

Morphological study of the submandibular gland in the type 2 diabetes mellitus model rat

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Morphological studies of the submandibular glands in the spontaneous type 2 diabetes mellitus (DM) model rats (Goto-Kakizaki : GK) are currently lacking. We investigated the morphological differences in the submandibular gland of two kinds of rats : spontaneous type 2 DM male rats and normal Wistar male rats (control). A total of 18 rats were used in the study : 9 GK rats in the DM group (fasting blood glucose level, 240.9 ± 58.0 mg/dL ; HbA1c level, $5.8 \pm 0.3\%$) and 9 Wistar rats in the control group (fasting blood glucose level, 108.4 ± 39.7 mg/dL ; HbA1c level, $5.1 \pm 0.3\%$). We investigated the cross-sectional area of the serous cells ($n=3$, light microscopic specimens), the acinus diameter ($n=3$, surface morphology specimens), and the diameter of the capillaries around the acinus ($n=3$, microvasucular corrosion cast specimens). The surface morphology and microvasucular corrosion cast specimens were investigated using a scanning electron microscope. Image and statistical analysis revealed that the cross-sectional area of the serous cell, the acinus diameter, and the capillary diameter around the acinus were significantly smaller in DM rats than in normal rats. We think that hyperglycemia caused these observed atrophic changes in the serous cells, the acinus, and the diabetic microangiopathy of capillaries around the acinus in the GK rats. (J Osaka Dent Univ 2014 ; 48(1) : 1–8)

Key words : Type 2 diabetes mellitus ; Submandibular gland ; HbA1c ; Microvascular corrosion cast ; GK rat

INTRODUCTION

Previous reports have shown that acinous cells of the submandibular gland are atrophic¹ and degenerating²⁻⁵ in streptozotocin (STZ)-induced type 1 diabetes mellitus (DM) model rats.¹⁻⁵ However, to our knowledge, no morphological study of the submandibular glands has been reported in spontaneous type 2 DM rats (Goto-Kakizaki : GK).⁶ Therefore, in this study we examined the morphological differences of the serous cells, acinus, and capillaries around the acinus in the submandibular glands of GK and normal (N) rats.

MATERIALS AND METHODS

Experimental animals

A total of 18 rats were used in this study (Shimizu Laboratory Supplies, Kyoto, Japan). The DM group contained 9 male GK rats (8 weeks old ; body weight,

204.4 ± 5.3 g) and the N group contained 9 Wistar male rats (8 weeks old ; body weight, 228.9 ± 9.3 g). Three rats from each group were used for light microscopy analysis, surface morphological analysis, and microvascular corrosion cast analysis. The surface morphology and microvasucular corrosion cast specimens were investigated using a scanning electron microscope (SEM ; JSM-5500[®], JEOL, Tokyo, Japan).

This animal study was approved by the Osaka Dental University Animal Research Committee (approval numbers 10-07002, 11-03015, 12-06001, and 13-02031) and complied with the guidelines for animal experiments.

Quantification of fasting blood glucose and HbA1c levels

Nine rats from each group were fasted for 20 hours,

after which their body weights were measured. Under isoflurane inhalation anesthesia (Forane[®]; Abbott Japan, Tokyo, Japan), the rats were intraperitoneally injected with 1,000 units of heparin sodium (Novo Heparin Injection 5000[®]; Mochida Pharmaceutical, Tokyo, Japan). After 30 minutes, the rats were euthanized with an excess intraperitoneal injection of sodium pentobarbital (Nembutal[®]; Dainippon Sumitomo Pharma, Osaka, Japan). The thorax was then dissected, and blood was collected from the left ventricle using a 5-mL Terumo Syringe[®] (Terumo, Tokyo, Japan). The fasting blood glucose and the HbA1c levels were then quantified using these samples.

Fasting blood glucose levels were measured in rat serum samples with the HK-G-6-PDH pack (Quick Auto Neo GLU-HK; Sino Test, Tokyo, Japan),⁷ according to the manufacturer's instructions. Serum was obtained from the blood samples by centrifugal separation. Based on the studies by Goto and Kakizaki,⁶ the DM group consisting of 9 GK rats was expected to have fasting blood glucose levels greater than 150 mg/dL.

