

Original Article

# Hyperglycemia simultaneously induces initial caries development and enhances spontaneous occlusal surface wear in molar teeth related to parotid gland disorder in alloxan-induced diabetic rats

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**Abstract:** Diabetes and salivary gland dysfunction are major factors that induce dental caries in experimental animals, but there are no reports analyzing the association of dental caries and salivary glands in an animal model of diabetes mellitus (DM). To clarify the initial development of dental caries and preceding salivary gland disorder, we performed a histopathological analysis on teeth and salivary glands in diabetic Wistar rats 7 weeks after alloxan treatment (DM group) in comparison with nondiabetic rats (Non-DM group) and functional analysis on saliva secretion during the experimental period. Pilocarpine-induced salivary fluid secretion in diabetic rats gradually decreased with continuous hyperglycemia from immediately after alloxan treatment to the time of autopsy. Histopathologically, Oil Red O-positive lipid droplets accumulated in the acinar cells of the parotid gland. No tooth was stereoscopically defined as having dental caries in any of the rats in either group; however, the external appearance remarkably changed owing to occlusal wear in almost all molars in the DM group. The initial lesions of dental caries, appearing as micro-defects in dentin with bacterial colonization on the molar surface, were identified using histopathological analysis, and the incidence in the DM group was more than twice that in the Non-DM group. In conclusion, hyperglycemia simultaneously induces initial caries development and enhances spontaneous occlusal wear in molar teeth of Wistar rats 7 weeks after alloxan treatment. The parotid gland dysfunction caused by hyperglycemia may be mostly involved in the pathogenesis of occlusal wear as well as in dental caries in this diabetic model. (DOI: 10.1293/tox.2016-0054; J Toxicol Pathol 2017; 30: 47–55)

**Key words:** diabetes, hyperglycemia, dental caries, hyposalivation, occlusal wear, rat

## Introduction

Dental caries occur in the presence of pathogenic bacteria and sugar in a predisposing host<sup>1</sup>. Thus far, since the onset of naturally occurring dental caries has not been reported in rodent models, the researchers have used a cariogenic diet containing large amounts of sugar and/or an inoculation of infectious cariogenic bacteria into the oral cavity to induce dental caries. Recently, we reported the development of dental caries in a caries-susceptible strain fed a noncariogenic diet with a low sugar concentration<sup>2</sup>. In addition, we clarified that diabetes is an important factor

inducing dental caries under the given noncariogenic diet<sup>3,4</sup>.

Salivary gland dysfunction in Sjögren's syndrome and radiation therapy are the major factors causing onset of dental caries in humans and experimental animals<sup>5–7</sup>. Hyposalivation and morphological abnormality of the salivary gland also occur in diabetic patients and animals<sup>8–12</sup>. Diabetes may be significantly associated with salivary gland dysfunction. Thus, the development of dental caries in a diabetic animal model may be correlated with salivary gland disorder; however, there are no reports sufficiently analyzing the relationship between dental caries and salivary gland disorder. To test this hypothesis, the development of dental caries and salivary gland disorder should be simultaneously analyzed in the same diabetic animals.

Alloxan (AL) is one of the chemicals that induce loss of pancreatic  $\beta$ -cells and cause a severe hyperglycemic condition, thereby inducing diabetes mellitus in animals. In AL-treated rats, progressive molar caries with collapse of the dental crown were observed after a 13-week hyperglycemic period<sup>13</sup>. Hyposalivation and morphological abnormality of the salivary gland were also reported after AL

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treatment<sup>14–16</sup>. However, to our knowledge, the association between dental caries and salivary gland disorders has not been investigated in AL-induced diabetic models or in other animal models of diabetes.

Although severe caries uniformly develop in rats at 13 weeks after the onset of hyperglycemia induced by AL treatment, there are no lesions present in the initial 4 weeks following induction of hyperglycemia<sup>13</sup>. Therefore, primary dental caries are expected to develop between 4 and 13 weeks after AL treatment in this model. In addition, we hypothesized that the salivary gland abnormalities in AL-treated diabetic rats might precede dental caries formation at an earlier stage. Thus, in this study, onset of the dental caries induced by AL treatment was estimated at 7 weeks (nearly half of 13 weeks) after hyperglycemia, and the autopsy point was set at this time. To this end, we examined the morphology of the teeth and salivary glands and performed serial measurements for evaluating salivary secretion ability; we also clarified the pathogenetic relationship between the dental caries development and salivary gland dysfunction in this diabetic model.

## Materials and Methods

### Animals and housing conditions

The rats were handled according to the principles outlined in the Guide for the Care and Use of Laboratory Animals by the Committee for Animal Experiments of Hiroshima International University and the Japanese Association for Laboratory Animal Science. Male Wistar (Crj:WI) rat pups born to female SPF Wistar rats supplied by Charles River Laboratories Japan, Inc. (Yokohama, Japan), were used in this study. They were reared in a barrier-sustained animal room maintained at a temperature of  $23 \pm 2^\circ\text{C}$  and a relative humidity of  $55 \pm 10\%$  with 12-h light/dark cycles and ventilation at least 10 times/h with high-efficiency particulate air-filtered fresh air. All rats were housed and reared in fiber-reinforced plastic (FRP) cages with a stainless (SUS304) wire-mesh floor. Rats had free access to tap water and a widely used noncariogenic standard pelletized diet for experimental mice and rats (CLEA Rodent Diet CE-2, CLEA Japan, Inc., Tokyo, Japan).

