Brief Report

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Comparison of salivary Mucin 5B (MUC5B) secretion between heat-not-burn tobacco users and non-smokers

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SUMMARY: The use of heat-not-burn (HNB) tobacco is rapidly increasing, particularly in Japan; however, its health effects, especially on innate immunity in the oral mucosa, remain unclear. This cross-sectional study aimed to compare the secretion levels of mucin 5B (MUC5B), a mucin that forms a protective barrier role on the oral mucosal surface, between HNB tobacco smokers and non-smokers. MUC5B levels in stored saliva samples from 208 males (147 in the non-smoking group, 32 in the HNB tobacco group, 15 in the paper cigarette group, and 14 in the dual-use group) were measured using enzyme-linked immunoassay (ELISA). The primary outcome was the MUC5B secretion rate (μg/min = MUC5B concentration × saliva secretion rate). Significant differences in MUC5B secretion rates were observed among the four groups, with multiple comparisons showing significantly lower rates in the HNB tobacco and dual-use groups than in the non-smoking group ($p = 0.042$ and $p < 0.001$, respectively). The observed decrease in salivary MUC5B secretion is a concern, as it may comprise oral hygiene and immune function. From a public health perspective, these findings provide a basis to discourage the use and combined use of HNB tobacco.

Keywords: saliva, innate immunity, heat-not-burn tobacco, mucin, mucin 5B

1. Introduction

Heat-not-burn (HNB) tobacco use has increased notably in Japan and is perceived as having less harmful than conventional paper cigarettes (*1*). Although HNB tobacco is reduces exposure to certain harmful components, concerns remain regarding exposure to chemical substances such as nicotine, cytotoxicity, and other adverse effects (*2*). Several studies have reported the link between HNB tobacco and oral immune components. For instance, a previous study reported that HNB tobacco users exhibit lower resting saliva secretion rates and significantly lower secretion rates of lactoferrin (Lac) and lysozyme (Lys) than non-smokers (*3*). Furthermore, significantly reduced salivary secretory immunoglobulin A (sIgA) levels have been observed in HNB tobacco users, especially females (*4*).

Saliva contains mucin, antimicrobial proteins (Lac and Lys), sIgA, and other protective factors. Furthermore, mucin 5B (MUC5B), the major gel-forming mucin, is secreted by the submandibular glands, sublingual glands, palatal, and minor salivary glands (*5*). As a highly glycosylated glycoprotein, MUC5B prevents pathogen adhesion, facilitates aggregation and detoxification, and contributes to mucosal barrier functions through lubrication and coating (*5,6*). Deficiency in MUC5B may compromise mucosal defense, increasing susceptibility to oral infections and inflammatory diseases (*5*).

We previously reported that the use of HNB tobacco reduces the secretion rates of Lac and Lys (*3*). However, few studies have quantitatively examined the association between HNB tobacco use and the secretion levels (especially secretion rate) of salivary MUC5B. Considering that HNB tobacco use reduces saliva secretion rates, as previously demonstrated (*3*), and that MUC5B contributes to the formation of oral salivary films (*7*), the hypothesis that the effective supply (secretion rate) of MUC5B may be relatively lower among HNB tobacco smokers warrants investigation.

This study aimed to quantitatively examine the association between HNB tobacco use and MUC5B secretion by comparing MUC5B secretion rates (as the primary endpoint) among four participant groups: a non-smoking, HNB tobacco, paper cigarette, and dual-use. While other mucins, including MUC1 (membrane-bound mucin) (*8*) and MUC7 (secretory and non-gel-forming mucin) (*9*), exist, this study focused on

MUC5B as the primary gel-forming mucin (*10*). To our knowledge, this study is the first to examine the association between MUC5B secretion rate in saliva and HNB tobacco use. This approach may provide novel insights into salivary innate immunity, with implications for preventive medicine and public health.

2. Materials and Methods

2.1. Study design and participants

This cross-sectional study included essential workers (firefighters, paramedics, and rescue workers) employed at five fire stations in Kasugai City, Aichi Prefecture, Japan. Participants were recruited *via* a research cooperation request form distributed through each manager of each station. Participants were selectively sampled because assessing immune factors in their saliva was considered critical, given their frontline contact with patients suspected of having infectious diseases. Saliva samples were collected in mid-June 2021.

