

Figure 1 The effect of 0.5% *Ginkgo biloba* extract (Ginkgo) diet on systolic blood pressure in normotensive Wistar Kyoto rats (WKY) and spontaneously hypertensive rats (SHR). The ordinate denotes systolic blood pressure, while the abscissa indicates the time course (days) after control diet (▲, WKY; ●, SHR) and 0.5% Ginkgo diet (△, WKY; ○, SHR). Each point represents the mean ± s.e.m. (n = 6). *P < 0.05 vs control SHR.

Ginkgo inhibited a rise in blood pressure by cortisol release during stress in healthy volunteers. In this study, 30-day administration of Ginkgo caused a significant inhibition of any increase in blood pressure in SHR, but had no effect on normotensive WKY. These results indicated that Ginkgo inhibited the development of hypertension.

It is well known that vascular resistance is regulated by the endothelium via the synthesis and secretion of a variety of vasoactive substances, such as nitric oxide, prostacyclin, endothelium-derived hyperpolarizing factors, and endothelium-derived contracting factor. The stable balance of these factors released from the endothelium is disturbed in diseases such as hypertension, atherosclerosis, and diabetes. In hypertension, endothelium-dependent relaxation induced by a variety of vasodilator agents, such as ACh, is markedly impaired; this has been documented by various investigators (Lockette et al 1986; Linder et al 1990; Jameson et al 1993; Taddei et al 1993; Vanhoutte & Boulanger 1995). The overproduction of vasoconstrictor prostanoids (Diederich et al 1990; Vanhoutte & Boulanger 1995) and super oxide anions generated in this pathologic process have been proposed as factors that contribute to the impaired relaxation of vessels to endothelium-dependent vasodilators (Fu-Xiang et al 1992; Jameson et al 1993). Akpaffiong & Taylor (1998) suggested that either excess production of oxidants or deficiency of antioxidant systems may have contributed to high blood pressure and vascular endothelial impairment in SHR.

In these in-vitro experiments, diminished relaxation in response to ACh was confirmed in SHR aorta, and relaxation

