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ORIGINAL RESEARCH

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Diurnal variation of salivary anaerobes correlates severe dental caries in children

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Abstract

Background: Salivary secretion has diurnal fluctuations and the saliva amount affects oral bacterial activity. In this study, the time dependence of number of anaerobic bacteria in saliva such as *Streptococcus mutans* (*S. mutans*) was investigated and examined its impact on caries severity. **Methods**: This study was conducted at university hospital in Japan. Twenty subjects (2–10 years old) with the primary dentition were asked to collect whole saliva every 1 hour after the supper while awake at home. Eighteen subjects delivered the collected saliva, which was cultured for *S. mutans* and total anaerobes in trypticase-yeast extract-cysteine sucrose-bacitracin (TYCSB) medium and the Gifu anaerobic medium (GAM), respectively. The numbers of severe dental caries were also analyzed from medical records. **Results**: A positive correlation was found between the number of colonies in GAM medium and the saliva collection time of the day. No significant correlation was observed between the number of colonies in TYCSB medium and the collection time. The patients were grouped based on whether or not they had experienced pulp treatment. The number of colonies of *S. mutans* and anaerobic bacteria were increased in the later hours of only the experienced group. **Conclusions**: The number of oral anaerobic bacteria of children fluctuated in time-dependent manner after dinner to bedtime, and was higher late in the night. Children with severe dental caries had increased *S. mutans* as the night progressed, whereas children without them did not.

Keywords

Dental caries; Circadian rhythms; Salivary anaerobes; *Streptococcus mutans*

1. Introduction

In modern society, individuals engage in variety of activities including eating, shopping and watching videos on internet at any time during the day. Visiting the dental clinic is one of them. Such activities allow substantial degree of convenience and offer lifestyle variations. However, it may lead to lifestyle-related problems such as sleep disorder. It has been reported that disruption of lifestyle rhythms induces lifestyle-related diseases in adults [1]. Dentists in the dental clinic inform children and their guardians that eating late at night may cause dental caries, which can be attributed to the decrease in saliva flow at nighttime. The flow and content of whole saliva show diurnal variation [2, 3]. Salivary flow gradually increases and peaks in the afternoon or early night and then declines. Diurnal variations in saliva flow and composition are because of the circadian rhythms, which are ~24-hour periodic variations shown by the salivary glands.

Circadian rhythms regulate range of physiological processes [4]. The circadian rhythms in mammals are centered in the suprachiasmatic nucleus of hypothalamus [5]. Oral tissues such as submandibular gland and tooth also show circadian rhythms in gene expression and are controlled by the circadian rhythm center [6–10]. Photic information is the most potent in adapting circadian rhythms to the environment [11]. It is conveyed from the retina to rhythm center via the retinohypothalamic tract, wherein the center adapts circadian rhythms to external day-night variation. Bedtime and waking time are vital for daily rhythm because the retina is exposed to light from the wake time till one goes to bed. Oral functions including the salivary flow has circadian rhythm [3] which is affected by the life routine such as sleep [12].

Risk factors of developing dental caries includes frequent carbohydrate consumption and low saliva flow [13]. Other contributions come from the social factors such as poverty, health literacy and lifestyle habits like diet, fluoride exposure and tooth brushing [14–17]. They influence caries risk by changing the physiological functions of body or bacterial activity. We have previously reported a correlation between the number of dental caries during primary dentition period and daily lifestyle habits [18]. The study has shown that children having eveningness lifestyle with late dinner and bedtime suffer more caries compared to those of morningness lifestyle. Furthermore, the study has predicted that they would have more caries than morningness because cariogenic bacteria such as *Streptococcus mutans* (*S. mutans*) increase later in the

night considering that children with eveningness chronotype eat meals and snacks late at night. A positive correlation has been reported between caries prevalence and the amount of salivary *S. mutans* [2, 19, 20]. *S. mutans* is the major cariogenic bacterium contributing most in developing dental caries, however they also develop because of the combined activities of various other bacteria in oral flora, especially the anaerobes [21]. This study was aimed to investigate whether the amount of *S. mutans* in children's saliva varied based on the time of day. Saliva was collected after the dinner for analysis. Whole saliva was collected every hour between the dinner and bedtime. The number of *S. mutans* and anaerobes was determined.

2. Materials and methods

2.1 Study population

Twenty outpatients (aged 2–10 years) of the Division of Dentistry for Children and Disabled Persons volunteered for this study. The subjects were children in the primary dentition stage with no systemic diseases.