A total of 219 individuals initially participated in this study; however, all seven female participants were non-smokers, so the analysis was limited to 212 males. Additionally, four samples with MUC5B concentrations outside the detection range were excluded, leaving 208 participants (147 in the non-smoking group, 32 in the HNB tobacco group, 15 in the paper cigarette group, and 14 in the dual-use group) in the final analysis. "Paper cigarettes" were defined as conventional combustible cigarettes. Dual users were those who used both HNB tobacco and paper cigarettes.

Smokers were defined as "individuals who had smoked continuously for at least six months" (*3*). None of the participants were e-cigarette users. Furthermore, as dental conditions could impact the results (*11,12*), individuals with periodontal disease were excluded in advance.

2.2. Ethics

All participants in this study were provided with a detailed explanation of the objectives, methods, sample collection procedures, and management of personal information in advance. Written informed consent was obtained from all participants. This study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Ethical Review Committee of Chubu University (Approval No.: 20200081).

2.3. Saliva collection

Stimulated saliva was collected uniformly in a quiet room between 9:00 am and 12:00 pm using a previously established standardized protocol (*3*). The participants were specifically instructed (1) not to eat, drink, brush teeth, or smoke 60 min before and after saliva collection; (2) to thoroughly rinse their oral cavity with distilled water (gargle thrice) immediately before saliva collection; (3) to rest for 5 min in a sitting position and swallow once any saliva accumulated in the oral cavity; and then (4) to start a timer and chew a piece of odorless, tasteless, sterile cotton swab (Salimetrics Oral Swab™; Salimetrics, Carlsbad, CA, USA) once per second for 1 min. We ensured that the participants strictly adhered to this procedure. Newly secreted saliva was absorbed by the sterile cotton and collected in a storage tube (Swab Storage Tube™; Salimetrics). After collection, the storage tubes were centrifuged at 1,400× *g* for 5 min to separate saliva from the swab, and the volume of saliva obtained was recorded to calculate the saliva secretion rate (mL/min). The samples were promptly divided into small portions and stored at -30°C. The samples were thawed only once for measurement (*13*).

Saliva secretion rate (mL/min) was calculated by dividing the volume collected by the duration of the collection period.

2.4. Measurement of MUC5B concentration

MUC5B concentration (μg/mL) was determined using an enzyme-linked immunoassay (ELISA) with the Human MUC5B ELISA Kit (Novus Biologicals, Centennial, CO, USA; NBP2-76705), according to the manufacturer's instructions (*14*). Absorbance was measured at 450 and 630 nm using a microplate reader (BioTek Instruments, Winooski, VT, USA), and the concentration of MUC5B was calculated from the standard curve after wavelength correction.

2.5. Definition of the primary outcome

The primary outcome of this study was the rate of MUC5B secretion (μg/min), calculated by multiplying the MUC5B concentration (μg/mL) by the saliva secretion rate (mL/min). This parameter was employed to reflect the effective supply of the mucosal defense. MUC5B concentration was not measured in our previous study (*3*) and was newly quantified and analyzed as the primary outcome in this study.

2.6. Survey

The background information of the participants, including age group, sex, smoking habits, and history of chronic diseases, was collected using a questionnaire.

2.7 Statistical analysis

The variables are expressed as the median (interquartile range: IQR). The Kruskal–Wallis test was used to

compare each marker among the four groups, and multiple comparisons were corrected using the Bonferroni method. Statistical significance was set at a two-sided p-value < 0.05. Statistical analysis was performed using IBM SPSS Statistics, Version 27 (IBM, Armonk, NY, USA).

3. Results and Discussion

3.1. Participant characteristics

The participant characteristics are summarized in Table 1. Among them, seven (3.4%) participants had a history of hypertension, seven (3.4%) had a history of hyperlipidemia, one (0.5%) had a history of diabetes, and one (0.5%) had a history of rheumatoid arthritis (data not shown). Neither of these medical histories nor age was considered a major confounding factor for the study outcomes.

