Table 4 Multinomial logistic regression analysis for outpatient medical fees.

	Outpatient medical fees					
	¥200 000–¥399 999			*≥¥400 000*		
	OR	*95% CI*	*p-values*	*OR*	*95% CI*	*p-values*
Eichner index						
A	1.00			1.00		
B1–B3	1.34	0.426–4.188	0.619	4.36	0.908–20.923	0.066
B4, C	0.96	0.364–2.525	0.931	2.44	0.580–10.282	0.223
Systemic disease						
Mild	1.00			1.00		
Moderate	3.41	1.655–7.013	0.001**	3.44	1.357–8.717	0.009*
Severe	3.84	1.704–7.013	0.001**	11.69	4.589–29.790	0.000**
Sex						
Female	1.00			1.00		
Male	0.59	0.313–1.126	0.110	0.81	0.386–1.679	0.563
Age						
65–69 years	1.00			1.00		
70–74 years	0.94	0.387–2.261	0.883	1.10	0.386–3.116	0.863
75–79 years	1.04	0.407–2.644	0.940	1.36	0.461–4.030	0.576
≥80 years	1.00	0.300–3.313	0.996	0.76	0.187–3.112	0.705
Current employment status						
Working	1.00			1.00		
Not working	1.65	0.799–3.390	0.176	1.36	0.600–3.099	0.459
Participate in social activities	1.00			1.00		
Do not participate in social activities	1.12	0.530–2.382	0.761	0.94	0.400–2.219	0.891
Educational background						
≥10 years	1.00			1.00		
<10 years	0.48	0.198–1.177	0.109	1.72	0.465–6.343	0.417
Subjective assessment of oral health						
Good	1.00			1.00		
Not good	1.36	0.677–2.738	0.387	1.77	0.808–3.886	0.153
Not good at all	5.27	1.489–18.625	0.01*	4.50	1.093–18.537	0.037*

OR (odds ratio) and 95% CI (confidence interval) for outpatient medical fees of ¥200 000–¥399 999 and ≥¥400 000 were calculated compared with ¥0–¥199 999. *$P < 0.05$; **$P < 0.01$.

reported that the number of times a subject receives medical treatment is influenced by medical needs[19]. Medical need has also been suggested as the primary factor determining outpatient medical expenses[20]. Thus, people who have good subjective assessment of oral health also might have good general health. Accordingly, they are less likely to seek consultation from their family doctor or local hospital, thereby incurring lower medical expenses, compared with subjects with poor assessment of oral and general health.

It has been suggested that subjective assessment of oral health has a significant independent effect on psychological well-being[17] and life satisfaction[21], while dental health behaviour has been shown to be associated with lifestyle[22–24]. Elderly subjects who have good psychological well-being and a good quality-of-life tend to find it easier to maintain their overall health. Medical expenditure in such elderly subjects will therefore be low.

It has also been reported that food selection is substantially affected by oral status[25] and that people who consume healthy foods tend to have lower medical expenses[26]. This is because people who have good subjective assessment of oral health also have good oral health status; that is, they have better nutrition[27], thus giving them stronger resistance to chronic or contagious diseases[28,29]. Consequently, they may not suffer from medical disorders so readily, which keeps their medical expenses low.

Having a chronic medical condition is considered a major contributing factor to the medical expenses of elderly persons; the present study supports this hypothesis. The relationship between subjective assessment of oral health and medical expenses was established after adjustment for chronic medical

conditions. This suggests that subjective self-assessment of oral health is significantly related to medical expenses through mechanisms other than chronic medical conditions. We previously demonstrated that people who have good self-assessment of masticatory ability also have good physical performance[30,31]. Self-assessed masticatory ability has been significantly related to subjective assessment of oral health[17]. For the elderly, maintaining muscular strength and balance is very important for protection against functional disorders, fragility, risk of falling and physical disabilities[32,33]. It has also been reported that muscular strength training reduces medical expenses[34]. One could argue that subjective assessment of oral health may therefore be related to the maintenance of physical strength, thus preventing a decline in general health. Consequently, subjective assessment of oral health may be related to a decrease in medical expenses.

Conclusion

We found that, after adjusting for confounding variables, subjective assessment of oral health was significantly and independently related to the medical expenses of community-dwelling elderly persons. These findings suggest that oral health status is an important parameter when elucidating the factors contributing to increased medical expenses among elderly persons.

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Original article

The short-term effects of various oral care methods in dependent elderly: comparison between toothbrushing, tongue cleaning with sponge brush and wiping on oral mucous membrane by chlorhexidine

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The short-term effects of various oral care methods in dependent elderly: comparison between toothbrushing, tongue cleaning with sponge brush and wiping on oral mucous membrane by chlorhexidine

Objectives: To explore the short-term effects from toothbrushing, tongue cleaning with sponge brush and wiping on oral mucous membrane by chlorhexidine.

Background: Numerous reports have been seen in recent years proving the effectiveness of mouth cleaning with a toothbrush for the prevention of respiratory infections among the dependent elderly. However, the short-term effects from each oral care method have not yet been clarified. Hence, an investigation was conducted by having each subject independently perform various oral care methods for five consecutive days.

Materials and Methods: The subjects consisted of 12 assistance-dependent elderly who have difficulties with tooth brushing by themselves, have 10 or more residual teeth and are not yet using plate dentures. After the pre-intervention examination, each of the following oral care methods were performed on the same subject on an approximately three week basis: 1) Tooth brushing 2)Tongue cleaning with sponge brush 3)Wiping on oral mucous with sponge brush by chlorhexidine. Each method was performed independently, once a day for 5 consecutive days and the subjects were reexamined on the sixth day for comparative verification.

Results: Consequently, toothbrushing decreased the plaque index and gingival index significantly and an improvement of oral malodour was also acknowledged ($p < 0.01$). Tongue cleaning with a sponge brush decreased the tongue coat score significantly ($p < 0.05$) and oral malodour was also improved ($p < 0.01$). Wiping on oral mucous with a sponge brush soaked in chlorhexidine significantly decreased opportunistic infections in the pharynx region ($p < 0.05$).

Conclusions: It was suggested that the use of not only a toothbrush but also chlorhexidine gluconate may be indicated for dependent elderly people in whom pathogens of opportunistic infection are detected.

Keywords: dependent elderly, oral care, the short-term effects, chlorhexidine, opportunistic infections.

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Introduction

The effects of oral care for the prevention of and treatment for dental diseases are widely known, and particularly, plaque control by brushing is useful to prevent and improve gingivitis and mild periodontitis[1–4]. It has also been reported that once-a-week professional oral health care (POHC) significantly reduced the number of pharyngeal bacteria within 5 months in dependent elderly people[5], reduced the detection rate of opportunistic infection-causing bacteria on the dorsum of the

tongue after 6 months[6], prevented pneumonia[7] and reduced the incidence of influenza[8]. The necessity of organic oral care centring on oral cleaning is clearly positioned in the 'guidelines for prevention of respiratory infections in inpatients' established by the Japanese Respiratory Society[9].

However, the intervention frequency was 1–2 times per month[3,10,11] or once a week[5–8,10,12–16] in most previous reports on the effect of oral care, and the results were obtained from a long-term intervention for 1 month or longer employing a series of professional oral care techniques for the teeth, mucosa, tongue and dentures using tools, such as tooth and interdental brushes, dental floss and a sponge brush. Sumi *et al.*[17] reported the results of 8-week daily intervention by wiping with drug solution and using an electric toothbrush, but no study compared the effect of short-term daily intensive care on oral malodour, periodontal disease and oral and pharyngeal bacteria among oral care methods: removal of the tongue coat using toothbrush and sponge brush and wiping with drug solution. Moreover, oral care by caregivers is essential, but some facilities have problems, such as limited staff, accompanying economic problems and a lack of attendants' knowledge concerning oral health, and oral care is not necessarily performed appropriately. Therefore, clarification of the short-term effect of each oral care method may facilitate the selection of an oral care method corresponding to the oral and systemic conditions of dependent elderly people and increase the efficiency of oral care.