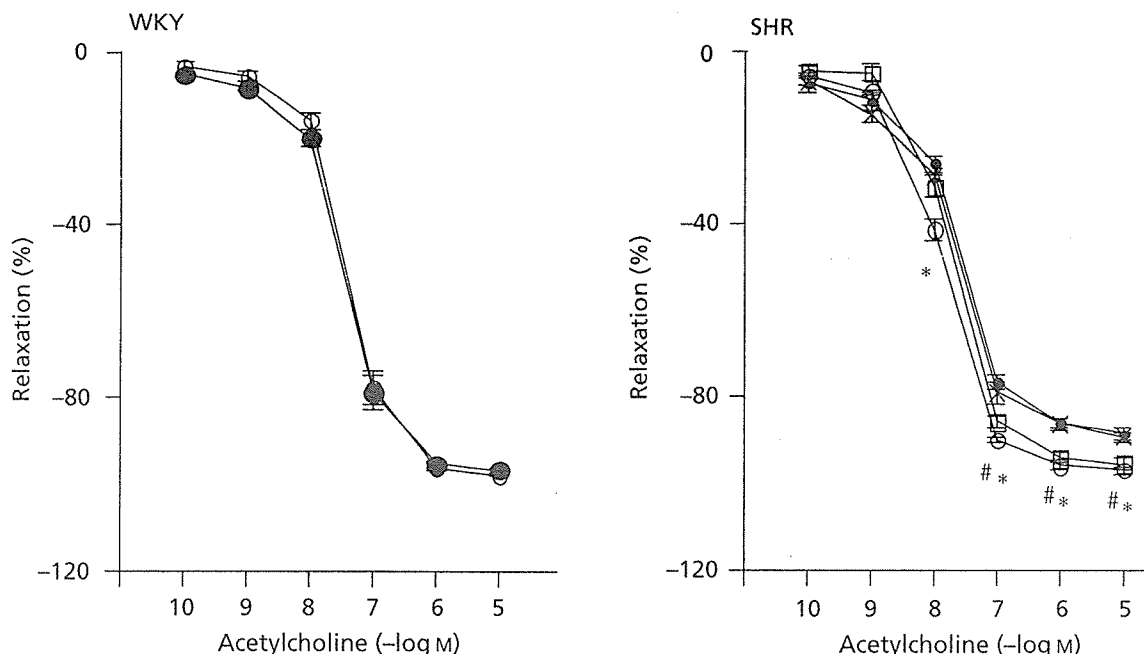


Figure 2 The effect of *Ginkgo biloba* extract (Ginkgo) diet on relaxation induced by acetylcholine in aortic rings precontracted with noradrenaline (10^{-7} M) isolated from normotensive Wistar Kyoto rats (WKY) and spontaneously hypertensive rats (SHR). The ordinate indicates the ratio of relaxation (%) to maximum relaxation in response to papaverine at 10^{-6} M and the abscissa shows the concentration of acetylcholine (M) in rats fed the control diet (▲, WKY; ●, SHR), the 0.05% Ginkgo diet (×, SHR), the 0.1% Ginkgo diet (□, SHR), and the 0.5% Ginkgo diet (△, WKY; ○, SHR). Each point represents the mean ± s.e.m. (n = 6). #P < 0.05 0.1% Ginkgo vs control. *P < 0.05 0.5% Ginkgo vs control.

Table 2 Effects of *Ginkgo biloba* extract (Ginkgo) treatment on EC50 of relaxation produced by acetylcholine in the aortic rings precontracted with noradrenaline (10^{-7} M) of spontaneously hypertensive rats

Diet	EC50 (nM)
Control	21.5 ± 2.6
Ginkgo 0.05%	16.2 ± 3.4
Ginkgo 0.1%	17.9 ± 2.5
Ginkgo 0.5%	12.6 ± 1.0*

Each value is the mean ± s.e.m. for six rats. * $P < 0.05$ vs control.

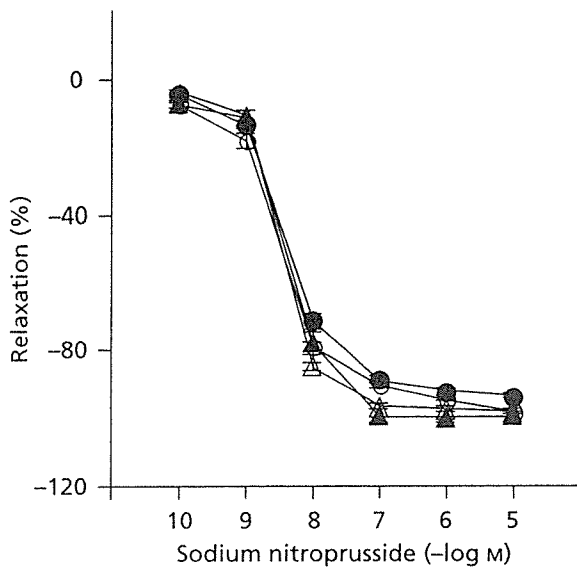


Figure 3 The effect of 0.5% *Ginkgo biloba* extract (Ginkgo) diet on relaxation induced by sodium nitroprusside in aortic rings precontracted with noradrenaline (10^{-7} M) isolated from normotensive Wistar Kyoto rats (WKY) and spontaneously hypertensive rats (SHR). The ordinate indicates the ratio of relaxation (%) to maximum relaxation in response to papaverine (10^{-6} M) and the abscissa denotes the concentration of sodium nitroprusside (M) in rats fed the control diet (\blacktriangle , WKY; \bullet , SHR) or the 0.5% Ginkgo diet (\triangle , WKY; \circ , SHR). Each point represents the mean ± s.e.m. (n=6).

was restored by long-term administration of a Ginkgo-containing diet. This effect of Ginkgo was not observed in WKY. However, a significant decrease in EC50 values was observed only with 0.5% Ginkgo diet and a significant increase in maximum relaxation values was observed only with 0.1% and 0.5% Ginkgo diets. These results indicated that ACh-induced relaxation at 10^{-7} – 10^{-5} M concentrations was enhanced by 0.1% and 0.5% Ginkgo diets in SHR. Furthermore, relaxation in response to nitric oxide, such as sodium nitroprusside-induced relaxation, was not affected by a Ginkgo-containing diet in either SHR or WKY. These findings suggested that enhanced relaxation resulting from Ginkgo administration was due to increased nitric oxide production/release from the endothelium or to greater nitric oxide bioavailability.