3.2. Saliva secretion rate

As shown in Figure 1, the median (IQR) saliva secretion rate was 1.4 (0.9–2.2) mL/min in the non-smoking group, 1.0 (0.7–1.5) mL/min in the HNB tobacco group, 1.1 (1.0–1.4) mL/min in the paper cigarette group, and 1.1 (0.7–1.5) in the dual-use group. A statistically significant difference was observed among the four groups (p = 0.02). Furthermore, multiple comparisons revealed a significantly lower saliva secretion rate in the HNB tobacco group compared with the non-smoking group (p = 0.04).

3.3. MUC5B concentration and secretion rate

Figure 2 shows the comparison of MUC5B concentrations among the four groups. The MUC5B concentration was 0.4 (0.2–0.7) μg/mL in the non-smoking group, 0.3 (0.2–0.6) μg/mL in the HNB tobacco group, 0.3 (0.2–0.9) μg/mL in the paper cigarette group, and 0.1 (0.1–0.3) μg/mL in the dual-use group, indicating a significant difference among the groups (p = 0.002). Multiple comparisons revealed a significantly lower MUC5B concentration in the dual-use group compared with the non-smoking group (p = 0.002).

Figure 3 shows the comparison of MUC5B secretion

Table 1. Participant characteristics

		%
Sex		
Male	208	100.0
Age group		
20–29	65	31.2
30–39	73	35.1
40–49	52	25.0
≥ 50	18	8.7
Smoking habit		
Non-smoker	147	70.7
HNB tobacco users	32	15.4
Cigarette smokers	15	7.2
Dual users (HNB + Cigarette)	14	6.7

HNB, Heat-not-burn.

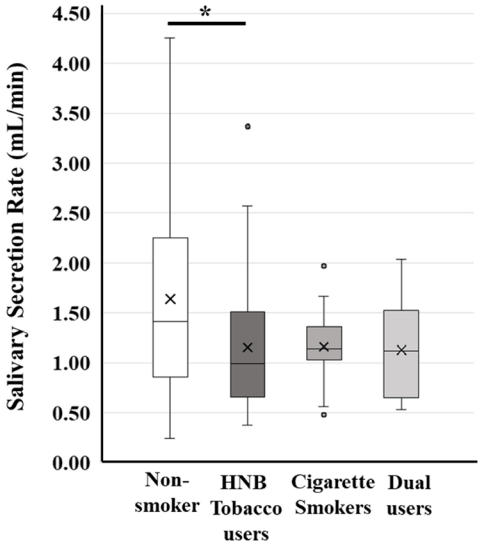

Figure 1. Comparison of saliva secretion rates. The Kruskal–Wallis test was applied, and Bonferroni's multiple comparison correction was performed. The boxes indicate the interquartile range, the line inside each box marks the median and the '×' denotes the mean. Whiskers represent the 10th to 90th percentiles, and values outside this range are shown as outliers. This result is based on analysis performed using a portion of samples employed in a previous study (Mori, Y, *et al*. 2022) (*3*). *, p < 0.05; HNB, Heat-not-burn; Dual users, HNB + Cigarette.

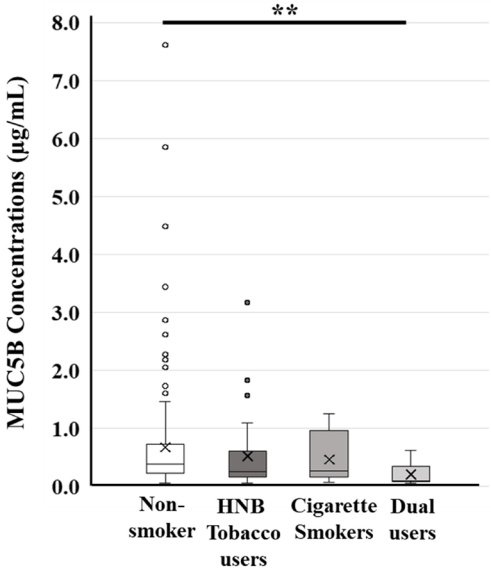

Figure 2. Comparison of MUC5B concentrations. The Kruskal–Wallis test was applied, and Bonferroni's multiple comparison correction was performed. The boxes indicate the interquartile range, the line inside each box marks the median and the '×' denotes the mean. Whiskers represent the 10th to 90th percentiles, and values outside this range are shown as outliers. **, p < 0.01; HNB, Heat-not-burn; Dual users, HNB + Cigarette.