In this study, we performed the following oral care individually for five consecutive days: (i) oral cleaning by toothbrushing alone, (ii) tongue coat removal using a sponge brush and (iii) wiping the oral mucosa with a gargling solution containing chlorhexidine gluconate, and the short-term effect of each method was clarified on the 6th day to investigate whether the oral condition of dependent elderly people is changed by short-term daily care and the oral care method which exhibits an earlier effect.

Subjects and methods

Subjects

The subjects were 12 totally dependent elderly nursing home residents with difficulty in self-brushing who had 10 or more remaining teeth and no dentures. There were three men and nine women, and the mean age was 80.2 ± 6.4 years.

In addition to the age and gender, underlying diseases and the nursing care level (Table 1) were investigated based on care records and by interviewing facility staff (Table 2).

Oral care methods

After prior examination, dentists or dental hygienists performed single oral care: (i) oral cleaning by toothbrushing alone, (ii) tongue coat removal using a sponge brush and (iii) wiping the oral mucosa with a gargling solution containing chlorhexidine gluconate, in the specified subjects for five consecutive days between lunch and dinner, and the effects were investigated on the 6th day. The intervention was performed three times at 3-week intervals in the same subjects (Fig. 1). To standardise the methods, oral care was performed following the procedures below:

In addition, oral care was performed in the time from the end of lunch to supper (13:00–15:00) by a dentist (years of experience 20 years) and three dental hygienists (each having 10, 5 and 4 years of experience).

Oral cleaning using a toothbrush. After staining using a plaque tester (Lion Corporation, Tokyo, Japan), the stained materials were removed using a toothbrush, Dent EX Slim head II 33(M) (Lion Corporation) (toothbrushing). The duration of brushing was 5 min.

Tongue coat removal using a sponge brush. Using a sponge brush (Toothette-plus®; Inoue Attachment Co., Tokyo, Japan), the left side, centre and right side of the dorsum of the tongue were wiped from the posterior to anterior region 10 times each to remove the tongue coat (tongue coat removal).

Wiping the oral mucosa with a chlorhexidine gluconate-containing gargling solution. The chlorhexidine gluconate concentration was adjusted to 0.0002% by placing 10 drops of ConCool F® (0.05% chlorhexidine gluconate solution; Weltech, Osaka, Japan) into 50 ml of water. The above sponge brush was soaked in the 0.0002% chlorhexidine gluconate solution, and the buccal, gingival and palatal mucosae were wiped twice each with this sponge brush from the posterior to anterior region in this order (drug solution).

Evaluation items before and after oral care

Oral examination. The severity of periodontal diseases was evaluated employing the plaque index (PlI, Silness & Löe), gingival index (GI, Silness & Löe) and community periodontal index (CPI), and

Table 1 Nursing care level.

Nursing care level 1	Requiring support from two states, reduced ability to carry out some way of life, the state will require partial care
Nursing care level 2	In addition to a frail state, the state will also require partial care activities of daily living
Nursing care level 3	Compared to the two states requiring care, significantly reduced both in terms of behaviour and instrumental activities of daily living life, the state will require an almost total care
Nursing care level 4	In addition to the three conditions requiring care, the ability is further reduced, without a care in difficult conditions and their daily lives
Nursing care level 5	And a reduced ability to work more than four states requiring care, a state without a care to make life almost impossible

Table 2 General examination.

Case	*Age*	*Sex*	*Underlying disease*	*Term care level*
1	78	M	Senile dementia – hypertension	5
2	79	F	Senile dementia	4
3	73	F	Senile dementia – angina pectoris	4
4	80	F	Senile dementia	5
5	78	M	Sequelae of cerebral infarction – hypertension	3
6	85	F	Senile dementia – depression	4
7	75	M	Sequelae of cerebral infarction – hypertension	4
8	84	F	Thrombotic thrombocytopenic purpura – hypertension	5
9	95	F	Senile dementia – osteoporosis	5
10	79	F	Senile dementia	5
11	70	F	Sequelae of cerebral infarction – hypertension	4
12	86	F	Senile dementia – hypertension	5

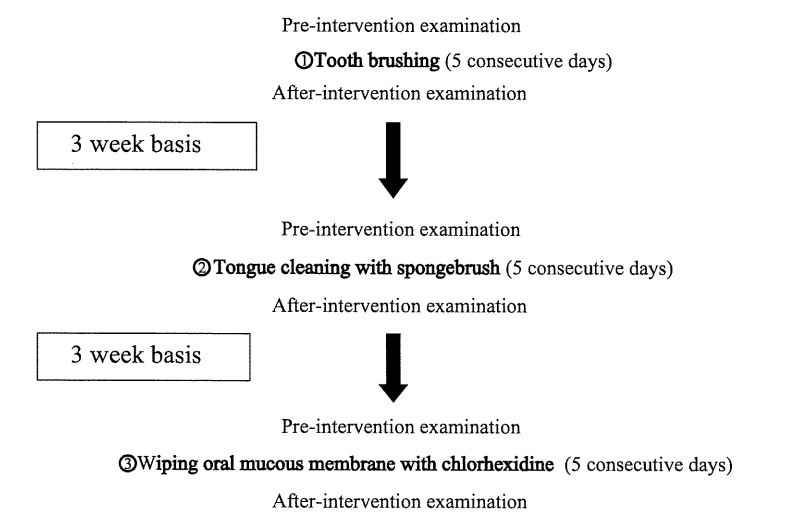

Figure 1 Study design.

the tongue coating condition was evaluated following the classification established by Miyazaki *et al.*[18] (Table 3).

The dryness of the mouth (saliva wetness) was measured at the dorsum of the tongue and hypoglottis, employing the method reported by

Table 3 Tongue coat score (standard of Miyazaki[18]).

0	Most of tongue coating is not accepted
1	Tongue coating 1/3 cover up under
2	Tongue coating covers up under 2/3 more than 1/3
3	Tongue coating 2/3 cover up the above

Kakinoki *et al.*[19] and a saliva wetness test paper (KISO Wet; KISOscience Co., Yokohama, Japan). Regarding oral malodour, two raters evaluated the expired air at a specific distance from the subject (10–20 cm) following the organoleptic test method[20–22] (Table 4).

Bacteriological test. (BML Inc., Tokyo, Japan) performed tests of periodontal disease-associated and dental caries-related bacteria, Streptococci, and pathogens of opportunistic infection using test kits of the company.

In the periodontal disease-related bacterial test, plaques on the upper frontal teeth were removed with sterile cotton balls and desiccation. A paper point was inserted into the tooth pocket and kept at the bottom for 10 s. The samples were sent for DNA detection of a periodontal disease-related bacterium, *Porphyromonas gingivalis*, employing the polymerase chain reaction invader (PCR invader) method.

Tests of *Staphylococcus mutans*, *Lactobacillus* and Streptococci as caries-related bacteria were also performed by BML Inc. Saliva retained in the hypoglottis was collected using a dropper and dripped on a cotton swab, and the swab was transferred into an exclusive tube and sent to the BML General Laboratory. In the measurement, the sample was transferred to a test tube containing 3 ml of PBS and vibrated on a direct mixer for 15 min to detach bacteria from the cotton swab to prepare a bacterial suspension. The bacterial suspension was then smeared on modified MS, modified MSB and lactobacillus media and anaerobically cultured for 48 h. *Staphylococcus mutans*, *Lactobacillus* and Streptococci were counted on modified MSB, *Lactobacillus* and modified MS media, respectively.

Table 4 Judgement standard of Organoleptic examination[20–22].

