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Morphological study of subepithelial connective tissue and capillaries in the palatal gingiva of the maxillary first molar in obese type 2 diabetes mellitus model rats

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Although obesity is a lifestyle-related disease and a major risk factor for type 2 diabetes mellitus, the effects of obese type 2 diabetes mellitus (ODM) on oral health have not been fully elucidated. We compared the subepithelial connective tissue and capillary network of the palatal gingiva of the maxillary first molar in ODM model rats and normal rats. The subepithelial connective tissue of the two groups differed substantially. In particular, the cross-sectional area of the subepithelial connective tissue, the cross-sectional area and height of the connective tissue papillae, and the capillary diameter were significantly lower in the ODM rats compared with the normal animals ($p < 0.01$). In conclusion, hyperglycemia in ODM rats is associated with atrophic changes, evidenced by reduced cross-sectional area of the subepithelial connective tissue, as well as reduced cross-sectional area and height of the subepithelial connective tissue papillae. Furthermore, it causes diabetic microangiopathy in the capillary network of the palatal gingiva of the maxillary first molar in the ODM rats. (J Osaka Dent Univ 2022; 56: 151-160)

Key words: Obese type 2 diabetes mellitus; Gingival capillary; Subepithelial connective tissue; Microvascular corrosion cast

INTRODUCTION

Obesity is a lifestyle-related disease and major risk factor for type 2 diabetes mellitus (DM),[1] and it is reported that approximately 33% of the world population has a body mass index (BMI) of 25 or higher.[2] Furthermore, approximately 24% of the Japanese population has a BMI of 25 or higher.[1] In Goto-Kakizaki (GK) rats, an animal model of non-obese spontaneous type 2 DM,[3] hyperglycemia causes atrophic changes and diabetic microangiopathy in the gingiva,[4-6] tongue,[7] palate,[8] submandibular glands,[9] and articular disc of the retrodiscal tissue in the temporomandibular joint,[10] and causes delayed wound healing in the palate.[11, 12] However, no such reports are available on oral health in Spontaneously Diabetic Torii (SDT) fatty rats, an animal model of obese type 2 DM (ODM).[13] Therefore, we investigated morphological differences in the subepithelial connective tissue of the palatal gingiva of the maxillary first molar among ODM model rats and normal (N) rats.

MATERIALS AND METHODS

Experimental Animals

A total of 18 rats were used in this study; nine 8-week-old male SDT fatty rats with a body weight of 303.2±14.1 g in the ODM group, and nine 8-week-old male Sprague Dawley rats with a body weight of 261.8±36.8 g in the N group (Fig. 1 and Table 1). The animals were purchased from CLEA (Tokyo, Japan). The study was approved by the Osaka Dental University Institutional Animal Care and Use Committee (Approval Nos. 21-01003 and 21-03008).

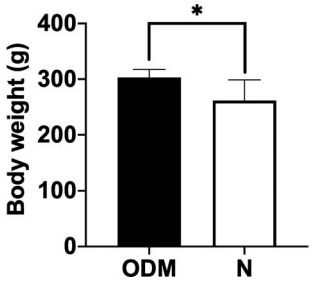

Fig. 1 Comparison of body weight in obese type 2 diabetes mellitus (ODM) and normal (N) rats (*p＜0.01).

Table 1 Comparison of body weight and serum levels of fasting blood glucose and hemoglobin A1c in obese type 2 diabetes mellitus (ODM) and normal (N) rats

Parameter	ODM	N
Body weight (g)	303.2±14.1*	261.8±36.8
Fasting blood glucose levels (mg/dL)	122.6±10.5*	72.8±15.4
Hemoglobin A1c levels (%)	5.3±0.6*	3.7±0.1

