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Title: Effect of mouth cleaning with hinokitiol-containing gel on oral malodor: a randomized, open-label pilot study

Kosaku Iha, DDS[1], Nao Suzuki, DDS, Ph.D.[1], Masahiro Yoneda, DDS, Ph.D.[2], Toru Takeshita, DDS, Ph.D.[3], and Takao Hirofuji, DDS, Ph.D.[1]

[1] Section of General Dentistry, Department of General Dentistry, Fukuoka Dental College

[2] Center for Oral Diseases, Fukuoka Dental College

[3] Section of Preventive and Public Health Dentistry, Division of Oral Health, Growth, and Development, Kyushu University Faculty of Dental Science

Correspondence to:

Dr. Nao Suzuki, DDS, Ph.D. Section of General Dentistry, Department of General

Dentistry, Fukuoka Dental College, 2-15-1 Tamura, Sawara-ku, Fukuoka 801-0193, Japan.

Tel: +81-92-801-0411; FAX: +81-92-801-4909.

E-mail: naojsz@college.fdcnet.ac.jp

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ABSTRACT

The aim of the study was to evaluate the effect of mouth cleaning with hinokitiol-containing gel on oral malodor. An open-label, randomized, controlled trial was conducted to assess oral malodor and clinical parameters related to oral malodor before and after mouth cleaning with hinokitiol-containing gel (n = 9) or with gel not including hinokitiol (n = 9). Mouth cleaning included the teeth, gingiva, and tongue and was carried out three times per day for 4 weeks. Organoleptic test (OLT) scores (P = .021), levels of hydrogen sulfide (P = .008) and methyl mercaptan (P = .020), frequency of bleeding on probing, average probing pocket depth, and plaque index significantly improved in the group using hinokitiol. In contrast, only the OLT score (P = .031) significantly improved in the control group after the treatment regimen. Mouth cleaning with hinokitiol-containing gel may be effective for reduction of oral malodor.

Short title: Effect of hinokitiol on oral malodor

Oral malodor, also called halitosis or bad breath, is a common problem in humans. Most oral malodor originates directly from the oral cavity, owing to conditions such as periodontitis, tongue debris, poor oral hygiene, deep caries, inadequately fitted restorations, and endodontic lesions.[1–5] Oral malodor is primarily the result of microbial metabolism of amino acids from local debris in the oral cavity.[6] The most common compounds associated with oral malodor are volatile sulfur compounds (VSCs), such as hydrogen sulfide (H_2S) and methyl mercaptan (CH_3SH).[7] VSCs are mainly produced through bacterial metabolism of sulfur-containing amino acids, such as cysteine and methionine.[7] Gram-negative strict anaerobes are important producers of VSCs.[8,9] Gram-positive oral bacteria, primarily streptococci, may also promote VSC production by Gram-negative bacteria.[10]

Different kinds of topical antimicrobial agents have been used to treat oral malodor. Mouth rinse and toothpaste with antimicrobial properties, such as chlorhexidine, triclosan, and cetylpyridinium chloride (CPC), can reduce oral malodor by chemically reducing the number of microorganisms.[11] Other chemical agents, such as zinc and chlorine dioxide, can reduce halitosis by chemically neutralizing VSCs.[12,13]

Combination of various chemical agents can also markedly reduce VSC concentrations.[14,15]

Hinokitiol $C_{10}H_{12}O_2$ (β-thujaplicin), a component of the essential oils isolated from Cupressaceae, shows antibacterial activity against various bacteria and fungi.[16–19] Hinokitiol has been used as a therapeutic agent against periodontal disease and oral *Candida* infections.[20,21] Periodontal disease causes oral malodor, while oral candidiasis causes malodor indirectly through decreased saliva production, along with localized inflammation of the oral mucosa. By eliminating the infectious agents causing disease, hinokitiol should be an effective treatment for oral malodor. However, no prior study had examined the effect of hinokitiol on oral malodor. In this study, we conducted an open-label, randomized, controlled trial to investigate the effect of mouth cleaning with hinokitiol-containing gel on oral malodor by comparison with CPC-containing gel, which does not include hinokitiol, as a control.

MATERIALS AND METHODS

Subjects

This study population consisted of 18 patients (4 males and 14 females; mean age, 54.7 ± 10.1 years; age range, 33–71 years) who complained of halitosis and presented to the Oral Malodor Clinic of Fukuoka Dental College Medical and Dental Hospital, Japan, between December 2011 and November 2012. Patients included in the study had not previously been receiving treatment for oral malodor and had oral malodor scores above questionable levels (OLT ≥ 1.5); they were not halitophobic and had no acute symptoms requiring immediate oral cavity treatment or antibiotic use within the last month; they did not smoke or consume alcohol above recommended levels (≤20 g/day)[22] and were not on any medications. All participating subjects understood the nature of the research project and provided informed consent. Permission for this study was obtained from the Ethics Committee for Clinical Research of Fukuoka Dental College and Fukuoka College of Health Sciences (approval number 191). All participants provided written informed consent to participate in this study.