Flavonoids are considered important dietary antioxidants (Robak & Gryglewski 1988). Ginkgo also has antioxidant properties (Haramaki et al 1994; Koc et al 1995; Miyajima et al 1997; Pietri et al 1997). Sasaki et al (2002) reported that Ginkgo produced antioxidant effects, increased nitric oxide metabolites, and increased the expression of endothelial nitric oxide synthase mRNA in stroke-prone SHR. One of the major active ingredients of Ginkgo is considered to be quercetin. Indeed, quercetin was reported to inhibit hypoxanthine-xanthine oxidase activity and scavenge superoxides, hydroxy radicals, and peroxynitrite in-vitro (Rice-Evans & Packer 1998). Moreover, metabolites of orally administered quercetin have been demonstrated to retain the antioxidant properties of the parent compound (Manach et al 1998). Duarte et al (2001a, b) demonstrated that while quercetin reduced elevated blood pressure and restored the endothelium-dependent vasodilation in response to ACh in SHR, no such effects were apparent in WKY. They suggested that the effects of quercetin were associated with a reduced oxidant status due to its antioxidant properties. Superoxide is generally recognized to impair endothelium-dependent vasodilation via inactivation of synthesis and/or release of nitric oxide and consequently to elevate blood pressure (Taniyama & Griendling 2003). Taken together, these findings suggested that the antioxidant properties of the flavonoids within Ginkgo, such as quercetin, may have participated in the effects of Ginkgo on blood pressure and endothelium-dependent relaxation observed in this study.

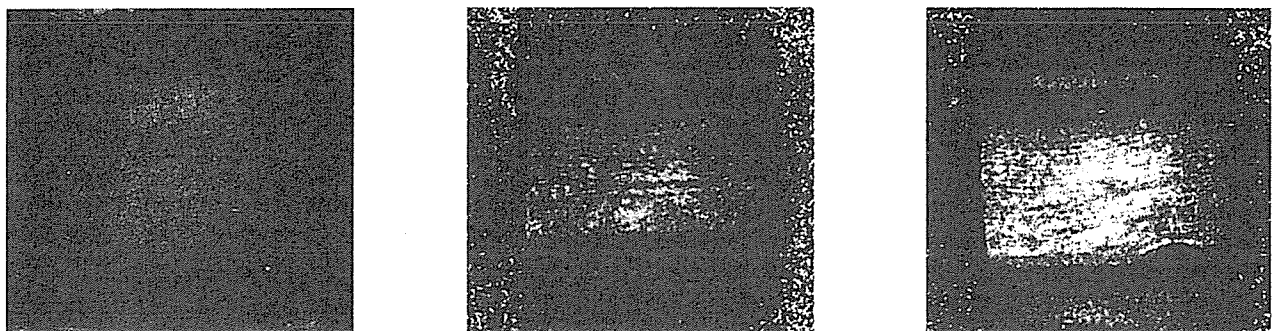


Figure 4 The effect of acetylcholine on intracellular calcium ion level in aortic endothelium isolated from spontaneously hypertensive rats (SHR). Left panel: phase-contrast microscopic image of the endothelial layer. Stainless steel wires are identified in the top and bottom of the image. The degree of vasoconstriction can be estimated from the distance between these two wires. The remaining panels show fluorescent confocal images of intracellular calcium ion level in the endothelial layer of the aorta precontracted with noradrenaline (10^{-6} M) in the absence (middle panel) and presence (right panel) of acetylcholine (10^{-6} M).

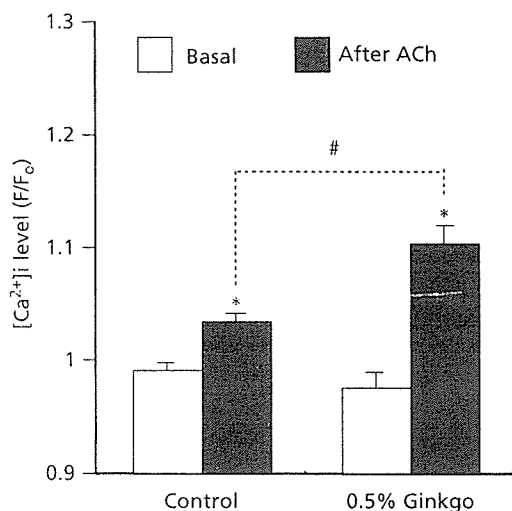


Figure 5 The effects of 0.5% *Ginkgo biloba* extract (Ginkgo) diet on the intracellular calcium ion increase induced by acetylcholine (ACh) in the aortic endothelium isolated from spontaneously hypertensive rats (SHR) fed with control and 0.5% Ginkgo diets. The ordinate shows the intracellular calcium ion level (F/F_0) (fluorescence intensity was the peak fluorescence intensity (F) divided by fluorescence intensity at the beginning of each experiment (F_0)). Each column represents the mean \pm s.e.m. ($n=6$). * $P < 0.05$ vs each baseline value. # $P < 0.05$ vs control acetylcholine.