*p＜0.01 between the ODM and N groups

Methods

Measurements of fasting blood glucose (FBG) and hemoglobin A1c (HbA1c) levels

All rats were fasted for 24 h and weighed. Under inhalation anesthesia with isoflurane (Forane®; Abbott Japan, Tokyo, Japan), the rats were administered intraperitoneal injections of heparin sodium (Novo Heparin Injection 5000®, Mochida Pharmaceutical, Tokyo, Japan). After 30 min, they were euthanized by intraperitoneal overdose of barbiturates (Somnopentyl®; Kyoritsu Pharmaceutical, Tokyo, Japan) under isoflurane inhalation anesthesia. After the thorax was opened, 4 mL of blood was collected from the left ventricle using a 5-mL Terumo® syringe (Terumo, Tokyo, Japan). After centrifugation of 2 mL of the collected blood at 3000 rpm for 5 min in a high-speed cooling centrifuge (H-201 FR; Kokusan, Saitama, Japan), the serum was collected and the FBG levels were measured via the hexokinase/glucose-6-phosphate dehydrogenase method using a Quick Auto Neo GLU-HK kit (Shino Test, Tokyo, Japan).[14] The HbA1c levels of the remaining 2 mL of blood were measured via a latex agglutination-based assay (RAPIDIA® Auto HbA1c-L kit; Fujirebio, Tokyo, Japan).[15] The HbA1c levels were determined using the National Glycohemoglobin Standardization Program values. A cannula was inserted from the left ventricle into the ascending aorta to rinse blood from the right atrium using a 0.1 M phosphate buffer solution (PBS) before specimen collection for specific assays, as described below.

Collection and preparation of connective tissue specimens

Three rats from each group were used to observe the surface morphology of the subepithelial connective tissue of the palatal gingiva of the maxillary first molar. A mixture of 2.5% (w/v) glutaraldehyde solution (Kishida Chemical, Osaka, Japan) and a 5% (w/v) neutral buffer formalin solution (Sigma-Aldrich, Osaka, Japan) adjusted with 0.1 M PBS was infused into the ascending aorta. A BS-3000 diamond band saw (EXAKT, Norderstedt, Germany) was used for en bloc removal of the maxillary first molars together with the surrounding mucosa and bone. The samples were fixed by soaking in the aforementioned glutaraldehyde/formalin solution at 4°C for 24 h. Next, the samples were soaked in a 4 N sodium hydroxide solution (Fujifilm Wako Pure Chemical, Osaka, Japan) at 22-26°C for 1 h to separate the mucosal epithelium according to a previously reported method.[5] After washing with 0.1 M PBS for 1 h, the samples were soaked in a 1% (w/v) tannic acid (Fujifilm Wako Pure Chemical) solution for 2 h for pretreatment. They were then washed in 0.1 M PBS for 2 h and further washed in 0.1 M PBS using an UT-105 HS® ultrasonic washer (Sharp, Osaka, Japan) at 35°C for 5 min. The samples were subsequently dehydrated using an ascending series of ethanol concentrations. The ethanol was replaced with *t*-butyl alcohol (Kishida Chemical) for 12 h and the samples were freeze-dried using a JFD-310® freeze-dryer (Jeol, Tokyo, Japan). The samples were fixed on a metal stage using conductive NEM TAPE® (Nisshin Em, Tokyo, Japan) and DOTITE® silver paste (Fujikura Kasei, Tochigi, Japan). Finally, an osmium coating was applied to the connective tissue specimens to a thickness of 2 nm using an HPC-20® osmium

coater (Vacuum Device, Ibaraki, Japan).

Collection and preparation of histological specimens

Three rats from each group were used to observe the histological morphology of the subepithelial connective tissue of the palatal gingiva of the maxillary first molar. A 10% (w/v) neutral buffered formalin solution (Sigma-Aldrich) was infused into the ascending aorta. The bandsaw was used for en bloc tissue removal as described for the connective tissue specimens. The samples were soaked in the aforementioned formalin solution at 4°C for 24 h, followed by decalcification with a 10% (w/v) ethylenediaminetetraacetic acid disodium solution (Kishida Chemical) for 14 days using an ML-77® microwave rapid sample processor (Azumaya, Tokyo, Japan). After washing with 0.1 M PBS, they were dehydrated with an ascending series of ethanol concentrations for 24 h, followed by soaking for 4.5 h in a low-toxicity solvent (G-Nox; Genostaff, Tokyo, Japan) as a xylene substitute. The specimens were subsequently embedded in a paraffin pellet (Genostaff) using a CT-Pro 20® paraffin embedding device (Genostaff). Serial frontal sections with a thickness of 5 μm were sliced distally from the medial surface of the maxillary first molar using an HM 430® sliding microtome (Thermo Fisher Scientific, Shanghai, China). The sections for histological analysis were prepared using a conventional hematoxylin-eosin staining method.