2.2 Data collection

The study objectives and recording procedures were explained to the subjects and their parents or guardians. Saliva was collected from the subjects after getting written informed consent. The following items were given to the subjects in the hospital: conical tubes, disposable funnels, sheet for recording sampling times, styrene foam box and delivery slip, ice pack and packing tape. Subjects collected resting saliva into conical tube immediately after the supper and mouthwash at home. Saliva was then collected every 1 hour while awake. Twenty children were asked to collect saliva samples, and saliva was received from 18 (mean age ± standard deviation (SD), 5.4 ± 1.9 years; 2–10 years age), wherein 7 had severe caries and 5 were without caries. Collected saliva was refrigerated, packed into styrene foam box with ice pack and delivered the next morning. After receiving parcels in afternoon at the university laboratory, samples were kept on ice until the subsequent experiments. Subjects were also asked to record daily life habits for 8 days on questionnaire described previously, *i.e.*, waking time, bedtime, mealtimes, snack time and tooth brushing time [18].

The number of dental caries was found out from subjects' medical records. Decayed or filled teeth (dft) index of children with primary dentition was employed for the number of caries. Subjects having "severe caries" needed root canal treatment. Diagnosis of dental caries was performed by the dentists attending subjects at university hospital. The diagnosis criterion was unified for dental caries requiring treatments. The first author conducted quick oral examination considering the stress of subjects and confirmed the number of teeth with caries. Therefore, the severity of cavities was determined based on whether root canal treatment was required.

2.3 Anaerobe culture

A trypticase-yeast extract-cysteine sucrose-bacitracin (TYCSB) medium in 10 cm-culture dish including 1.5% trypticase, 0.5% yeast extract, 1.6% agar (214010, Becton Dickinson, Sparks, MD, USA), 0.02% l-cysteine, 0.01% sodium sulfate, 0.1% sodium chloride, 0.2% disodium hydrogen phosphate dodecahydrate, 0.2% sodium bicarbonate, 2% sodium acetate trihydrate, 5% sucrose and 0.2 U/mL bacitracin (BIB0106, Wako Pure Chemical, Osaka, Japan) was used to selectively culture *S. mutans* [22]. The Gifu anaerobic medium (GAM) (05426, Nissui Pharmaceutical, Tokyo, Japan) was used to culture whole anaerobes. Then, 100 μL of 40- or 20,000-fold diluted saliva were seeded in TYCSB medium or GAM, respectively. The dishes were placed in anaerobic jars (GasPak100 or GasPak150, Becton Dickinson, Sparks, MD, USA) with oxygen scavenger (Anaeropack, Mitsubishi Gas Chemical, Tokyo, Japan) and cultured at 37 °C.

2.4 Data analysis

Images of the dishes were taken after 24 hours of culture using digital camera. Number of colonies in the dishes were counted using ImageJ software (version 1.53, National Institutes of Health, Bethesda, MD, USA). The correlation between number of colonies and time was determined from Pearson's correlation coefficient using Microsoft Excel (ver. 2016, Microsoft Corporation, Redmond, WA, USA).

3. Results

The number of colonies in *S. mutans* and whole anaerobes varied based on the saliva collection time (Fig. 1). Upon quantifying the number of colonies and analyzing the correlation with collection time, the whole anaerobes however not *S. mutans*, showed statistically significant change with time (Fig. 2A,B). The whole anaerobes' concentration in saliva became higher in the later hours. Upon classifying and analyzing the subjects according to the presence or absence of severe caries, it was shown that the number of *S. mutans* colonies in the subjects with severe caries also fluctuated with time (Fig. 2C). The number of whole anaerobes also fluctuated in the severe caries group (Fig. 2D). Neither *S. mutans* nor whole anaerobes changed with time in the group without severe caries (Fig. 2E,F). Fig. 3 showed changes in the number of individual *S. mutans* colonies and the caries experience.

4. Discussion

Our previous report had shown that there was greater caries experience in primary dentition period with later and irregular bedtime and dinner time [18]. This study reported for the first time that the factors affecting circadian rhythms such as timing of sleep and meal were correlated with caries prevalence. Multiple regression analysis of the life habits investigated for children in the primary dentition period revealed snacking frequency as the most influencing factor on number of caries. This result endorsed the impact of sugar consumption reported previously [14, 15]. However, life habits related to circa-