HbA1c levels were measured by the mean latex agglutination method (RAPIDIA[®]; AUTO HbA1c-L, Fujirebio, Tokyo, Japan),⁸ according to the National Glycohemoglobin Standardization Program guidelines. After the fasting blood glucose and HbA1c levels were measured in the 9 rats from each group, the specimens were prepared according to the methods described below.

Light microscopy analysis of specimens

In order to study the histological differences between the submandibular glands of rats, we inserted a cannula through the left ventricle into the ascending aorta and then infused physiological saline into the ascending aorta. Blood was drawn from right atrium to measure the fasting blood glucose and HbA1c levels. Physiological saline solution containing 4% (W/V) paraformaldehyde solution (Formaldehyde Solution[®]; Merck K GaA, Darmstadt, Germany) was infused into the ascending aorta. Specimens of the submandibular glands were collected and fixed by soaking in physiological saline solution containing 4% (W/V) paraformaldehyde solution at 4°C for 24 hours.

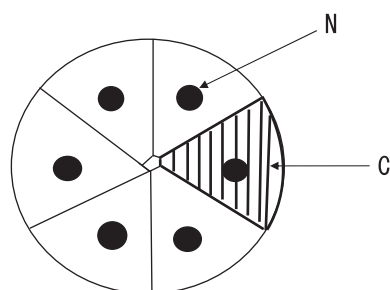


Fig. 1 Measurement of the cross-sectional area (C : shaded area) of serous cells.
N : nucleus

After washing, the specimens were dehydrated through graded alcohols. The specimens were soaked in xylene (Kishida Chemical, Osaka, Japan) at room temperature (r.t.) for 8 hours and incubated at 63°C for 6 hours in a paraffin-embedding oven (TE-HER[®]; Hirasawa Works, Tokyo, Japan).

The specimens were embedded using a paraffin-embedding machine (AP-280[®]; Carl Zeiss Japan, Tokyo, Japan) and continuous 8- μ m thick sections were sliced using a microtome (Microm HM 350[®]; Carl Zeiss). These sections were stained with a hematoxylin-eosin solution and imaged using a light microscope equipped with a digital camera (BZ-9000[®]; Keyence, Osaka, Japan). Five digital images in the middle portion of the submandibular gland were randomly selected from each group. The cross-sectional area of 10 serous cells located in the terminal regions of the submandibular glands were measured using imaging analysis software (Image-Pro Plus[®] 5.1 J; Nippon Roper, Tokyo, Japan) (Fig. 1).

Surface morphology of specimens

In order to investigate the morphological surface differences of the submandibular glands, we inserted a cannula from the left ventricle into the ascending aorta and then infused physiological saline into the ascending aorta. Physiological saline solution containing 2.5% (W/V) glutaraldehyde solution (Kishida Chemical) and 2% paraformaldehyde solution (Merck K GaA) was infused into the ascending aorta. The submandibular glands were removed and fixed by soaking them in the same solution at 4°C for 24 hours. The specimens were washed using an ultrasonic cleaner

(UT-105 HS[®]; Sharp, Osaka, Japan) and then soaked in 8 N hydrochloric acid solution (Matsuno Chemicals, Osaka, Japan) for 1.1 hours at 60°C in a water bath (Thermo Regulator; CTR-320, Iwaki, Tokyo, Japan). Specimens were then washed with 0.1 mol phosphate buffer (PB) for 2 hours and soaked with 0.2% collagenase (Sigma-Aldrich Japan, Tokyo, Japan) at 37°C for 5.5 hours. Only the capsules of the submandibular glands could be removed by the abovementioned steps.

The specimens were then washed with 0.1 M PB at r.t. for 48 hours and soaked with 1% tannic acid solution (Kishida Chemical) at r.t. for 2 hours. Next, the specimens were washed again with 0.1 M PB for 2 hours and then treated with 1% osmium tetroxide solution (Kishida Chemical) for 2 hours at r.t. in order to prepare them for conductive staining. The specimens were dehydrated through graded alcohols and soaked in t-butyl alcohol (Kishida Chemical) for 12 hours at r.t. Specimens were then frozen and dried using a freezing-drying machine (JFD-310[®]; JEOL). Each specimen was mounted on a metal stage with conductive tape (NEM Tape[®]; Nisshin EM, Tokyo, Japan) and silver paste (Dotite[®]; Fujikura Kasei, Tochigi, Japan). Digital images of the surface morphology of the submandibular gland specimens were obtained using SEM at an acceleration voltage of 5 KV and working distance of 47 mm. The diameter of 10 acinus from the surface morphology digital image was quantified using the previously mentioned software. The major and minor axes of the acinus were measured, and the mean values were defined as the acinus diameter (Fig. 2).