Collection and preparation of microvascular corrosion cast specimens

Three rats from each group were used to observe the capillary network and microvascular architecture of the subepithelial connective tissue of the palatal gingiva of the maxillary first molar. An acrylic resin (low and high-viscosity) for injection into the ascending aorta was prepared according to a previously described method.[10] After ligating the descending aorta, low-viscosity acrylic resin (45 mL at 5 mL/min) was injected via a cannula into the ascending aorta using a 50-mL Terumo® syringe (Terumo) and a KDS 200 precision syringe pump (Muromachi Kikai, Tokyo, Japan). High-viscosity acrylic resin (5 mL at 1 mL/min) was subsequently injected in the same manner. Following the injection, the specimens were heated to the polymerization temperature of 40°C for 24 h in a water bath fitted with a BF 201® immersion-type constant temperature device (Yamato Scientific, Tokyo, Japan). After curing the acrylic resin, a bandsaw was used for en bloc tissue removal as described for the connective tissue specimens. The soft tissues were disintegrated by soaking in a 12% sodium hypochlorite solution (Honcho Chemical, Tokyo, Japan) at 40°C for 72 h. The specimens were then cleaned using tap water in the ultrasonic washer (40°C/5 min), dried naturally, and fixed on the metal stage using conductive tape and silver paste. Finally, the microvascular corrosion cast specimens were coated with platinum to a thickness of 4-5 nm using a JEC-3000 FC® ion-sputtering coating device (Jeol) and an EC-30020 RTS® sample rotation tilting apparatus (Jeol).

Imaging analysis and statistical processing of connective tissue specimens

In the connective tissue specimens, the subepithelial connective tissue of the palatal gingiva of the maxillary first molar was photographed from the palatal side using a JSM-5500® scanning electron microscope (SEM) (Jeol) and the obtained digital images were used for evaluation of the surface morphology of the subepithelial connective tissue.

Imaging analysis and statistical processing of histological specimens

In the histological specimens, the palatal gingiva of the maxillary first molar of the ODM and N group rats was photographed using a BZ-9000® digital light microscope (Keyence, Osaka, Japan) and ten digital images per specimen were selected for morphological evaluation of the subepithelial connective tissue. The cross-sectional area and height of the tissue were measured using Image-Pro Plus® 5.1 J image analysis software (Nippon Roper, Tokyo, Japan) (Figs. 2 A and B). The cross-sectional area (Co) of the subepithelial connective tissue in the

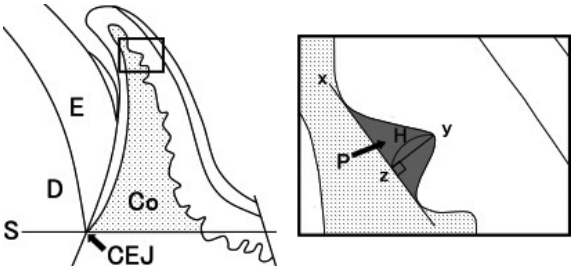

Fig. 2 Schematic illustration of the palatal gingiva of the maxillary first molar where (A) shows the dimensions of the cross-sectional area (Co) of the subepithelial connective tissue, and (B) shows a higher magnification of the black frame in (A). The cross-sectional area (P) is defined as the area bounded by line x, running tangential to the adjacent mucosal epithelial papillae. With the top of the connective tissue papillae defined as point y, (H) is the length of the perpendicular line drawn from point y to line x, and intersecting at point z. CEJ: Cementoenamel junction; D: Dentin; E: Demineralized enamel; S: Standard line.

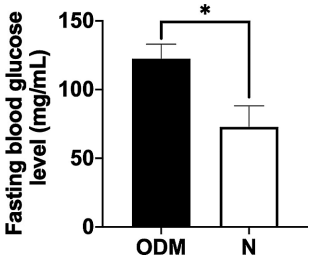

Fig. 3 Comparison of fasting blood glucose levels in the ODM and N rats (*$p<0.01$).

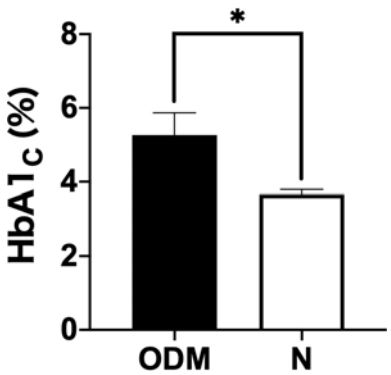

Fig. 4 Comparison of hemoglobin A1c (HbA1c) levels in the ODM and N rats (*$p<0.01$).

digital images is described in Fig. 2 A. The cross-sectional area (P) and the height (H) of the connective tissue papillae (CTPe) are described in Fig. 2 B. Ten CTPe were randomly selected from one photograph for analysis.