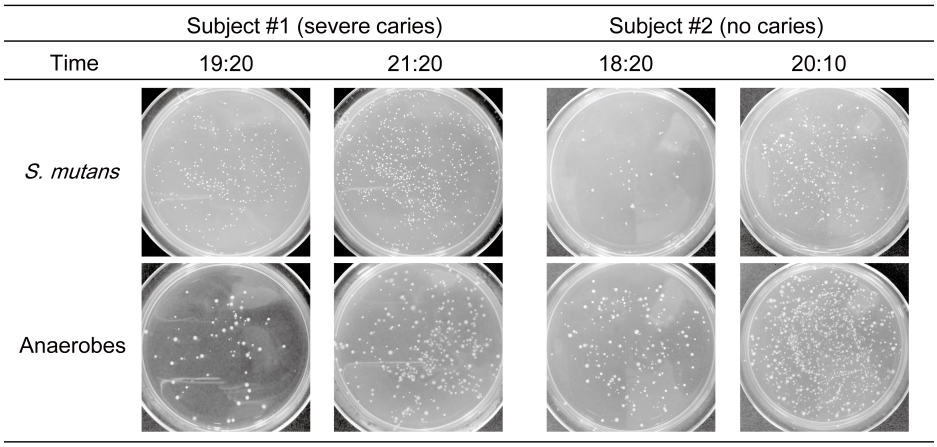

FIGURE 1. The representative culture plates of *S. mutans* and anaerobes. Whole saliva collected at the indicated times was cultured for 24 hours. Resultant plates from the two subjects (with severe caries and no caries) are shown. Upper images show the culture for *S. mutans* with selective medium, and the lower images show the culture for anaerobes. *S. mutans*: *Streptococcus mutans*.

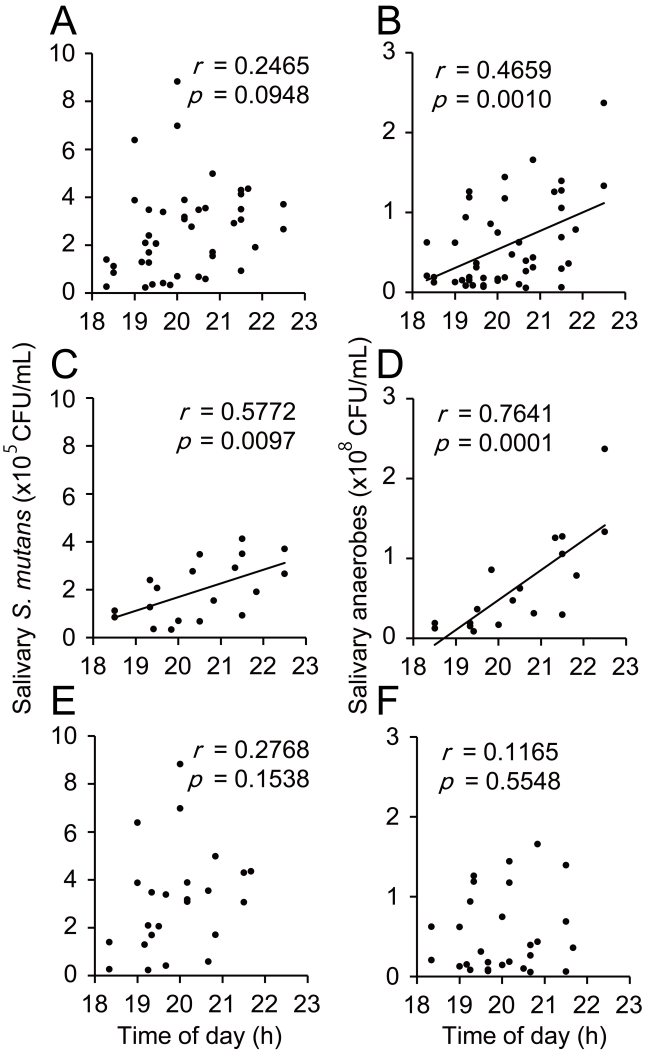

FIGURE 2. Correlations of salivary cariogenic microorganisms with time. Correlations between the time of saliva collection and the number of colonies of *S. mutans* (A,C,E) and anaerobes (B,D,F) in all subjects (A,B), and in subpopulations with (C,D) or without (E,F) severe caries. Subjects undergone root canal therapy were classified as "severe caries". Linear regression lines indicate significant correlations. *S. mutans*: *Streptococcus mutans*; CFU: colony forming unit.