0	No smell
1	Very slight (sense a smell, but it is not a bad smell)
2	Slight (it is barely admitted that it is a bad smell)
3	Medium (it is recognised with a bad smell easily)
4	Severe (the strong bad smell that I can endure it)
5	Very severe (the strong bad smell that I cannot endure it)

Regarding pathogens of opportunistic infection, 10 species were investigated: methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-sensitive *Staphylococcus aureus* (MSSA), *Pseudomonas aeruginosa*, β haemolytic *Streptococcus*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Moraxella (Branhamella) catarrhalis* and *Candida* sp. The pharyngeal region was swabbed laterally from right to left four times at different sites using a culture swab with a sterile cap, transferred to an exclusive tube and promptly sent to the BML General Laboratory by postal service. For the measurements, bacterial species other than *H. influenzae* were cultured on blood agar medium. For selection culture, *P. aeruginosa*, *K. pneumoniae* and *S. marcescens* were cultured on BTB medium, a Gram-negative bacteria-selective medium. The 10 bacterial species including *H. influenzae* were also cultured on chocolate agar medium. OPA medium was used for the selection of MRSA, PASA medium for the selection of *P. aeruginosa* and Sabouraud medium for fungi. The sample was smeared on the above six media and incubated in a CO_2 incubator for 24–48 h, and the target bacterial colonies were identified with confirmation media and through using identification kits. The bacterial species were identified using the following materials: MRSA, ps Latex (Eiken Chemical, Tokyo, Japan), rabbit plasma (Eiken Chemical) and MRSA screening medium (BD Japan, Tokyo, Japan); MSSA, ps Latex, rabbit plasma and MSSA screening medium (BD Japan); *P. aeruginosa*, VITEK (Sysmex kobe, Japan); β haemolytic Streptococcus, Seroidenstrepto kit (Eiken Chemical), API Strepto (BVJ, Tokyo, Japan) and VITEK (BVJ); *S. pneumoniae*, *S. pneumoniae* identification disk, Taxo P disk (BD Japan) and Strepto (BVJ); *H. influenzae*, Haemophilus ID4 fraction (BD Japan); *K. pneumoniae*, VITEK (BVJ); *S. marcescens*, VITEK (BVJ); *M(B) catarrhalis*, ID test HN20 (Nissui , Tokyo, Japan); and *Candida* sp., CHROMagar Candida (BD Japan).

Statistical analysis

The conditions before and after oral care were compared using the Wilcoxon signed-rank test. Specific oral care in the same 12 subjects was performed every 3 weeks, and the condition before and after intervention was compared using the Friedman test. The significance level was set at 5% in all tests.

This study was approved by the Matsumoto Dental University Ethics Committee (approval number

0048), and informed consent was obtained after the study objective and content were orally explained using documents to all subjects and their families.

Results

Evaluation before intervention

Oral examination items. No significant differences were noted in the GI, CPI or saliva wetness at the hypoglottis or dorsum of the tongue among the toothbrush, tongue coat removal and drug solution groups before intervention. The PlI was significantly higher in the toothbrush than in the tongue coat removal group, but no significant difference was noted between the tongue coat removal and drug solution groups (Table 5).

Regarding the condition of the tongue coat (Fig. 2) and oral malodour (Fig. 3), no significant differences were observed among the toothbrush, tongue coat removal and drug solution groups.

Bacteriological test. There were no significant differences in the counts of *P. gingivalis*, *S. mutans*, *Lactobacillus* or Streptococci among the toothbrush, tongue coat removal and drug solution groups before intervention (Table 6), nor was there a significant difference in the number of patients carrying pathogens of opportunistic infection among the groups (Table 7).

Effects of interventions

Oral examination. The PlI and GI significantly decreased after intervention compared to those before intervention in the toothbrush group ($p < 0.01$), but no significant changes were observed in the other groups (Figs 4 and 5). The CPI did not significantly change after intervention in any group (Fig. 6). The tongue coat score decreased in significantly more subjects in the tongue coat removal group, but no significant change was observed after intervention in the toothbrush or drug solution group (Fig. 7). The saliva wetness was not significantly changed after intervention at the hypoglottis or the dorsum of the tongue in any of the three oral care groups (Fig. 8).

Regarding oral malodour, no subject was judged as showing 'no smell' before intervention in the toothbrush group, but eight subjects were judged as

Table 5 Oral examination before intervention.

Examination	*Before toothbrush use*	*Before the tongue coating removal*	*Before wiping on oral mucous membrane by chlorhexidine*	*Significance*
PI I	1.7 ± 0.5	1.3 ± 0.5	1.5 ± 0.3	0.03*
GI	1.4 ± 0.4	1.2 ± 0.4	1.4 ± 0.2	0.10 (NS)
CPI	2.0 ± 1.0	1.9 ± 0.9	2.0 ± 0.9	0.93 (NS)
Saliva wetness (hypoglottis) mm	9.2 ± 3.2	9.3 ± 4.6	8.8 ± 4.0	0.97 (NS)
Saliva wetness (on the tongue) mm	6.4 ± 3.0	6.3 ± 4.3	5.1 ± 2.6	0.92 (NS)

Friedman test *$p < 0.05$.

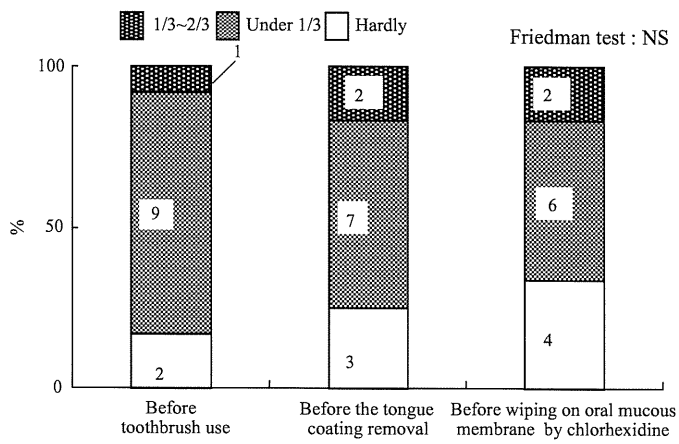

Figure 2 The condition of the tongue coat before intervention.

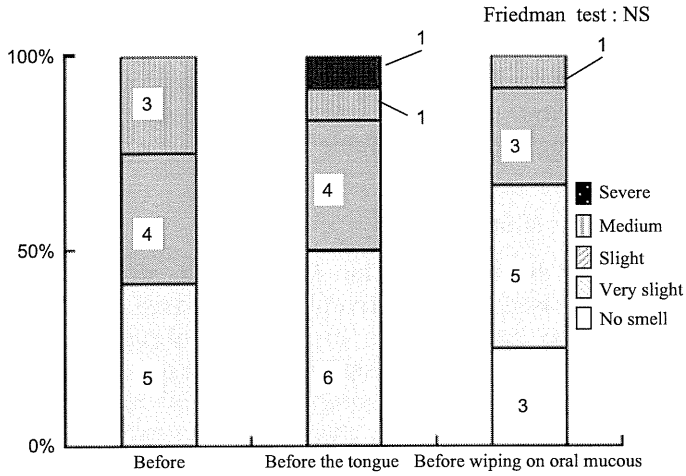

Figure 3 The condition of oral malodour before intervention (Organoleptic examination).

Table 6 Bacteriological test (*Porphyromonas gingivalis, Streptococcus mutans, Lactobacillus, Streptococci*) before intervention.

Bacteria	*Before toothbrush use*	*Before the tongue coating removal*	*Before wiping on oral mucous membrane by chlorhexidine*	*Significance*
Porphyromonas gingivalis $\log_{10}$/10 μl	2.6 ± 1.3	2.5 ± 1.4	2.7 ± 1.7	0.91 (NS)
Streptococcus mutans $\log_{10}$ CFU/ml	2.9 ± 0.5	3.1 ± 0.9	3.0 ± 0.4	0.51 (NS)
Lactobacillus $\log_{10}$ CFU/ml	3.1 ± 0.8	3.3 ± 0.7	3.1 ± 0.5	0.55 (NS)
Streptococci $\log_{10}$ CFU/ml	5.3 ± 0.9	5.2 ± 0.7	5.4 ± 1.0	0.76 (NS)

Friedman test.

such after intervention, revealing a significant improvement ($p < 0.01$). Significant improvement after intervention was also noted in the tongue coat removal and drug solution groups (Fig. 9).

