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Hinokitiol Inhibits *Candida albicans* Adherence to Oral Epithelial Cells

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Abstract : *Candida* spp. are an opportunistic pathogen causing serious local and systemic infections, especially in immuno-compromised hosts such as the elderly and HIV-positive patients. Hinokitiol $C_{10}H_{12}O_2$ (β-thujaplicin) is a component of the essential oils isolated from Cupressaceae and shows antibacterial activities for various bacteria. The aim of this study was to demonstrate the preventive effects of hinokitiol on the adherence of seven species of *Candida* to oral epithelial cells and to establish a safe and useful method for oral hygiene. A short-time treatment (30 min) with 0.25 mM hinokitiol showed 30—70% inhibition of adherence of *Candida* spp. to oral epithelial cells, inhibited about 11% biofilm formation, and did not inhibit the cell growth of *Candida* spp. Furthermore, short treatment with 0.25 mM hinokitiol was a safe method for oral hygiene against *Candida* infection because it did not inhibit the cell growth of commensal bacteria, such as oral streptococci existing in normal flora, or damage the epithelial cells. However, long-time treatment and a high concentration of hinokitiol demonstrated both the adherence inhibition of *Candida* and damage to commensal bacteria and epithelial cells. Our data suggest an appropriate procedure to apply hinokitiol that may be beneficial for the prevention of opportunistic pathogens such as *Candida* spp. in the oral cavity. The clinical and daily use of hinokitiol under an appropriate procedure may be a preventive and realistic therapy for *Candida* infection in immune-compromised hosts.

Introduction

Candida spp. are human commensals and can participate in biofilm formation on mucosal and cutaneous surfaces as well as on device surfaces, *e. g.*, dentures and catheters[1—7]. Many manifestations of candidiasis are associated with the formation of biofilms on host tissues (*e. g.*, oral thrush) and on indwelling medical devices (central venous catheter-associated candidemia)[8]. Biofilms are a community of microor-

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ganisms attached to a surface and surrounded by an extracellular matrix. A clinically significant characteristic of microbial biofilms is their enhanced resistance to antimicrobial agents[9,10]. Anti-fungal agents include ketoconazole, fluconazole, itraconazole, ravuconazole, miconazole, amphotericin B, nystatin, chlorhexidine, hydrogen peroxide and povidone-iodine. Long-term treatment of oropharyngeal candidiasis with anti-fungal therapy, such as fluconazole, itraconazole, and ketoconazole, sometimes leads to the emergence of drug-resistant *Candida albicans*[11]. Chlorhexidine, hydrogen peroxide and povidone-iodine are potent anti-microbial and chemical agents that reduce various pathogens. Their clinical application is also limited because they induce various adverse reactions, such as anaphylactic shock[12,13], damage to human tissues, and destruction of normal beneficial flora including the oral streptococci that exist in high concentrations in the oral cavity.

C. albicans, one of the *Candida* spp., is the primary causative agent of candidiasis, among the most important nosocomial infections of humans. The microorganism is an opportunistic pathogen that can cause serious local as well as systemic infections, especially in immuno-compromised hosts such as the elderly and HIV-positive patients[14]. *C. albicans* is a pleiomorphic fungus having the ability to transition between yeast cell and filamentous forms that is essential for virulence ; and therefore constitutes a central component in *C. albicans* pathogenesis. Various models have been established to study candidiasis : *Candida*-epithelial cell interactions using immortalized human epithelial cell lines in tissue culture[15—17] where *C. albicans* adheres to the epithelial cells of the oral mucosa and also invades these cells. Invasion into the epithelial cell limit of the oral mucosa is characteristic of both human and experimental animal models of oropharygeal candidiasis[18—21].

Hinokitiol $C_{10}H_{12}O_2$ (β-thujaplicin) is a component of the essential oils isolated from Cupressaceae and shows antibacterial activities against various bacteria : MRSA, Legionella, colon bacillus and oral bacteria[22—27]. It is reported as being used in Japan as a preservative for flowers, mushrooms and vegetables, a plant growth stimulator (inhibit bacteria), and antibacterial or fungicidal additive in food, cosmetics, toothpaste, hair tonics and tick repellents[28]. However, the clinical application of hinotikiol may be limited because it has anti-fungal and broad spectrum anti-bacterial activities, and killing activities of human tissues. Conditioning studies for the preventive effects of hinokitiol on *Candida* infection and biofilm formation are needed and the aim here was to demonstrate the preventive and safe effects of hinokitiol on adherence in seven species of *Candida* on oral epithelial cells. Our data indicate the beneficial effects of hinokitiol in preventing *Candida* from colonizing human tissues. The clinical use of hinokitiol may be considered for use in preventive therapy for *Candida* infections.

Materials and Methods

1. Strains and culture conditions

The strains used were *C. albicans* SC5314, *Candida tropicalis* IFO0618, *Candida dubliniensis* CD36, *Candida dubliniensis* CD57, *Candida parapsilosis* ATCC 22019, *Candida parapsilosis* FRCP-0201, *Candida glabrata* 850821, *Streptococcus mutans* MT6229, *S. mutans* OMZ175, *Streptococcus sobrinus* 6715, *Streptococcus salivarius* TCM5707, *Streptococcus sanguinis* ATCC 10556 and *Streptococcus mitis* ATCC 6249. *Candida* spp. were provided by Dr. Masakazu Niimi of the National Institute of Infectious Diseases. *Candida* spp. were incubated for 24 h at 37℃ in Yeast Peptone Dextrose (YPD ; 2% Bacto peptone, 2% dextrose and 1% yeast extract) broth. Streptococci were grown in Brain Heart Infusion broth (BHI ; Difco Laboratory, Detroit, MI, USA) in an aerobic atmosphere of 5% CO_2, 75% N_2 and 20% O_2 (GasPack CO_2 ; Mitsubishi, Tokyo) at 37℃ for 24 h before beginning each experiment.

2. Effects of hinokitiol on growth

Cell suspensions 1×10^8 CFU/mL of *Candida* strains and various concentrations (0.0625, 0.125, 0.25 and 0.5 mM) of hinokitiol (Wako Pure Chemical Industries Ltd., Osaka, Japan) were mixed and incubated in YPD for 24 h at 37℃ using aerobic conditions. Cell

growth was measured as absorbance at 660 nm at 0, 4, 6, 8, 10 and 24 h during incubation. Treatment assays were performed independently three times.

3. Effects on adherence and cytotoxic activity on epithelial cells of hinokitiol

C. albicans SC5314 1×10^8 CFU were treated using 0.25 mM hinokitiol for 0, 1, 2, 5, 10, 20, 30 and 60 min at 37℃ in 1 mL sterile phosphate-buffered saline (PBS), and washed using sterile PBS two times. Pellets after the last centrifugation were suspended in 1 mL sterile PBS, added to a monolayer of Ca9-22 epithelium cells in 24-well polystyrene plates that contained 70—90% confluent, and incubated for 24 h at 37℃ under aerobic conditions. Cells attached to *Candida* were washed using sterile PBS three times and detached using 0.05% trypsin-EDTA. The detached cell suspensions were collected in 0.05% trypsin-EDTA using pipetting, and plated on YPD using an EDDY JET spiral plating system (IUL, S. A., Barcelona, Spain).

Short-treatment effect of hinokitiol on *C. albicans* viability was also tested. After treatment with 0.25 mM hinotikiol for 0, 1, 2, 5, 10, 20, 30 and 60 min and washing with sterile PBS two times, they were then poured onto a YPD agar plate using an EDDY JET spiral plating system and incubated for 24 h at 37℃ under aerobic conditions.

Other *Candida* strains at 1×10^8 CFU were also treated using various concentrations of hinokitiol : 0, 0.0625, 0.125, 0.25, 0.5, 1.0 and 5.0 mM for 30 min in PBS at 37℃, and added to a monolayer of Ca9-22 epithelium cells in 24-well polystyrene plates, washed with sterile PBS two times, and incubated for 30 min at 37℃. The effects of hinokitiol on *Candida* species viabilities were also observed in a previously described method. Moreover, it was tested whether hinokitiol has effects on the viability of commensal bacteria, such as oral streptococci, *S. mutans* MT6229, *S. mutans* OMZ175, *S. sobrinus* 6715, *S. salivarius* TCM5707, *S. sanguinis* ATCC 10556 and *S. mitis* ATCC 6249. They were treated with 0.25 mM or 1.0 mM for 30 min and poured onto a Mitis-Salivarius agar plate (MS, Becton/Dickinson, Sparks, MD, USA).

After incubation for 24 h at 37℃ under aerobic or 5% CO_2 aerobic conditions (Gaspack CO_2 ; Becton/Deckinson), the colonies of *Candida* or streptococci in all experiments were counted on the YPD or MS agar plate and the colony number rates of tested organisms were calculated in comparison with the control : non-treated organisms (100%) or time zero.

4. Biofilm formation

C. albicans SC5314 incubated for 24 h at 37℃ in YPD broth was adjusted to OD = 0.5 at 660 nm ; harvested using centrifugation ; and washed in sterile PBS two times. *C. albicans* suspensions were diluted using RPMI1640, treated using various concentrations (0, 0.125, 0.25, 0.5, 1.0 and 5.0 mM) of hinokitiol, and added to 96-well microtiter plates. Chemically defined RPMI1640 medium containing minimal (0.2%) glucose was used as a nutrient-poor condition for the biofilm formation assay. After incubation for 24 h at 37℃, biofilms formed in wells were washed using sterile PBS two times. Biofilm formation was tested using the XTT assay at 492 nm. XTT reduction has been widely used to measure biofilm activity and allows the detection of small differences in metabolic activity between strains[29—31].