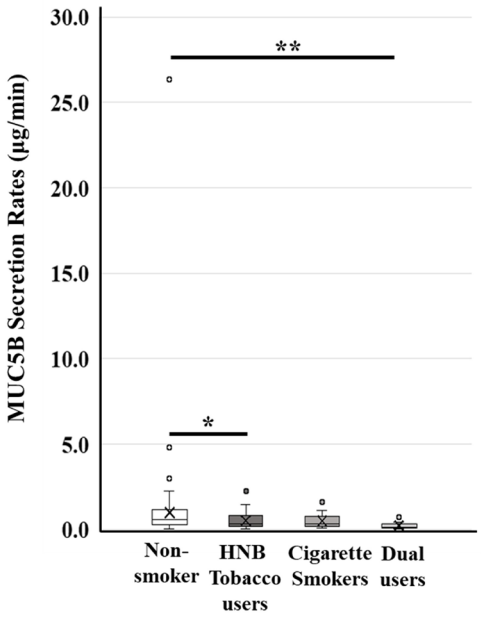

Figure 3. Comparison of MUC5B secretion rates. The Kruskal–Wallis test was applied, and Bonferroni's multiple comparison correction was performed. The boxes indicate the interquartile range, the line inside each box marks the median and the '×' denotes the mean. Whiskers represent the 10th to 90th percentiles, and values outside this range are shown as outliers. *, $p < 0.05$; **, $p < 0.01$; HNB, Heat-not-burn; Dual users, HNB + Cigarette.

rates among the four groups. The MUC5B secretion rate was 0.6 (0.3–1.1) μg/min in the non-smoking group, 0.3 (0.2–0.8) μg/min in the HNB tobacco group, 0.3 (0.2–0.7) μg/min in the paper cigarette group, and 0.1 (0.1–0.3) μg/min in the dual-use group. A significant difference in MUC5B secretion rates was observed between the four groups ($p < 0.001$), with multiple comparisons revealing a significantly lower MUC5B secretion rate in the HNB tobacco and dual-use groups than in the non-smoking group ($p = 0.04$ and $p < 0.001$, respectively). One sample exhibited an outlier MUC5B secretion rate (26.3 μg/min), although it was within the detection range. Nonetheless, the statistical conclusions remained unchanged when this sample was excluded.

As described above, this study revealed that, in an adult male population, MUC5B secretion rates were significantly lower in the HNB tobacco and dual-use groups than in the non-smoking group. This academically significant finding suggests that the use of HNB tobacco may be associated with the secretion dynamics of MUC5B, a key component of the innate immune system that is present in saliva.

HNB tobacco aerosols contains nicotine and other chemicals (*15*). Research on biomarkers in the oral cavity region has indicated that HNB tobacco use can alter inflammatory and immune indices in saliva (*16*). These exogenous stresses can alter the function of the salivary glands and regulation of MUC5B secretion by inducing chronic stress in the oral cavity and upper airway epithelium. However, the specific mechanisms through which HNB tobacco use is linked to MUC5B secretion have not yet been elucidated.

Salivary secretion is regulated by the autonomic nervous system (*17*). Nicotine-related autonomic nervous system changes associated with HNB tobacco smoking (*18*) may reduce salivary flow and contribute to decreased MUC5B secretion. Furthermore, reactive carbonyl compounds detected in HNB aerosols in previous studies, such as formaldehyde, acetaldehyde, and acrolein (*19,20*) have been shown in cell experiments to potentially promote increases in inflammation-related substances (*e.g.*, IL-8) (*21*). Furthermore, substances involved in inflammation (IL-6/IL-17 and IL-8) have been shown to potentially affect mucin secretion, including MUC5B, a major component of mucus (*22,23*). Therefore, a hypothetical pathway exists where HNB components alter the balance of inflammation-related substances, consequently affecting mucin production and secretion. However, since this study did not measure IL-6/IL-8 or similar substances in saliva, this mechanism could not be directly confirmed and requires future verification.