Bacteriological test. No significant changes were noted after intervention in the counts of *P. gingivalis* (Fig. 10), *Lactobacillus, S. mutans* (Fig. 11) or Streptococci (Fig. 12). Regarding pathogens of opportunistic infection, no significant change was noted after intervention in the toothbrush or tongue coat removal group, but the number of carrier subjects significantly decreased in the drug solution group (Table 8).

Discussion

Plaque control by toothbrushing

Self-care is difficult for dependent elderly people with reduced Activities of Daily Living (ADL), and so brushing by caregivers is essential. The effect of toothbrushing alone was not clear in previous reports on oral care in dependent elderly subjects because toothbrushing was performed while concomitantly using other cleaning tools[3,5–8,10–17], and the interval was long: once a week[5–8,10,12–16] or 1–2 times a month[3,10,11]. Biofilm is formed within several hours or several days[23]. Löe *et al.*[24] also reported that gingivitis developed within 2–3 days, suggesting that the educational effect of intervention on facility staff was greater than the effects of cleaning tools and methods in previous reports. It has been reported that the effect of toothbrushing on experimental gingivitis in animals was exhibited after brushing for 4[1] or 7[2] days as a significant reduction in the GI representing an improvement in gingivitis. In the dependent elderly subjects, the PlI decreased after 5 days, showing significant improvement in gingivitis, but the CPI or number of *P. gingivalis* was not decreased, in contrast to previous reports in which the numbers of periodontal pockets[1] and submarginal gingival bacteria[25,26] were decreased by marginal plaque

Table 7 The number of patients carrying pathogens of opportunistic infection before intervention.

Opportunistic infections	*Before toothbrush use*	*Before the tongue coating removal*	*Before wiping on oral mucous membrane by chlorhexidine*
MRSA	0	0	0
MSSA	1	1	2
Pseudomonas aeruginosa	3	2	1
β-*Streptococcus*	0	0	0
Streptococcus pneumoniae	0	0	0
Haemophilus influenzae	0	0	0
Klebsiella pneumoniae	0	0	0
Serratia marcescens	1	1	1
M(B) catarrhalis	0	0	0
Candida sp.	0	0	3
Total	5	4	7

Friedman test: NS.

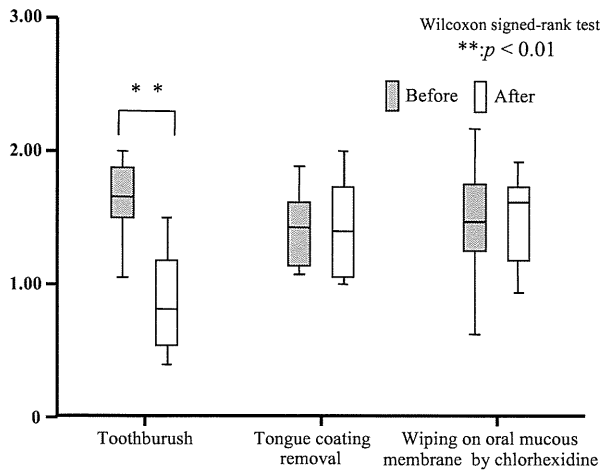

Figure 4 Comparison of Plaque Index before and after intervention.

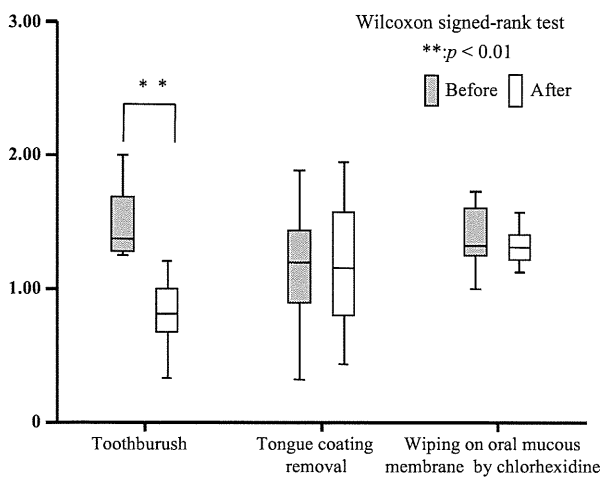

Figure 5 Comparison of Gingival Index before and after intervention.

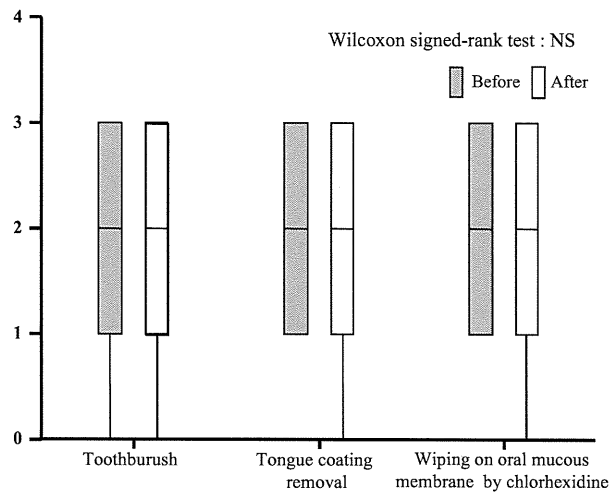

Figure 6 Comparison of Community Periodontal Index before and after intervention.

removal. Cercek *et al.*[27] reported that the depth of periodontal pockets was not decreased by oral cleaning alone when advanced periodontal disease was already present, pointing out the necessity of periodontal disease treatment. Although the CPI value does not directly reflect the pocket depth, our study also confirmed that it is difficult to improve periodontitis by toothbrushing alone. Moreover, the 5-day oral care did not affect the number of *P. gingivalis* because the subgingival region was not treated, suggesting that a prolonged duration of plaque control is necessary to reduce the number of subgingival bacteria. Once-a-day plaque removal did not influence the number of salivary caries-related bacteria. There is within-day variation in the salivary bacterial count: the number of bacteria is temporarily reduced by meals and brushing but

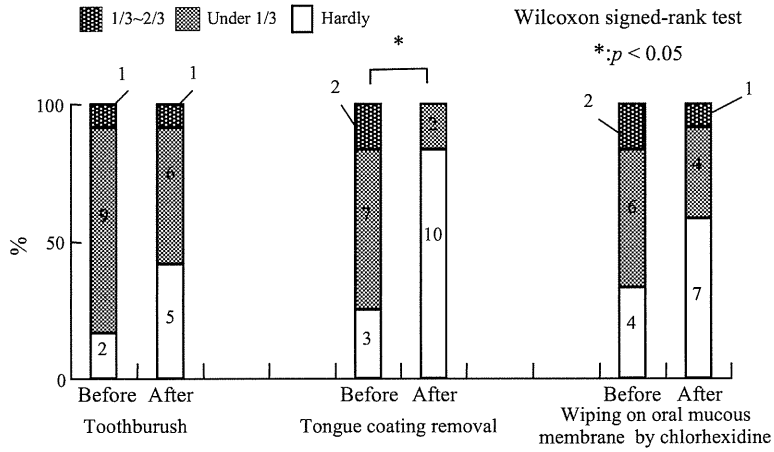

Figure 7 Comparison of Tongue coat before and after intervention.