5. Epithelial cell monolayer damage assay

Epithelial cell (monolayer cells of Ca9-22) damage caused by hinokitiol was determined by the release of lactate dehydrogenase (LDH) into the medium using a Cytotoxicity Detection Kit-LDH (Roche Diagnostics K. K., Tokyo). The assays were performed according to the manufacturer's instructions and the measurements were performed in triplicate.

6. Statistical analysis

All data were analyzed using the Statistical Package for SPSS for Windows (version 100 ; Chicago, IL, USA). Student's t-test with the Bonferroni method was used to compare control data and the data of *Candida* spp. or streptococci after incubation. p-Values less than 0.05 were considered to be significant.

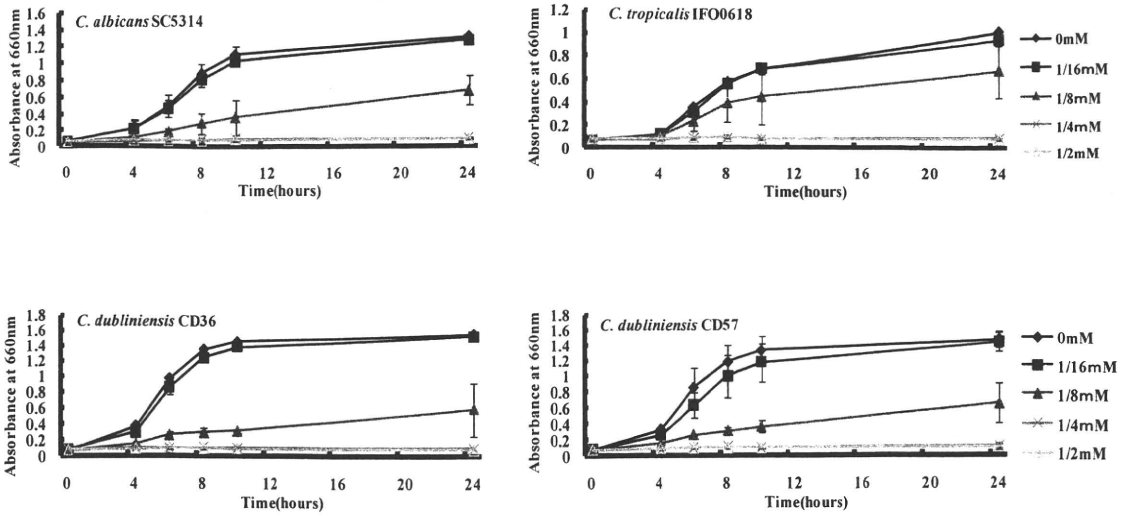

Fig. 1 Long-term treatment using hinokitiol on *Candida* growth
Various *Candida* strains were incubated in YPD broth containing each concentration (0, 0.0625, 0.125, 0.25 or 0.5 mM) of hinokitiol. Absorbance at 660 nm was measured at 0, 4, 6, 8, 10, and 24 h incubation with *C. albicans* SC5314, *C. tropicalis* IFO0618, *C. dubliniensis* CD36 and CD57 suspensions. Results are the mean ± standard deviation of three independent experiments, each performed using triplicate assays.

Results

1. Effects of hinokitiol on *Candida* growth and adherence

At first, to clarify the effects of hinokitiol on *Candida* growth, various concentrations of hinokitiol were added for 24 h in the growth of *Candida* species. The 0 and 0.0625 mM hinokitiol did not inhibit the growth of *C. albicans* SC5314, *C. tropicalis* IFO0618 and *C. dubliniensis* CD36 and CD57, whereas 0.125 mM hinokitiol inhibited growth intermediately, and 0.25 and 0.5 mM hinokitiol completely inhibited fungal cell growth of all *Candida* strains at all measurement times (Fig. 1). Similar inhibition data were also observed for other *Candida* species (*C. parapsilosis* ATCC 22019, *C. parasilosis* FRCP-0201 and *C. glabrata* 850821) (data not shown). If hinokitiol is used in the oral cavity, long-time application may damage oral flora and epithelial cells. Short-term treatment of hinokitiol is better for application in the oral cavity than long-time treatment. Therefore, short-term (1, 2, 5, 10, 20, 30 and 60 min) treatment with 0.25 mM hinokitiol was performed in the *C. albicans* viability experiment. Inhibition effects were not observed at all treatment times (Fig. 2). Further, it was tested whether short-term treatment inhibited the adherence of *C. albicans* to a monolayer of Ca9-22 epithelial cells. Inhibition effects were significantly observed at 10, 20, 30 and 60 min using 0.25 mM hinokitiol ; further, the inhibition levels increased in a treatment time-dependent manner (Fig. 2). It was also observed whether short-time treatment damaged epithelial cells. Cytotoxic activities (LDH activities) against epithelial cells were less than 2% with treatment of 0.25 mM hinokitiol for less than 30 min and increased slightly with the treatment time (60 min) in comparison with other treatment times (Fig. 3). Taken together, treatment for 30 min using 0.25 mM hinokitiol is appropriate for not only inhibition of the adherence of *C. albicans* but also safeguarding epithelial cells in the oral cavity.

Short-term treatment effects of hinikitiol were also tested with the adherence of other *Candida* species on monolayers of oral epithelial cells. To check the viability of *Candida* after treatment, the treated cells were immediately poured onto a YPD agar plate, and colony counts were performed after overnight incubation at 37℃. Short-term treatment (30 min) of 0.125—0.25 mM hinokitiol inhibited adherence of

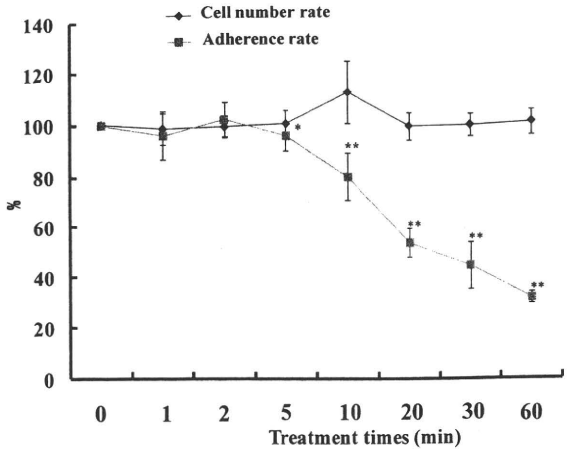

Fig. 2 Short-term treatment of hinokitiol on *Candida* growth and attachment

C. albicans SC9314 were pretreated using 0.25 mM hinokitiol for 1, 2, 5, 10, 20, 30 and 60 min. Pretreated *Candida* were poured onto a YPD agar plate and applied on Ca9-22 monolayer cells. The *C. albicans* attached to Ca9-22 cells were also poured onto a YPD agar plate. The colony-forming units (CFU) were counted and the percent of hinokitiol-treated *C. albicans* CFU was recalculated as 100% to control data (non-treatment). Results are the mean ± standard deviation of three independent experiments, each performed using triplicate assays. ; and compared to the control (0 mM hinokitiol) (* : $p<0.05$, ** : $p<0.01$).

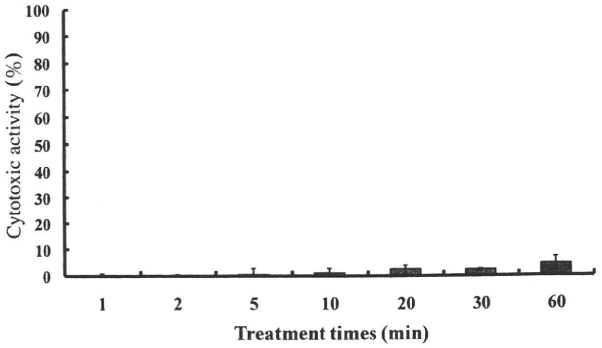

Fig. 3 Short-term treatment of hinokitiol on YPD monolayer cells

YPD monolayer cells were treated using 0.25 mM hinokitiol for 1, 2, 5, 10, 20, 30 and 60 min. Cell damage caused by hinokitiol was determined by the release of lactate dehydrogenase (LDH) into the medium. The assays were performed according to the manufacturer's instructions and the measurements were performed in triplicate. To measure epithelial cell damage, the following calculation was used : (ECa − C1 − C2) / (100 L − C1) × 100 = relative cytotoxicity (%). Absorbance measured at OD 490—600 directly correlates with LDH activity. ECa, epithelial cells treated with hinokitiol ; C1, control 1, non-treated epithelial cells ; C2, control 2, only *C. albicans* ; 100 L, 100% lysis (0.2% Triton-X 100). Controls 1, 2 and 100% lysis were determined individually for each treatment.

Candida spp. to Ca9-22 cells by 30—70%, but showed little effect on the cell numbers of *Candida* (Fig. 4). More than 1 mM hinokitiol markedly inhibited both the adherence and cell number of *Candida* spp. Treatment using 0.5 mM hinokitiol inhibited adherence by more than 60% and the cell number of *Candida* spp. by about 20%, except for *C. grabrata* 850821. Short-treatment using 1 mM hinokitiol did not show a different appearance of the cell surface from non-treated *C. albicans* using electron microscope observation (data not shown).