### Experimental design

The experimental design is shown in Fig. 1. A total of 23 rats were randomly divided into 2 groups: diabetic rats (DM group;  $n=13$ ) and nondiabetic rats (Non-DM group;  $n=10$ ) at 7 weeks of age. In the DM group, a single dose of 50 mg/kg of AL (Sigma-Aldrich Japan, Tokyo, Japan) was administered via the tail vein, whereas the rats in the Non-DM group were administered a single dose of 0.9% saline. All of the rats were euthanized at 14 weeks of age, and their teeth and salivary glands were histopathologically examined.

### Glucosuria and glycemia monitoring

Urinary glucose levels were measured semiquantitatively with a urine test paper (Wako Pure Chemical Indus-

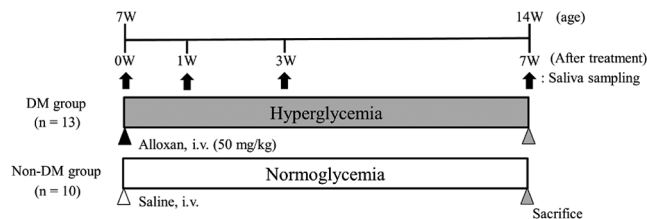


Fig. 1. Study design.

tries, Osaka, Japan) using fresh urine. Blood glucose levels were also measured semiquantitatively by the glucose oxidase method (Glutest PRO R, Sanwa Kagaku Kenkyusho, Aichi, Japan) using non-fasting blood samples collected from the tail vein. Urinary and blood glucose levels were measured at 0, 1, 3 and 7 weeks after AL treatment. Samples of blood from the tail vein and fresh urine were collected between 13:00 and 16:00. Hyperglycemia and glucosuria were defined as glucose levels greater than 200 mg/dL and 250 mg/dL, respectively.

### Measurement of food intake

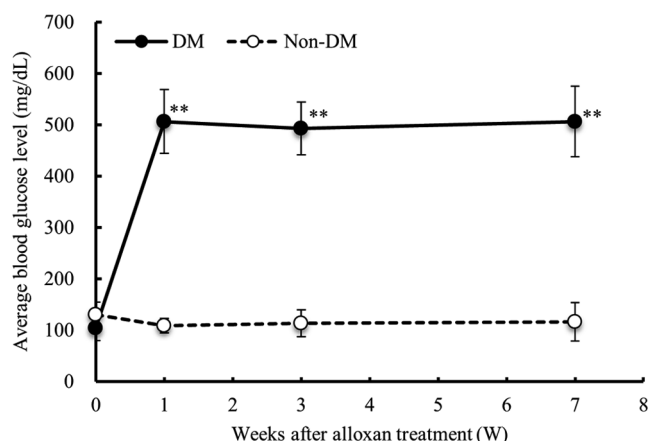
Food intake over a 24-hour period was measured using rats from each group (DM group,  $n=8$ ; Non-DM group,  $n=5$ ) at 0, 1, 3, and 7 weeks after AL treatment.

### Saliva collection

Saliva secretion was induced by subcutaneous injection of pilocarpine (2 mg/kg body weight). The saliva was collected using cotton balls between 3 to 8 min after pilocarpine injection under deep anesthesia with pentobarbital (25 mg/kg body weight, i.p.). The total weight of the secreted saliva (difference in the weight of the cotton balls before and after collection) was measured at 0, 1, 3, and 7 weeks after AL treatment.

### Stereoscopic and soft X-ray examination of the mandibular and maxillary molars

At the end of study period, the animals were euthanized by exsanguination from the abdominal aorta under deep anesthesia induced using isoflurane. Subsequently, the mandible and maxilla were removed and fixed in 10% neutral buffered formalin solution (pH 7.4). After a 24-h fixation, the occlusal, buccolingual, and proximal surfaces of all teeth were examined under a binocular stereoscope. The stereoscopic change scores of each molar obtained using a binocular stereoscope were classified into 4 grades: grade 0, no change in the molar tooth; grade 1, mild wear of the cuspis dentis; grade 2, moderate wear of the cuspis dentis; grade 3, severe wear of the cuspis dentis. Following stereoscopic examination, a soft X-ray examination was performed, and images of the mesiodistal plane were obtained at 35 kV and 2 mA for 4 min. The cusp height of the all molars on soft X-ray images was measured using the scale function in Adobe Photoshop Elements 9 (Adobe Systems, San Jose, CA, USA).



**Fig. 2.** The blood glucose changes in the DM and Non-DM groups. Severe hyperglycemia (>400 mg/dL) persisted from the day after alloxan treatment in the DM group. The blood glucose level in the Non-DM group was consistently <200 mg/dL. The data are shown as the mean  $\pm$  SD. Significant difference compared with the Non-DM group (\*\* $P$ <0.01).