Imaging analysis and statistical processing of the microvascular corrosion cast specimens

In the microvascular corrosion cast specimens, the microvascular architecture of the palatal gingiva of the maxillary first molar was photographed using a SEM and digital images were obtained. Ten digital images per specimen were randomly selected from each rat to observe the morphology of the capillary network at the upper margin of the palatal gingiva of the maxillary first molar. Ten capillaries at the upper margin of the palatal gingiva of the maxillary first molar were randomly selected from each image, and the capillary diameter was measured using the image analysis software described above. The mean and standard deviation of the measured levels were calculated, and Student's *t*-test was performed for analysis of differences among the groups at a significance level of 1%.

RESULTS

Measurements of FBG and HbA1c levels

The FBG levels were significantly higher in the ODM group than in the N group (122.6 ± 10.5 mg/dL vs. 72.8 ± 15.4 mg/dL, $p<0.01$, Fig. 3 and Table 1). Similarly, the HbA1c levels were significantly higher in the ODM group than in the N group (5.3 ± 0.6 % vs. 3.7 ± 0.1 %, $p<0.01$, Fig. 4 and Table 1).

Specimen Findings

Connective tissue specimens

In the palatal gingival margin of the maxillary first molar, the CTPe in the ODM group (Fig. 5 A) had a wave-like appearance with few undulations and minimal height differences among protrusions (Fig. 5 B). In contrast, the CTPe in the N group (Fig. 5 C) had many protrusions of different heights, and a rough wave-like appearance with many undulations (Fig. 5 D).

Histological specimens

No infiltration of inflammatory cells was observed in the subepithelial connective tissue of the palatal gingiva of the maxillary first molar in either group (Figs. 6 A and C). The cross-sectional area of the

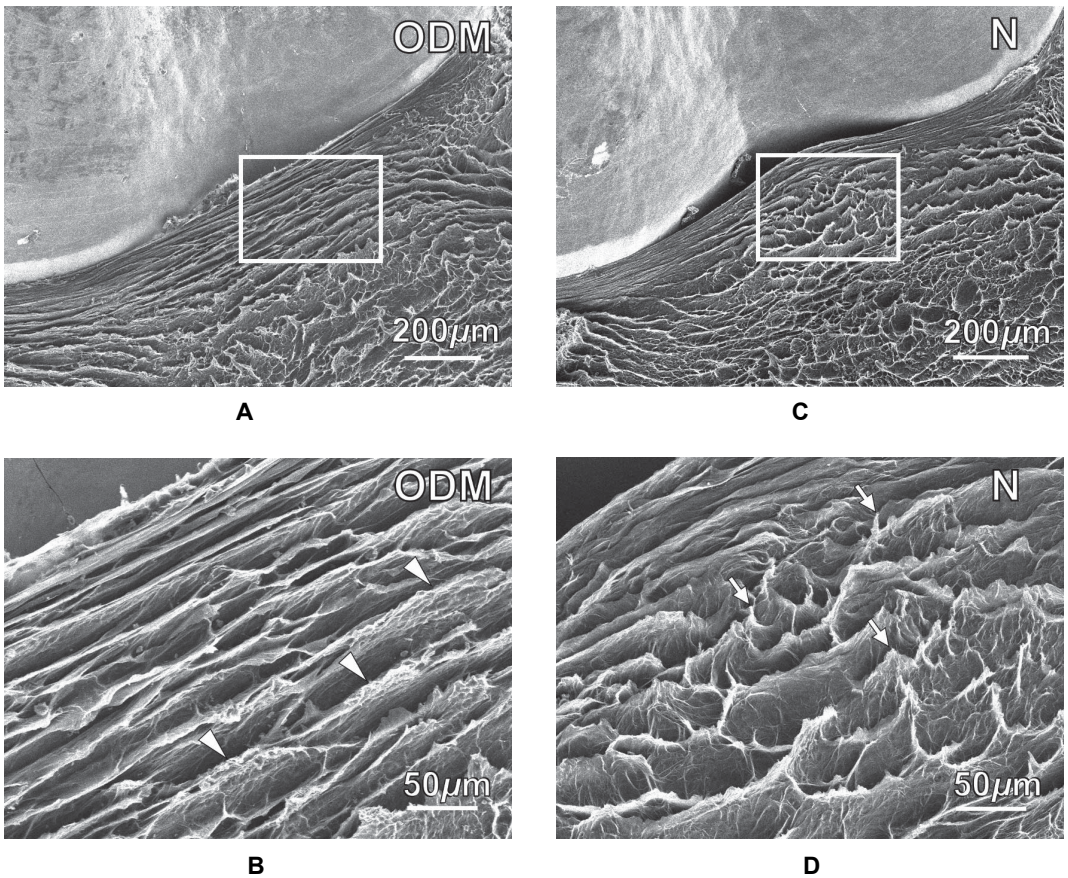

Fig. 5 Scanning electron micrographs of connective tissue specimens from ODM rats (A) and higher magnification (B) of the white frame in (A). (C) and (D) show similar low and high magnification images of connective tissue specimens from the N rats.