2. Effects of hinokitiol on *C. albicans* biofilm formation and streptococci viability

To determine the effects of hinokitiol on *Candida* biofilm formation, various concentrations of hinokitiol were applied during biofilm formation with *C. albicans*. Hinokitiol inhibited biofilm formation in a dose-dependent manner (Fig. 5) ; however, treatment with 0.25 mM hinokitiol, inhibiting the adherence of *Candida* species on oral epithelial cells by 30—70%, inhibited biofilm formation by about 11%. Treatment with 0.5 mM and 1.0 mM hinokitiol inhibited biofilm formation by about 45% and 75%. Therefore, 0.25 mM hinokitiol has significant ability to inhibit both adherence and biofilm formation ; but no bactericidal effects against *Candida* species were observed. The effect of using 0.25 mM hinokitiol is a mild treatment for *Candida* infection. To determine the effects on commensal bacteria of hinokitiol in the oral cavity, 0.25 mM and 1.0 mM hinokitiol were applied to vari-

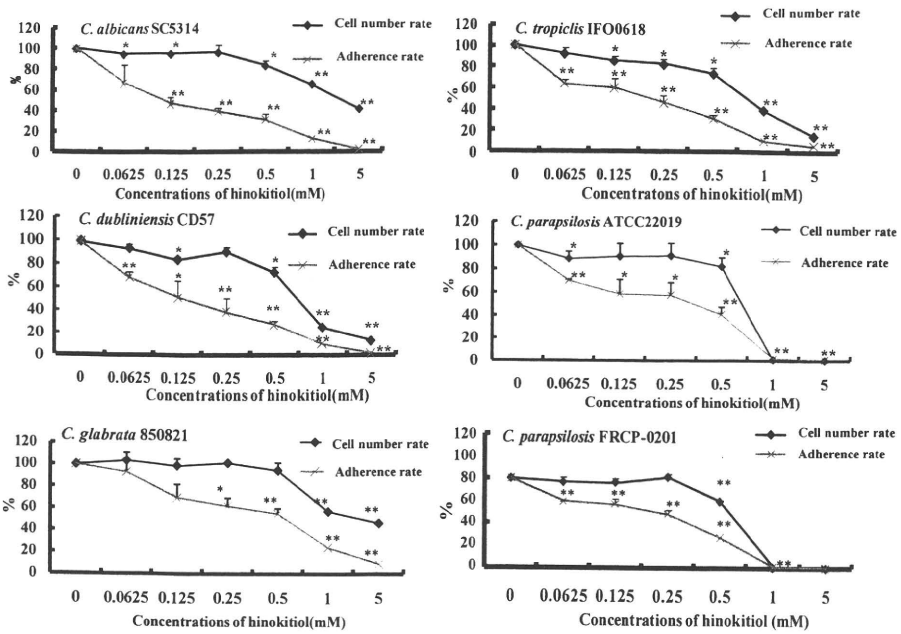

Fig. 4 Short-term treatment of hinokitiol on *Candida* adherence
Various *Candida* strains were pretreated using each concentration (0, 0.0625, 0.125, 0.25, 0.5, 1.0 and 5.0 mM) of hinokitiol for 30 min. Pretreated *Candida* strains were applied to YPD or Ca9-22 monolayer cells. Ca9-22 cells attached to a *Candida* strain were collected in 0.05% trypsin-EDTA and poured onto the YPD agar plate. Colony formation on YPD was counted 24 h after incubation with *C. albicans* SC5314, *C. tropicalis* IFO0618, *C. dubliniensis* CD57, *C. parapsilosis* ATCC22019, *C. glabrata* 850821, *C. parapsilosis* FRCP-0201 suspensions. Percent of hinokitiol-treated *Candida* CFU was calculated in comparison to non-treated *Candida* (100%). Results are the mean ± standard deviation of three independent experiments each performed using triplicate assays ; and compared to the control (0 mM hinokitiol) (* : $p<0.05$, ** : $p<0.01$).

ous oral streptococci. Treatment of 1.0 mM inhibited all streptococci, whereas treatment with 0.25 mM did not significantly inhibit the viability of streptococci (Fig. 6).

Discussion

Here we show that *Candida* adhesion to oral epithelial cells was reduced with lower concentrations (0.0625—0.25 mM) of hinokitiol. Short 30-min treatment with 0.25 mM hinokitiol is safe, and may be useful for the prevention of *Candida* infections in human tissues. Further, 0.25 mM hinokitiol may not destroy normal fungal and bacterial flora or damage epithelial cells. In contrast, at higher concentrations (>0.5 mM), hinokitiol prevented adhesion and biofilm formation and also prevented the cell viability of *Candida* spp. and oral commensal streptococci. Higher concentrations of hinokitiol have anti-fungal and -microbial broad spectrum activities and may have side effects allowing damage to human tissues and microbial substitution of the normal microflora, which causes infection by opportunistic pathogens[32,33]. Therefore, daily oral care with 0.25 mM hinokitiol is safe and may be effective for continuing prevention of *Candida* infections on human tissues.

Several studies concerning *C. albicans* biofilms have examined the activities of CHX, ethanol, hydrogen peroxide, betadine, and povidone-iodine ; however, the findings are not consistent[10,34—36]. CHX was uniformly effective against strains of common borne microorganisms, but was disqualified for clinical use

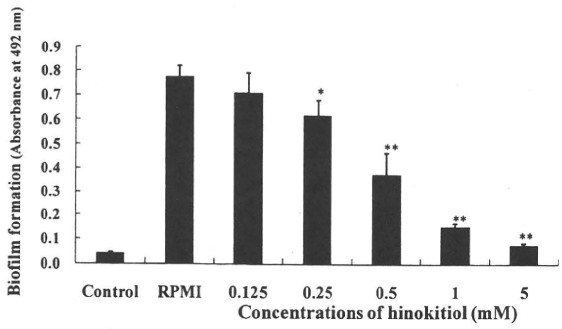

Fig. 5 Effects of hinokitiol on *Candida* biofilm formation

C. albicans SC5314 was incubated in chemically defined RPMI1640 medium containing each concentration (0, 0.125, 0.25, 0.5, 1 and 5 mM) of hinokitiol for 24 h. Biofilm formation was tested using the XTT assay. Absorbance at 492 nm was measured 24 h after incubation with *C. albicans*. Results are the mean ± standard deviation of three independent experiments each performed using triplicate assays ; and are compared to the control (non-treatment) (* : $p<0.05$, ** : $p<0.01$).

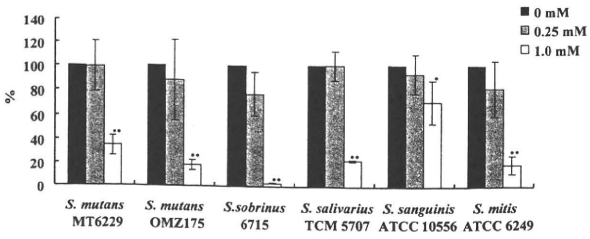

Fig. 6 Short-term treatment of hinokitiol on streptococci growth

S. mutans MT6229, *S. mutans* OMZ175, *S. sobrinus* 6715, *S. salivarius* TCM5707, *S. sanguinis* ATCC 10556 and *S. mitis* ATCC 6249 were treated with each concentration (0, 0.25 or 1.0 mM) of hinokitiol. The treated strains were then poured onto an MS agar plate. After incubation for 24 h, the colonies of streptococci were counted. Percent of hinokitiol-treated streptococci CFU was calculated in comparison with non-treated streptococci (100%). Results are the mean ± standard deviation of three independent experiments each performed using triplicate assays, and compared to the control (0 mM hinokitiol) (* : $p<0.05$, ** : $p<0.01$).

because it was highly cytotoxic[37]. Sanchez *et al.* investigated the effectiveness of using 30-min exposure to dilutions of CHX in PBS on canine embryonic fibroblasts and on cultures of *S. aureus*. They found that the antiseptics tested were lethal to canine fibroblasts at bactericidal concentrations *in vitro*[38]. Hydrogen peroxide at concentrations of 3% and 5% was the most effective to reduce the biofilm density of *C. albicans* and *Staphylococcus epidermidis* in the elimination of biofilms and killing fungi and bacteria[39,40]; however, a drawback of hydrogen peroxide is its toxicity and irritation to the skin or tissue when used for longer contact periods[41]. Thus, several reports have proposed the cytotoxic effects of anti-fungal and microbiological agents on human cells.

Hinokitiol has cytotoxic effects on human cell lines. Yamano *et al.* evaluated the toxic effect of hinokitiol on Hela 229 cells[42]. Although no decrease in activity was observed at hinokitiol concentrations of less than 1 μg/mL, activity decreased to 50% of the control value with long exposure (over 72 h) to 32 μg/mL hinokitiol ; no morphological changes were apparent by Giemsa Staining at 32 μg/mL. The agent must be effective against bacteria within a short exposure time without harming host cells or tissues to use in the oral cavity[33]. Here we show that short treatment (5—30 min) at 0.25 mM (42.05 μg/mL) hinokitiol, a higher concentration than 32 μg/mL, significantly inhibited *Candida* adherence, and poor cytotoxicity to the monolayer Ca9-22 cells (Fig. 2). In particular, 30-min treatment was a convenient or appropriate time for treatment using 0.25 mM hinokitiol (Figs. 2, 3).

Taken together, our data show that it is important for anti-fungal and anti-bacterial agents to be used at the appropriate concentrations in practice, at the correct concentrations, and for adequate contact times[43]. In this study, short treatment using 0.25 mM hinokitiol showed a preventive effect on *Candida* adherence to oral epithelial cells rather than an anti-fungal effect, and this concentration prevented *Candida* biofilm formation. The clinical and daily use of hinokitiol under the appropriate procedure may be a preventive and realistic therapy for *Candida* infection in

immune-compromised hosts. However, a definitive conclusion regarding the mechanism of the inhibition effect using short treatment of 0.25 mM on the adherence of *Candida* spp. to the monolayer of epithelial cells requires further investigation, changing interactions between the surface adhesins of *Candida* and receptors on epithelial cells.