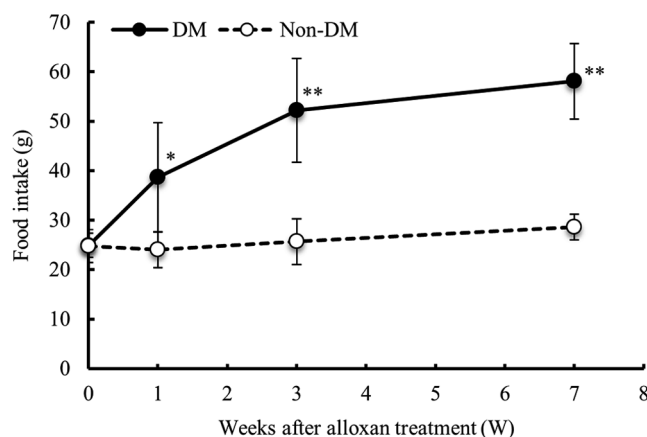
### Histopathological examination

For histopathological examination, the jaw, salivary glands, and tongue were removed and fixed in 10% neutral buffered formalin solution (pH 7.4). After formalin fixation for 24-h, the parotid gland, submandibular gland, and tongue, including the von Ebner's glands, were trimmed, dehydrated in a sequential ethanol series by using an automated processor, and embedded in paraffin. The mandible and maxilla were decalcified in K-CX (Falma Co., Ltd., Tokyo, Japan) for 24 h and immersed in a 1:1 mixture of 10% formic acid solution and neutral buffered formalin solution for 5 days after formalin fixation. After decalcification, the specimens were trimmed, dehydrated, and embedded in paraffin. These paraffin tissue sections (5- $\mu$ m thick) were stained with hematoxylin/eosin. The vacuolation scores of sections of each salivary gland were classified into 5 grades: grade 0, no or rare vacuoles; grade 1, small vacuoles present in less than 50% of the total gland; grade 2, small vacuoles present in more than 50% of the total gland; grade 3, in addition to the small vacuoles, large vacuoles present in less than 50% of the total gland; grade 4, large vacuoles present in more than 50% of the total gland.

To determine the contents of the vacuoles observed within the salivary glands, a part of the left parotid gland and a part of the tongue, including the von Ebner's glands, were embedded in OCT Compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan) for preparing frozen tissue specimens, and these frozen tissue sections (10- $\mu$ m thick) were stained with Oil Red O.

### Statistical analysis

The differences between the groups in mean blood glucose levels, body weight, food intake, saliva weight, and cusp height were determined using a Student's *t*-test or Welch's *t*-test. The chi-square test was used to compare the



**Fig. 3.** The food intake changes in the DM and Non-DM groups. The data are shown as the mean  $\pm$  SD. Significant difference compared with the Non-DM group (\* $P$ <0.05; \*\* $P$ <0.01).

incidence of macroscopic and histopathologic changes in the molars. The Mann-Whitney test was used to compare the mean lesion scores of the salivary glands between the groups.  $P$ <0.05 was regarded as statistically significant.

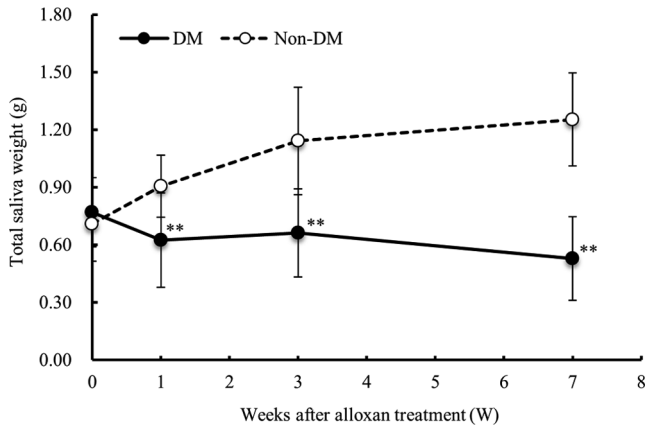
## Results

### Glucosuria, glycemia, and general condition monitoring

The mean blood glucose levels of each group are shown in Fig. 2. All rats in the DM group showed severe hyperglycemia (>400 mg/dL) and glucosuria (>2,000 mg/dL) from the day after AL injection till the end of the study. In contrast, glucosuria was not observed in any rat in the Non-DM group, and normoglycemia persisted throughout the study (Fig. 2). The mean body weight in the DM group decreased over time after AL treatment and was significantly lower than that in the Non-DM group at 3 and 7 weeks following treatment. However, the mean food intake after AL treatment in the DM group was significantly higher than that in the Non-DM group at each time point and at the final time point was more than twice that of the Non-DM group (Fig. 3). All of the rats used in this study survived until the end of the experimental period.