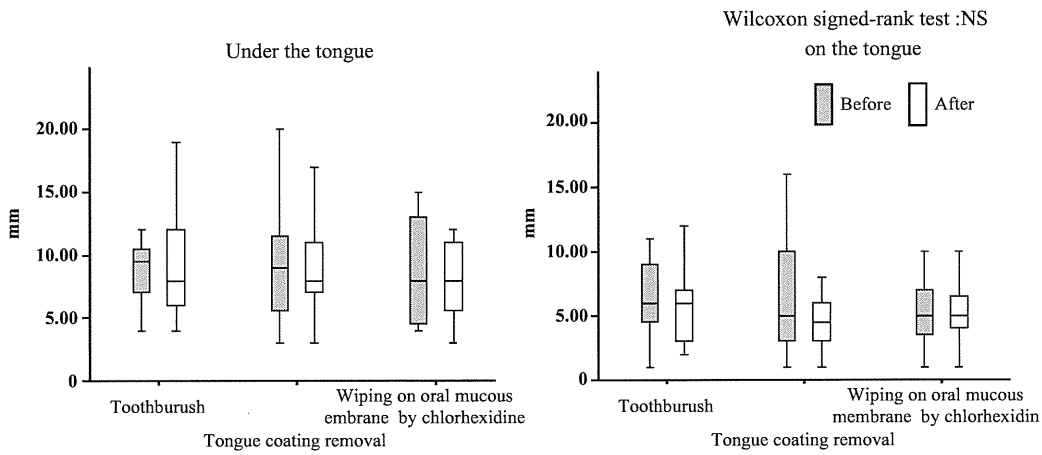

Figure 8 Comparison of Saliva Wetness before and after intervention.

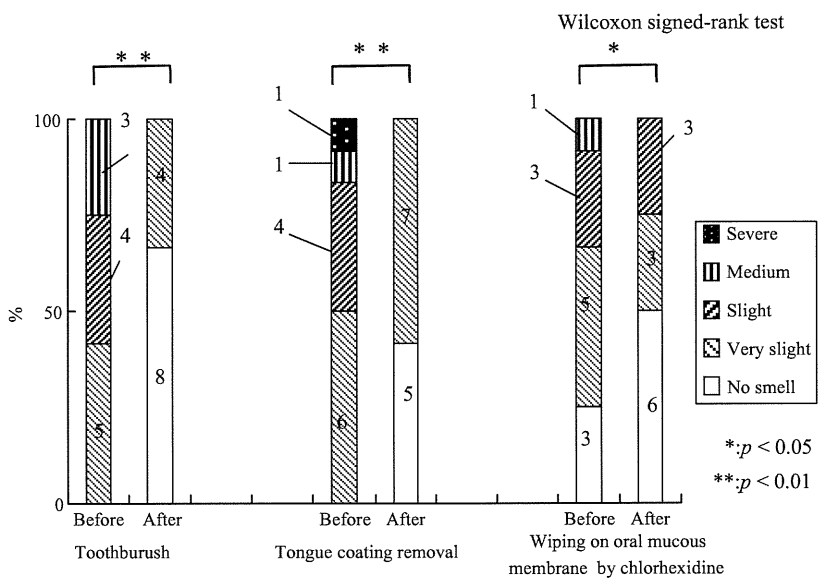

Figure 9 Comparison of oral malodour (Organoleptic examination) before and after intervention.

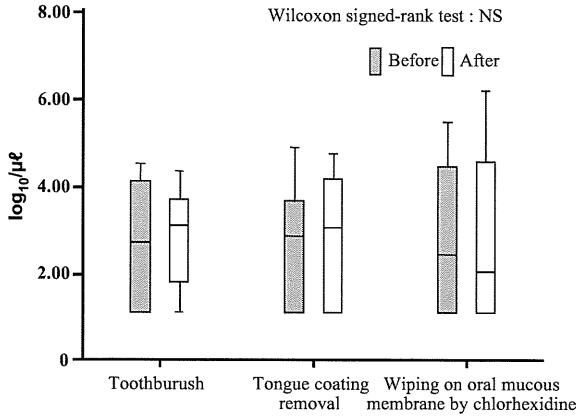

Figure 10 Comparison of *Porphyromonas gingivalis* before and after intervention.

increases again before meals and at night-time. The absence of a significant difference in the salivary bacterial count may have been due to the time passage after plaque control on the previous day.

Plaque control by brushing can significantly improve oral malodour. A physiological oral malodour is present in all people and is increased on awakening and in strained and fasting states, but is decreased by eating, drinking and oral cleaning[28]. The oral malodour was mild before intervention in many subjects, and plaque control by toothbrushing improved this malodour.

The subjects were dependent elderly people incapable of following investigational instructions. Because the measurement of saliva secretion was difficult, wetness of the oral mucosa was measured following Kakinoki's method[18] using KISO Wet®. For the treatment of dryness of the mouth, salivary gland massage[29,30], oral moisturiser application[31] and saliva secretion stimulator administration[32,33] are available. An increase in the wetness by massage applying vibration of a sonic toothbrush three times a week for 4 weeks in patients with dry mouth has been reported[18], but no influence of brushing with a toothbrush on the oral wetness was observed in our study. This may have been due to the fact that we did not select subjects with reduced saliva wetness at the hypoglottis or dorsum of the tongue, and dryness of the mouth was within the normal range. Therefore, it is unclear whether plaque control by brushing is effective for the treatment of xerostomia.

Regarding pathogens of opportunistic infection, *P. aeruginosa* was frequently detected in the pharyngeal region before intervention, as reported by Nagaosa *et al.*[34] and Tsugayasu *et al.*[6]. Kimizuka *et al.*[8] reported that the prevalence of *P. aeruginosa* and *Candida* sp. was significantly decreased by once-a-week professional care over 2 years, although the tools used were not described. In a study reported by Hirota *et al.*[5], dentists or dental hygienists performed oral care using a toothbrush, interdental brush and dental floss, and the number of pharyngeal bacteria was unchanged after 1–2 months but decreased after 5 months, although the interval of oral care was not described. A lower number of detected MRSA in patients treated with oral care was also reported[35,36], suggesting that plaque control by brushing reduces the number of bacteria causing opportunistic infection in the pharyngeal region. However, plaque control by 5-day brushing alone did not influence pathogens of opportunistic infection in the pharynx. For pathogens of opportunistic infection detected in dependent elderly patients with dysphagia, the instruction of facility staff to use a toothbrush alone is insufficient and inappropriate to prevent aspiration pneumonia.

Tongue coat removal using a sponge brush

The tongue coat is bacterial biofilm accumulated on the dorsum of the tongue, in which *Candida* sp.,

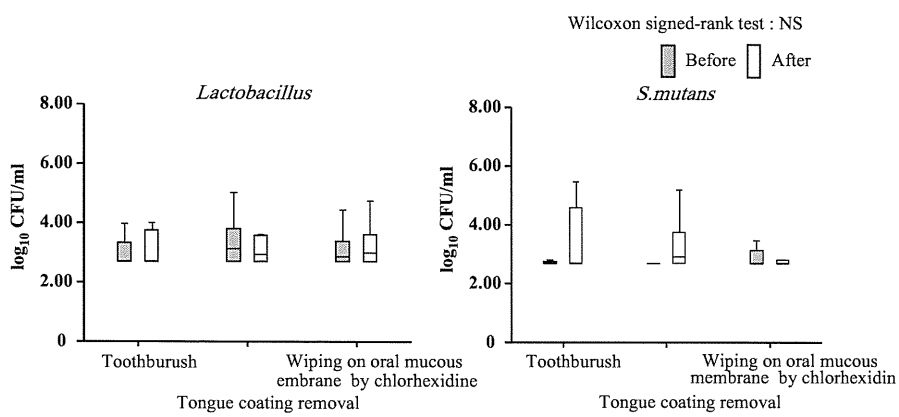

Figure 11 Comparison of *Lactobacillus* and *Streptococcus mutans* before and after intervention.

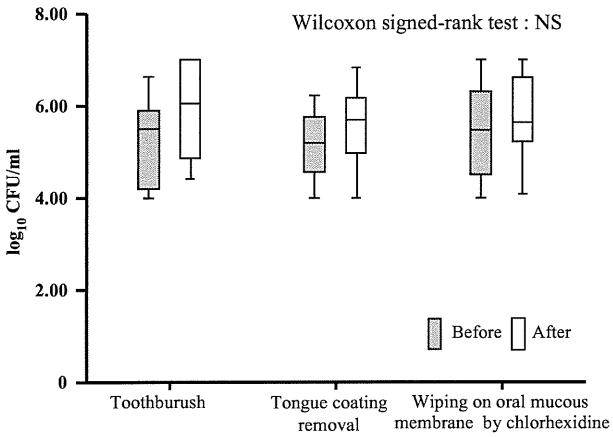

Figure 12 Comparison of *Streptococci* before and after intervention.

