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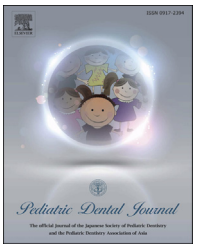

Original Article

# Study on factors that affect caries susceptibility in mice

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ABSTRACT

In our previous research, we discovered a C57BL/6CrSlc mouse strain (B6) that is highly susceptible to caries and a C3H/HeSlc mouse strain (C3H) that is highly resistant to caries, and reported that genetic factors play a role in caries susceptibility. In a study on the B6 and C3H mouse strains, a difference was found in their saliva flow rates, as well as their caries scores. In the present study, we focused on saliva secretion, which may influence differences in caries susceptibility. We examined temporal changes in stimulated saliva secretion volume in B6 and C3H mice, and found that secretion volume was significantly higher in C3H mice than in B6 mice even at 30 min after stimulation, and that total saliva volume was significantly higher in C3H mice. In addition, histological comparisons of the submandibular gland in the two strains revealed the ratio of acinar cells area to be significantly higher in C3H mice than in B6 mice. We then examined the gene expression of the muscarinic acetylcholine receptors M1R, M2R and M3R, which are thought to be regulatory factors of water secretion from acinar cells in the parasympathetic nervous system, but did not find any significant differences between the two strains. Enamel hardness was significantly higher in C3H mice than in B6 mice. The acinar cell ratio in the submandibular gland may be a factor associated with differences in salivary flow rate, and enamel hardness may be a determining factor of tooth decalcification; two factors that affect caries susceptibility.



## 1. Introduction

Differences in caries susceptibility arise from complex interactions among environmental factors and genetic factors. Environmental factors can be standardized for conducting research on mice. In addition to the extent of enamel calcification, saliva flow rate that greatly affects the oral environment is also a host factor affecting caries susceptibility.

In our previous research, we used mice in an experiment in which we induced caries with oral inoculation of *Streptococcus mutans*, a bacterium that is cariogenic in humans [1–4]. We

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discovered a C57BL/6CrSlc strain (B6) that is highly susceptible to caries and a C3H/HeSlc strain (C3H) that is highly resistant to caries, and reported that genetic factors play a role in caries susceptibility [1–4]. When we conducted caries-inducing experiments on these mice and analyzed their saliva secretion, we found caries score to be significantly lower and pilocarpine-stimulated saliva flow rate higher in C3H mice than in B6 mice, but we did not assess temporal changes in saliva flow rate [5]. In a study on gene expression in the submandibular glands of these two strains, Kokubun et al. suggested that *Capn3* and *Tmem87* may affect water secretion and transport in the salivary glands [6].

In the present study, we focused on salivary secretion and enamel hardness, which appear to influence caries susceptibility. We compared stimulated saliva secretion over time, histology of the submandibular gland and muscarinic acetylcholine receptor gene expression in the B6 and C3H mouse strains. We also used a hardness meter to measure and compare enamel hardness, which is a host factor for developing caries, between the two strains.

## 2. Materials and methods

### 2.1. Mice

Two strains of mice (B6, C3H) were used in this study. Twenty-day-old B6 and C3H mice were purchased from Sankyo Lab Service Co. (Tokyo, Japan). All mice were kept in clean racks at room temperature (25 ± 1 °C), with a relative humidity of 55 ± 5%, and a 12-h light/dark cycle. Mice were fed *ad libitum* on a commercial diet (MR Breeder; Nihon Nohsan Co, Kagawa, Japan), and were provided with pure bottled water. The animal-use protocols of this study were reviewed and approved by the Nihon University Institutional Review Board (AP12MD010).

### 2.2. Comparison of stimulated saliva secretion volume over time

At age 49 days, six male B6 mice and five male C3H mice were intraperitoneally administered pentobarbital diluted 1:10 with normal saline (40 mg/ml). After 5 min, they were intraperitoneally administered isoproterenol (0.20 μg/100 g body weight) and pilocarpine (0.05 μg/100 g body weight). For each 10-min period following administration (0–10 min, 10–20 min, 20–30 min) saliva was collected from the oral cavity of the mice with a pipette. Collected saliva was weighed and measurements were tested for significant differences by F test and Student's *t*-test.

### 2.3. Histological comparison of submandibular gland

At age 49 days, three male B6 mice and three male C3H mice were anesthetized with pentobarbital diluted 1:10 with normal saline (40 mg/ml) and fixed by perfusion with 1× PBS and 4% paraformaldehyde. The left submandibular glands were extracted and paraffin-embedded blocks were prepared. Blocks were sliced into 4-μm sections with a microtome and stained with hematoxylin-eosin (HE). Slices were examined and photographed with an All-in-One Fluorescence Microscope (BIOREVO BZ-9000 Generation II®; KEYENCE, Japan). Ducts were traced on the histological images. A scanner was used to re-digitize the tracings with an image analysis resolution of 300 dpi, and Image J image analysis software was used to take measurements. For measurement, the area of a given region on the image was measured as (*a*), and the area of the ducts in (*a*) was measured as (*b*). The ratio of duct cells in the given region was calculated as (*b*/*a*). The ratio of acinar cells in the given region was calculated as ((*a*−*b*)/*a*) (Fig. 1). In one mouse of each strain, the area was measured at 10 sites on histological images and the average was calculated. Results were tested for significant differences by F test and Welch's *t*-test.

### 2.4. Comparison of muscarinic acetylcholine receptor gene expression in submandibular gland

At the age of 49 days, the submandibular glands were extracted from three male B6 mice and three male C3H mice using the same methods as for histological comparisons. The extracted submandibular glands were immersed in RNA*later*® Solution and stored at 4 °C, after which RNA was extracted. RNA extracted by real-time PCR was analyzed for expression of muscarinic acetylcholine receptor subtypes 1–3 (M1R, M2R and M3R). The primers used are shown in Table 1. Primers

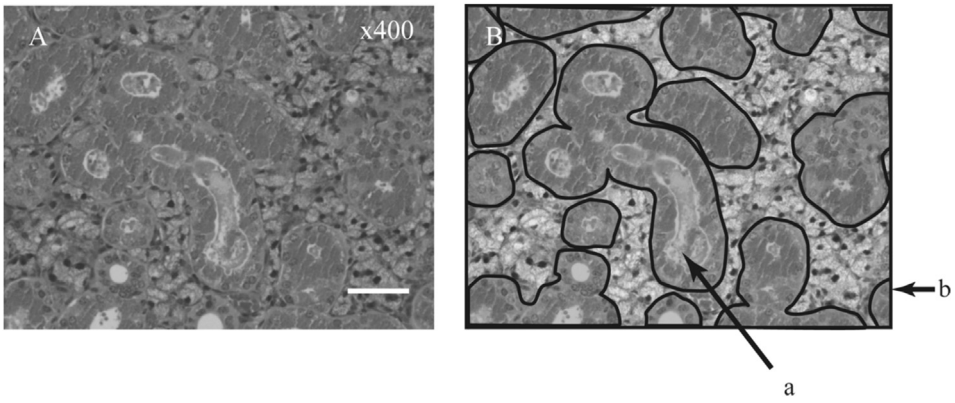


**Fig. 1 – Histological analysis of submandibular gland A: Submandibular gland histology (HE: ×400). B: Trace on the organization's image. a: The area of a given region on the image. b: The area of ducts in (a). Scale bar was indicated 20 μm.**