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Effects of mucosal care on oral pathogens in professional oral hygiene to the elderly

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ABSTRACT

To elucidate the effects of mucosal care on colonization of mutans streptococci and infection of *Candida* species, we randomized 50 functionally dependent nursing home residents, mean age 76.1 (±7.8 years), 76% females, to receive oral professional care with or without mucosal care. During 12 months of follow-up, significant reduction in the numbers of mutans streptococci in saliva and dental plaque samples were observed in both treatment groups. However, there was no reduction in the numbers of mutans streptococci in tongue samples in the non-mucosal group ($p = 0.150$). In the comparison between the treatment with and without mucosal care, the treatment with mucosal care showed a significant inhibition effects on numbers of nutans streptococci on the tongue surface at 6 months after the treatment as compared with the treatment without mucosal care ($p = 0.043$). Mucosal care was not associated with any significant reduction in the rate of opportunistic infection with *Candida* species in any of the three sites. However, more subjects without mucosal care had *Candida* infection in dental plaque during the follow-up ($p = 0.046$). Professional dental care with (versus without) mucosal care had no effect on mutans streptococci colonization in saliva or plaque. Lack of mucosal care seemed to be associated with higher rates opportunistic infections with *Candida* species.

1. Introduction

Institutionalized elderly individuals who need systemic care have poorer oral health than those who live independently at home (Simons et al., 1999; Yoneyama et al., 1999; Senpuku et al., 2003). Diminished oral health, in turn, may affect their quality of life (Karuza et al., 1992). Poor oral health increases the risk of systemic disease such as aspiration pneumonia and septicemia (Scannapieco, 1999; Shay, 2002) as well as for dental diseases such as caries (Tada et al., 2004).

Although various species of bacteria have been found in oral cavity, mutans streptococci (mutans streptococci, *Streptococcus mutans* and *Streptococcus sobrinus*) are shown to be the principal causative pathogens related to dental caries in humans (Köhler and Persson, 1991; Kolenbrander and London, 1993; Loesche et al., 1995). The fungus *Candida albicans* and other *Candida* species constitute a part of the normal microbial flora in the oral cavity. However, some carriers with a high density of *Candida* species may become infected (oral Candidosis) and has a higher prevalence in the elderly; and may prompt the dentist or clinician to look out for underlying pathology changes in the seemingly healthy individuals. Therefore, oral *Candida* may be an indicator of pneumonia because of fungal aspiration from the oral cavity.

Oral professional care is an important practice to maintain the oral health of the elderly (Yoneyama et al., 1999; Abe et al., 2001). However, little is known about how mucosal oral care controls mutans streptococci colonization and infection of *Candida* in the oral cavity. To elucidate the effects of mucosal care on colonization of mutans streptococci and infection of *Candida* to maintain oral health of elderly, we studied two groups of elderly patients treated using oral professional care with or without mucosal care.

2. Materials and methods

2.1. Human subjects

Fifty elderly residents from an Itabashi-ward Tokyo nursing home who are dependently living in long-term nursing care participated in this study. The study was conducted from June 2006 to June 2007. The subjects were randomly selected from the residents using a random-numbers table and their identity was held blind from the investigators. Prior to the survey, the aim and details of the study were explained and consent was obtained from all subjects before registration. The study was approved by the Ethics Committee of the Tokyo Medical and Dental University and performed according to the rules of the Helsinki Declaration. One

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subject dropped out at 2 months, one at 6 months and four at 12 months after beginning the study where they moved to other institutions. Forty subjects (75.9 ± 7.5 years old; 29 females, 11 males) with more than 10 teeth were selected to compare the infections with streptococci and lactobacilli after oral professional care and with or without mucosal care, because the numbers of teeth influence the amount of colonization of streptococci and lactobacilli in the oral cavity. To study *Candida* species infections, all 50 subjects (76.1 ± 7.8 years old; 38 females, 12 males) were sampled before and after professional care. Dental examinations to determine the presence of dental caries, periodontal inflammation, denture usage, oral hygiene level, coated tongue and other typical oral conditions were performed under artificial white light by trained dentists before the study; and then routine oral professional care began. Oral examinations were performed according to WHO oral examination procedures (WHO, 1986). Coronal caries status indicators were: the number of coronal and root decayed teeth, the number of coronal and root filled teeth, the number of coronal and root surface decayed teeth and the number of coronal and root surface filled teeth. The general oral status of the subjects before the investigation is shown in Table 1. The subjects were randomly divided into two groups; Groups treated with professional oral care with and without mucosal care. The care givers and other staff at the institution remained unchanged during the study period.

Table 1
Baseline characteristics in subjects.

	Total	With mucosal care	Without mucosal care
Age	76.1 ± 7.8	75.6 ± 8.4	76.8 ± 6.1
Gender (female:male)	38:12	21:6	18:6
Tooth brush/day	2.5 ± 2.0	2.5 ± 1.0	2.5 ± 1.0
Existing tooth	17.0 ± 7.7	17.8 ± 7.6	16.3 ± 7.9
Decayed tooth	0.9 ± 2.0	0.8 ± 1.2	1.0 ± 2.5
Missing tooth	10.9 ± 7.8	10.2 ± 7.7	11.8 ± 7.9
Filled tooth	9.5 ± 5.3	9.4 ± 4.2	9.6 ± 6.4
Partial denture usage			
Do	29	14	15
Do not	21	12	9
Status of oral hygiene			
Good	9	5	4
Moderate	35	17	18
Bad	6	14	2
Inflammation of gingival			
No	0	0	0
Mild	17	9	8
Moderate	27	15	12
Heavy	6	2	4
Coating of tongue			
–1/3	11	6	5
1/3–2/3	23	10	13
2/3–	12	6	6
Oral dryness			
Normal	27	15	15
Mild	2	2	2
Moderate	10	6	6
Severe	11	3	3
Medical problem			
Sinus problem	4	1	3
Pneumonia	9	4	5
GI disorder	7	4	3
Diabetes mellitus	7	5	2
Liver complaint	7	5	2
Blood disorder	2	2	2
Auto immune disease	2	1	1
Cancer	2	0	2
Cardiac disease	10	6	4
High-blood pressure	20	9	11
Kidney diseases	2	2	0
Smoking habit	5	4	1

Values indicated mean (±standard deviation) of age, tooth brush/day, existing tooth, decayed tooth, missing tooth and filled tooth. Other values indicated numbers of person.

2.2. Oral care

During the initial examination, all patients had a routine dental and medical check-up. Oral care techniques by dental hygienists were standardized before beginning oral care. For daily oral care, all subjects who were able used the sink facilities in their room performed daily care 2–3 times a day after each meal. Further, dental hygienists provided professional care for 20 min once per week, i.e. dental brushing of teeth surfaces, mucosal cleaning with a sponge brush (NIPPON ZETTOC, Tokyo, Japan), tongue cleaning with tongue brush (Ebis Co., Ltd, Nara, Japan), denture cleaning, and oral washing with tap water in addition to the daily oral care. After professional care, mucosal cleaning was performed by sponge brush soaked in top water for 1 min in oral professional care group with mucosal care. The routine oral care including professional care was performed for 12 months. The subjects received no standard or professional care prior to entering the institution and the first sampling. No antibiotic therapy was administered during the 2 weeks prior to this study and during the 12-month study period; and none of these subjects suffered from severe infections. There was no significant difference in systemic disease incidence between the two groups with and without mucosal care prior to beginning the study (Table 1). There was no information concerning antibiotic therapy of the subjects before entering the institution.

2.3. Sampling of saliva, plaque and tongue

Subjects were asked not to brush their teeth 2 h prior to sampling. All sampling was performed before oral professional care and at 0, 1, 2, 3, 6 and 12 months after beginning the study. Whole saliva was stimulated by chewing paraffin gum for 5 min. For bacterial counts, a sterile cotton swab (Seedswab No. 1, Eiken Chemical Co. Ltd., Tokyo) was immersed in the saliva for 10 s and placed in transport fluid (0.4% agar, 0.15% thioglycolate/phosphate). Supragingival plaque samples were collected by swabbing five times from the posteroanterior buccal surface of the upper right second premolar and first molar using a cotton swab. Subjects having partial dentures ($n = 29$) and not having the above teeth were sampled from the opposite side or other remaining teeth. Tongue samples were collected by swabbing five times from the center of the tongue. Totally two swabs were acquired to analyze the bacteria and fungi in each sampling. After placing in transport fluid, all samples were immediately taken to the Biomedical Laboratory (BML, Tokyo, Japan) for analysis to detect mutans streptococci, total Streptococci (TS), Lactobacilli (LB) and *Candida* species.

2.4. Counting of bacteria and identification of fungi

Each sample was poured onto Mitis-Salivarius agar (Nippon Becton Dickinson Co. Ltd, Tokyo, Japan) to count TS, modified Mitis-Salivatius agar containing 0.2 U/m bacitracin to count mutans streptococci, and Rogosa selective lactobacillus agar to count LB using an EDDY JET spiral plating system (IUL, S.A., Barcelona, Spain); incubated at 37 °C using anaerobic conditions for 48 h (Tsuha et al., 2004); and the numbers of colony forming units (CFU) were counted. Colonies of mutans streptococci were identified by their characteristic appearance.

For *Candida* species identification, each sample was poured directly onto sabouroud dextrose agar plate and incubated for 48 h

at 35 °C to identify *Candida* species colonies, which were identified by their characteristic morphological appearance and color, and using Candida check (Iatron Laboratories Inc., Tokyo, Japan).

2.5. Statistical procedures

All data were analyzed using the Statistical Package for SPSS for Windows (version 100, Chicago, IL, USA). The repeated data for bacterial number (Log10) and *Candida* species identification were compared using the one way ANOVA and Friedoman test in Table 2. *T*-test with the Bonferroni method was used to compare primary data with data after oral professional care at each month or the two groups of oral professional care with and without oral mucosal care. In comparison between the treatment with and without oral mucosal care, the data subtracted data after the treatment from primary data in the numbers of mutans streptococci were used for analyses in the *T*-test with the Bonferroni method. The data of parameters excepting for mean ± SD in Table 1 and all data in Table 3 were compared between two groups using a χ^2 test for equal and unequal variations. *p*-Values less than 0.05 were considered to be significant.