Study design

The subjects were randomly assigned to one of two groups by simple randomization

using computer-generated random numbers. Random numbers were generated by a third party, Professor H. Anan, Department of Odontology, Fukuoka Dental College, and the randomization code was not broken until the start of each intervention. One group (n = 9) cleaned their mouths with an oral care gel including hinokitiol as an active ingredient (REFRE-CARE H; EN Otsuka Pharmaceutical Co. Ltd., Iwate, Japan) for 4 weeks, whereas another group (n = 9) performed mouth cleaning with a CPC-containing control gel that did not include hinokitiol. The hinokitiol concentration of REFRE-CARE H is not listed; however, as an unregulated drug, Japanese law states that the concentration must be between 0.01% and 0.2%. The concentration of CPC in the controlgel is 0.01%. Both commercialized products were gels for oral care that had humidity retention power and did not include blowing agents or abrasives. Mouth cleaning included the teeth, gingiva, and tongue and was carried out three times daily (after every meal) for 4 weeks using the test gel or the control gel. First, subjects placed a 1-cm strip of gel on a toothbrush, brushed their teeth as normal, and rinsed with water. Next, subjects placed 1 cm of gel on a finger and performed a gingival massage. Finally, subjects placed 1 cm of gel on a tongue scraper and rubbed it against the dorsal surface

of the tongue five times. Subjects did not rinse, eat, or drink for 30 min after cleaning. All subjects used the same toothbrush (DENT. MAXIMA medium soft; Lion, Tokyo, Japan) and tongue scraper (MS Tongue Cleaner; Morita, Osaka, Japan) during the intervention period. Malodor and clinical assessments were performed on days 0 and 28 at the Oral Malodor Clinic of Fukuoka Dental College Medical and Dental Hospital.

Malodor assessment

Malodor was assessed for each patient, and a clinical examination was performed at the same time of day, at least 5 h after eating, drinking, chewing, and brushing or rinsing the mouth. The severity of oral malodor was determined using an organoleptic test (OLT) and gas chromatography (model GC2014; Shimadzu Works, Kyoto, Japan). For the OLT, patients were instructed to exhale through the mouth with moderate force into a Teflon sampling bag (GL Science, Tokyo, Japan) for 2-3 s. This procedure was repeated until ~1 L of breath sample was obtained. OLT scores were estimated by two of the three evaluators (with training and experience in calibration tests) using a scale of 0 to 5 (0, absence of odor; 1, questionable odor; 2, slight malodor; 3, moderate malodor;

4, strong malodor; 5, severe malodor)[23]; the mean of the scores given by the evaluators was used. The presence of OLT scores ≥2 among the three evaluators always exceeded 75.0% ($\kappa = 0.50$). Gas chromatography was used to measure the concentrations of H_2S and CH_3SH in mouth air.[24] The presence of oral malodor was defined as a mean OLT score ≥1.5.

Clinical examination

Periodontal health, plaque control, and degree of tongue coating were evaluated as major clinical outcomes. Periodontal health was assessed using the average of probing pocket depth (PPD) and the number of bleeding on probe (BOP) sites. PPD and BOP were measured at six points around each tooth in all subjects. Plaque control was evaluated using the Silness and Löe Plaque Index (PlI).[25] The degree of tongue coating was determined by the tongue coating score (TCS) using a scale of 0 to 4 (0, no tongue coating; 1, thin tongue coating covering less than one-third of the tongue dorsum; 2, thick tongue coating covering approximately one-third of the tongue dorsum or thin tongue coating covering one-third to two-thirds of the tongue dorsum; 3, thick tongue

coating covering one-third to two-thirds of the tongue dorsum or thin tongue coating covering more than two-thirds of the tongue dorsum; 4, thick tongue coating covering more than two-thirds of the tongue dorsum).[26] The moisture level of the tongue surface was evaluated using the moisture checker Mucus® (Life, Saitama, Japan) according to the manufacturer's instructions.[27] Briefly, the subject was directed to push out the tongue, and the examiner placed a sensor perpendicular to the center of the tongue dorsum to measure the level of impedance. Saliva was collected using chewing gum[28]; subjects were asked to spit into a vessel throughout the 5-min collection period.

Microbial quantitative analysis

Quantities of ubiquitous bacteria and *Candida albicans* present in the saliva of test subjects were determined. Ubiquitous bacteria were quantified using a polymerase chain reaction (PCR)-invader assay performed by BML (Tokyo, Japan). Quantitative real-time PCR for *Candida albicans* DNA was performed using a QuantiFast SYBR Green PCR Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The sequences of the primers for *C. albicans* and the details of the procedure have been

previously described.[29]

Statistical analysis

Baseline demographics were evaluated by chi-square test and *t*-test. A Wilcoxon signed-rank test was used to compare major outcomes, including OLT score, the concentrations of H_2S and CH_3SH, number of BOP sites, average PPD, PlI, and microbial number between days 0 and 28. TCS was evaluated by a chi-square test. The Mann-Whitney *U* test was used to compare major outcomes between the two groups. Statistical significance was set at $P < .050$. All statistical analyses were conducted using the R software package, version 2.15.2.[30]

RESULTS

Baseline characteristics of subjects

Simple randomization allocated nine subjects to the hinokitiol group and the other nine to the control group. Baseline demographics are shown in Table I. No significant differences were noted between the two groups at baseline with respect to malodor

assessments (OLT score and the concentrations of H_2S and CH_3SH) and clinical parameters (sex, age, number of teeth, average PPD, TCS, moisture level of the tongue surface, and stimulated salivary flow). The conclusion was made that the two groups were balanced in terms of baseline characteristics.