Indirect and direct experimental evidence demonstrated that the entry of extracellular Ca^{2+} and the liberation of Ca^{2+} from intracellular stores could contribute to an increase in free cytoplasmic Ca^{2+} concentration in endothelial cells, which was an essential step in the synthesis and release of nitric oxide (Rubanyi & Vanhoutte 1988). Endothelial nitric oxide synthase is constitutively expressed in endothelial cells lining the blood vessels and heart. Its activity is tightly controlled by an intramolecular auto-inhibitory element that hinders calmodulin binding and this molecular hindrance is removed by elevated intracellular Ca^{2+} levels (Wu et al 2002; Fleming & Busse 2003). Thus, many studies have indicated that the release of nitric oxide must require an increase in cytoplasmic Ca^{2+} within endothelial cells. Pogan et al (2001) have shown that the Ca^{2+} signalling process in SHR endothelial cells was affected by increased oxidative stress, resulting in a depletion of releasable Ca^{2+} from inositol 1,4,5-trisphosphate-sensitive and -insensitive Ca^{2+} pools. They suggested a possible beneficial action of antioxidants on Ca^{2+} signalling in endothelial cells from models of hypertension. In our study, a greater ACh-induced increase in intracellular Ca^{2+} level was observed in endothelial cells of the aorta isolated from SHR that had been fed a Ginkgo-containing diet than in those isolated from control SHR. This accelerated response may have been caused by recovery of an intracellular Ca^{2+} mobilization mechanism in endothelial cells resulting from the antioxidative action of Ginkgo flavonoids. However, further study is necessary to elucidate the site of action and mechanism of Ginkgo.

In conclusion, the results confirmed that Ginkgo reduced the elevation of blood pressure and improved the dysfunction of the endothelial nitric oxide synthase/nitric oxide pathway in the

endothelium in SHR. Ginkgo enhanced the increase in intracellular Ca^{2+} level to ACh in endothelial cells. This acceleratory influence of Ginkgo on intracellular Ca^{2+} mobilization may have participated in the restoration of endothelium-dependent vasodilation in response to ACh. To our knowledge, this was the first study to have investigated the influence of long-term Ginkgo administration on intracellular calcium mobilization in vascular endothelium. The pharmacological activity was considered to contribute to the possible beneficial properties of Ginkgo in clinical practice, including the regulation of hypertension.

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Effect of Disaccharide Xylosylfructoside on Sucrose Cariogenicity in an Artificial Mouth System

人工口腔装置におけるスクロースのう蝕原性に及ぼす
二糖類キシロシルフルクトシドの影響について

Takeshi KAMODA, Toshio IMAI, Tsutomu SATO, Susumu IMAI,
Tosiki NISIZAWA and Nobuhiro HANADA

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Abstract : It has been reported that xylosylfructoside (XF) effectively inhibited the synthesis of water-insoluble glucan (WIG) and adherence of mutans streptococci to smooth surfaces. The purposes of this study were to examine the cariogenicity of XF and assess the inhibitory effect of XF against the cariogenicity of sucrose using culture and artificial mouth systems. *S. mutans* MT8148 was cultured in heart infusion broth containing 1% sucrose with 0-5% XF in a glass test tube. The amounts of WIG and bacterial cells in the adherent fraction were significantly inhibited by XF in a dose-dependent manner. In the artificial mouth system, 1% XF did not inhibit the formation of artificial biofilm and enamel demineralization in the presence of 1% sucrose, but 2.5% XF significantly inhibited the formation of biofilm on enamel slabs, pH decrease underneath the biofilm, and enamel demineralization, suggesting that an appropriate concentration of XF could inhibit, in part, the cariogenicity of sucrose.

