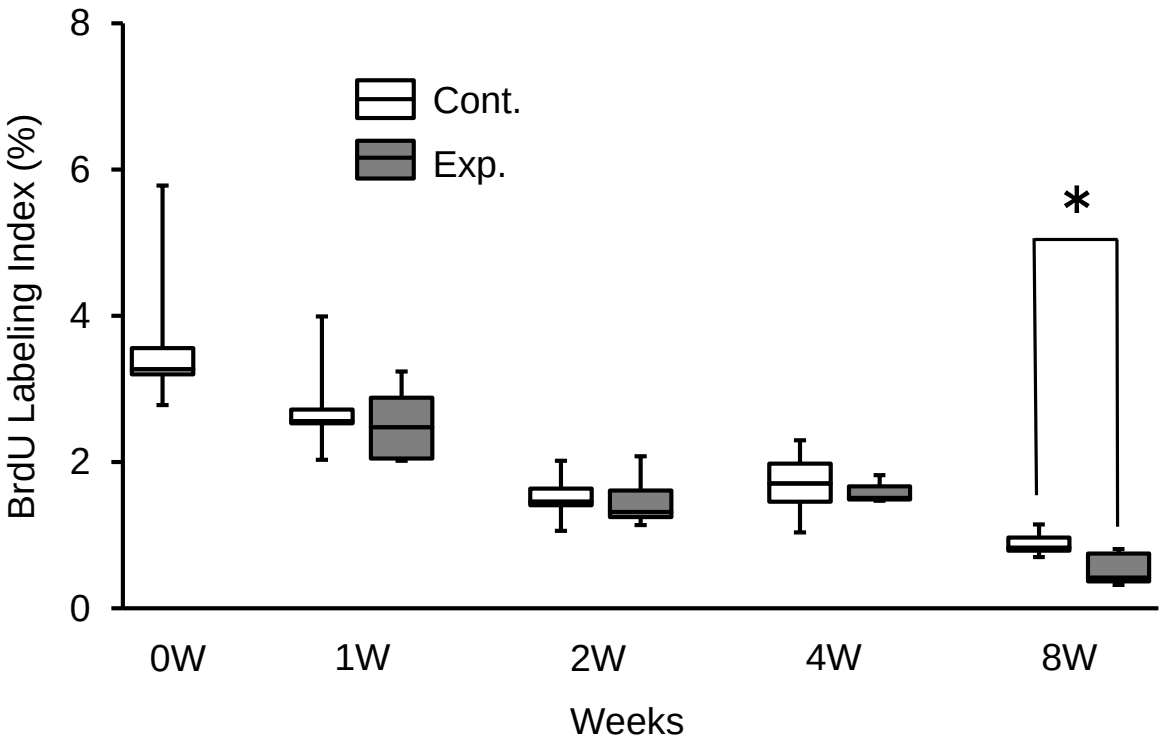
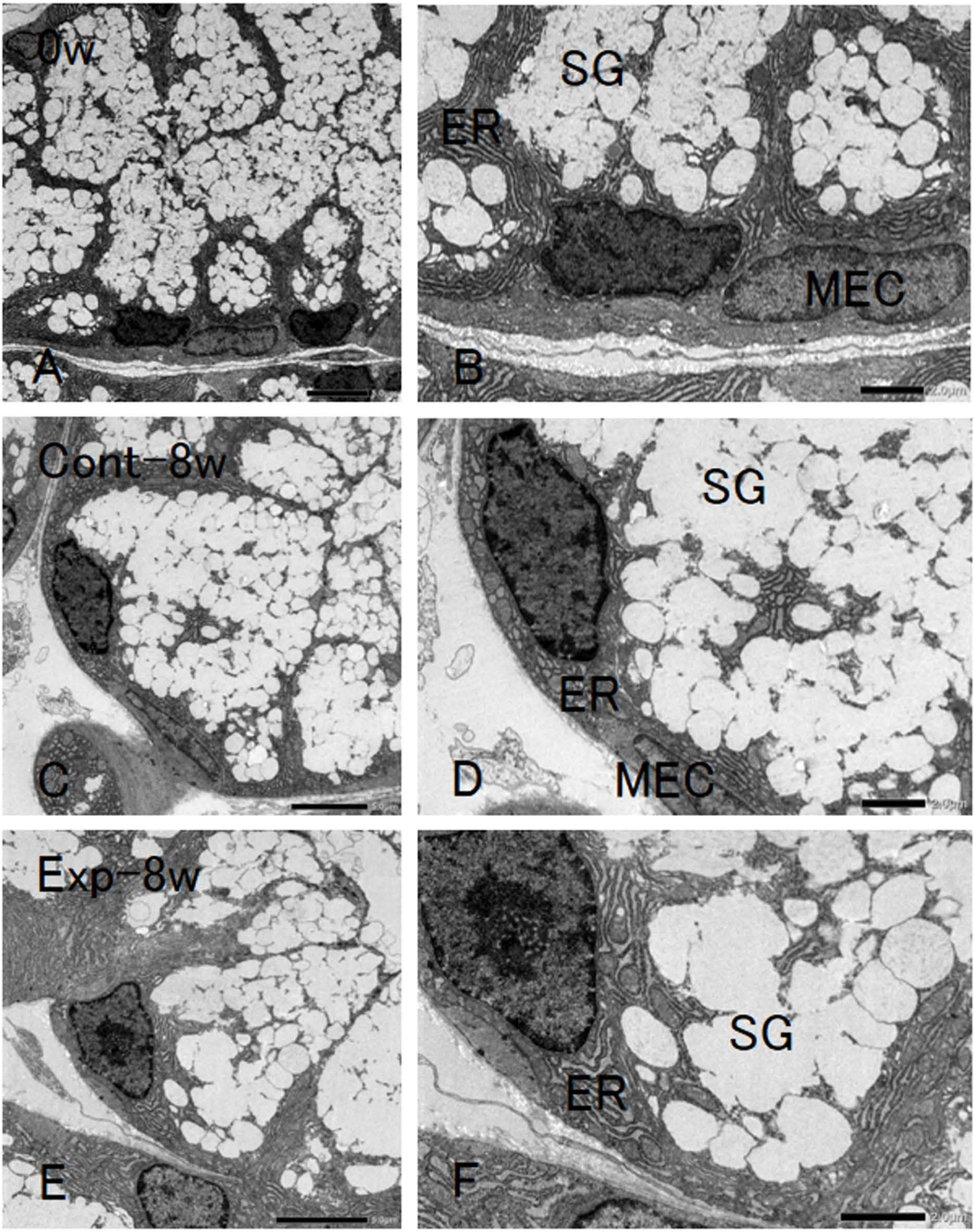
50
40
30
20
10
0
Gland Weight (mg)
Cont.
Exp.
0W
1W
2W
4W
8W
Weeks


0w
Cont-8w
Exp-8w
HE
PAS
AB
A
B
C
D
E
F
G
H
I
30μm


240
220
200
180
160
140
Acinar Cell Area (μm²)
*
Cont.
Exp.
0W
1W
2W
4W
8W
Weeks


0w
Cont−8w
Exp−8w
BrdU
Casp−3
A
B
C
D
E
F
30 μm
30 μm
30 μm
30 μm
30 μm
30 μm


8
6
4
2
0
BrdU Labeling Index (%)
Cont.
Exp.
*
0W
1W
2W
4W
8W
Weeks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SG
ER
MEC
B
Cont-8w
C
SG
ER
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D
Exp-8w
E
SG
ER
F