Changes in oral malodor parameters

After 28 days, OLT scores significantly decreased in both the hinokitiol and control groups compared with their respective baseline scores ($P = 0.021$ and $P = 0.031$, respectively; Figure 1). No significant differences were observed between the two groups ($P = .352$). The OLT scores of two subjects in the hinokitiol group improved to <1, indicating no oral malodor. Compared with the values at day 0, the concentration of H_2S measured by gas chromatography was significantly lowered in the hinokitiol group at day 28 (3.6 [2.8-8.9] ng/10 mL on day 0 and 2.0 [1.1-2.6] ng/10 mL on day 28, $P = .008$), but not in the control group (2.2 [1.7-7.2] ng/10 mL on day 0 and 3.6 [3.3-8.9] ng/10 mL on day 28, $P = .203$; Figure 2, *A*); the difference was statistically significant ($P = .004$). In addition, CH_3SH, a major component of oral malodor derived from

periodontitis, showed significant reduction (P = .003) in the hinokitiol group (2.5 [0.9-2.7] ng/10 mL on day 0 and 0.9 [0.4-1.2] ng/10 mL on day 28, P = .020), but not in the control group (2.2 [1.9-3.8] ng/10 mL on day 0 and 3.0 [1.5-5.0] ng/10 mL on day 28, P = .203; Figure 2, *B*).

Changes in clinical and microbial parameters

Among the major clinical outcomes, the number of BOP sites (P = .014), the average PPD (P = .014), and the PlI (P = .039) improved significantly in the hinokitiol group (Table II). Additionally, the number of subjects with PPD ≥ 4 mm also decreased significantly in the hinokitiol group (5.6 ± 4.7 on day 0 and 2.8 ± 3.3 on day 28, P = .022). TCS and moisture level of the tongue surface did not change after 28 days in the hinokitiol group. No changes were observed in clinical parameters in the control group, although the number of BOP sites showed a decreasing trend (P = .059).

A microbial quantitative analysis of saliva showed no significant changes in the hinokitiol group, although the number of *C. albicans* on day 28 was lower than that on day 0 (Table II). In the control group, the number of ubiquitous bacteria significantly

increased at day 28 (P = .039) compared with day 0.

DISCUSSION

In this study, oral care with hinokitiol-containing gel resulted in reduction of oral malodor parameters after 28 days, and with regard to clinical parameters, periodontal parameters (the number of BOP sites and average PPD) and the plaque index improved significantly. The main cause of oral pathologic malodor is periodontal disease.[31] The principal periodontopathic bacteria, such as *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, and *Prevotella intermedia*, can highly produce H_2S and CH_3SH.[8] Nonsurgical periodontal treatment played an important role in reducing oral malodor in patients with periodontitis.[32] Oral management using antimicrobial and probiotic agents that targeted periodontopathic bacteria also contributed to reducing oral malodor.[24,33–35] Tooth brushing using 0.1% hinokitiol-containing medicine (Hinoporon™; Showa Yakuhinnkako Co., Ltd., Tokyo, Japan) has been reported to show statistically meaningful, improved BOP compared with tooth brushing without medicine in dental school students with gingivitis.[20] The present study is the first report to evaluate and

reveal the reduction effect of hinokitiol on oral malodor clinically.

Hinokitiol shows antibacterial activity against various oral microorganisms in *in vitro* studies.[18,19,36] Saeki *et al*. reported that hinokitiol inhibited the growth of 25 oral bacterial strains at a concentration of 0.1%.[18] At 0.01%, hinokitiol inhibited the growth of 14 strains, including periodontopathic bacteria, and did not inhibit the growth of six streptococcal strains tested.[18] A report stated that the growth of *Candida* strains was inhibited by long-term treatment with more than 0.25 mM (4.2×10^{-3}%) hinokitiol for 24 h, and that the adherence of these bacteria to epithelial cells was inhibited 30–70% after short-term treatment with 0.25 mM hinokitiol for 30 min.[36] Quantitative microbial analysis of saliva samples in the present study showed a reduction of *C. albicans* in the hinokitiol group, but not in the control group. The number of whole oral bacteria did not change in the hinokitiol group, whereas it significantly increased in the control group. The composition of salivary bacterial populations has been reported to be stable against shifts in the supragingival microbiota.[37] Further studies will be necessary to examine changes in the bacterial composition of supra/subgingival plaques and tongue debris to clarify the antimicrobial effect of hinokitiol. Recently, the inhibition effect of hinokitiol

on LPS-induced inflammation was found in *in vitro* and *in vivo* studies.[38] Hinokitiol inhibited LPS-induced nitric oxide (NO), prostaglandin E2, interleukin-6, and tumor necrosis factor-α (TNF-α) production in an *in vitro* study and was also shown to be effective *in vivo* for inhibiting LPS-induced NO and TNF-α production as well as significantly decreasing the mortality rate of mice suffering from septic shock.[38] The remarkable improvement of periodontal parameters was assumed to be a result of both the antimicrobial and anti-inflammatory actions of hinokitiol.

Tongue coating scores did not change at day 28 in either group, although the subjects performed tongue cleaning. Tongue coating is understood to be an important factor of oral malodor in both physiological and oral pathological halitosis.[39] Tongue cleaning contributed to a lesser extent to reduction in oral malodor in patients with periodontitis.[32] With regard to patients with gingivitis, tongue cleaning alone could be the primary approach to reducing oral malodor.[32] The subjects in the current study did not show high TCS (only one patient in the control group had a score >3, while the other patients had scores <2) at baseline. In addition to TCS, the moisture level of the tongue surface did not change, although both commercialized products were gels for

oral care and had humidity retention power. The moisture level of the tongue surface measured using Mucus® was judged to be sufficient if it was greater than 25.0, according to the manufacturer. The subjects in our study had sufficient moisture levels on the tongue surface at baseline in both groups (28.1 ± 3.7 in the test group and 27.0 ± 1.5 in the control group). However, moisture levels on day 28 (26.0 ± 3.9) were lower than at baseline (28.1 ± 3.7) in the hinokitiol group. Perhaps the method and power of mechanical cleaning of the tongue should be regulated depending on the degree of tongue coating.