3. Results

3.1. General and oral status

Comparing systemic diseases such as sinus problems, pneumonia, cardiac disorders, diabetes mellitus and others showed there were no significant differences between oral mucosal care-treated and -non-treated groups (Table 1). The dental status of the groups with and without oral mucosal treatment is shown in Table 1 where there were no significant differences between the two groups in all parameters.

3.2. Effects of oral mucosal treatment on oral bacteria colonization

The roles of oral mucosal treatment for the oral pathogenic bacteria, mutans streptococci, lactobacilli and total streptococci were studied for the tongue, plaque and saliva samples from both groups at various time points after oral professional care. Significant differences ($^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$) in the decrease in the repeated data of mutans streptococci numbers were shown using the one way ANOVA in all samples from both groups except for the tongue sample from elderly patients without oral mucosal care (Table 2). Further, in the two data comparisons using the *T*-test with the Bonferroni method; the numbers of mutans streptococci decreased significantly in the plaque samples at 6 and 12 months after the treatment of elderly patients without oral mucosal care in comparison to the primary data (at 0 month, $^{*}p < 0.05$, Table 3); whereas, a significant decrease in the saliva sample was observed throughout the study from 1 to 12 months ($^{*}p < 0.05$ and $^{**}p < 0.01$, Table 2). However, there were no significant differences in tongue samples (Table 2). Further, a significant decrease in mutans streptococci numbers was also observed throughout the study from 1 to 12 months in all samples from the elderly patients with oral mucosal care ($^{*}p < 0.05$ and $^{**}p < 0.01$, Table 2). In the comparison between the treatment with and without oral mucosal care in Table 2, a significant difference was only recognized at 6 months in tongue sample (*T*-test with the Bonferroni method, $p = 0.043$) but not at other months and in other samples. The treatment with mucosal care showed a significant inhibition effects on numbers of mutans streptococci on the tongue

Table 2
Numbers of mutans streptococci in elderly subjects with and without oral mucosal care.

Subject group	After start of oral professional care (month)						One way ANOVA (*p*)
Sample	0	1	2	3	6	12	
With mucosal care (*N*=21)							
Saliva	5.0±2.1	3.5±2.2*	3.5±2.0**	3.4±2.1**	3.0±1.9**	3.0±1.9**	0.000††
Plaque	5.3±2.0	4.1±2.5*	4.2±2.3*	3.7±2.5*	3.5±2.3**	3.7±2.0*	0.000††
Tongue	5.1±2.0	3.5±2.2**	3.8±2.2*	3.5±2.2**	2.9±1.9**	3.2±2.2**	0.003††
Without mucosal care (*N*=19)							
Saliva	5.1±1.8	4.1±1.4**	4.0±1.7*	4.1±1.4*	3.4±1.5**	3.5±1.7**	0.000††
Plaque	5.2±2.1	4.9±1.3	4.8±2.0	4.6±2.0	4.0±2.0*	4.0±2.2**	0.019†
Tongue	4.5±2.4	4.0±1.6	4.1±1.5	4.0±1.7	3.6±1.5	3.7±1.5	0.150

The results are expressed as mean (±standard deviation) of number (Log10/ml) of mutans streptococci. Asterisks indicate significant differences between 0 month and other months in *T*-test with Bonferroni method ($^{*}p < 0.05$; $^{**}p < 0.01$) and among 0–12 months in one way ANOVA ($^{\dagger}p < 0.05$; $^{\dagger\dagger}p < 0.01$). *N*: numbers of subjects with mucosal care or without mucosal care.

Table 3
Numbers of subjects which detected *Candida* species.

Subject group	After start of oral professional care (month)						Friedoman test (*p*)
Sample	0	1	2	3	6	12	
With mucosal care (*N*=26)							
Saliva	1 (3.8)	3 (11.5)	0 (0.0)	1 (3.8)	2 (7.7)	1 (3.8)*	0.487
Plaque	2 (7.7)	3 (11.5)	0 (0.0)	0 (0)**	1 (3.8)	0 (0)**	0.127
Tongue	2 (7.7)	3 (11.5)	0 (0.0)	2 (7.7)	0 (0)	2 (7.7)	0.298
Total	2 (7.7)	4 (15.4)	0 (0.0)	2 (7.7)*	3 (11.5)	3 (11.5)*	0.437
Without mucosal care (*N*=24)							
Saliva	2 (8.3)	2 (8.3)	2 (8.3)	4 (16.7)	4 (16.7)	6 (25.0)	0.299
Plaque	3 (12.5)	4 (16.7)	1 (4.2)	5 (20.8)	5 (20.8)	8 (33.3)	0.046†
Tongue	2 (8.3)	4 (16.7)	0 (0)	4 (16.7)	2 (8.3)	5 (20.8)	0.133
Total	3 (12.5)	4 (16.7)	2 (8.3)	8 (33.3)	5 (20.8)	9 (37.5)	0.006††

The results are expressed as numbers (% to total number) of subjects which detected *Candida* species. Total was counted subject numbers which detected *Candida* species in saliva, plaque or tongue. Asterisks indicate significant differences between professional treatment with mucosal care and without mucosal care in χ^2-test ($^{*}p < 0.05$; $^{**}p < 0.01$) in each sample and month, and among 0–12 months in Freidoman test ($^{\dagger}p < 0.05$; $^{\dagger\dagger}p < 0.01$). *N*: numbers of subjects with mucosal care or without mucosal care.

surface at 6 months after the treatment as compared with the treatment without mucosal care. Significant differences for the repeated data of lactobacilli were not observed in all samples from both groups (data not shown). Mucosal care had no significant association with reduction in lactobacilli count. Significant differences in the decrease in the repeated data of total streptococci numbers were indicated in all samples from both groups (data not shown). Dental care with or without mucosal care had significant association with reduction in total streptococci count.

3.3. Effects of oral mucosal treatment on Candida species infection

The roles of oral mucosal treatment for the *Candida* species were also studied for the same samples from both groups at various time points after oral professional care. The infection of *Candida* species was qualitatively assessed in each sample. Significant differences ($^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$) in the increase in the repeated data of isolation frequency of *Candida* species were shown using the Friedoman test in plaque samples and total of samples from elderly patients without oral mucosal care (Table 3). However, in elderly patients with oral mucosal care, there were no significant differences. Significant differences between elderly with and without oral mucosal care were also observed in isolation frequency of *Candida* species from total of saliva, plaque and tongue at 3 and 12 months after treatment with professional oral hygiene ($^{*}p < 0.05$, Table 3). In particular, the differences were observed in saliva at 12 months ($^{*}p < 0.05$) and plaque at 3 and 12 months ($^{**}p < 0.01$). However, in other months and tongue, there were no significant differences.

Microbiology studies previously suggested the colonized denture acted as a possible reservoir of *Candida* infection (Budtz-Jøgensen, 1974). However, we found no significant difference in denture usage between groups treated by professional care with or without mucosal care.

4. Discussion

Long-term decreasing effects of mucosal care on mutans streptococci number were recognized on the tongue surface in which physical attachment of bacteria is due to existence of many papillaes. On the other hand, the decreasing effects of mucosal care appeared shortly after start of the professional treatment, but were lost during long-term treatment on the tooth surface in which bacterial attachment is supported by salivary binding receptors absorbed on the enamel surface to oral streptococci. The difference of efficacies might be dependent on physical and biochemical interactions between the tissue surfaces and bacteria. The mucosal care might have more benefit to reduce mutans streptococci numbers in the physical attachment to tongue surface than in the biochemical attachment to tooth surface during long-term treatment. We suggest at least oral mucosal care reduces infection directly and reduces the attachment of mutans streptococci on the tongue in the oral cavity; and this was further reduced in the biofilm using professional treatment. However, the effects of mucosal care on the planktonic cell numbers of mutans streptococci in saliva samples were not observed in comparison with data before start of oral professional care. Oral hygiene procedures with mucosal care are used to remove the biofilm and micro-colonies formed by oral microorganisms on teeth and tongue surfaces, and mucosal epithelial cells attached to oral microorganisms on mucosal surfaces. Further, a healthy and fresh environment is created in the oral cavity. Oral mucosal care may be more effective in reducing reattachment of remaining oral microorganisms including pathogens such as mutans streptococci attached to tongue after professional oral treatment, and supports the effects of professional treatment in the oral cavity without the biofilm after professional treatment than by using professional care without mucosal care that does not completely remove microorganisms on oral mucosal tissues.

In contrast, the oral mucosal care may not affect the decrease or increase of lactobacilli numbers during treatment using oral professional care. The mucosal care may loosen adhesive bacteria such as mutans streptococci and some of the oral streptococci on the tissues but not non-adhesive bacteria such as lactobacilli or some of the planktonic cells in the oral cavity.

The oral streptococci are the first to attach in early dental plaque formation and have a specific temporal and spatial distribution that is essential for the development of the oral biofilm (Rickard et al., 2003). Reports show early colonizers of oral streptococci species (mitis group streptococci) can suppress the growth of *S. mutans* by producing hydrogen peroxide (Kreth et al., 2005). The streptococci compete for adhesion-binding sites on the saliva-coated tooth surface (Nobbs et al., 2007). Recently, *Streptococcus oligofermentans* was found as a new oral commensal species and has an inverse correlation with *S. mutans* within human dental plaque (Tong et al., 2007). In combination cultures, *S. salivarius* strongly inhibits biofilm formation when cultured with *S. mutans* (Tamura et al., 2009). This inhibition occurs in the early phase of biofilm formation and was dependent on inactivation of the competence stimulating peptide of *S. mutans*. Therefore, some oral streptococci may function as competitive strains to inhibit *S. mutans* colonization. When the oral streptococci are refreshed by professional treatment with oral mucosal care, this treatment may create a potential for a protective environment to inhibit infection by mutans streptococci.