Streptococci and *Staphylococcus* are indigenously present. It markedly influences the total number of oral bacteria because of a wide adhering area[37]. Changes in this are reflect variation in the total number of overall oral bacteria, and the amount of adhering tongue coat is closely associated with the cause of oral malodour, the volatile sulphur compound (VSC) level[38]. There have been several reports on the association between the tongue coat and oral malodor[19–21], and the posterior dorsum of the tongue was suggested as the cause of this malodor[19]. Because tongue coats are reservoirs of pharyngeal bacteria and cause aspiration pneumonia and halitosis[28], tongue coat removal is necessary for dependent elderly people. Removal methods using tongue and sponge brushes have been reported[39]. Sumi *et al.*[17] reported that lightly scrubbing the tongue with a tongue brush from the posterior to the anterior region 10 times reduced the amount of tongue coat and oral malodour. We also performed tongue cleaning using a low-price sponge brush (Toothette-plus®; Inoue Attachment Co.), which is also useful for the cleaning of other mucosae.

In another report, a sponge brush was used for tooth surface cleaning but the interdental plaque removal rate was low[40], suggesting that sponge brushes should be used for mucosal cleaning. Mechanical cleaning of the dorsum of the tongue using a sponge brush reduced the tongue coat score and significantly improved oral malodour. However, no influences were noted on the PlI, GI, number of caries-related or periodontal disease-associated bacteria, or wetness of the dorsum of the tongue or hypoglottis.

The tongue coat level can be reduced using a sponge brush but not completely as pathogens of opportunistic infection are indigenously present on the tongue. Moreover, treatment was performed for only 5 days, which may have been insufficient to affect the detection of pathogens of opportunistic infection.

The effect of tongue coat removal on oral malodour appeared within short-term treatment even though oral malodour was severe. The use of a sponge brush to remove the tongue coat may be recommended for dependent elderly people with halitosis.

Table 8 Comparison of the number of patients carrying pathogens of opportunistic infection before and after intervention.

	Toothbrush		*Tongue coating removal*		*Wiping on oral mucous membrane by chlorhexidine**	
Opportunistic organisms	*Before*	*After*	*Before*	*After*	*Before*	*After*
MRSA	0	0	0	0	0	0
MSSA	1	1	1	0	2	1
Pseudomonas aeruginosa	3	0	2	0	1	0
β-*Streptococcus*	0	0	0	0	0	0
Streptococcus pneumoniae	0	0	0	0	0	0
Haemophilus influenzae	0	0	0	0	0	0
Klebsiella pneumoniae	0	1	0	1	0	0
Serratia marcescens	1	1	1	0	1	0
M(B) catarrhalis	0	0	0	0	0	0
Candida sp.	0	1	0	2	3	1
Total	5	4	4	3	7	2

Wilcoxon signed-rank test $*p < 0.05$.

Cleaning of oral mucosa with chlorhexidine gluconate

Chlorhexidine gluconate reduces pellicle formation and bacterial adsorption and adhesion to the teeth[41]. Chlorhexidine is strongly cationic and impairs bacterial cell membrane permeability by adsorbing to the negatively charged bacterial surface and penetrating the cell wall. It also exhibits a bacteriostatic effect by inhibiting membrane-bound enzymes at a low concentration and bactericidal and persistent antimicrobial effects by coagulating ATP or nucleic acid at a high concentration[42]. About a 60% reduction in plaque and gingivitis with the use of chlorhexidine gluconate for 4 months has been reported[43–45], but no influence on the PlI, GI or number of *P. gingivalis* was noted in our study. In reports from Western countries, chlorhexidine gluconate was used at 0.12% or higher, whereas a 0.05% or higher concentration is prohibited in Japan because of a risk of anaphylactic shock, and we used it at 0.0002%. This difference in the concentration and the short duration of intervention may have affected the results as the drug solution generally does not reach 3 mm or deeper periodontal pockets[46]. The number of *P. gingivalis* was not reduced by wiping the mucosa with chlorhexidine gluconate. One reason for this may have been that the periodontal pockets not reached by the drug solution. In addition, the number of caries-related bacteria was small before intervention, which may not have been reduced further.

The numbers of subjects with the tongue 'not' coated and '<1/3' of the tongue coated increased after intervention, but the changes were not significant. As the dorsum of the tongue was not wiped, no direct effect was exhibited, but the oral malodour was significantly improved, suggesting that not only mechanical cleaning of the buccal, gingival and palatal mucosae but also the pharmacological effect of chlorhexidine gluconate affected the oral bacteria, improving oral malodour.

As pathogens of opportunistic infection cause aspiration pneumonia in dependent elderly people, the control of these pathogens is important for their survival. The number of opportunistic infection-causative pharyngeal bacteria significantly decreased after wiping the oral mucosa with chlorhexidine gluconate for 5 days. DeRiso *et al.*[47] treated the oral cavity and pharynx with chlorhexidine gluconate in intubated patients and achieved a reduction in the rate of respiratory and hospital infections. Our study showed that the pharmacological effect was exhibited on the pharynx by 5-day application to the entire oral cavity. However, the Japanese Respiratory Society pointed out a risk of resistant bacterial colonisation[48] and development of infectious disease[10], although no emergence of resistant bacteria induced by the long-term use of chlorhexidine gluconate has been demonstrated[45,49]. This study suggested that when pathogens of opportunistic infection are detected in dependent elderly patients with dysphagia, which is likely to cause aspiration pneumonia, wiping of the oral mucosa with chlorhexidine gluconate is effective, but a more appropriate method should be proposed.

Conclusion

The effects of short-term oral care for 5 days employing various methods (oral cleaning by toothbrushing alone, tongue coat removal using a sponge brush and wiping the oral mucosa with a gargling solution containing chlorhexidine gluconate) in dependent elderly subjects were clarified to investigate the effect and indication of each method:

1. Plaque control by toothbrushing significantly reduced the PlI and GI and improved oral malodour.

2. Scrubbing the dorsum of the tongue with a sponge brush significantly reduced the tongue coat score and improved oral malodour.

3. Wiping the oral mucosa with chlorhexidine gluconate significantly reduced the number of subjects in whom pathogens of opportunistic infection were detected in the pharynx.

4. It was suggested that the use of not only a toothbrush but also chlorhexidine gluconate may be indicated for dependent elderly people in whom pathogens of opportunistic infection are detected.

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ORIGINAL PAPER

Expression and cellular localizaion of melatonin-synthesizing enzymes in rat and human salivary glands

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Abstract Melatonin, discovered in 1958, is secreted by the pineal gland primarily during the night. Its secretion is controlled by the light/dark cycle of the environment. Melatonin is also produced in and secreted by various extrapineal organs, tissues and cells and its synthesizing enzyme arylalkylamine *N*-acetyltransferase (AANAT) is expressed in various extrapineal organs, tissues and cells. Recently, it was reported that melatonin is present in saliva, but it is not certain where melatonin was synthesized and whether it was secreted into saliva and what function it may have in saliva. The present study was performed to investigate where melatonin was synthesized and whether it was secreted by salivary glands into saliva. We performed immunohistochemical analysis of the expression of AANAT in rat parotid, submandibular and sublingual glands and the expression of both AANAT and hydroxyindole-*O*-methyltransferase (HIOMT) in human submandibular glands. We evaluated the expression of AANAT and HIOMT mRNA in rat submandibular glands by quantitative reverse transcription-polymerase chain reaction. As a result, we observed expression of AANAT in epithelial cells of striated ducts in rat salivary glands and expression of AANAT, HIOMT and melatonin in epithelial cells of striated ducts in human submandibular glands. In addition, we also confirmed the expression of the most potent melatonin receptor, melatonin 1a receptor, in rat buccal mucosa. Our findings suggest that melatonin might be produced and secreted by salivary glands directly into saliva and that it might play some physiological role in the oral cavity.