Many flavors and natural botanic extracts have been put in food and medicine for reducing oral malodor. These compounds have been generally used as traditional Chinese medical therapy or phytotherapy. A few reports have tried to clarify scientific evidence regarding the reduction effects on oral malodor of compounds such as green tea powder, *Eucalyptus* extract, and the pericarp extract of *Garcinia mangostana* L.[35,40,41] Unlike synthetic antimicrobial components, medicinal plants, including hinokitiol, have very low toxicity and fewer side effects.[42,43] At the same time, Japanese law requires the concentration of synthetic antimicrobial agents combined with

dentifrice to be lower than their effective concentrations. For example, the concentrations of chlorhexidine and CPC combined with dentifrice are limited to 0.05% and 0.01%, respectively, although foreign studies examining the effectiveness of these compounds on oral malodor were performed using 0.1-0.2% chlorhexidine and 0.05-0.07% CPC.[33,44,45] In this study, oral care with the 0.01% CPC-containing control gel showed an improvement in the OLT score; however, this improvement was not seen for concentrations of H_2S and CH_3SH, or periodontal conditions. Whether OLT scores improved as a result of CPC activity, or simply an increased consciousness of mouth cleaning due to participation in a clinical trial, is uncertain. Control of oral malodor using natural antimicrobial ingredients is likely to increase in the future as interest in natural foods and products becomes more popular.

The present study had several limitations. The study included a small study population. After being allocated randomly by a third party, the subjects and the examiner knew the kind of gel that each was using. Individual differences in techniques of mouth cleaning were not considered in the statistical analysis. To clarify the clinical effects of hinokitiol on oral malodor, more extensive double-blinded, crossover,

randomized studies in a larger number of subjects are required.

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References

1. Morita M, Wang HL. Association between oral malodor and adult periodontitis: a review. J Clin Periodontol 2001;28:813-9.
2. Koshimune S, Awano S, Gohara K, Kurihara E, Ansai T, Takehara T. Low salivary flow and volatile sulfur compounds in mouth air. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2003;96:31-41.
3. Yoneda M, Naito T, Suzuki N, Yoshikane T, Hirofuji T. Oral malodor associated with internal resorption. J Oral Sci 2006;48:89–92.
4. Garrett NR. Poor oral hygiene, wearing dentures at night, perceptions of mouth dryness and burning, and lower educational level may be related to oral malodor in denture wearers. J Evid Based Dent Pract 2010;10:67-9.

5. Yoneda M, Suzuki N, Mocedo SM, Fujimoto A, Iha K, Koga C, Matsuura M, Hirofuji T. The variable etiology of oral pathologic halitosis: a case series. Smile Dent J 2012;7:50-6.

6. Scully C, Porter S, Greenman J. What to do about halitosis. BMJ 1994;308:217–18.

7. Kleinberg I, Westbay G. Oral malodor. Crit Rev Oral Biol Med 1990;1:247–59.

8. Persson S, Edlund MB, Claesson R, Carlsson J. The formation of hydrogen sulfide and methyl mercaptan by oral bacteria. Oral Microbiol Immunol 1990;5:195-201.

9. Washio J, Sato T, Koseki T, Takahashi N. Hydrogen sulfide-producing bacteria in tongue biofilm and their relationship with oral malodor. J Med Microbiol 2005;54:889-95.

10. Sterer N, Rosenberg M. Effect of deglycosylation of salivary glycoproteins on oral malodor production. Int Dent J 2002;52(Suppl 3):229-30.

11. Cortelli JR, Barbosa MD, Westphal MA. Halitosis: a review of associated factors and therapeutic approach. Braz Oral Res 2008;22(Suppl 1):44-54.

12. Wåler SM. The effect of some metal ions on volatile sulfur-containing compounds originating from the oral cavity. Acta Odontol Scand 1997;55:261-4.

13. Lynch E, Sheerin A, Claxson AWD, Atherton MD, Rhodes CJ, Silwood CJL, Naughton DP, Grootveld M. Multicomponent spectroscopic investigations of salivary antioxidant consumption by an oral rinse preparation containing the stable free radical species chlorine dioxide (ClO_2). Free Radical Res 1997;26:209-34.

14. Winkel EG, Roldán S, Van Winkelhoff AJ, Herrera D, Sanz M. Clinical effects of a new mouthrinse containing chlorhexidine, cetylpyridinium chloride and zinc-lactate on oral halitosis. A dual-center, double-blind placebo-controlled study. J Clin Periodontol 2003;30:300-6.

15. Thrane PS, Young A, Jonski G, Rolla G. A new mouthrinse combining zinc and chlorhexidine in low concentrations provides superior efficacy against halitosis compared to existing formulations: a double-blind clinical study. J Clin Dent 2007;18:82-6.

16. Southam CM. Antibiotic activity of extract of western red ceder heartwood. Exp Biol Med 1946;61:391-6.

17. Erdtman H, Gripenberg J. Antibiotic substances from the heart wood of Thuja plicata. Nature 1948;161:719.

18. Saeki Y, Ito Y, Shibata M, Sato Y, Okuda K, Takazoe I. Antimicrobial action of natural substances on oral bacteria. Bull Tokyo Dent Coll 1989;30:129-35.

19. Osawa K, Matsumoto T, Maruyama T, Takiguchi T, Okuda K, Takazoe I. Studies of the antibacterial activity of plant extracts and their constituents against periodontopathic bacteria. Bull Tokyo Dent Coll 1990;31:17-21.