It is generally considered the mechanisms employed when a healthy microflora is present may interfere with the adhesion of invading pathogens and includes competitive exclusion (Reid and Tieszer, 1993), displacement (Millsap et al., 1994), production of antibacterial compounds (Klaenhammer, 1988), and release of biosurfactants (Frandsen et al., 1991). Oral streptococci have an antagonistic effect toward many bacterial species (Holmberg and Hallander, 1973; Willcox and Drucker, 1988; Bormard and Stinson, 1999) and have an inverse correlation with the detection of opportunistic pathogens (Tada et al., 2007). Therefore, we consider oral professional care with mucosal care cleanses the tongue and mucosal surface where the treatment allows re-establishment and growth by fresh commensal bacteria such as total streptococci; and disturbs the infection by opportunistic pathogens such as *Candida* in the oral cavity of the elderly. In this study, the significant effects of treatment with mucosal care on *Candida* species were observed in saliva and plaque samples but not in tongue sample. The results of inverse influence on samples were shown in the effects of mucosal care on mutans streptococci. It was also considered that the difference of efficacies on *Candida* species and mutans streptococci infections might be dependent on physical and biochemical interactions between the tissue surfaces and pathogens. However, to attain these potential effects treatment may be required for long periods to disturb increase of *Candida* species infections because the differences of isolate frequency of *Candida* between treatment with and without oral mucosal care were observed after 3 months after professional care treatment began, and the increases of repeated data were also observed during 12 months in the treatment without mucosal care. The treatment with mucosal care might disturb the increase of *Candida* species infection in the oral cavity. In other studies, long duration of professional care treatment was suggested to be effective in decreasing the risk of oral infection and inflammation in institutionalized elderly (Adachi et al., 2002; Yoneyama et al., 2002; Kokubu et al., 2008). Dental care of the institutionalized elderly is often limited to emergency care and is not aimed at

retaining teeth by means of restorative treatment and daily oral care (Ekelund, 1991; Wårdh et al., 2000). Updated information is therefore needed for planning oral care to meet the needs of the long-term hospitalized elderly.

The combination of professional oral treatment and mucosal care is very useful for improvement of oral as well as general health in the institutional elderly. In conclusion, here we demonstrate the beneficial effects of mucosal care on infection control of pathogens such as mutans streptococci and *Candida* during oral professional care. However, the oral professional care is limited by the high costs of odontologic assistance, the oral and general health status of the elderly, the lack of time available for caregivers to carry out the tasks, etc., in the institutionalized elderly. The preventive attitude offered by repetition of oral professional care is based on nursing professionals and institutional resources. Moreover, there are limitations of this study in a small sample size, no group treated with only mucosal care, the influences to other oral pathogens, etc. Further investigation is warranted based upon these findings to evaluate whether mucosal oral care can support prevention of multiple oral pathogens in the expanded study plan.

Conflict of interest statement

None.

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Inhibition of *Streptococcus mutans* adherence and biofilm formation using analogues of the SspB peptide

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ABSTRACT

Objective: *Streptococcus gordonii* is a pioneer colonizer of the enamel salivary pellicle that forms biofilm on the tooth surfaces. Recent reports show the surface protein analogue peptide {400 (T) of SspB 390–402 is substituted to K forming SspB (390–T400K–402)} from *S. gordonii* interacts strongly with salivary receptors to cariogenic bacteria, *Streptococcus mutans*. To characterize the analogue peptide biological activities, we investigated its binding and inhibiting effects, and the role of its amino acid moities.

Methods: We measured binding activity of analogue peptides to salivary components using the BIAcore assay; assayed inhibition activities of peptides for bacterial binding and growth on saliva-coated hydroxyapatite beads (s-HA); and describe the peptides interfering with biofilm formation of *S. mutans* on polystyrene surfaces.

Results: The SspB (390–T400K–402 and –401) peptides significantly bound with salivary components and inhibited the binding of *S. mutans* and *S. gordonii* to s-HA without bactericidal activity; but did not inhibit binding of *Streptococcus mitis*, a beneficial commensal. Further, the lack of D and E-L at position 390 and 401–402 in the peptide, and substituted peptide SspB (D390H– or D390K–T400K–402) did not bind to salivary components or inhibit binding of *S. mutans*. The SspB (390–T400K–402) peptide inhibited biofilm formation on salivary components-coated polystyrene surfaces in absence of conditioned planktonic cells.

Conclusions: We found constructing the peptide to include positions 390(D), 400(K) and 401(E), two surface positive and negative connective charges, and at least 12 amino acids are required to bind salivary components and inhibit the binding of *S. mutans* and *S. gordonii*.

1. Introduction

Oral bacterial communities known as biofilms are characterized by species composition, surface or substratum composition, and the conditioning films coating the surfaces on which they form.[1,2] The interaction between oral streptococci and the salivary pellicle plays a large role in early colonization, aggregation and biofilm formation. *Streptococcus gordonii* colonizes the oral cavity very early in life[3] and interacts with other species such as *Porphyromonas gingivalis*, a periodontal pathogen, to form the predominant biofilm on the tooth surface.[4] *Streptococcus mutans* is the etiologic agent of human dental caries[2] and is shown to be able to colonize the mouth only after acquiring the enamel pellicle surfaces of the erupted tooth and to form part of the biofilm on tooth surfaces.[5] *S. mutans* and *S. gordonii* produce surface protein antigens (PAc, AgI/II, B, P1, SpaP, and MSL-1); and SspB (SspA), respectively, that have respective molecular masses of approximately 190[6–10] and

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180 kDa.[11,12] These proteins interact with salivary components that include lysozyme,[9,13] amylase,[9] 18,000 and 38,000 Da proline-rich proteins, and an agglutinin.[11]

We previously demonstrated the molecular mechanisms of the cell surface adhesion with the alanine-rich repeating region (residues 219–464, the A-region) of the SpaP (PAc) from *S. mutans* at the N-terminal region (residue 1–429) homologous to the SspB from *S. gordonii*.[5–12,9,13–17] The analogue peptide, position 400 T of SspB 390–402 was substituted with K forming SspB (390–T400K–402), had the highest binding activity to salivary components[17] and to the agglutinin (gp340/DNBT1) peptide (Scavenger Receptor Cystein-Rich domain Peptide 2; SRCRP2)[18] in comparison to various other SspB peptides and the PAc (365–377) peptide. This analogue peptide has two surface positive charges correlating with positively charged residues.[17] Recently, we reported the lysine position was important for binding between the SspB peptide and SRCRP2; and inhibited binding of *S. mutans* to saliva-coated hydroxyapatite (s-HA).[19] Therefore, the position of the charged amino acid residues in the peptide is essential for binding and inhibition.

Here we demonstrate inhibition of adherence of *S. mutans* and *S. gordonii* to surfaces previously treated with SspB (390–T400K–402) peptide, a truncated analogue peptide, compared to lysine alone using *in vitro* assays on s-HA, and biofilm formation on microtitre plates. Our data show the role of the analogue and truncated peptides and the connection to the surface's positive and negative charge results in the inhibition of the adherence. This provides important information about the initial phase of oral biofilm formation; and may yield preventive therapies for oral diseases.

2. Materials and methods

2.1. *Bacterial strains and culture conditions*

S. mutans MT8148, *S. gordonii* ATCC 10558 and *S. mitis* ATCC 6249 were grown in Brain Heart Infusion broth (BHI; Difco Laboratory, Detroit, MI) using 5% CO_2, 75% N_2 and 20% O_2 (GasPack CO_2, Becton Dickinson, Sparks, MD) at 37 °C prior to incubation with s-HA beads and in 96-well microtitre plates.

2.2. *Peptide synthesis*

The sequences of the analogue and truncated peptides are from a previous report[17] and were modified using the sequence of the SspB gene from *S. gordonii* M5[11] (Table 1). The peptides were synthesized using a stepwise solid-phase procedure at Scrum, Inc. (Tokyo, Japan). Purity was determined as greater than 95% using HPLC analysis. The peptides were dissolved in sterile de-mineralized water at 1 mg/ml; and aliquots were freeze-dried and stored at −20 °C. For each experiment, the freeze-dried peptides were re-dissolved to 1 mg/ml in sterile distilled-water (dH_2O).

2.3. *Human saliva collection*

Prior to saliva collection, the aim and details of the study were explained and consent was obtained from all subjects. Whole saliva from five human volunteers (27–44 years-old) was stimulated by chewing paraffin gum for 5 min; and the saliva was collected in an ice-chilled sterile bottle. After the saliva was clarified by centrifugation at 10,000 × *g* for 10 min at 4 °C, the supernatant without debris was taken and filter sterilized using a 0.45 μm filter (Millex-HV, Millipore, Cark, Co., Ireland); and used immediately to coat HA for the binding assay and the 96-well microtitre plates for the biofilm assay.

2.4. *Bactericidal assay*

S. mutans or *S. gordonii* was grown at 37 °C for 18 h using 5% CO_2, 75% N_2 and 20% O_2 (GasPack CO_2). The bacteria were harvested and washed with sterilized PBS three times; suspended in BHI; and adjusted to 5×10^7 CFU in a 1.5 ml bacterial suspension. Six hundred and fifty μM of SspB (390–T400K–402) peptide and 0.04% chlorhexidine (CHX) were, respectively, added into separate bacterial cultures. The mix suspensions were incubated for 16 or 22 h using 5% CO_2, 75% N_2 and 20% O_2 (GasPack CO_2). The bacterial concentration at 550 nm was measured using a spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) and compared with control (no addition of peptide or CHX).