### Weight of secreted saliva

The changes in the mean weights of pilocarpine-induced saliva in each group are shown in Fig. 4. The mean weight of secreted saliva after AL treatment in the DM group was significantly lower than that in the Non-DM group at each time point, and the final weight at 7 weeks after AL treatment was less than 50% of the weight in the Non-DM group. In the Non-DM group, the mean total saliva weight at 0, 1, 3, and 7 weeks after treatment was 0.71, 0.91, 1.14, and 1.25 g, respectively, and showed a gradual increase over time. In contrast, a decreasing trend (0.77, 0.62, 0.66, and 0.53 g at 0, 1, 3 and 7 weeks, respectively) was observed



**Fig. 4.** The changes in total saliva weight in the DM and Non-DM groups. The data are shown as the mean  $\pm$  SD. Significant difference compared with the Non-DM group (\*\* $P < 0.01$ ).

in the DM group; the values after AL treatment were also significantly lower than those in the Non-DM group at each time point ( $P < 0.01$ ).

#### Macroscopic morphological examination by stereoscope and soft X-ray film

In this study, the typical manifestations of dental caries, such as collapse of the molar crown or a hole in the tooth surface with bacterial attachment, were not stereoscopically observed in any rats in the DM and Non-DM groups. Thus, no tooth was identified as having dental caries after stereoscopic morphological examination. Apart from the development of dental caries, external changes due to occlusal surface wear were apparent in almost all the teeth in the DM group, and the stereoscopic changes (maxilla, 98.7%; mandible, 97.4%) in the DM group were significantly more common than those in the Non-DM group (maxilla, 15.0%; mandible, 30.0%) (Table 1). Furthermore, these changes were remarkably enhanced in the DM group (Grade 1, 46.1–56.4%; Grade 2, 38.5–43.6%; Grade 3, 3.8–7.7%) compared with the Non-DM group (Grade 1, 15.0–30.0%; Grade 2, 0%; Grade 3, 0%) (Table 1, Fig. 5).

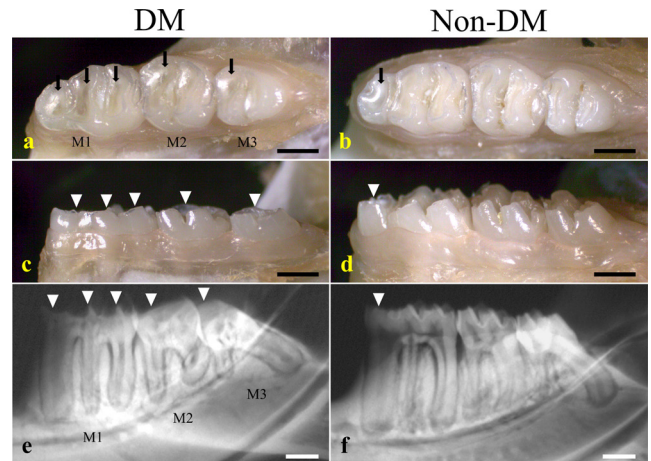
Soft X-ray examination showed wear of the enamel and dentin on the occlusal surface at the cusp, which was classified as a Grade-3 stereoscopic change under a microscope (Fig. 5e).

#### Morphometric examination on occlusal surface wear of molar teeth

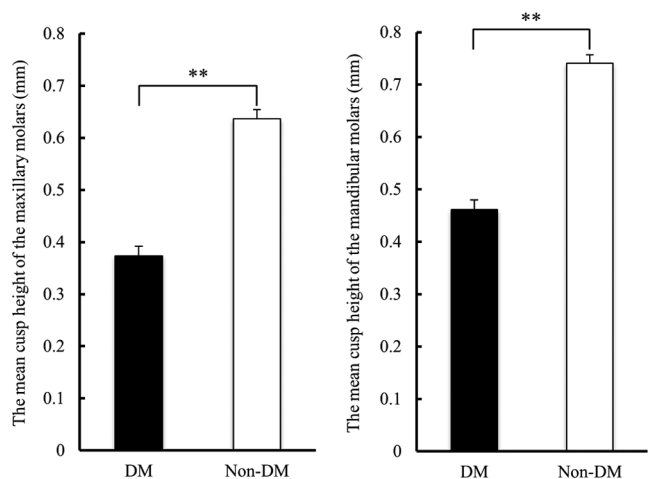
The mean cusp heights of the maxillary and mandibular molars on the soft X-ray images are shown in Fig. 6. The mean cusp height in the DM group was approximately half that of the Non-DM group, and the difference between them was significant ( $P < 0.01$ ) (Fig. 6).

#### Histopathological examination

Mild dental caries that were not evident on stereoscopic examination could be identified on histological exami-



**Fig. 5.** Stereoscopic and soft X-ray images of the molars in the DM and Non-DM groups. M1, the 1st molar; M2, the 2nd molar; and M3, the 3rd molar. (a, b) Stereoscopic features of the overhead view of the mandibular molars in the DM group (a) and the Non-DM group (b). (a) Obvious occlusal wear is observed on the occlusal surface (arrows). (b) Mild occlusal wear is observed on a part of the occlusal surface of the 1st molar (arrow). (c, d) Stereoscopic lateral views of (a) and (b), respectively. (c) The height of molar cusps in the DM group is reduced (arrowheads) compared with that in the Non-DM group (d). (d) Mild occlusal wear is observed on the 1st molar (arrowhead). (e, f) Soft X-ray images of (c) and (d), respectively. (e) The disappearance of enamel and dentin at the cusp (arrowheads) is apparent. (f) The enamel and dentin of the cusp are clearly visible. Black scale bar (a–d) = 1 mm. White scale bar (e, f) = 1 mm.