CTPe was small in the ODM group (Fig. 6 B) and large in the N group (Fig. 6 D). Similarly, the height of the CTPe was lower in the ODM group (Fig. 6 B), and higher in the N group (Fig. 6 D).

Microvascular corrosion cast specimens

In the ODM group, the capillaries of the superior margin ran in an undulating pattern (arrowheads in Fig. 7 A), and the shapes of the capillary networks in the palatal gingival margin of the maxillary first molar were oval (indicated by the circles in Fig. 7 A). In the N group, the capillaries of the superior margin ran in a straight pattern (arrows in Fig. 7 B), and the shapes of the capillary networks in the palatal gingival margin of the maxillary first molar were rectangular (indicated by the squares in Fig. 7 B). A schematic of the mesh structure of the capillary network at the superior margin is shown in Fig. 8.

Imaging analysis and statistical processing

Histological specimens

The cross-sectional area of the subepithelial connective tissue of the palatal gingiva of the maxillary first molar was significantly lower in the ODM group than the N group ($6.5\times10^4\pm3.1\times10^4$ μm^2 vs. $13.7\times10^4\pm4.5\times10^4$ μm^2, $p<0.01$, Fig. 9 and Table 2). The cross-sectional area of the CTPe was significantly lower in the ODM group than the N group (167.4 ± 85.9 μm^2 vs. 392.3 ± 228.9 μm^2, $p<0.01$, Fig. 10 and Table 2). The height of the CTPe was significantly lower in the ODM group than the N group (17.8 ± 7.3 μm vs. 28.7 ± 9.6 μm, $p<0.01$, Fig. 11 and Table 2).