2.5. *Inhibiting effects of the SspB (390–T400K–402) peptide to bind oral streptococci to s-HA*

The s-HA binding assay was originally described by Liljemark et al.,[20] Koga et al.[21] and Arakawa et al.[22] with our modifications. To determine the effects of the SspB (390–T400K–402) peptide, truncated SspB (390–T400K–402) peptides, and various amino acids were used to examine *S. mutans*, *S. gordonii* and *S. mitis* adherence: 20 mg of HA (Sangi Co., Ltd., Tokyo, Japan) was equilibrated in phosphate buffered saline, pH7.4 (PBS); soaked in 1.5 ml of sterilized whole saliva; incubated for 60 min at room temperature; and washed twice with sterile PBS. s-HA was suspended and incubated for 60 min at 37 °C in 6.5, 32.5, 65.0, 650.0 and 3,250 μM of the test peptides or 68.5, 685.0, and 6,850.0 μM of lysine, threonine or aspartic acid solutions in PBS. Oral streptococci were grown at 37 °C for 18 h using 5% CO_2, 75% N_2 and 20% O_2 (GasPack CO_2) in BHI broth containing [methyl-^{3}H] thymidine (ICN Radiochemicals, Irvine, CA) at 10 μCi/ml having a specific activity of between 2.5×10^{-2} and 0.6×10^{-3} cpm/cell. The bacteria were harvested and washed with sterilized PBS three times; suspended in PBS; sonicated on ice for 10 s; and adjusted to 5×10^7 CFU in

Table 1

Position	Sequence
SspB (T400K–401)	KE
SspB (399–T400K)	QK
SspB (399–T400K–401)	QKE
SspB (398–T400K–402)	YQKEL
SspB (391–T400K–402)	YQAKLAAYQKEL
SspB (390–399)	DYQAKLAAYQ
SspB (390–T400K)	DYQAKLAAYQK
SspB (390–T400K–401)	DYQAKLAAYQKE
SspB (390–T400K–402)	DYQAKLAAYQKEL
SspB (D390H–T400K–402)	HYQAKLAAYQKEL
SspB (D390K–T400K–402)	KYQAKLAAYQKEL

a 1.5-ml bacterial solution. The s-HA specimens were suspended in the [^{3}H]-labelled bacterial solution and incubated with shaking for 90 min at 37 °C 5% CO_2, 75% N_2 and 20% O_2 (GasPack CO_2). Unattached cells were removed where the beads with bound [^{3}H]-labelled bacteria were washed five times with sterilized PBS and transferred to a scintillation vial. After the addition of 10 ml of Ultimagold Scintillation Cocktail (Packard Co., Downers Grove, IL), the radioactivity was determined using a liquid scintillation counter (LSC-5000, Aloka Co., Ltd., Tokyo, Japan). In an assay without the peptide with an input of 7.5×10^7 cells, 60% of the cells attached to the s-HA and showed radioactivity was from 36,500 to 42,500 cpm. Background values were less than 100 cpm.

2.6. *Binding of analogue peptides to salivary components using the BIAcore Biosensor System*

To confirm binding activities of the analogue peptides to salivary components, BIAcore Biosensor System (BIAcore 2000, BIAcore AB, Uppsala, Sweden) analysis for peptide binding was modified and performed as described previously.[17,20,15] We used a standard CM5 sensor chip with a carboxymethylated dextran-coated gold sensor chip activated with 70 μl of a solution containing 400 mM N-ethyl-N′-(3-diethylaminoprophyl) carbodiimide and 100 mM N-hydroxysuccinimide at a flow rate of 10 μl/min. Following activation, 70 μl of 1/4 diluted and sterilized whole saliva in 10 mM sodium acetate buffer (pH 5.0) was applied to the chip to immobilize it on the surface. Residual N-hydroxysuccinimide esters were then inactivated using 70 μl of 1 M ethanolamine hydrochloride. We used a flow rate of HBS-EP buffer saline (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20) at 10 μl/min throughout the immobilization procedure. The peptide solutions at 0.625, 1.25 and 2.5 mM in PBS (pH 6.5) were exposed to the immobilized ligand(s) on the CM5 sensor chip (flow rate, 10 μl/min); and for the dissociation phase we injected HBS-EP at a rate of 10 μl/min. All binding experiments were conducted at 25 °C. At the end of each binding cycle, the surface of the sensor chip was regenerated using 50 mM glycine-NaOH (pH 9.5) for 60 s. Before re-using it, the returning to baseline of resonance unit was confirmed. The binding assays were performed less than three times using a single immobilized surface of the sensor chip because the reactivity was reduced by repeated use. Association (K_a) and dissociation (K_d) rate constants were determined using BIA-evaluation software (BIAcore AB).

2.7. *Biofilm formation assay using microtitre plates*

Biofilm formation by *S. mutans* was assayed using the method of Motegi et al.[23] The assay was performed as an *in vitro* experiment to study whether the inhibition effects by peptide on the adherence was useful for the prevention of biofilm formation which associated with development of dental caries in the condition including sucrose. The wells of a 96-well (flat bottom) micro titre plate (Sumitomo Bakelite, Tokyo, Japan) were coated with sterilized whole saliva for 30 min at 4 °C. To perform the inhibition assay using the SspB (390–T400K–402) peptide, 100 μl of the analogue peptide solution (650 μM in PBS) was added to the wells and incubated for 1 h at 4 °C. After removing the peptide solution and washing three times with sterile PBS, 20 μl (4.0×10^4 CFU) of a bacterial cell suspension was added to the wells with 160 μl tryptic soy broth without dextrose supplemented with 0.25% sucrose (TSBS). The plates were incubated at 37 °C for 1 h under 5% CO_2 in aerobic conditions; then the liquid medium including planktonic cells was removed by decantation; and the wells were rinsed twice with sterile PBS. Each well then received 200 μl fresh TSBS and the culture was incubated for 5, 8, 11 and 15 h at 37 °C aerobically with 5% CO_2. The liquid medium was removed and the wells were rinsed twice with sterile PBS. The plates were then air-dried and stained with 0.25% safranin for 15 min. After staining, the plates were rinsed with dH_2O to remove excess dye and air-dried. The biofilm mass was measured quantitatively using an ELISA microplate reader at A_{492} (Multiskan Bichromatic Laboratory Japan, Tokyo, Japan) where the biofilms were uniformly spread on the bottoms of the wells in the 96-well plates.[15]

2.8. *Modeling of the secondary structure using surface charge*

The A-region of SspB is thought to assume an α-helical structure.[11,23] Modeling of the secondary structures using the surface charge of the SspB (390–T400K–402), truncated SspB (390–T400K–402), SspB (D390H–T400K–402) and SspB (D390K–T400K–402) peptides were performed using a MOE (Molecular Operating Environment, Version 2006-08, Chemical Computing Group. Inc., Montreal, Canada) with the developing platform of PPh4Dock and the MMFF94 s force field subroutines used for energy evaluation.[24] The proteins and protein analogues were evaluated and analyzed based on an α-helical structure using the MOE system. An α-helical structure was constructed using a Phi-angle of −65° and Psi-angle of −39° with energy minimization of possible structures in these peptides; and was performed using atom size, bond stretch, angle bend, stretch-bend, out-of-plane, torsion, and charge.

3. Statistics

Comparisons of bacterial adherence and biofilm formation using peptide or amino acid treatment and non-treatment were performed using the ANOVA and Fisher's PLSD tests. Differences at $p = 0.05$ or less were considered to be statistically significant.

4. Results

4.1. *Effects of SspB analogue peptide on bacterial binding to s-HA*

The adherence of *S. gordonii* and *S. mutans* were significantly inhibited using 650 μM SspB (390–T400K–402) peptide; but not at 6.5 and 65 μM (Fig. 1A and B). Adherence of *S. mitis* was not inhibited by the peptides (Fig. 1C). This suggests the analogue peptide is a significant and specific inhibitor for the binding of *S. gordonii* and *S. mutans* to s-HA. The analogue peptide contains lysine which plays an important role in the binding of

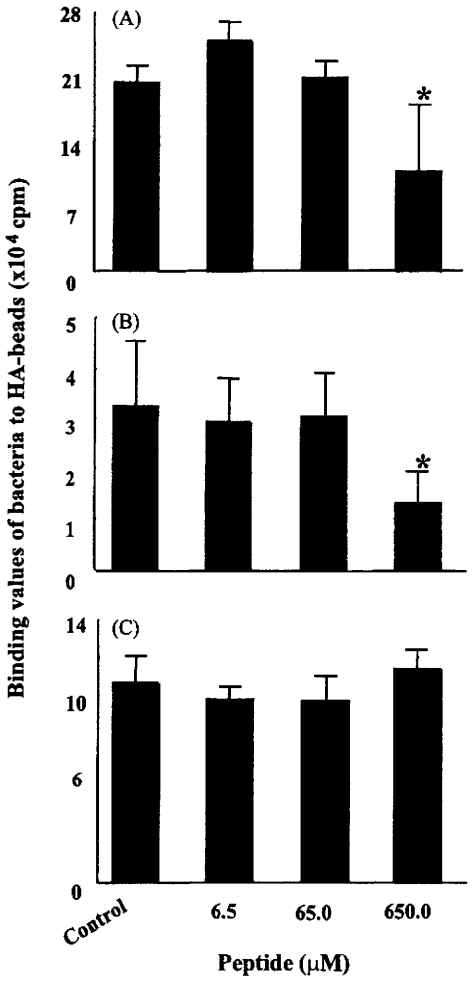

Fig. 1 – SspB peptide inhibits streptococcal adherence to s-HA. The adherence levels (cpm) of *S. gordonii* ATCC 10558 (A), *S. mutans* MT8148 (B) and *S. mitis* ATCC 6249 (C) to s-HA treated with 6.5, 65.0 and 650.0 μM of SspB (390–T400K–402) peptide are shown. The data are the means ± SD of three independent assays. Asterisks show significantly different relative levels of bacterial adherence ($p < 0.05$ vs. control: non-treated and saliva-coated hydroxyapatite).