**Fig. 6.** The mean cusp height of the maxillary and mandibular molars in the DM and Non-DM groups. The data are shown as the mean  $\pm$  SD. Significant difference compared with the Non-DM group (\*\* $P < 0.01$ ).

nation of serial sections of each molar in both groups. The incidence and severity of the dental caries in the DM group was significantly higher than that in the Non-DM group (Table 2). The majority of dental caries in both groups (DM,

**Table 1.** Incidence and Grading of Stereoscopic Changes in the Molars

Group		DM (n = 13)	Non-DM (n = 10)
Maxillary molars	No. examined	78	60
	No. of teeth with changes	77 (98.7%) **	9 (15.0%)
	Grade 0	1 (1.3%)	51 (85.0%)
	Grade 1	44 (56.4%)	9 (15.0%)
	Grade 2	30 (38.5%)	0
	Grade 3	3 (3.8%)	0
Mandibular molars	No. examined	78	60
	No. of teeth with changes	76 (97.4%) **	18 (30.0%)
	Grade 0	2 (2.6%)	42 (70.0%)
	Grade 1	36 (46.1%)	18 (30.0%)
	Grade 2	34 (43.6%)	0
	Grade 3	6 (7.7%)	0

Significant difference compared with the Non-DM group (\*\*P<0.01).

**Table 2.** Incidence of Histopathologic Caries in the Molars

Group	DM (n = 13)		Non-DM (n = 10)	
	Maxillary molars	Mandibular molars	Maxillary molars	Mandibular molars
No. examined	78	78	60	60
No. of teeth with caries	28 (35.9%) **	31 (39.7%) **	5 (8.3%)	6 (10.0%)
Micro-defects of dentin with bacterial colonization	25 (32.1%)	20 (25.6%)	5 (8.3%)	6 (10.0%)
Expanded defects of dentin with bacterial colonization	3 (3.8%)	11 (14.1%)	0	0

Significant difference compared with the Non-DM group (\*\*P<0.01).

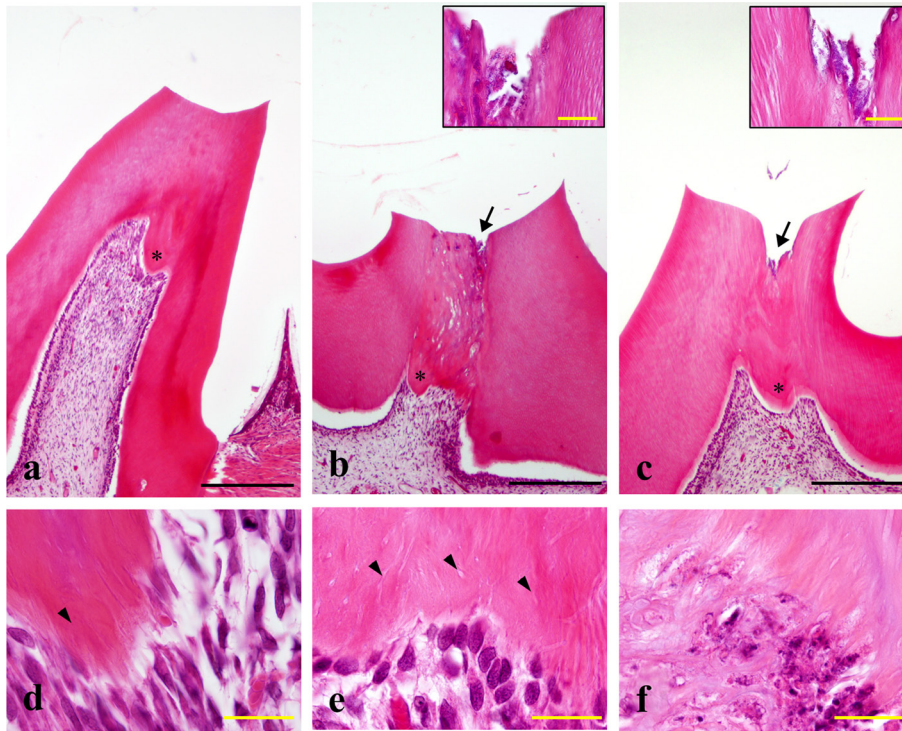
25.6–32.1%; Non-DM, 8.3–10.0%) were diagnosed as micro-defects in dentin with bacterial colonization (Fig. 7b), and the incidence in the DM group was more than twice that in the Non-DM group. Furthermore, advanced dental caries such as expanded defects with bacterial colonization (Fig. 7c) were detected at a low rate (3.8–14.1%) in the DM group alone (Table 2). In some of the teeth with advanced caries, hypertrophic odontoblasts (Fig. 7e) and an abscess and/or necrosis of the dental pulp were observed (Fig. 7f). Meanwhile, mild to severe occlusal surface wear was histopathologically confirmed in almost all the teeth in the DM group, corroborating the results of morphometric examination for cusp height determined using soft X-ray photographs; the lesions were seen as a smooth dent in the occlusal surface of dentin. In many of the teeth with advanced occlusal wear, expanded dental caries were observed on the occlusal surface (Table 3). In addition, at the dentin–pulp junction just below the site of occlusal wear in the DM group, disappearance of the predentin layer or irregular arrangement of odontoblasts was observed accompanied by reparative dentin (Fig. 7d). Reparative dentin, where the dentinal tubules had locally become tortuous or unclear (Fig. 7e), was formed in almost all of the teeth in both DM and Non-DM groups (93.3–100%), and yet the presence of bacteria in the dentinal tubules in the DM group (maxilla, 79.5%; mandible, 76.9%) was remarkably higher than that in the Non-DM group (maxilla, 30.0%; mandible, 46.7%; Table 4).