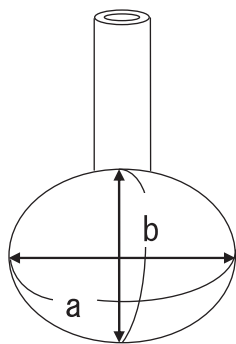


Fig. 2 Measurement of acinus diameter.
a : Major axis, b : Minor axis.

Microvascular corrosion cast specimens

In order to investigate the microvascular architecture of the submandibular gland, we inserted a cannula through the left ventricle into the ascending aorta and then infused physiological saline into the ascending aorta. The acrylic resin was prepared and injected using the methods described by Suwa *et al.*⁹ A polymerization inhibitor (hydroquinone) was contained in the commercial methyl methacrylate (Acrylic Ester M[®]; MMA, Mitsubishi Rayon, Tokyo, Japan). Hydroquinone was removed using sodium hydroxide solution.¹⁰ One liter commercial methyl methacrylate was placed in a 3-L beaker, at which 1 L of 0.5% (w/v) sodium hydroxide solution was added. This was mixed for 5 minutes using a magnetic stirrer (HVE-S; As One, Osaka, Japan) and placed in a 3-L separation funnel (Funnel Separatory Globe with Teflon Stopcock; Sanyo, Tokyo, Japan). The sodium hydroxide solution (an aqueous solution that became brown with the sodium salt of hydroquinone) and the methyl methacrylate (oil) were then separated. The brown-colored aqueous solution was removed using a separation funnel, and this process was repeated three times until the brown solution became transparent.

One liter of the remaining oil was placed in the 3-L beaker. To adjust for neutrality, 1 L of distilled water was placed in the oil and mixed for 5 minutes; this process was repeated until the pH was neutral, as determined by a pH meter (Basic pH meter; Denver Instrument, Göttingen, Germany). To remove any remaining water, 100 g anhydrous magnesium sulfate (Nacalai Tesque, Kyoto, Japan) was added to the oil, and the solution was filtered to remove anhydrous sodium sulfate. The commercial methyl methacrylate (liquid monomer), which removed the hydroquinone, was transferred to a dark glass bottle and stored at 4°C.

We prepared a low-viscosity acrylic resin by mixing polymethyl methacrylate (solid polymer; Sigma-Aldrich Japan, Tokyo, Japan) and a liquid monomer at a 1 : 9 ratio. High-viscosity acrylic resin was prepared from a 3 : 7 ratio of polymer to monomer. We added 0.5% polymerization promoter (benzoyl peroxide, Kishida Chemical), 5% plasticizer (di-n-butyl phthalate, Kishida Chemical), pigment (Cromophthal

Red; Ciba Japan, Tokyo, Japan), and 0.5% polymerization initiator (N,N-dimethylaniline, Kishida Chemical) to these mixtures to initiate polymerization. First, we injected the low-viscosity acrylic resin (45 mL, 5 mL/minute) into the ascending aorta of rats using a precision syringe pump (KDS 200; Muromachi Kikai, Tokyo, Japan). We then used the same pump to inject 5 mL of high-viscosity acrylic resin at a rate of 1 mL/minute into the aorta. The injected carcasses were allowed to polymerize at 40°C for 24 hours in a water bath.

Specimens were collected from the polymerized animals. The submandibular glands were removed, and the soft tissues were removed by soaking the specimens in 10% aqueous sodium hydroxide for 24 hours at 42°C. The specimens were then washed in an ultrasonic cleaner under running water (42°C). Each specimen was air-dried, mounted on a metal stage using silver paste and conductive tape, and coated with gold using an ion-sputtering coating device (JFC-1500; JEOL, Tokyo, Japan). Using a SEM, we obtained digital images of the microvascular architecture of the submandibular glands. Three digital images were selected from each group. We randomly selected 10 capillaries around the acinus. We used the abovementioned imaging software to measure the diameter of the capillaries of the microvascular corrosion cast specimens.