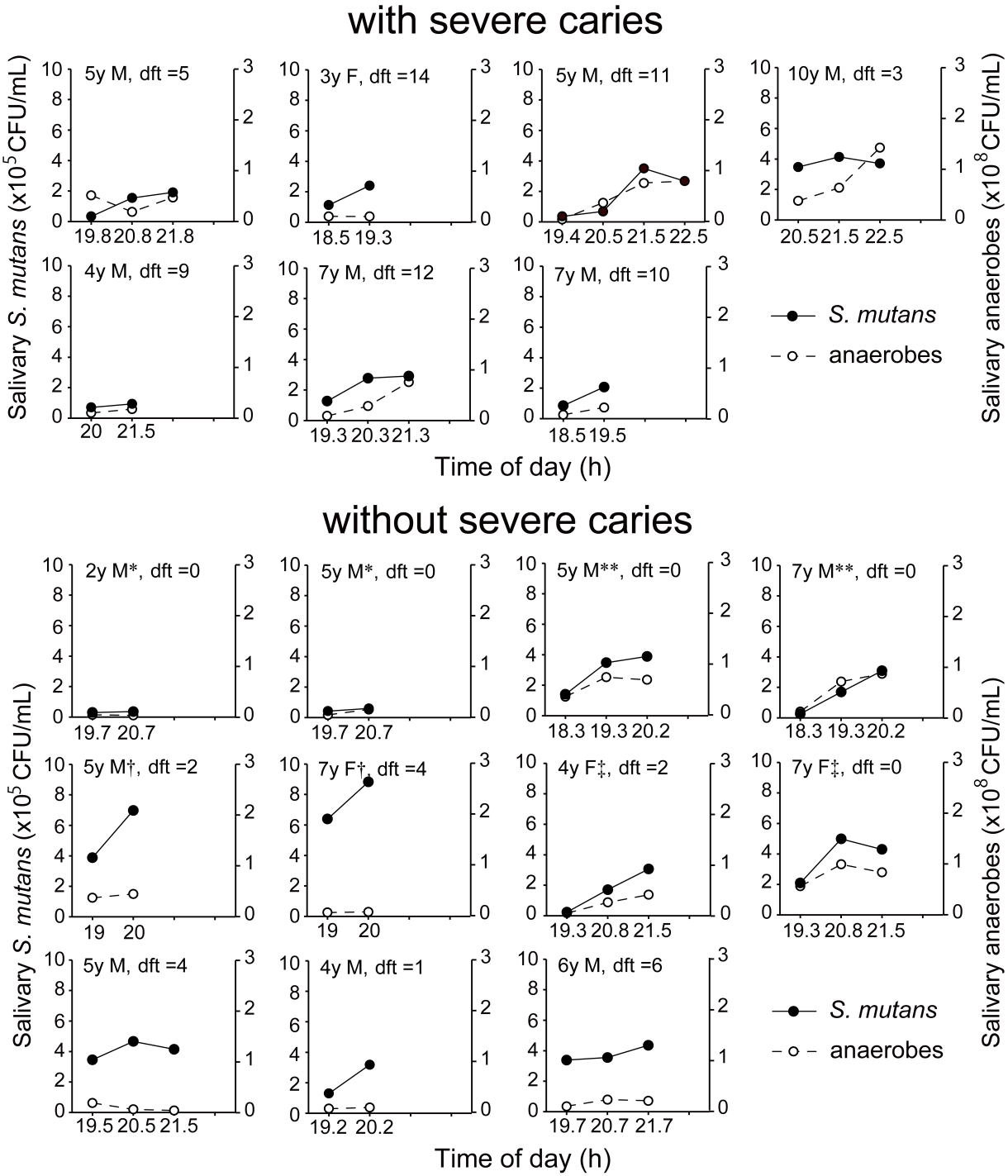

FIGURE 3. Data of salivary *S. mutans* and anaerobes in individual subjects. Abscissa indicates saliva collection time. Each graph label indicates subject age, sex and number of tooth caries. dft: decay, filled teeth index. Graphs with same symbols (*, **, †, ‡) show data from siblings. *S. mutans*: *Streptococcus mutans*; CFU: colony forming unit; dft: Decayed or filled teeth.

dian rhythms were not correlated with the snacking frequency [18]. Moreover, the bed timing was a risk factor for caries development, which was independent of sugar intake. Other subsequent studies had also supported that bedtime induced the cavities development [23, 24].

Oral *S. mutans* had been the main cause of caries emergence and its amount in saliva could be used as the index for caries risk [2, 19, 20]. The diurnal variation of *S. mutans* in saliva (Fig. 2) meant that caries risk changed over time. Changes in the number of *S. mutans* after dinner were shown in this study, however studying changes in the number of *S. mutans* throughout the day could prevent caries and their risk. Current results showed that the number of *S. mutans* in saliva varied with the time of measurement. Saliva sampling time required to be fixed for accurate caries risk determination. Furthermore, analyzing the patient's tendency of morningness-eveningness (chronotype) and the circadian rhythm pattern of physiological functions could determine the caries risk.