20. Suzuki J, Tokiwa T, Mochizuki M, Ebisawa M, Nagano T, Yuasa M, Kanazashi M, Gomi K, Arai T. Effects of newly designed toothbrush for the application of periodontal disease treatment medicine (Hinoporon™) on the plaque removal and the improvement of gingivitis. J Jpn Soc Periodontol 2008;50:30-8.

21. Nagao Y, Sata M. Effect of oral care gel on the quality of life for oral lichen planus in patients with chronic HCV infection. Virol J 2011;8:348. Doi: 10.1186/1743-422X-8-348.

22. Inoue M, Nagata C, Tsuji I, Sugawara Y, Wakai K, Tamakoshi A, Matsuo K, Mizoue T, Tanaka K, Sasazuki S, Tsugane S. Impact of alcohol intake on total mortality and mortality from major causes in Japan: a pooled analysis of six large-scale cohort studies. J Epidemiol Community Health 2012;66:448-56.

23. Miyazaki H, Arao M, Okumura K, Kawaguchi Y, Toyofuku A, Hoshi K, Yaegaki K. Tentative classification for halitosis patients and its treatment needs. Niigata Dent J (Japanese) 1999;32:11-5.

24. Iwamoto T, Suzuki N, Tanabe K, Takeshita T, Hirofuji T. Effects of probiotic *Lactobacillus salivarius* WB21 on halitosis and oral health: an open-label pilot trial. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2010;110:201-8.

25. Silness J, Löe H. Periodontal disease in Pregnancy II. Correlation between oral hygiene and periodontal condition. Acta Odontol Scand 1964;22:121–35.

26. Suzuki N, Yoneda M, Naito T, Iwamoto T, Hirofuji T. Relationship between halitosis and psychologic status. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2008;106:542–7.

27. Yamada H, Nakagawa Y, Nomura Y, Yamamoto K, Suzuki M, Watanabe NY, Saito I, Seto K. Preliminary results of moisture checker for Mucus in diagnosing dry mouth. Oral Dis 2005;11:405-7.

28. Suzuki N, Tanabe K, Takeshita T, Yoneda M, Iwamoto T, Oshiro S, Yamashita Y., Hirofuji T. Effects of oil drops containing *Lactobacillus salivarius* WB21 on

periodontal health and oral microbiota producing volatile sulfur compounds. J Breath Res 2012;6:017106. doi:10.1088/1752-7155/6/1/017106.

29. Hsu MC, Chen KW, Lo HJ, Chen YC, Liao MH, Lin YH, Li SY. Species identification of medically important fungi by use of real-time LightCycler PCR. J Med Microbiol 2003;52:1071-6.

30. The R project homepage. http://www.r-project.org 2010.

31. Yaegaki K, Sanada K. Biochemical and clinical factors influencing oral malodor in periodontal patients. J Periodontol 1992;63:783-9.

32. Pham TA, Ueno M, Zaitsu T, Takehara S, Shinada K, Lam PH, Kawaguchi Y. Clinical trial of oral malodor treatment in patient with periodontal disease. J Periodontal Res 2011;46:722-9.

33. Roldán S, Herrera D, Santa-Cruz I, O'Connor A, Gonzalez I, Sanz M. Comparative effects of different chlorhexidine mouth-rinse formulations on volatile sulphur compounds and salivary bacterial counts. J Clin Periodontol 2004;31:1128-34.

34. Farrell S, Baker RA, Somogyi-Mann M, Eitt JJ, Gerlach RW. Oral malodor reduction by a combination of chemotheraperutical and mechamical treatments. Clin

Oral Investig 2006;10:157-63.

35. Tanaka M, Toe M, Nagata H, Ojima M, Kuboniwa M, Shimizu K, Osawa K, Shizukuishi S. Effect of eucalyptus-extract chewing gum on oral malodor: a double-masked, randomized trial. J Periodontol 2010;81:1564-71.

36. Nakamura M, Fujibayashi T, Tominaga A, Satoh N, Kawarai T, Shinozuka O, Watanabe H, Yamazaki T, Senpuku H. Hinokitiol inhibits *Candida albicans* adherence to oral epithelial cells. J Oral Biosci 2010;52:42-50.

37. Yamanaka W, Takeshita T, Shibata Y, Matsuo K, Eshima N, Yokoyama T, Yamashita Y. Compositional stability of a salivary bacterial population against supragingival microbiota shift following periodontal theraphy. PLoS One 2012;7:e42806.

38. Shih MF, Chen LY, Tsai PJ, and Cherng JY. In vitro and in vivo therapeutics of β-thujaplicin on LPS-induced inflammation in macrophages and septic shock in mice. Int J Immunopathol Pharmacol 2012;25:39-48.

39. Delanghe G, Ghyselen J, Bollen C, van Steenberghe D, Vandekerckhove BN, Feenstra L. An inventory of patients' response to treatment at a multidisciplinary

breath odor clinic. Quintessence Int 1999;30:307–10.

40. Lodhia P, Yaegaki K, Khakbaznejad A, Imai T, Sato T, Tanaka T, Murata T, Kamoda T. Effect of green tea on volatile sulfur compounds in mouth air. J Nutr Sci Vitaminol 2008;54:89-94.

41. Rassameemasmaung S, Sirkulsathean A, Amornchat C, Hirunrat K, Rojanapanthu P, Gritsanapan W. Effects of herbal mouthwash containing the pericarp extract of Garcinia mangostana L on halitosis, plaque and papillary bleeding index. J Int Acad Periodontol 2007;9:19-25.