Measurements are represented as the mean and standard deviation (SD), and Student's t-test was used to test for differences between the two groups, with significance set at $p < 0.01$.

RESULTS

Fasting blood glucose levels

The fasting blood glucose level in the DM group (240.9 ± 58.0 mg/dL) was significantly higher than in the N group (108.4 ± 39.7 mg/dL) ($p < 0.01$, Fig. 3).

HbA1c levels

The HbA1c level in the DM group ($5.8 \pm 0.3\%$) was significantly higher than in the N group ($5.1 \pm 0.3\%$) ($p < 0.01$, Fig. 4).

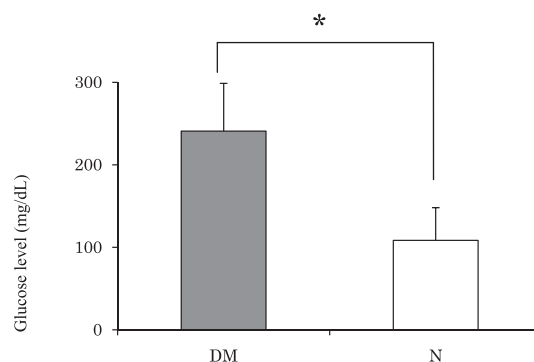


Fig. 3 Comparison of fasting blood glucose levels in the DM and N groups (* $p < 0.01$).

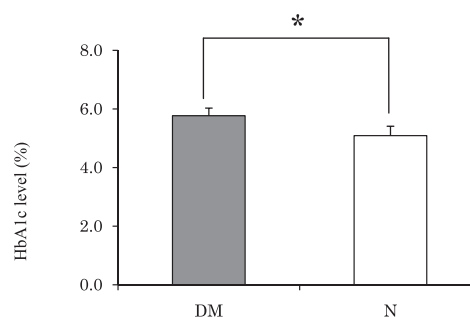


Fig. 4 Comparison of HbA1c levels in the DM and N groups (* $p < 0.01$).

Light microscopy analysis

The submandibular glands in both groups were the seromucous type that contained many serous cells, and were composed of tubuloalveolar glands. The submandibular gland was divided into terminal and duct portions. The serous and mucous cells were located in the terminal portion of the specimens, and the intercalated and striated ducts were found in the duct portions of both the DM and N groups. With regard to morphology, the serous cells in the terminal portion were sector- and polygon-shaped. The nucleus was round and was located at the center of the cell (Fig. 5 A–D).

Surface morphology

The acinous form of the submandibular gland was ellipsoid for both groups (Fig. 6 A–D).