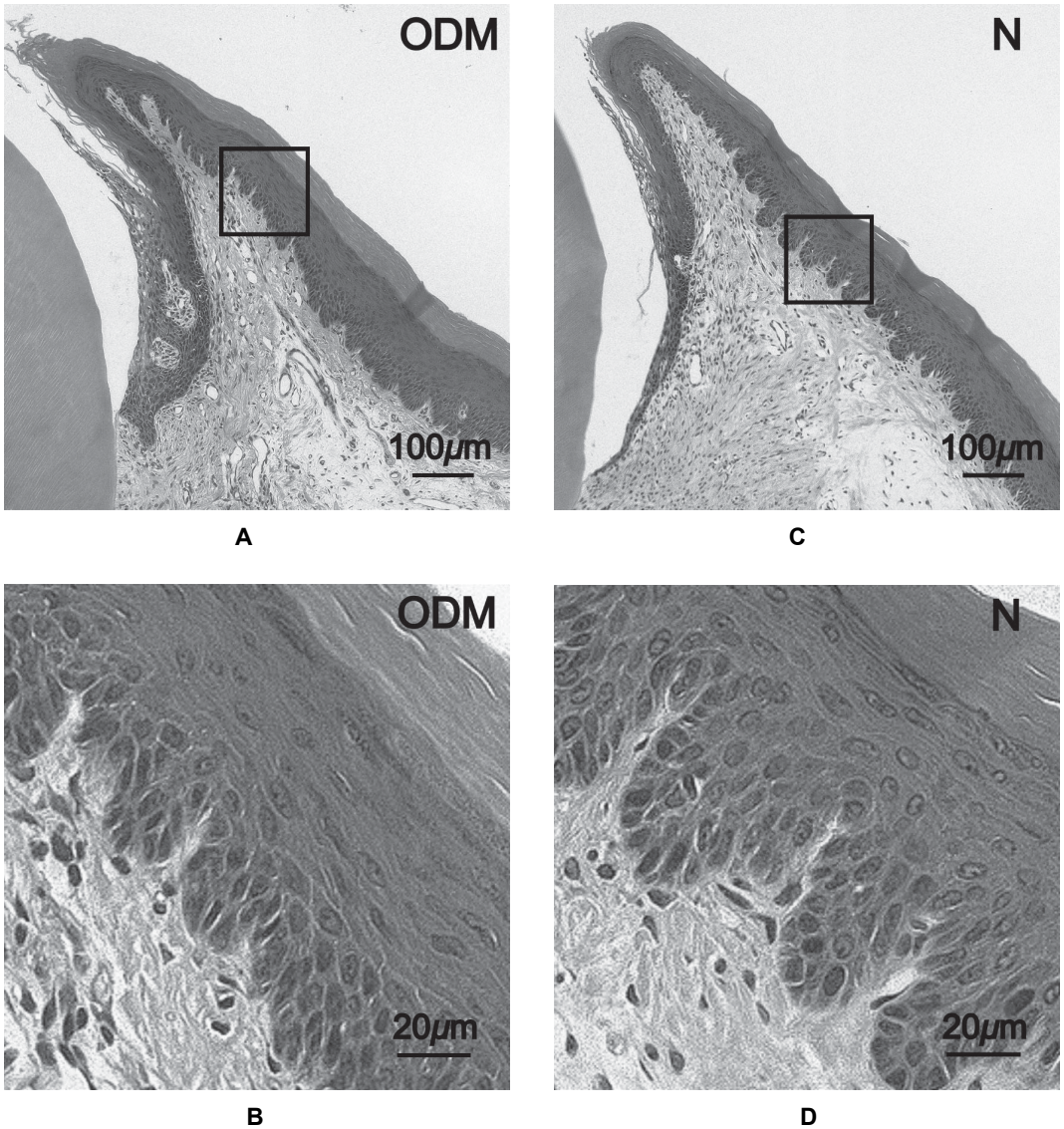

Fig. 6 Light micrographs of histological specimens from ODM rats (A) and a higher magnification (B) of the black frame area in (A). Similar low- and high-magnification and high-magnification images of histological specimens from the N rats are shown in (C) and (D).

Microvascular corrosion cast specimens

The capillary diameter of the microvascular corrosion cast specimens was significantly lower in the ODM group than the N group (4.7±0.6 μm vs. 5.4 ±0.7 μm, $p < 0.01$, Fig. 12 and Table 3).

DISCUSSION

Experimental Animals

In DM research, streptozotocin (STZ)-induced diabetic rats are studied as a model of type 1 DM. STZ destroys the β-cells of the pancreatic islets, resulting in the induction of the type 1 DM, and has also been reported to affect the kidneys and liver.[16, 17] Extremely high FBG levels ranging from 421-573 mg/dL have been reported in STZ-induced diabetic rat models.[18, 19] Comparatively lower FBG levels of 186.27±17.10 mg/dL have been reported in GK rats,[12] along with atrophic changes in the CTPe,[5-8] and diabetic microangiopathy in the capillaries of the oral organs.[6-9]

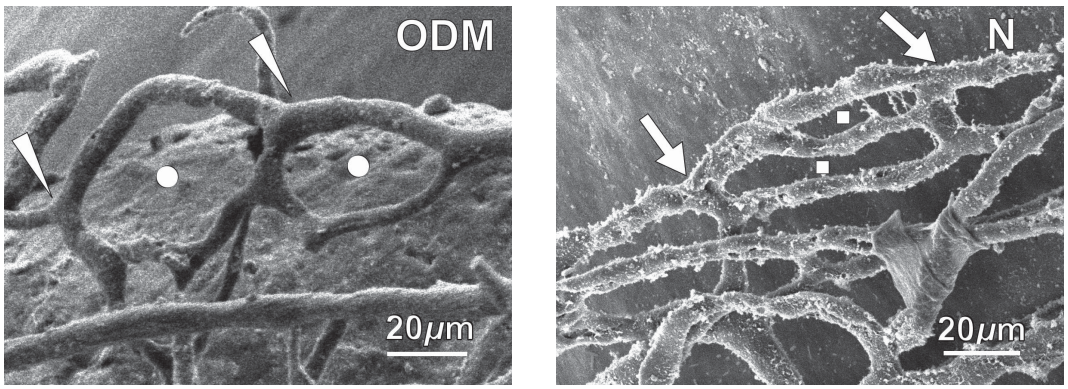

Fig. 7 Scanning electron micrographs of the microvascular corrosion cast specimens in ODM rats (A) and in normal (N) rats (B).

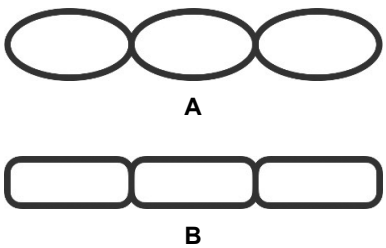

Fig. 8 Schematic of the capillary mesh structure and arrangement at the upper palatal gingival margin in ODM rats (A) and N rats (B).