42. Ema M, Harazono A, Fujii S, Kawashima K. Evalution of developmental toxicity of beta-thujaplicin (hinokitiol) following oral administration during organogenesis in rats. Food Chem Toxicol 2004;42:465-70.

43. Pomenta AS, Bayona JM, García MT, Solanas AM. Evaluation of acute toxicity and genotoxicity of liquid products from phyrolysis of Eucalyptus grandis wood. Arch Environ Toxicol 2000;38:169-75.

44. Witt J, Bsoul S, He T, Gibb R, Dunavent J, Hamilton A. The effect of toothbrushing regimens on the plaque inhibitory properties of an experimental cetylpyridinium

chloride mouthrinse. J Clin Periodontol 2006;33:737-42.

45. Berchier CE, Slot DE, Van der Weijden GA. The efficacy of 0.12% chlorhexidine mouthrinse compared with 0.2% on plaque accumulation and periodontal parameters: a systematic review. J Clin Periodontol 2010;37:829-39.

Figure legends

Figure 1. Changes in the organoleptic test score. *A significant difference was observed between days 0 and 28 by Wilcoxon signed-rank test ($P < .050$).

Figure 2. Changes in H_2S (A) and CH_3SH (B) levels measured by gas chromatography (ng/10 mL mouth air, median [IQR]). *A significant difference was observed between days 0 and days 28 by Wilcoxon signed-rank test ($P < .050$).

Clinical Relevance

In an open-label, randomized, controlled trial involving a limited number of nonsmokers, cleaning with hinokitiol-containing gel significantly improved oral

malodor, the level of volatile sulfur compounds, sites of bleeding on probing, average probing pocket depth, and plaque index after 4 weeks.

Table I. Baseline demographics (average ± SD).

Demographics	Hinokitiol group (n = 9)	Control group (n = 9)	*P* value*
Males/female (n)	3/6	1/8	NS
Age (years)	52.2 ± 11.4	57.2 ± 8.6	NS
Number of teeth (n)	27.3 ± 3.3	23.7 ± 5.6	NS
Average of probing pocket depth (mm)	2.8 ± 0.5	2.4 ± 0.6	NS
Tongue coating score ≥ 2 (%)	44.4 (n = 4)	77.8 (n = 7)	NS
Salivary flow rate (ml/5 min)	7.5 ± 3.3	7.0 ± 3.1	NS
Moisture level of tongue surface	28.1 ± 3.7	27.0 ± 1.1	NS
Organoleptic score ≥ 2 (%)	88.9 (n = 8)	100 (n = 9)	NS
H_2S concentration (ng/10 mL mouth air)	6.1 ± 5.0	4.9 ± 4.7	NS
CH_3SH concentration (ng/10 mL mouth air)	2.1 ± 1.2	2.6 ± 1.6	NS

PPD, probing pocket depth; VSCs, volatile sulfur compounds; NS, not significant.

*Comparisons between the groups were conducted by *t*-test and chi-square test.

Table II. Changes in the clinical and microbial parameters between days 0 and 28.

	Hinokitiol group (n = 9)			Control group (n = 9)		
	0 day	28 days	*P* value	0 day	28 days	*P* value
Clinical parameters with regard to oral malodor						
Sites of bleeding on probing (%)	11.0 ± 8.8	4.1 ± 3.1	0.014	10.2 ± 8.0	6.1 ± 6.3	0.059
Average of probing pocket depth (mm)	2.8 ± 0.5	2.6 ± 0.4	0.014	2.4 ± 0.6	2.4 ± 0.7	0.203
Tongue coating score ≥ 2 (%)*	44.4 (n = 4)	33.3 (n = 3)	0.629	77.8 (n =7)	55.6 (n = 5)	0.317
Plaque Index	0.5 ± 0.3	0.3 ± 0.1	0.039	0.4 ± 0.3	0.4 ± 0.2	0.944
Moisture level of tongue surface	28.1 ± 3.7	26.0 ± 3.9	0.250	27.0 ± 1.1	27.9 ± 4.1	0.375
Microbial parameters in the saliva (log copies/mL)						
Ubiquitous bacteria	8.9 ± 0.2	8.9 ± 0.3	0.910	8.9 ± 0.3	9.0 ± 0.2	0.039
Candida albicans	1.1 ± 1.4	0.9 ± 1.4	1	1.1 ± 1.3	1.3 ± 1.3	0.787

* Chi-square test. The other parameters were evaluated by Wilcoxon signed-rank test.