Microvascular corrosion cast specimens

The microvascular architecture of the submandibular

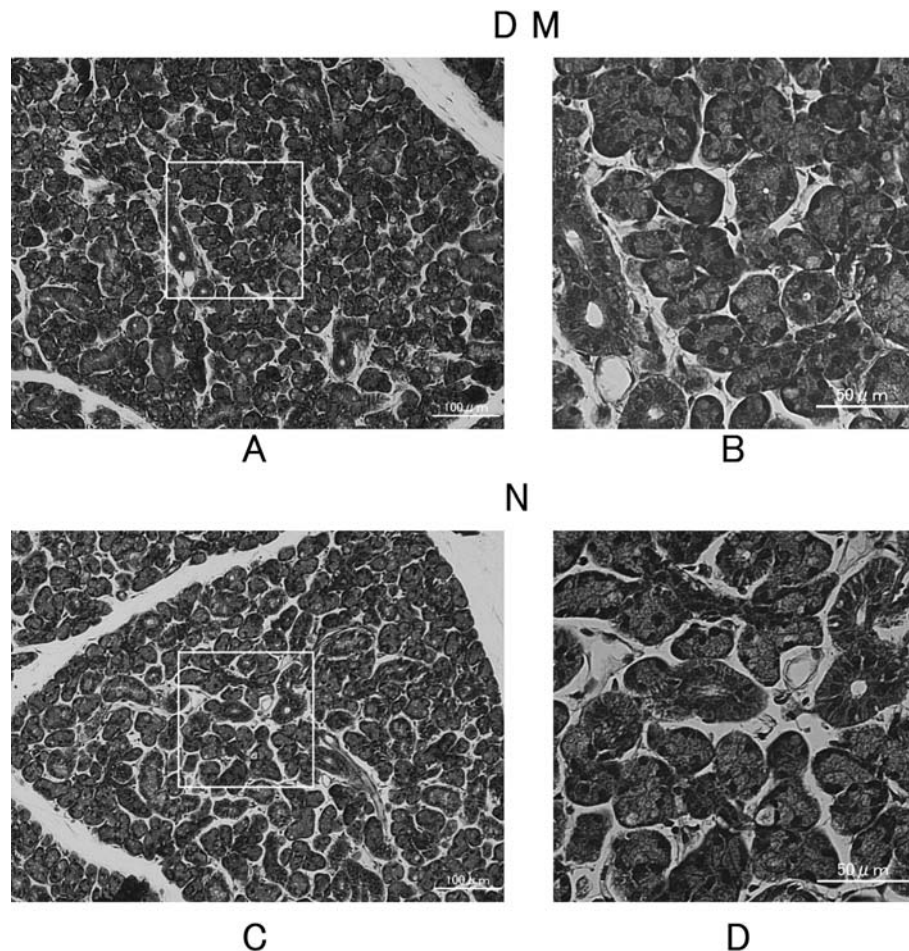


Fig. 5 Light micrographs in the DM and N groups. (A) Lower magnification in the DM group ; (B) Higher magnification of the white frame of Fig. 5 A ; (C) Lower magnification in the N group ; (D) Higher magnification of the white frame of Fig. 5 C.

gland exhibited reticulation in both the DM and N groups. Moreover, the reticulation of the capillaries in the DM group was smaller than that of the N group (Fig. 7 A–D).

Cross-sectional area of the serous cell

The cross-sectional area of the serous cells in the DM group ($47.9 \pm 11.1 \mu\text{m}^2$) was significantly smaller than in the N group ($59.3 \pm 13.8 \mu\text{m}^2$) ($p < 0.01$, Fig. 8).

Acinus diameter

The diameter of the acinus of the serous cells in the DM group ($19.4 \pm 0.2 \mu\text{m}$) was significantly smaller than in the N group ($23.6 \pm 0.9 \mu\text{m}$) ($p < 0.01$, Fig. 9).

Capillary diameter around the acinus

The capillary diameter around the acinus in the DM group ($3.1 \pm 0.8 \mu\text{m}$) was significantly smaller than in the N group ($4.3 \pm 0.9 \mu\text{m}$) ($p < 0.01$, Fig. 10).

DISCUSSION

The acinous cells of the submandibular glands are known to be atrophic¹ and undergo degenerative changes²⁻⁵ in the STZ-induced type 1 DM rat. However, in GK rats, no morphological studies of the serous cell or the acinus of the submandibular gland have been reported thus far. In the oral mucosa of the GK rats, studies have shown that hyperglycemia caused microangiopathy in the capillaries;¹¹⁻¹⁴ however, no reports on the morphology of the capillaries