Key words : Xylosylfructoside, Artificial mouth system, Cariogenicity, Biofilm formation, Mutans Streptococci

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Introduction

Dental caries are analogous to bacterial infectious diseases involving a variety of factors^{1,2)}. A causal relationship between sucrose and dental caries has been clarified by epidemiologic and animal studies^{3,4)}. Sucrose is transported into mutans streptococci and other oral bacteria and metabolized into organic acids, resulting in enamel demineralization. Moreover, sucrose is utilized by mutans streptococci as a substrate for glucosyltransferases (GTFs) which are involved in the synthesis of the adhesive water-insoluble glucan (WIG), responsible for the formation of cariogenic biofilm⁴⁻⁹⁾. Consequently, numerous attempts directed at the prevention of dental caries have been performed using either sugar substitutes that do not ferment easily or GTF inhibitors.

Various non- or low-cariogenic sweeteners including oligosaccharides and sugar alcohols have been developed and applied worldwide¹⁰⁾. Xylosylfructoside (O- α -

D-Xylp-(1 \leftrightarrow 2)- β -D-Fruf, XF) is a non-reducing disaccharide composed of xylose and fructose that has the same glycosidic bond as sucrose¹¹⁾. It was reported that XF effectively inhibited WIG synthesis and the adherence of mutans streptococci¹¹⁾, as well as competitively inhibiting the sucrose degradation activity of GTF as a sucrose analog and reducing sucrose consumption¹²⁾. Although XF was not easily fermented by mutans streptococci, the cariogenicity of XF itself and inhibitory effect of XF against sucrose have not yet been examined.

The aims of this study were thus to examine the cariogenicity of XF and to assess the inhibitory effect of XF on the cariogenicity of sucrose using an artificial mouth system (AMS).

Materials and Methods

1. Bacterial strain and culture conditions

Streptococcus mutans MT8148 was used for this study. Following pre-culture, *S. mutans* was cultured in brain

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heart infusion broth (BHI : Difco Laboratories, Detroit, MI) at 37°C for 16 hours. Bacterial cells were collected by centrifugation (6,000 rpm, 10 min), and washed once with phosphate-buffered saline, pH 7.2 (PBS). The cell suspensions of bacteria in PBS solutions were adjusted to an optical density of 2.0 at 500 nm (5.0×10^8 colony forming units/ml).

2. XF

XF was synthesized by the method of Avigad et al.¹³⁾ using levan-sucrase of *Bacillus subtilis* var *saccharolyticus* and provided by Dr. Kitahata (Osaka Municipal Technical Research Institute).

3. Adherence assay in culture system

The effect of XF on the synthesis and adherence of WIG and adherence of mutans streptococci to a glass surface were examined by batch culture. Twenty μ l of subcultured *S. mutans* MT8148 was inoculated into 2 ml of heart infusion broth (HI : Difco Laboratories) containing 1% sucrose and 0.5% XF, and the bacterium was cultured at 37°C for 17 hr in a small glass test tube at a 45° angle. After cultivation, bacterial cells and WIG were fractionated into firm-adherent, loose-adherent, and non-adherent fractions. The cultured tube was rotated gently 3 times and non-adherent culture was poured into another test tube. The original test tube was washed with 2 ml of PBS. Washing was combined with non-adherent culture and this fraction was referred to as the non-adherent fraction. Two ml of PBS was added to the original test tube and shaken by a mixer (Scientific Industries, NY, USA) for 10 sec. The upper fraction by this procedure was the loose-adherent fraction, and the residual fraction on the glass surface was the firm-adherent fraction. In non-adherent and loose-adherent fractions, bacterial cells and WIG were precipitated by centrifugation (25,000 rpm, 20 min) at 4°C. Two ml of 0.5 N NaOH was added to each adherent fraction to resolve WIG on the bacterial cell surface, and cells and WIG were separated by centrifugation (25,000 rpm, 20 min) at 4°C. The amount of cells in each fraction was measured by turbidity at 500 nm after suspension in 1 ml of PBS. WIG in the supernatant was determined colorimetrically at 492 nm by the phenol-sulfuric acid method¹⁴⁾ using glucose as a standard.

4. Biofilm formation and enamel demineralization in artificial mouth system

1) Preparation of enamel slabs

Enamel slabs with a flat surface, 3.5×3.5×1.5 mm in size, including dentine were prepared from the central part of the labial surface of unerupted bovine lower incisors. The enamel slabs were gradually polished using wet abrasive paper and finally with 1 μ m grade polishing film. Nine symmetrical points on the surface of each slab were measured for hardness with a diamond Vicker's indenter on a microhardness tester (Akashi Seisakusho Ltd., MVK-E) loaded with 200 g. Slabs with mean Vicker's hardness values of 280 to 320 were used in the experiments. The area of each enamel slab was measured using a micrometer caliper (Mitsutoyo, CD-15, Kawasaki, Japan).