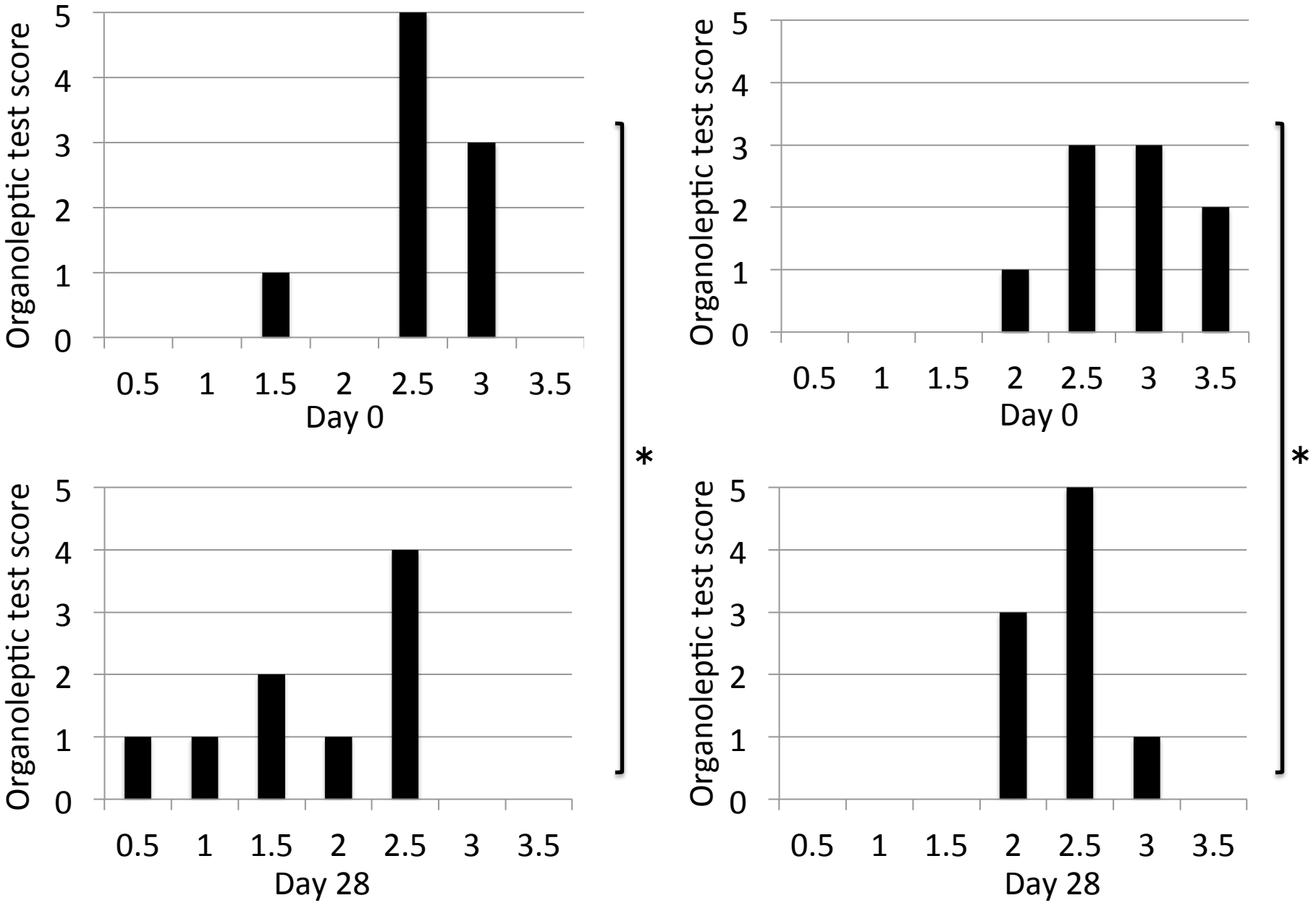

Organoleptic test score
5
4
3
2
1
0
0.5
1
1.5
2
2.5
3
3.5
Day 0
Day 28
*
Hinokitiol group (n = 9)
Control group (n = 9)