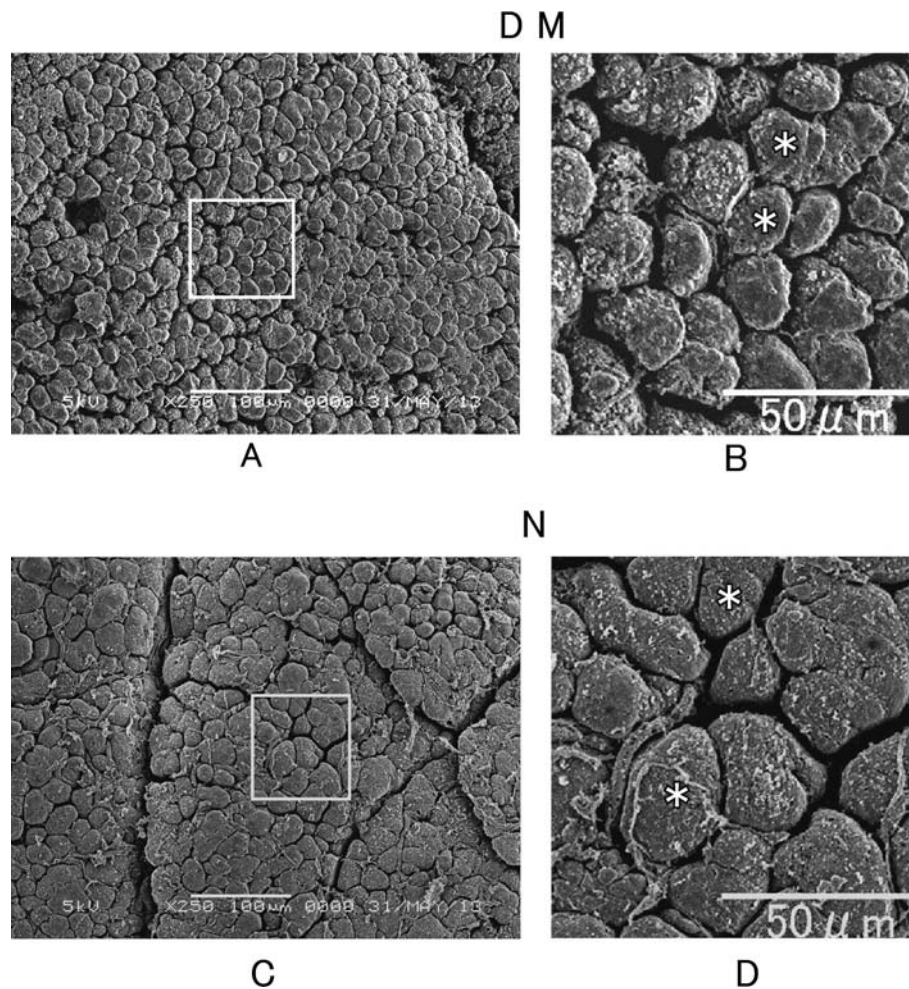


Fig. 6 Scanning electron micrographs of the surface morphology specimens in the DM and N groups (Asterisk : Acinus). (A) Lower magnification in the DM group ; (B) Higher magnification of the white frame of Fig. 6 A ; (C) Lower magnification in the N group ; (D) Higher magnification of the white frame of Fig. 6 C.

around the acinus in GK rats have been published. In this study, we investigated morphology of the serous cell, the acinus, and the capillaries around the acinus in both GK and N rats.

Light microscopy analysis

The acinus cells of the submandibular gland have been found to be atrophic in STZ-induced rats.¹ Moreover, transmission electron microscopy and histological analysis has shown that atrophic changes of the acinus in the STZ-induced rats were caused by damage to the membrane system required for cell structure maintenance, the rough-surfaced endoplasmic

reticulum, and the Golgi apparatus, resulting in a reduced number of gland cell secretory granules.¹⁵ In this study, the cross-sectional area of serous cells from the DM group was significantly smaller than that from the N group by approximately 80%. These data indicate that the atrophic changes in the acinus cells of the submandibular gland of GK rats are caused by hyperglycemia.

Surface morphology

To our knowledge, no studies have used SEM examination for STZ-induced rats and GK rats. In this study, the diameter of the acinus in the DM group was