A particularly notable finding is that the dual-use group exhibited the most negative association with MUC5B secretion. This aligns with a previous study focusing on the cellular effects of dual exposure, which demonstrated that it adversely affects the function of airway epithelial cells *in vitro*, causing decreased cell viability, increased oxidative stress, and other functional abnormalities (*24*). A similar phenomenon may occur in the salivary glands; however, the association with decreased MUC5B secretion requires further investigation. Nevertheless, from a public health perspective, these findings provide a basis for strongly discouraging the combined use of HNB tobacco with paper cigarettes. Because mucins such as MUC5B suppress the growth of opportunistic pathogens (*5*), decreased salivary secretion of MUC5B, as observed in HNB tobacco users, raises major concerns regarding oral hygiene and immune function.

However, this study has several limitations. First, its cross-sectional nature limits causal inferences, and we could not completely exclude residual confounding factors (*e.g.*, water intake, exercise, oral cleaning, and stress). For instance, water loss has been suggested to affect salivary flow rate, potentially affecting the concentration and secretion rate of immune-related components in saliva (*25*). However, in the present study, it was difficult to determine the hydration status of the participants. Second, the sample size of this study depended on the availability of stored samples; consequently, the power was not designed in advance, and the sample size was relatively small. Additionally, approximately half the smokers were HNB tobacco users, which differs from the general usage trend in Japan (*1*). Therefore, further research is needed to examine whether the findings of this study can be

directly applied to the general population. Third, although the selection criterion of this study included "smokers who had smoked for a certain period of time," we were unable to obtain information regarding both the duration and quantity of smoking for the participants. This prevented us from performing a multivariate analysis that accounts for these factors as covariates. Moreover, it has been reported that the dual use of HNB tobacco and paper cigarettes "may have additive and synergistic disadvantages" (*24*); thus, prospective studies that accurately capture exposure indices (number of cigarettes, frequency of inhalation, and nicotine dependence) are needed. Fourth, the study population was biased toward males and firefighters; thus, the reproducibility of the findings needs to be verified in a population with more diverse age, sex, underlying diseases, medications, and other factors. Additionally, a link between circadian rhythm and saliva secretion has been reported (*26*); given that several participants in this study had irregular work schedules, their circadian rhythms may differ from those of the general population. Furthermore, the occupation of firefighters involves high levels of occupational stress during fire responses (*27*), as well as the risk of exposure to toxic gases in combustion products (*28,29*), alongside characteristic physical activity patterns (*30*). It cannot be denied that these factors may influence variations in salivary secretion volume and immune-related components in saliva. Therefore, the results of this study are preliminary and require validation in diverse populations.

A key strength of this study is that the rate of MUC5B secretion (μg/min), which was calculated using both MUC5B concentration and saliva secretion rate, was used as the primary outcome. This measure represents the effective functional supply of MUC5B to the oral mucosa and provides greater physiological relevance to mucosal barrier function than a simple concentration index. Furthermore, this method is highly practical and useful in clinical settings. Furthermore, from a public health perspective, demonstrating the quantitative association between HNB tobacco use and the innate immune system (MUC5B) in saliva is expected to encourage behavioural change and dispel the misconception that "HNB tobacco is safe because exposure levels are relatively low".

In conclusion, this study investigated the association between HNB tobacco use and salivary MUC5B secretion levels using a secretion index that integrated MUC5B concentration and saliva secretion rate. The MUC5B secretion rates were lower in the HNB tobacco and dual-use groups than those in the non-smoking group, suggesting an impairment of the innate immune barrier within the oral cavity. The novelty of this study lies in its focus on HNB tobacco and the direct comparison of MUC5B secretion levels. Our findings contribute valuable insights for assessing the risks associated with oral diseases and oral/upper respiratory tract infections among HNB tobacco users and aid in optimizing smoking cessation strategies. Therefore, this study contributes valuable knowledge for the fields of preventive medicine and public health.

Given the limitations of the cross-sectional analysis approach used in this study and the male-only study population, future studies should focus on establishing accurate exposure assessments and integrative associations with disease mechanisms and clinical outcomes.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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