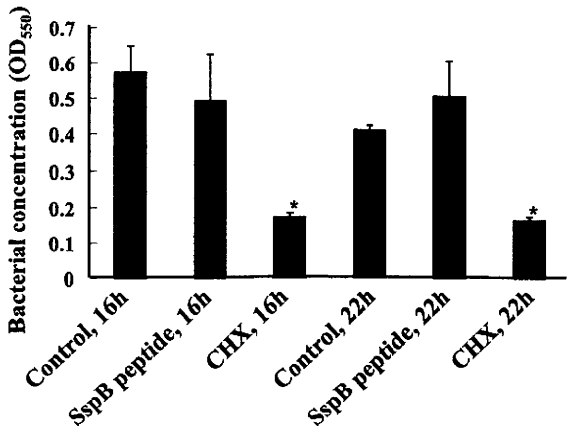

Fig. 2 – Bacterial growth in the presence of the SspB peptide or CHX. To determine whether 650 μM the SspB peptide has cationic anti-microbial activity, SspB (390–T400K–402) peptide and a positive control, 0.04% CHX, were applied into *S. mutans* cultures grown in BHI. The mixed suspensions were incubated for 16 or 22 h; and the bacterial concentrations at 550 nm were measured using a spectrophotometer. The data are the mean ± SD of three independent assays. Asterisks show significantly different relative levels of bacterial growth ($p < 0.05$ vs. control: PBS-treated *S. mutans*).

the SspB peptide to salivary components where we constructed an analogue protein with two positive surface charges in connection with the positively charged residues. These peptides commonly adopt an amphipathic conformation in which positively charged and hydrophobic groups segregate onto opposing faces of an α-helix, a β-sheet, or some other tertiary structure and may have anti-microbial activities.[25] SspB (390–T400K–402) peptide has cationic amino acid and α-helix structures;[19] and, therefore, to determine whether the peptide has cationic anti-microbial activity we performed the bacteriocidal test. One mg/ml SspB (390–T400K–402) peptide and positive control, 0.04% chlorhexidine (CHX), were added to growing *S. mutans* cultures. CHX inhibited both 16 and 22 h growth of *S. mutans*; whereas the peptide did not show inhibition of growth (Fig. 2).

To determine the influence of the positive charged amino acid, lysine, in the binding of *S. mutans*, lysine alone was used in a competitive inhibition assay. At 685 μM lysine alone, higher than the molarity (650 μM) of the SspB (390–T400K–402) peptide, lysine alone did not show inhibition; whereas at an excess molarity of 6850 μM lysine alone significantly inhibited the binding activities (Fig. 3). Members of other similar amino acids such as glutamine and aspartic acid that are negatively charged and threonine, the original residue before substitution by lysine in SspB (390–402) did not inhibit the binding of *S. mutans* (data not shown). To determine the effects of changes in the secondary structure of the analogue peptide, we examined if the numbers of amino acids and two surface positive charges and their connection with the positive charged residues are required for the inhibition. We constructed various truncated peptides, substituted amino acids in the preparations, and made small peptides (Table 1). The dimer amino acid peptides (KE or QK) of lysine and glutamic acid or glutamine did not show significant inhibition as shown by SspB (390–T400K–402) peptide at the same molarities (Fig. 4). The trimer amino acid peptide (QKE) of lysine, glutamic acid and glutamine, and the pentamer amino acid peptide (YAKEL) containing lysine at its centre did not show significant inhibition. The central chain (amidohydrogen) of SspB has a positively charged aspartic acid that is partially exposed on the surface of the α-helical peptide structure determined in a previous crystallography analysis.[17] The authors suggest the two surface positive charges at residues 390(D) and 400(K) were important for the binding to the salivary components. To study the role of the positively charged residues for the inhibition activity, we constructed a peptide without aspartic acid and substituted a positive charge

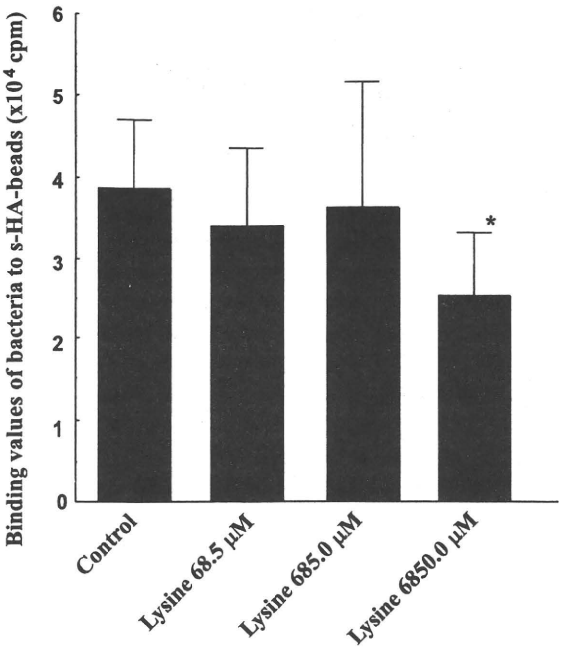

Fig. 3 – Inhibition using lysine alone for *S. mutans* adherence to s-HA. The adherence levels (cpm) of *S. mutans* MT8148 to s-HA treated with 68.5, 685.0 and 6850.0 μM lysine are shown. Control: binding values of *S. mutans* to non-treated s-HA. The data are the means ± SD of three independent assays. Asterisks show significantly different relative levels of bacterial adherence ($p < 0.05$ vs. control: non-treated s-HA).

amino acid at position 390 (Table 1). SspB (391–T400K–402) and SspB (D390H or D390K–T400K–402) peptides were synthesized and used in the inhibition assay. We found substituted peptides lacking aspartic acid did not show significant inhibition (Fig. 4). Expression of the positive and negative charge at position 390 was required for the inhibition effects of the peptide in *S. mutans* adherence (Fig. 5). After removing leucine at position 402 (Table 1), the SspB (390–T400K–401) peptide was synthesized and used in the inhibition assay. The SspB (390–T400K–401) peptide showed significant inhibition at similar levels as the SspB (390–T400K–402) peptide. *S. gordonii* and *S. mutans* adherence had dose-dependent inhibition by the SspB (390–T400K–402) peptide; but not after removing more amino acids using the SspB (390–399) peptide (Fig. 6AB). *S. mitis* adherence was not inhibited by any of these peptides (Fig. 6C). Removing more amino acids from the SspB (390–T400K) peptide removed significant inhibition (Fig. 4). This shows the negative charged amino acid, glutamic acid, at position 401 was required for the inhibition effect (Fig. 5).

To further quantitatively confirm the binding activity of analogue and truncated peptides to salivary components, an adhesion binding assay was performed using the BIAcore Biosensor System. Peptides (1.25 mM) were applied to the sensor chip having the immobilized salivary components. The K_a and K_d were determined using the BIA-evaluation software (BIAcore). The K_a of peptides were lower than K_a levels (1×10^4–10^9 M^{-1}) seen with monoclonal antibodies to antigens; however, our values were significant (Table 2). There were no significant differences between SspB (390–T400K–402) and SspB (390–T400K–401) peptides in K_a and K_d. Other peptides and peptides at less than 650 μM were below the detectable K_a and K_d using the BIAcore assay. Taken together, the connection of the positively and negatively charged residues involving aspartic acid, lysine and glutamic acid at positions 390, 400 and 401; and the 12 amino acid sequence from positions 390 to 401 were required for binding and the inhibition activity of the SspB (390–T400K–402) peptide.

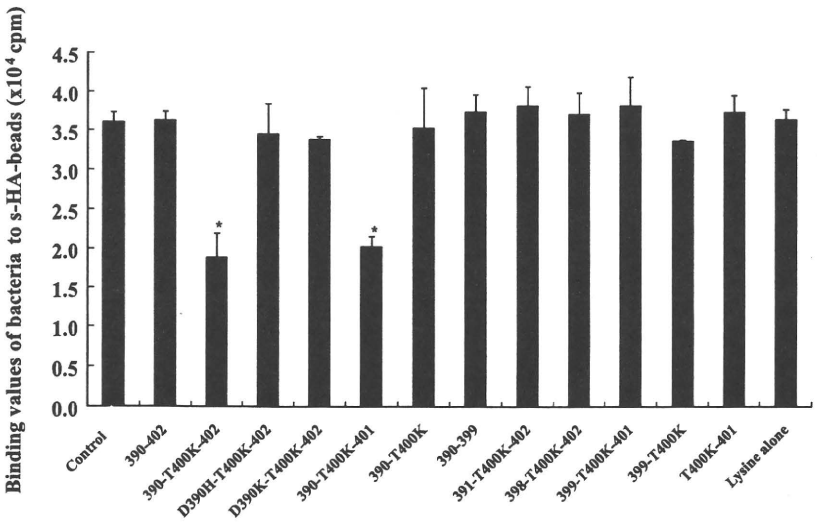

Fig. 4 – Inhibition using SspB truncated peptides for *S. mutans* adherence on s-HA. The adherence levels (CPM) of *S. mutans* MT8148 to s-HA treated using 650.0 μM lysine alone, SspB (T400K–401), SspB (399–T400K), SspB (399–T400K–402), SspB (398–T400K–402), SspB (391–T400K–402), SspB (390–399), SspB (390–T400K), SspB (390–T400K–401), SspB (390–T400K–402), SspB (D390H–T400K–402) and SspB (D390K–T400K–402) peptides are shown. Control: binding values of *S. mutans* to non-treated s-HA. The data are the means ± SD of three independent assays. Asterisks show significantly different relative levels of bacterial adherence ($p < 0.05$ vs. control: non-treated and saliva-coated hydroxyapatite).