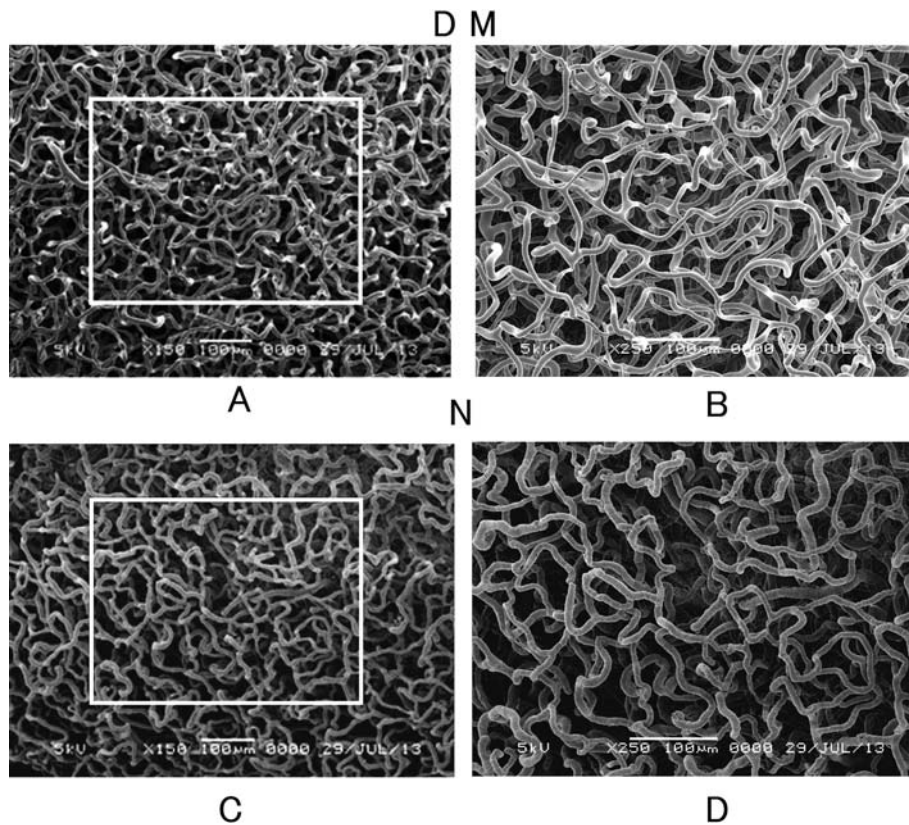


Fig. 7 Scanning electron micrographs of the microvascular corrosion cast specimens in the DM and N groups. (A) Lower magnification in the DM group ; (B) Higher magnification of the white frame of Fig. 7 A ; (C) Lower magnification in the N group ; (D) Higher magnification of the white frame of Fig. 7 C.

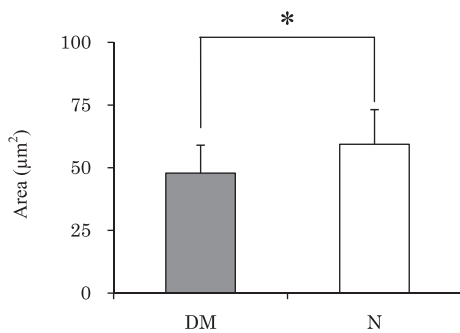


Fig. 8 Comparison of the average cross-sectional area of the serous cells in the DM and N groups (*p<0.01).

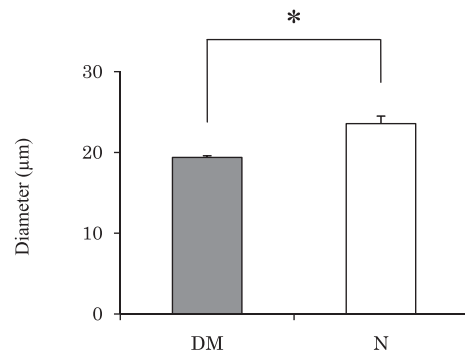


Fig. 9 Comparison of average acinus diameter in the DM and N groups (*p<0.01).

approximately 80% smaller than that of the N group, and this difference was statistically significant. Therefore, we believe that hyperglycemia caused the atrophy in the submandibular gland acinus of GK rats.

Microvascular corrosion cast specimens

Reports have shown that proliferation of vascular endothelial cells was suppressed when placed in a media containing high glucose content *in vitro*.¹⁶ Previous studies have also indicated that hyperglycemia in-

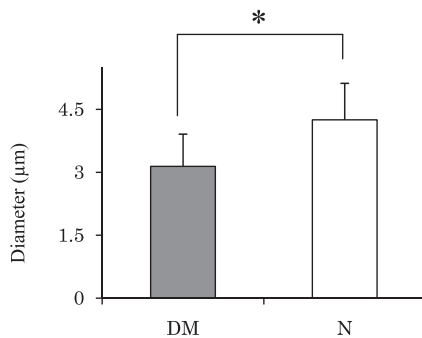


Fig. 10 Comparison of average capillary diameter in the DM and N groups (* $p < 0.01$).

duces microangiopathy in the capillary loops of the filiform papillae in the lingual dorsum,¹¹ the lingual gingiva of the mandibular first molar,¹² the palatal gingiva of the maxillary first molar,¹³ and the palatine mucosa.¹⁴ In this study, we found that the mean capillary diameter around the acinus was approximately 70% smaller in the DM group than in the N group. The mean diameter of the capillaries around the acinus of the DM group was also significantly smaller than that of the N group. This suggests that hyperglycemia causes microangiopathy around the acinus of the submandibular gland and the capillaries of the oral cavity. In addition, we believe the dimension of the acinus in the DM groups was small, because the capillary net in the DM group was smaller than that in the N group.