In the salivary glands, vacuolation of the acinar cells of the parotid gland and the von Ebner's gland was seen in the DM group, and the incidence and severity were sig-

**Table 3.** Occlusal Wear and Caries Grades of Each Molar in Individual Rats

	No.	Maxillary molars						Mandibular molars					
		Left			Right			Left			Right		
		M1	M2	M3	M1	M2	M3	M1	M2	M3	M1	M2	M3
DM	1	2	1	1	2	2	1	2	1	1	2	1	0
	2	1	1	1	2	1	2	2	1	1	1	1	1
	3	2	2	1	2	2	2	3	1	1	3	1	1
	4	1	1	1	2	1	1	2	1	2	2	1	2
	5	3	3	2	3	2	2	3	2	2	3	2	2
	6	1	1	2	1	1	1	2	1	1	1	2	1
	7	1	1	1	2	1	2	2	1	1	3	2	1
	8	1	0	1	1	1	2	1	1	0	1	1	1
	9	1	1	1	2	1	2	2	1	2	2	2	1
	10	2	1	1	1	2	1	2	1	2	2	2	1
	11	1	1	1	2	2	1	2	2	1	2	1	2
	12	1	2	1	1	2	1	2	2	1	2	1	1
	13	2	2	2	2	1	1	3	2	1	2	2	2
Non-DM	1	0	0	0	0	0	1	0	0	1	0	0	
	2	0	0	0	0	0	1	0	0	0	0	0	
	3	0	0	0	1	0	0	1	0	0	1	0	
	4	0	0	0	1	0	1	1	0	0	1	0	
	5	0	0	0	0	1	0	1	0	0	1	0	
	6	0	0	0	1	0	1	1	0	0	1	0	
	7	0	0	1	0	0	0	1	0	1	1	0	
	8	0	0	0	1	0	0	1	0	0	1	0	
	9	0	0	0	0	0	0	1	0	0	1	0	
	10	0	0	0	0	0	0	1	0	0	1	0	

Numbers indicate the grade of occlusal wear. Yellow indicates slight dentin caries. Red indicates expanded dentin caries.



**Fig. 7.** Histopathological characteristics of molars. (a–c) Typical example of morphological changes in the Non-DM group (a) and DM group (b, c). The inset is an enlarged view of the arrow. (a) No caries. Reparative dentin is formed in part of the cusp (asterisk). (b) Mild dental caries (arrow, inset). The occlusal surface is roughened by micro-defects of dentin with bacterial infection (arrow, inset). Reparative dentin is formed in part of the cusp (asterisk). (c) Advanced dental caries (arrow, inset). Expanded collapse with partial caving of the occlusal surface is observed (arrow, inset). Reparative dentin is formed in a part of the cusp (asterisk). (d–f) Typical example of the morphological changes in the dentin-pulp junction of the Non-DM group (d) and the DM group (e, f). (d) Disappearance of the predentin layer (arrowhead) and irregular arrangement of odontoblasts can be observed. (e) The dentinal tubule is tortuous or unclear (arrowheads). Hypertrophic odontoblasts are also observed. (f) Abscess and necrosis of the dental pulp are observed. Black scale bar = 250 µm. Yellow scale bar = 20 µm. HE stain.