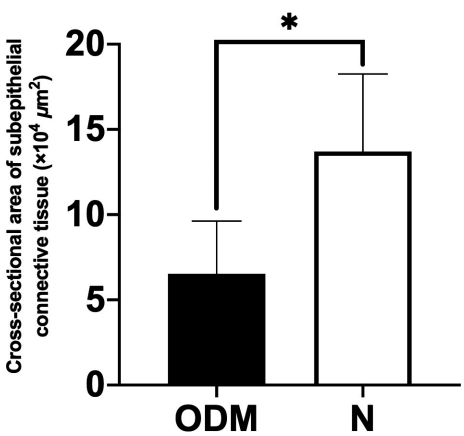

Fig. 9 Comparison of the cross-sectional area of the subepithelial connective tissue of the palatal gingiva of the maxillary first molar in ODM and N rats (*p＜0.01).

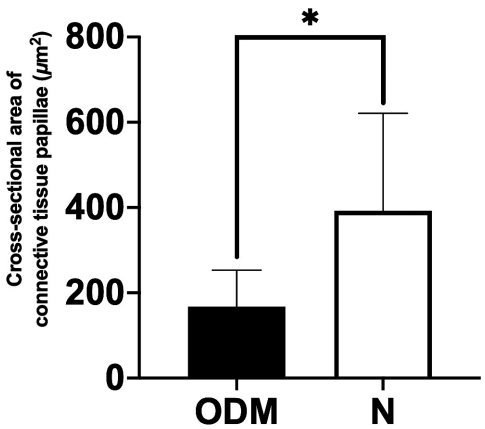

Fig. 10 Comparison of the cross-sectional area of the connective tissue papillae in the palatal gingiva of the maxillary first molar in ODM and N rats (*p＜0.01).

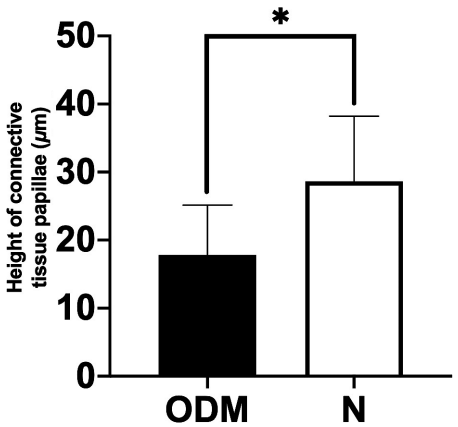

Fig. 11 Comparison of the height of the connective tissue papillae of the palatal gingiva of the maxillary first molar in ODM and N rats (*p＜0.01).

Table 2 Comparison of the cross-sectional area of the subepithelial connective tissue, the cross-sectional area of the connective tissue papillae, and the height of the connective tissue papillae in ODM and N rats

Parameter	ODM	N
Cross-sectional area (μm^2) of subepithelial connective tissue	$6.5\times10^4\pm3.1\times10^4$*	$13.7\times10^4\pm4.5\times10^4$
Cross-sectional area (μm^2) of the connective tissue papillae	167.4±85.9*	392.3±228.9
Height (μm) of the connective tissue papillae	17.8±7.3*	28.7±9.6

*p＜0.01 between the ODM and N groups

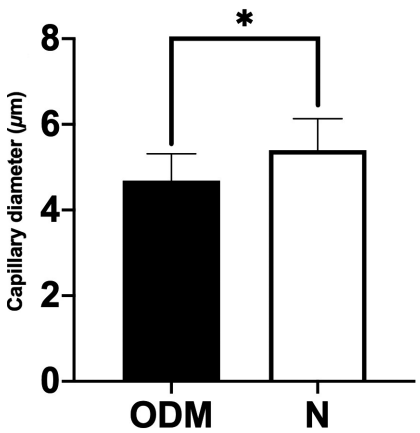

Fig. 12 Comparison of the capillary diameter in the palatal gingiva of the maxillary first molar in ODM and N rats (*p＜0.01).

Table 3 Comparison of the capillary diameter in the palatal gingiva of the maxillary first molar in the ODM and N rats

Parameter	ODM	N
Capillary diameter (μm)	4.7±0.6*	5.4±0.7

*p<0.01 between the ODM and N groups

Although previous studies have investigated metabolic disturbances in STZ-induced type 1 DM and non-obese type 2 DM animal models, the effects of co-morbid obesity on oral health in rat models with type 2 DM have not been elucidated. STD fatty rats (FBG levels: 122.6±10.5 mg/dL, $HbA1_C$ levels, 5.3±0.6 %) in this study were used as an animal model of ODM. The SDT fatty rat is a congenic rat line established by crossing SDT rats, a non-obese type 2 DM model, with the leptin receptor obesity gene (*Lepr^{fa}*) from Zucker fatty rats, an animal model of obesity.[13] SDT fatty rats exhibit obesity, hyperglycemia, and dyslipidemia; therefore, they served as a suitable animal model to investigate ODM-induced morphological differences in the subepithelial connective tissue of the palatal gingiva of the maxillary first molars. The following compares our present findings in ODM and normal rats (N) with previously reported findings in GK and Wistar rats.