On the basis of these data, we conclude that the amount of the salivary secretion in the DM group was less than that in the normal group. This is because the hyperglycemia caused atrophy of the acinus cells, which resulted in diabetic microangiopathy around the acinus. However, quantification of the amount of salivary secretion with no stimulation in rats is still required, and this is an active area of investigation for our group.

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REFERENCES

- Okumura K. Light microscopical, electron microscopical and histochemical studies on the submandibular glands of streptozotocin-induced rats. *Bull Josai Dent Univ* 1987; **16**: 228–254. (Japanese, English Abstract)
- Takai N, Uchihashi K, Yoshida Y, Kakudo Y. Salivational and histological damage of submandibular and sublingual glands in streptozotocin-induced diabetic rats. *J Osaka Dent Univ* 1983; **17**: 65–72.
- Watanabe M, Yamagishi-Wang H, Kawaguchi M. Lowered susceptibility of muscarinic receptor involved in salivary secretion of streptozotocin-induced diabetic rats. *Jpn J Pharmacol* 2001; **87**: 117–124.
- Anderson LC, Suleiman AH, Garrett JR. Morphological effects of diabetes on the granular ducts and acini of the rat submandibular gland. *Microsc Res Tech* 1994; **27**: 61–70.
- Cutler LS, Pinney HE, Christian C, Russotto SB. Ultrastructural studies of the rat submandibular gland in streptozotocin induced diabetes mellitus. *Virchows Arch A Path Anat and Histol* 1979; **382**: 301–311.
- Goto Y, Kakizaki M. The spontaneous-diabetes rat: A model of noninsulin dependent diabetes mellitus. *Proc Jpn Acad* 1981; **57**: 381–384.
- Slein M. D-glucose determination with hexokinase and glucose-6-phosphate dehydrogenase. In: Bergmeyer HU, ed. *Methods of enzymatic analysis*. New York and London: Academic Press, 1963: 117–123.
- Hirata M, Takanashi N, Oka M, Tsukada Y. Application of unsensitized soap-free latex to a new assay principle for HbA_{1c} and its evaluation. *Jpn J Med Pharm Sci* 1995; **34**: 125–136. (Japanese)
- Suwa F, Uemura M, Takemura A, Toda I, Fang YR, Xu YJ, Zhang ZY. Acrylic resin injection method for blood vessel investigations. *Okajimas Folia Anat Jpn* 2013; **90**: 23–29.
- Sandler S, Karo W. *Polymer syntheses volume 1 second edition 3. Reactants and reaction conditions A. Inhibitors 3–1. General procedure for removal of phenolic inhibitors*. Boston: Academic Press, 1992: 323–324.
- Uemura M, Tamada Y, Suwa F. Morphological study of the connective tissue papillae and the capillary loops on the lingual dorsum in the type 2 diabetes mellitus model rats. *Okajimas Folia Anat Jpn* 2009; **85**: 139–149.
- Hata Y, Suwa F, Imai H, Takemura A, Ueda M. Microvasculature of the sub-sulcus epithelium in type 2 diabetic rat. *The Preliminary Program for IADR/AADR/CADR 82nd General Session 2004*; Program No.2595.
- Yasuda K, Uemura M, Suwa F. Morphological study of the palatal gingiva of the maxillary first molar in the type 2 diabetes mellitus model rat. *Okajimas Folia Anat Jpn* 2011; **88**: 65–74.
- Akai K, Uemura M, Suwa F. Morphological study of the palatine mucosa in the type 2 diabetes mellitus model rat. *J Osaka Dent Univ* 2013; **47**: 185–194.
- Tsunoda T. Effect of streptozotocin-induced diabetes on glycoprotein synthesis in the rat submandibular gland. *J Osaka Odont Soc* 1988; **51**: 275–290. (Japanese, English Abstract)
- Nelson DM, Curran EM. High glucose levels decrease proliferation of cultured human fetal cells from placenta. *Am J Obstet Gynecol* 1989; **161**: 1553–1558.