Keywords Melatonin · Arylalkylamine *N*-acetyltransferase (AANAT) · Hydroxyindole-*O*-methyltransferase (HIOMT) · Salivary glands · Melatonin 1a receptor · Oral mucosa

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Introduction

Melatonin was discovered by Lerner et al. in 1958 and is secreted by the pineal gland primarily during the night. Its secretion is controlled by the light:dark cycle, so that it is released in large amounts during the night, but only minimally during the day (Lynch et al. 1987; Reiter et al. 1991; Reiter et al. 1996). Melatonin is also produced and secreted in various extrapineal organs, tissues and cells such as retina (Hamm and Menaker 1980), lens (Itoh et al. 2007), ovarium (Itoh et al. 1999), stomach and gut (Stefulj et al. 2001) and lymphocytes (Carrillo-Vicoa et al. 2004). Melatonin is synthesized enzymatically from L-tryptophan by the sequential actions of four enzymes: Tryptophan hydroxylase, 5-hydroxytryptophan amino acid decarboxylase, arylalkylamine *N*-acetyltransferase (AANAT) and hydroxyindole-*O*-methyltransferase (HIOMT) (Klein and Joan 1970; Axelrod and Weissbach 1960). Of these enzymes, AANAT, which acetylates serotonin (Klain 2007), is the most potent rate-limiting enzyme in the melatonin synthesis pathway. *N*-acetylserotonin subsequently is converted to melatonin by HIOMT (Axelrod and Weissbach 1960).

Melatonin has a variety of physiological actions such as control of circadian rhythms (Rednab et al. 1983; McArthur et al. 1997; Sack et al. 2000), regulation of body temperature (Dollins et al. 1994), hormone secretion influencing sexual development (Esquifino et al. 1987; Batmanabane and Ramesh 1996) and the reproductive cycle in seasonally breeding animals (Kennaway and Rowe 1995), activation of immune system (Garcia-Mauriño et al. 1997; Barjavel et al. 1998; García-Mauriño et al. 2000), anti-tumor activity (Hill and Blask 1988; Martínez-Campa et al. 2006), free-radical scavenging and antioxidation (Eşrefoğlu et al. 2005; León et al. 2005; Reiter et al. 2003). In addition, melatonin was suggested to play an important physiolosical role in bone formation (Roth et al. 1999; Satomura et al. 2007) and tooth development (Kumasaka et al. 2010). Recently, it was reported that melatonin existed in saliva as well as serum (Kennaway and Voultsios 1998). However, it is not certain where salivary melatonin is synthesized and secreted or what function melatonin has in saliva. Judging from the fact that melatonin plays various biological roles in a variety of tissues and organs, it is not difficult to imagine that salivary melatonin may be produced and secreted by salivary glands. In the present study, to elucidate this possibility, we examined the expression and localization of AANAT and HIOMT in rat and human salivary glands. In addition, to explore the possibility that the oral mucosa is a target tissue for salivary melatonin, we also examined the expression of the most potent melatonin receptor, melatonin 1a receptor (Mel1aR) in rat buccal mucosa.

Materials and methods

Rat tissue samples

The housing care and experimental protocol were approved by the Animal Care and Use Committee of the School of Dental Medicine, Tsurumi University. The salivary glands (parotid glands, submandibular glands and sublingual glands), brains and buccal mucosa of 8-week-old male Sprague–Dawley rats were extirpated, fixed with 4% paraformaldehyde (PFA, Wako Pure Chemical Industries Ltd, Osaka, Japan) and embedded in paraffin. Sections were cut 5 μm thick, deparaffinized and stained with hematoxylin and eosin.

Human tissue samples

The experimental protocol and the use of archival formalin-fixed, paraffin-embedded human submandibular gland samples were approved by the Ethical Review Committee of the Tsurumi University. Sections were cut 5 μm thick, deparaffinized and stained with hematoxylin and eosin.

Immunohistochemistry

Some sections of rat salivary glands (parotid glands, submandibular glands, sublingual glands) and human submandibular glands were transferred onto poly-L-lysine-coated glass slides. After deparaffinization with xylene and rehydration with descending concentrations of ethanol, endogenous peroxidase was blocked by treatment with 3% H_2O_2 in methanol for 1 h at room temperature (RT). After treatment with 20% normal goat serum at RT for 1 h, sections were incubated with the primary antibody (rabbit polyclonal antibody against rat AANAT; Santa Cruz Biotechnology, Inc., CA, USA, rabbit polyclonal antibody against human HIOMT; Santa Cruz Biotechnology, Inc., rabbit polyclonal antibody against human melatonin; Thermo Fisher Scientific, Rockford, USA, or mouse polyclonal antibody against human Mel1aR; Abnova Corporation, Taipei, Taiwan) diluted 1:500 in phosphate-buffered saline (PBS, pH7.4) containing 1% bovine serum albumin at 4°C overnight. After washing with PBS, the sections were incubated with biotinylated secondary antibody (Histofine SAB-(R) or Histofine SAB-(Multi)) for 30 min at RT. The location of AANAT, HIOMT, melatonin and Mel1aR was visualized by the method by Adams (1981). Sections were counterstained with hematoxylin and mounted. The specificity of the immunoreaction was confirmed by incubation with normal rabbit or mouse IgG and normal rabbit or mouse serum instead of primary antibody.

Some sections of rat buccal mucosa were also treated in the same manner as mentioned earlier. Sections were incubated with the primary antibody (mouse polyclonal antibody against rat Mel1aR; Abnova Corporation) diluted 1:1000 in phosphate-buffered saline (PBS, pH7.4) containing 1% bovine serum albumin at 4°C overnight. After washing with PBS, the location of Mel1aR was visualized using a Histofine SAB-PO(M) Kit (Nichirei) and a 3,3′-diaminobenzidine (DAB) Substrate Kit (Nichirei). Sections were counterstained with hematoxylin and mounted. The specificity of the immunoreaction was confirmed by incubation with normal mouse IgG and normal mouse serum instead of primary antibody.

Semi-quantitative reverse transcription-polymerase chain reaction

The expressions of mRNA encoding AANAT and HIOMT in rat submandibular glands and Mel1aR in rat buccal mucosa were examined by reverse transcription-polymerase chain reaction (RT–PCR). Submandibular glands and brains were obtained from rats during the dark period (extirpated at 23:30–24:00 h) or the light period (extirpated at 15:00–16:00 h). Total RNA was extracted using TRIzol® reagent (Invitrogen Corp., Carlsbad, CA, USA), and

the cDNA was generated from 1 μg of total RNA using the Superscript III First-Strand Synthesis System (Invitorogen) after DNase I treatment. PCR was carried out in a 50 μl reaction mixture using Thermo ReddyMix PCR Master Mix (Thermo Fisher Scientific, Rockford, IL, USA). Conditions and primer sequences for PCR amplification are shown in Table 1. The GAPDH gene was used as an internal control for the quantity and quality of cDNA. PCR products were analyzed by ethidium bromide staining after separation by electrophoresis through a 2% agarose gel. The brain was used as positive control for mRNA expression of AANAT, HIOMT and Mel1aR.

Real-time reverse transcription polymerase chain reaction

The expression of mRNA encoding AANAT and HIOMT in rat submandibular glands was also examined by real-time PCR. PCR was performed with SYBR® Premix Ex Taq™ (Takara Bio Inc., Shiga, Japan) using an Applied Biosystems StepOne™ Real-Time PCR System (Applied Biosystems Inc., Carlsbad, CA, USA). Conditions and primer sequences for PCR amplification are shown in Table 2. The GAPDH gene was used as an internal control for the quantity and quality of cDNA. The brain was used as positive control for mRNA expression of AANAT and HIOMT.