**Table 4.** Incidence of Histopathologic Reparative Dentin and Presence of Bacteria in the Molars

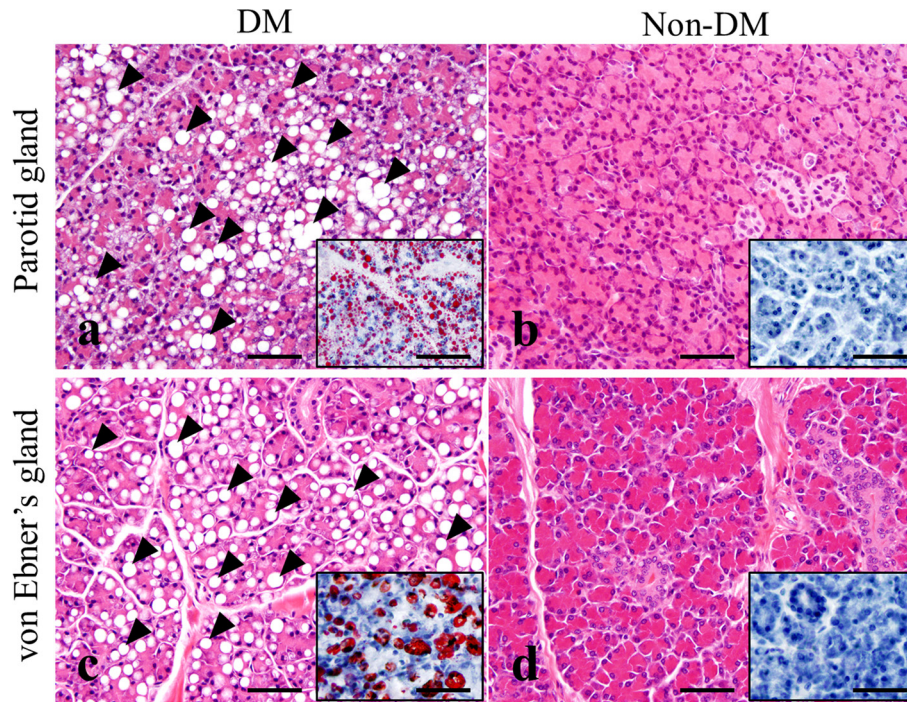
Group	DM (n = 13)		Non-DM (n = 10)	
	Maxillary molars	Mandibular molars	Maxillary molars	Mandibular molars
No. examined	78	78	60	60
Reparative dentin	78 (100%)	76 (97.4%)	56 (93.3%)	58 (96.7%)
Presence of bacteria in dentinal tubule	62 (79.5%) **	60 (76.9%) **	18 (30.0%)	28 (46.7%)

Significant difference compared with the Non-DM group (\*\*P<0.01).

**Table 5.** Incidence and Grading of Vacuolation of the Acinar Cells in Each Salivary Gland

Group	Parotid gland		Von Ebner's gland		Submandibular gland	
	DM (n = 13)	Non-DM (n = 10)	DM (n = 13)	Non-DM (n = 10)	DM (n = 13)	Non-DM (n = 10)
Incidence	13 (100%)	3 (30.0%)	13 (100%)	4 (40.0%)	13 (100%)	10 (100%)
Grade 0	0	7 (70.0%)	0	6 (60.0%)	0	0
Grade 1	1 (7.7%)	3 (30.0%)	0	3 (30.0%)	9 (69.2%)	6 (60.0%)
Grade 2	2 (15.4%)	0	1 (7.7%)	1 (10.0%)	2 (15.4%)	2 (20.0%)
Grade 3	6 (46.1%)	0	0	0	2 (15.4%)	1 (10.0%)
Grade 4	4 (30.8%)	0	12 (92.3%)	0	0	1 (10.0%)
Mean score	3.0 **	0.3	3.8 **	0.5	1.5	1.7

Significant difference compared with the Non-DM group (\*\*P<0.01).



**Fig. 8.** Histopathological changes in the parotid and von Ebner's glands in the DM and Non-DM groups. (a, b) Parotid. (a) Typical example of vacuolation (Grade 4) of the acinar cells of the parotid gland in the DM group (arrowheads). Vacuoles are positive for Oil Red O stain (inset). (b) Normal parotid gland in the Non-DM group. (c, d) Von Ebner's glands. (c) Typical example of vacuolation (Grade 4) of the acinar cells of the von Ebner's gland in the DM group (arrowheads). Vacuoles are positive for Oil Red O stain (inset). (d) Normal von Ebner's gland in the Non-DM Group. Scale bar (a–d, inset) = 50  $\mu$ m. HE stain. Oil Red O stain (inset).

nificantly higher than in the Non-DM group (Table 5). The vacuolation of the DM group progressed from focal lesions with a few small vacuoles to diffuse lesions with numerous small and large vacuoles. Some cells contained a large well-defined single round vacuole similar to a white adipocyte (Fig. 8a, 8c). The vacuoles were stained with Oil Red O (Fig. 8a, 8c inset). Mild vacuolation of the acinar cells in the submandibular gland was also observed, but there was no clear difference between the DM and Non-DM groups (Table 5). In addition, there was no difference in the size of the acinar cells of the salivary glands between the two groups.

## Discussion

Long-lasting hyperglycemia inevitably induces dental caries in both chemically induced and spontaneous diabetic rodent models<sup>3, 4, 13</sup>. However, there are few reports regarding the initial formation of the dental caries in these diabetic models. In our previous study, progressive molar caries were induced in diabetic rats 13 weeks after alloxan administration<sup>13</sup>. The dental caries in these diabetic models reach the dental pulp from the dentin surface, develop into pulpitis with bacterial colonization, and expand through the entire crown or to the dental root, thereby destroying the crown<sup>13, 17</sup>. On the other hand, development of dental caries has not been reported yet in rats 1 month after AL treatment<sup>18</sup>. Therefore, in this study, to investigate the process of

initial caries formation, the autopsy point was set at 7 weeks after AL treatment, which corresponded to approximately half the time taken to reach the symptomatic stages of the dental caries. The present study revealed that the initial lesion of the dental caries, which appeared as micro-defects in dentin with bacterial colonization on the molar surface, could be identified using histopathological analysis, but not stereomicroscopic analysis, after 7 weeks of hyperglycemia.