Body weight

The mean body weight was significantly greater (approximately 1.2 times) in ODM rats than in normal rats. In a previous study using GK rats, the mean body weight was significantly lower (approximately 0.9 times) in the diabetic rats compared with the normal rats[9], and the total fat weight, representing the combined weight of visceral and subcutaneous fat, was approximately five times greater in six-week-old SDT fatty rats than in non-obese type 2 DM SDT rats.[20] Thus, the greater body weight of the ODM group rats compared with the normal rats in this study was probably due to the higher total fat weight associated with the obese model.

FBG and $HbA1_C$ levels

The FBG and $HbA1_C$ levels of the experimental animals in this study were significantly higher (approximately 1.7 and 1.4 times, respectively) in the ODM group than the N group. In previous experiment using GK rats, the FBG levels were significantly higher (approximately 1.5 times) in the DM group than the N group, but the $HbA1_C$ levels were no significant difference.[12] In addition, it has been reported that serum levels of tumor necrosis factor alpha (TNF-α), which inhibits insulin action, have a positive correlation with insulin resistance.[21] Thus, our observations of higher FBG and $HbA1_C$ levels in the ODM group compared with the N group may be attributed to the ODM disease model. Furthermore, insulin resistance may have increased as a

result of elevated serum levels of TNF-α.

Subepithelial connective tissue

In previous experiments using GK rats, the cross-sectional area of the subepithelial connective tissue of the palatal gingiva of the maxillary first molar in the DM group was approximately half that of the N group, representing a significant decrease, and the atrophic changes were attributed to hyperglycemia.[6] In addition, it has been reported that the proliferation of fibroblasts from GK rats is inhibited under high glucose conditions *in vitro.*[22] Our analysis of CTPe in the connective tissue specimens of ODM rats showed less pronounced height differences in the protrusions and a smoother appearance compared with specimens from normal rats. This was consistent with our histological observations of reduced cross-sectional area and height of the CTPe in the ODM group compared with the N group. Indeed, imaging analyses of the histological specimens indicated that the cross-sectional area of the subepithelial connective tissue of the palatal gingiva of the maxillary first molar in the ODM group was approximately half that of the N group (Fig. 8 and Table 2), representing a significant decrease. Furthermore, the cross-sectional area and height of the CTPe in the ODM group were approximately 40% and 60% that of the N group, respectively (Figs. 9 and 10, and Table 2).

These results suggest that hyperglycemia-induced suppression of fibroblast proliferation leading to atrophic changes may be one of the reasons for the smaller cross-sectional areas of the subepithelial connective tissue and CTPe, as well as the reduced height of CTPe, in ODM rats compared with normal rats.

Capillaries

Previous *in vitro* studies have shown that vascular endothelial cell proliferation is suppressed under hyperglycemic conditions.[23] Furthermore, studies using GK rats have reported that the capillary diameter in DM group rats was approximately 60% that of normal rats, and diabetic microangiopathy was observed in the capillaries of the palatal gingiva of the maxillary first molar.[6] In this study, the capillary diameter in the palatal gingiva of the maxillary first molar in the ODM group was approximately 90% that of the N group, representing a significant reduction. Our observation of reduced capillary diameter in ODM rats is in accordance with similar observations in previous studies using GK rats, and may be attributed to hyperglycemia-induced suppression of vascular endothelial cell proliferation, resulting in characteristic diabetic microvascular disorders.

These findings suggest that the capillaries in the palatal gingiva of patients with ODM may undergo atrophic changes similar to those in subepithelial connective tissue, and that oral health should be monitored carefully in these patients. The results of this study suggest that ODM is associated with above-normal body weight and elevated serum levels of FBG and $HbA1_C$. Our analyses of connective tissue specimens and histological specimens from ODM and normal rats indicated hyperglycemia-induced atrophic changes and a significant reduction in the cross-sectional area of the subepithelial connective tissue of ODM rats, as well as significant reductions in the cross-sectional area and height of CTPe in the palatal gingiva of the maxillary first molars of these rats. Furthermore, in the ODM group, the subepithelial capillary networks were oval-shaped and hyperglycemia caused diabetic microangiopathy, evidenced by thinning of the capillaries.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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