Results herein showed that the number of anaerobic bacteria in children's oral cavity increased as the night progressed (Fig. 2). The number of *S. mutans* also increased in children with severe dental caries. Increase in the number of *S. mutans* and anaerobic bacteria might be because of the decrease in saliva secretion as the night progressed, and bacteria were no longer washed away by the saliva. Moreover, the changes in cell division rate of bacteria and the activity of immune cells in oral cavity might change because of the circadian rhythms. It had been reported that the bacteria in human intestinal tract or oral cavity exhibited circadian rhythm [25, 26]. It was thus likely that the amount of *S. mutans* in saliva fluctuated throughout the day. The resting saliva was used in this study because the saliva flow rate factor would disappear, otherwise the caries risk in oral cavity was not accurately determined in stimulated saliva. The number of *S. mutans* in some children increased as the night progressed, while in others it did not. It could be predicted that the child whose bacterial count fluctuated depending on the time of day had developed severe caries. The number of *S. mutans* and whole anaerobic bacteria colonies in most subjects increased over time (Fig. 3). However, the caries experience (dft) varied among subjects, and influenced by fluoride usage, genetic factors and others. Dental caries might be suppressed in children by elucidating the reasons of fluctuations in *S. mutans* through immunological and bacteriological approaches.

The first null hypothesis of this study was that the number of *S. mutans* and whole anaerobes in children's saliva would not vary with time of day. The number of total anaerobic bacteria significantly increased with the later time of day (Fig. 2D), and the null hypothesis was rejected. However, there was no correlation between *S. mutans* and time of day, and the null hypothesis was not rejected. Therefore, we adopted the second null hypothesis that the number of *S. mutans* in saliva would not vary with time of day in the subpopulation with severe caries. As a result, this null hypothesis was rejected (Fig. 2B).

Some peripheral organs such as salivary glands were required to have high levels of function at certain times of the day. When the cellular rhythms in an organ were synchronized, the rhythm amplitude of organ was increased and functions (*e.g.*, salivary flow) could be sufficiently improved at required times. Organs shifted their internal circadian rhythms to adapt new schedule when sleep routine changed rapidly. Since the magnitude of phase shift in circadian rhythms was organ-specific, "jet lag" occurred where the phase differed between the organs [27, 28]. Inter- or intra-organ desynchronization reduced the rhythm amplitude of individual or organ, respectively, which minimized the physiological functions of individuals and organs. Circadian rhythms were sensitive in the developmental period and physiological functions were thus declined more due to external stimuli [29, 30]. For instance, changes in the offspring's rhythm to synchronize with that of mothers in rodents were large immediately after birth, but gradually disappeared as they developed. Children could synchronize their rhythms with those of mothers, however children's rhythms were affected by environmental factors. Furthermore, the perturbation impact of circadian rhythms in the early developmental period could last for long period of life [31].

This study had certain limitations. It was performed in the metropolitan Sapporo city in Japan. Children in urban areas were exposed to more light in the evening. Staying up late disrupted their circadian rhythms and sleep compared to those in rural areas. Another limitation was that the subjects' dietary patterns were not investigated. Selective media was used to quantify the bacteria, while accurate bacterial identification required genetic analysis. The highly reliable (but time-consuming) methods were not used since this study aimed to easily determine the caries risk. Oral bacteria such as *S. mutans* might change with the dietary patterns. Children should be encouraged for early bedtime to avoid light exposure and food intake at night when circadian rhythms were more susceptible. Careful tooth brushing was not enough to prevent emergence of caries in children with daily life habits of eveningness.

5. Conclusions

The later in the night, the higher the number of *S. mutans* in the saliva of children with severe caries, and the number of anaerobes in children overall. This indicates that the risk of developing caries increases the later at night. The customized oral health counseling considering the individual's circadian rhythm should be promoted in future.

AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

AUTHOR CONTRIBUTIONS

SN and YY—designed the research study. SN and HH—performed the research. TY and TK—provided help and advice on clinical research and bacterial culture, respectively. SN, HH, TY and TK—analyzed the data. SN, TY and YY—wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by Ethical Review Board for Life Science and Medical Research, Hokkaido University Hospital (No. 013-0393). The purpose of the study and the contents of the experiment were explained to the subjects and their guardians, and the experiment was conducted after obtaining written consent from the guardians.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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