Results

Localization of AANAT in rat salivary glands

To examine whether AANAT is expressed in rat salivary glands, we first examined the cellular location of AANAT protein in rat parotid glands, submandibular glands and sublingual glands by immunohistochemical analysis. In parotid glands, serous acini and secretory ducts were observed (Fig. 1a). Epithelial cells of striated ducts were positive for AANAT (Fig. 1b). In submandibular glands, seromucous acini and secretory ducts were observed (Fig. 1d). The striated ductal cells were noted to be positive for AANAT (Fig. 1e). In sublingual glands, mucous acini and secretory ducts were observed (Fig. 1g). Epithelial cells of striated ducts were also positive for AANAT (Fig. 1h).

Localization of AANAT, HIOMT, melatonin and Mel1aR in human submandibular glands

Next, we immunohistochemically confirmed AANAT, HIOMT, melatonin and Mel1aR location in human submandibular glands. In human submandibular glands, seromucous acini and secretory ducts were observed (Fig. 2a, b). Similar to rat submandibular glands, epithelial cells of striated ducts were positive for AANAT (Fig. 2c). Importantly, these ductal cells were noted to be positive for HIOMT (Fig. 2e) and melatonin (Fig. 2g). In contrast, no significant expression of Mel1aR was detected in human submandibular glands (data not shown).

Expression of mRNA for AANAT and HIOMT in rat submandibular glands

Semi-quantitative RT–PCR analysis revealed that mRNA for AANAT was expressed in rat submandibular glands. Interestingly, the expression level of mRNA for AANAT and HIOMT in submandibular glands appeared to be higher in the dark period compared with the light period (Fig. 3a). So in the next step, we performed real-time RT–PCR for quantitative analysis. As a result, no significant difference in the expression level of AANAT was detected between light and dark period (Fig. 3b). In contrast, and interestingly, the expression level of HIOMT mRNA was higher in the dark period compared with the light period (Fig. 3c). Besides, in brains, the expression

Table 1 Oligonucleotide primers used in semi-quantitative RT–PCR

Primers	GenBank accession no.	Size (bp)	Sequences	Annealing temperature	Cycles
AANAT	DQ075321	325	F: 5′-GCG CCA CAC ACT COC TGC CAG TGA-3′ R: 5′-GOC CTT GOC CTG CTG OCG GAA GGT TC-3′	65	40
HIOMT	L78306	363	F: 5′-GGT AGC TOC GTG TGT GTC TT-3′ R: 5′-AGT GGC CAG GTT GCG GTA GT-3′	63	40
GAPDH	NM-017008	452	F: 5′-ACC ACA GTC CAT GOC ATC AC-3′ R: 5′-TCC ACC ACC CTG TTG CTG TA-3′	55	25
Mel1aR	UI4409	360	F: 5′-(57)GTGCTAOGTGTTCCTGATATGG-3′ R: 5′-(416)GGATCTGAGGCCACAATAAGAC-3	55	40

F forward, *R* reverse

Table 2 Oligonucleotide primers used in real-time quantitative RT–PCR

Primers	GenBank accession no.	Size (bp)	Sequences	Annealing temperature	Cycles
AANAT	DQ075321	148	F: 5′-CGA AGC CTT TAT CTC AGT CT-3′ R: 5′-CTC CTT GTC OCA AAG TGA AC-3′	60	40
HIOMT	L78306	108	F: 5′-GGT AGC TCC GTG TGT GTC TT-3′ R: 5′-GAA GTC ACC AGC GAC AAA OC-3′	60	40
GAPDH	NM-017008	112	F: 5′-ACC ACA GTC CAT GOC ATC AC-3′ R: 5′-GGA TGC AGG GAT GAT GTT CT-3′	60	40

F forward, *R* reverse

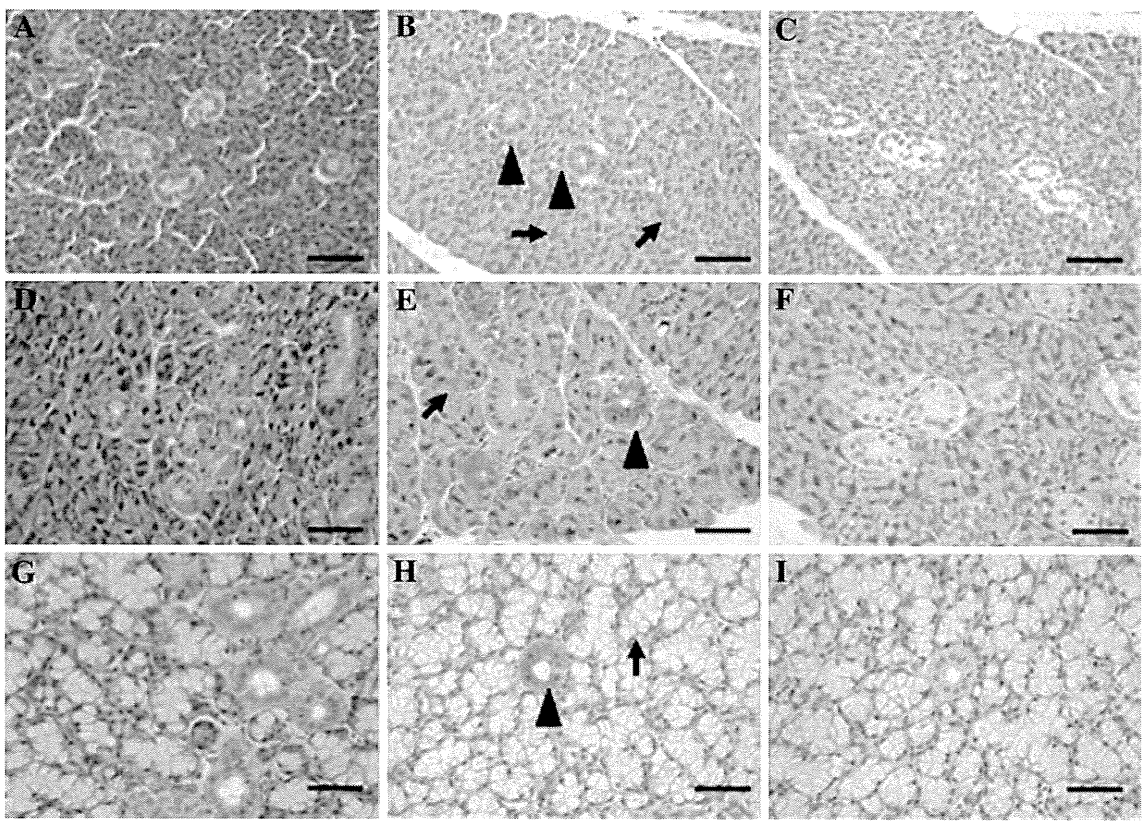

Fig. 1 Immunohistochemical localization of AANAT in rat salivary glands. **a**, **b**, **c** parotid gland, **d**, **e**, **f** submandibular gland, **g**, **h**, **i** sublingual gland. **a**, **d**, **g** General histology. Hematoxylin and eosin staining. **b**, **e**, **h** Immunohistochemical staining for AANAT (DAB and hematoxylin). **c**, **f**, **i** Negative control. Expression of AANAT (*arrowheads*) was identified in epithelial cells of striated ducts of parotid gland (**b**), submandibular gland (**e**), sublingual gland (**h**), whereas serous and mucous acini (*arrows*) showed no expression of AANAT in any salivary glands. *Bars* 50 μm

level of AANAT mRNA was higher in the dark period (Fig. 3d) and no significant difference was detected in HIOMT expression (Fig. 3e).

Localization and expression of Mel1aR in rat oral mucosa

To explore the possibility that oral mucosa is a possible target tissue for salivary melatonin, the localization of Mel1aR in rat buccal mucosa was also investigated by immunohistochemical analysis. As a result, the location of Mel1aR protein was confirmed in epithelial cells in the prickle cell layer of buccal mucosa (Fig. 4a). RT–PCR analysis also confirmed the expression of mRNA for Mel1aR (Fig. 4c).

Discussion

The inner surface of the oral cavity is constantly coated with saliva. Saliva is a complex fluid produced and secreted by the salivary glands such as parotid,