Fig. 1

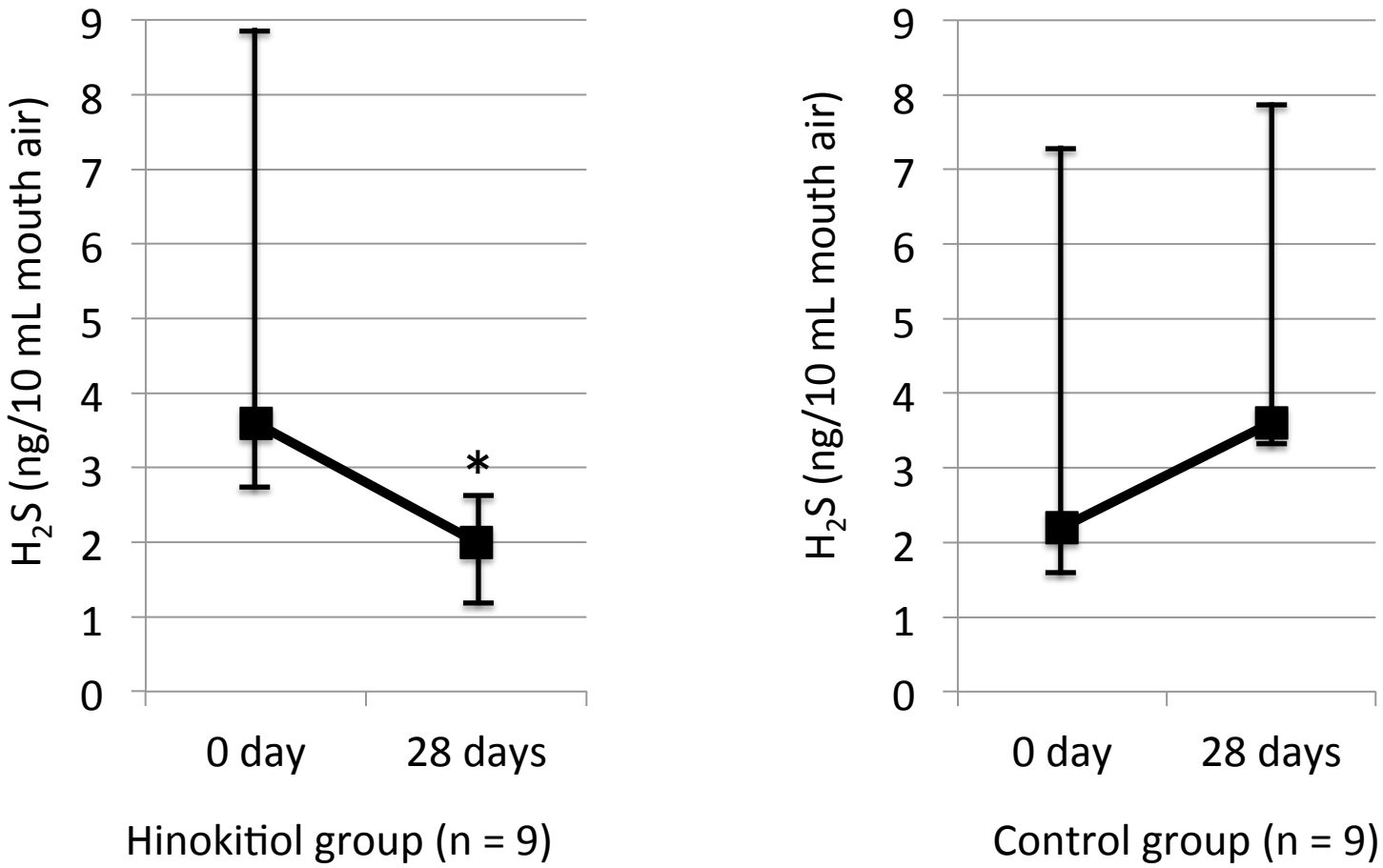
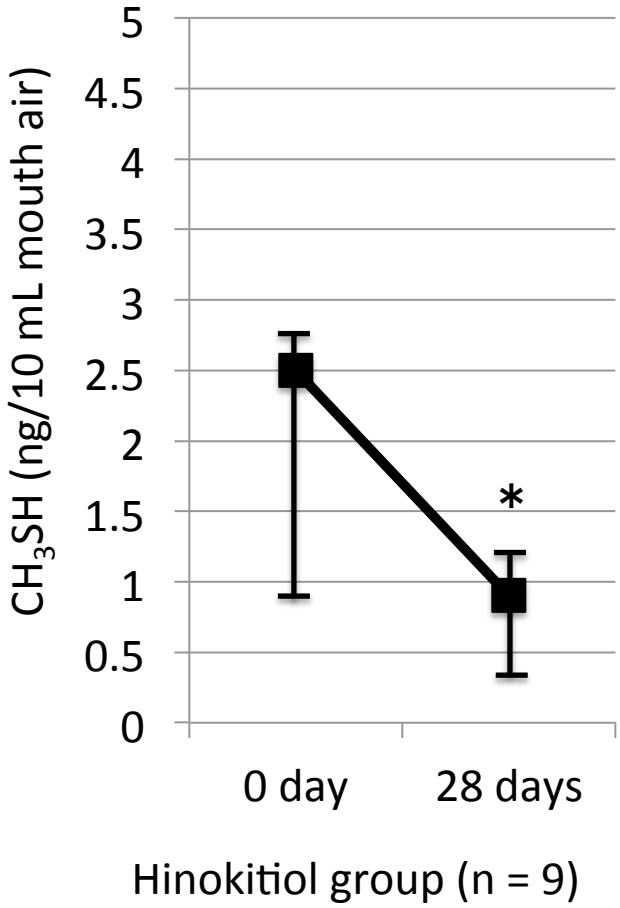

9
8
7
6
5
4
3
2
1
0
H_2S (ng/10 mL mouth air)
*
0 day
28 days
Hinokitiol group (n = 9)
9
8
7
6
5
4
3
2
1
0
H_2S (ng/10 mL mouth air)
0 day
28 days
Control group (n = 9)

Fig. 2 (A)


5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
CH_3SH (ng/10 mL mouth air)
*
0 day
28 days
Hinokitiol group (n = 9)

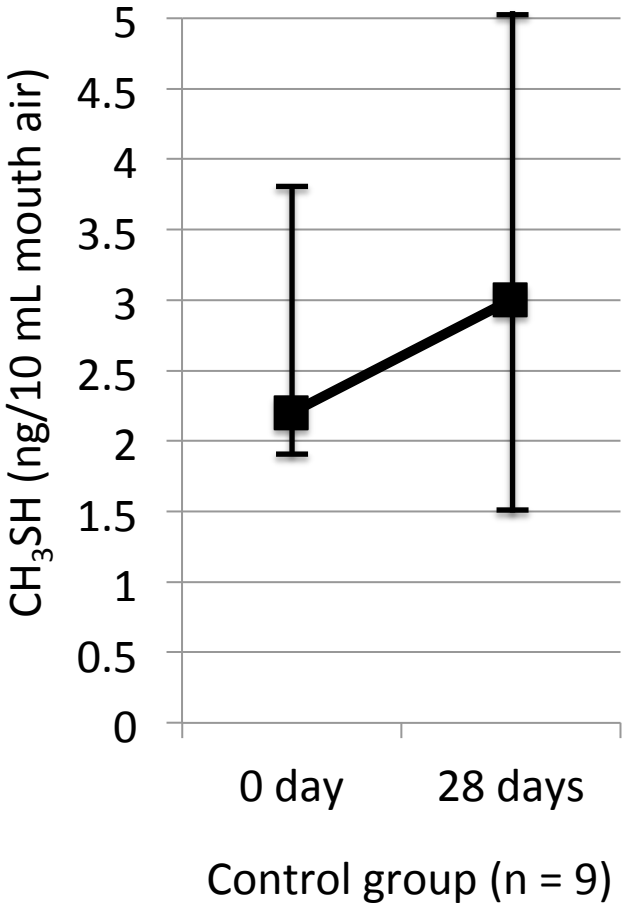

5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
CH_3SH (ng/10 mL mouth air)
0 day
28 days
Control group (n = 9)

Fig. 2 (B)