We hypothesized that salivary gland dysfunction, which is one of the most important factors in the development of dental caries, might be latent before the initial formation of the dental caries in diabetic rats. The present study revealed that pilocarpine-induced saliva secretion in diabetic rats gradually decreased with continuous hyperglycemia starting immediately after AL treatment up to the point of autopsy when the dental caries were initially formed. Furthermore, large Oil Red O-positive lipid droplets accumulated in the acinar cells of the parotid glands in these diabetic rats. AL- and streptozotocin (STZ)-induced diabetic rats reportedly show a significant reduction in muscarinic agonist-induced salivary fluid secretion, such as in the case of pilocarpine, from 2 weeks to 6 weeks after the onset of hyperglycemia<sup>8, 12, 19, 20</sup>. In addition, in rats, small lipid droplets were observed in the acinar cells of the parotid glands 24 h after STZ treatment, and lipid accumulation progressed and large lipid droplets appeared at 4.5 months after STZ treatment<sup>21, 22</sup>. The point of onset of salivary gland

dysfunction and the resultant morphological changes in hyperglycemia status were in agreement with our findings in this study.

Serous saliva secretion is induced by the muscarinic receptor stimulation<sup>23</sup>, and serous saliva is secreted into the oral cavity mainly from the parotid and submandibular glands<sup>24</sup>. In this study, saliva secretion induced by muscarinic receptor stimulation was remarkably reduced in the DM group, and morphological abnormalities were observed in the parotid glands but not in the submandibular glands. In rats, the parotid duct opens opposite the molar teeth<sup>25</sup>, and the effect of selective removal of the salivary glands on the incidence of dental caries was highest when the parotid glands were removed compared with that after removal of the other major salivary glands<sup>26</sup>. Thus, parotid gland dysfunction may be involved in the initial development of molar dental caries in AL-induced diabetic rats. Interestingly, in the Non-DM group, bacteria were present in the dentinal tubules of the molar surfaces, and minute collapse of the dentin with bacterial colonization appeared in a few cases; however, these changes were apparently enhanced in the DM group. These findings suggest that the salivary functions such as flushing of bacteria and remineralization of enamel and dentin by saliva<sup>27, 28</sup> may constantly protect the teeth against bacterial infection in normal rats and that hyposalivation caused by hyperglycemia may inhibit these salivary defensive functions against caries formation in AL-induced diabetic rats.

In the present study, we discovered that hyperglycemia caused the development of the dental caries and enhanced occlusal surface wear in AL-induced diabetic rats. In rodents, enamel does not exist at the occlusal surface of the cusp of the molar teeth<sup>29</sup>, and occlusal wear spontaneously progresses with age after the eruption of tooth buds<sup>30-32</sup>. The histological findings of mild tortuosity of the dentinal tubules in the Non-DM group was consistent with that reported in a previous study on occlusal wear due to aging in young intact rats<sup>33</sup>. In the DM group, progressive molar occlusal wear stereoscopically formed a mortar-like shape, where histologically severe tortuosity and disappearance of the dentinal tubules were observed and the cusp height was morphometrically half that in the Non-DM group. From these findings, it is evident that the occlusal surface wear was further enhanced by hyperglycemia in the DM group. In addition, the odontoblast reaction and disarrangement in the pulp reportedly occur with occlusal wear in rats<sup>33</sup>, and these changes may have been similarly elicited by enhanced occlusal wear in the DM group in this study.

The pathogenesis of spontaneous occlusal surface wear mainly depends on mastication in both humans and rats<sup>31, 32, 34</sup>. Diabetic animals consume a lot of food<sup>35, 36</sup>, and in this study, food intake in the DM group was significantly greater than that in the Non-DM group. Thus, increase in food intake may naturally increase mastication in diabetic rats. In addition, saliva is a lubricant that reduces friction between the dental occlusal surfaces during mastication<sup>34</sup>. In the diabetic rats in the present study, hyposalivation and increased

mastication may have increased the friction, thereby resulting in progressive occlusal surface wear. Furthermore, a number of mineral ions and antibacterial proteins in the saliva reportedly fluctuate in diabetic patients<sup>37-39</sup>. It is possible that remarkable hyposalivation in the diabetic rats in this study caused quantitative changes in salivary electrolytes and antibacterial proteins, inducing failure of tooth remineralization or caries formation. The hyperglycemic condition suppresses the formation of enamel and dentin in STZ-induced diabetic rats<sup>40</sup>. In AL-induced diabetic rats, metabolic disturbance caused by hyperglycemia could directly weaken the tooth substance, thereby enhancing occlusal surface wear as well as formation of dental caries.

In conclusion, hyperglycemia simultaneously induces initial caries development and enhances spontaneous occlusal surface wear of the molars in rats 7 weeks after alloxan treatment. The parotid gland dysfunction caused by hyperglycemia may be involved in the pathogenesis of occlusal wear as well as in dental caries in this diabetic model.

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