2) AMS

The AMS consisted of two identical columnar artificial mouths, thermostatic incubator, multiple pH meter, peristaltic pump, and cooling stirrer (Fig. 1): The artificial mouth (60 mm in diameter, 140 mm in height) and surrounding water jacket (140 mm in diameter) were made from transparent vinyl chloride. Warm water was circulated by the thermostatic incubator (Taitech, Saitama, Japan) to maintain the temperature of the artificial mouths at 37°C. A pH electrode with a flat bulb (9 mm in diameter, TOA-DKK, Tokyo, Japan) was set upside down in the center of the artificial mouth with a silicon plug. Four enamel slabs were arranged on the flat surface of a special Teflon holder (24 mm in diameter) around the bulb of the electrode. Another silicon plug with five stainless steel tubes and a thermometer was set on the upper part of the artificial mouth to constantly supply HI medium supplemented with sucrose, PBS, or XF in PBS, and bacterial cell suspension with a peristaltic pump (Furue Science, Tokyo, Japan). The cell suspension ($OD_{500}=2.0$) was maintained at a low temperature using a cooling stirrer (Iwaki Glass, Tokyo, Japan) during the experiment. Changes in pH underneath the artificial biofilm were continuously monitored with a multiple pH recorder (TOA-DKK, Tokyo, Japan). All procedures were conducted under aseptic conditions.

3) Evaluation of quantity of artificial biofilm and enamel demineralization

After stopping the operation of the AMS, the entire

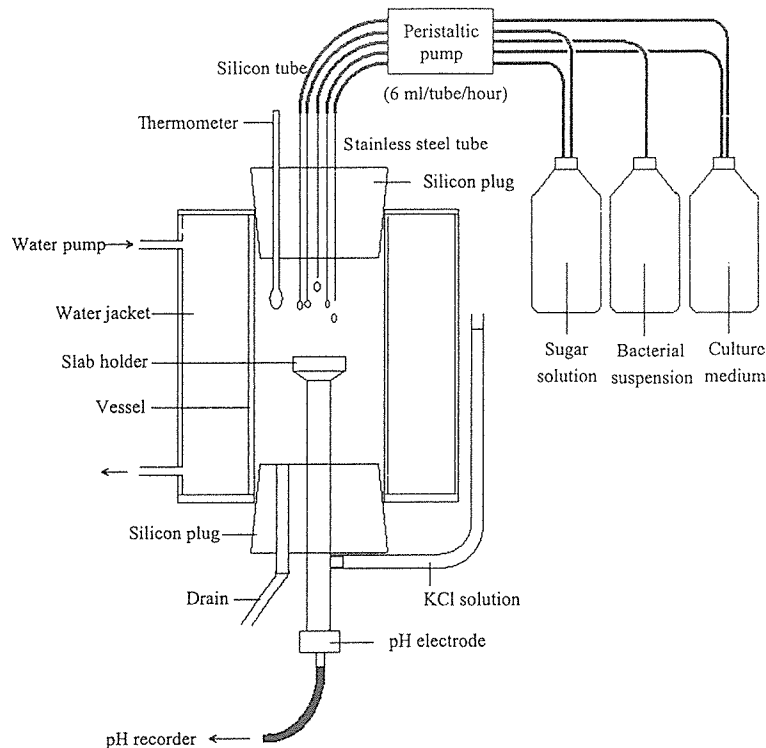


Fig. 1 Cross-sectional appearance of the artificial mouth system

flat electrode and enamel slabs were photographed. The biofilm formed on each enamel slab and flat bulb was carefully removed with a silicon scraper before being immersed in a 0.5 N NaOH solution to resolve the WIG on ice. This was followed by centrifugation (6,000 rpm, 20 min) to separate the bacterial cells from the WIG. Bacterial cells were re-suspended in PBS and the turbidity at 500 nm was measured. In addition, the amount of WIG in the NaOH solution was determined colorimetrically at 492 nm by the phenol-sulfuric acid method. Both cell turbidity and the amount of WIG were used as indicators of the amount of biofilm.

Vicker's hardness values of the enamel slabs were then determined after the experiment and compared with those before the experiment. The differences in hardness (ΔH) were used to infer the degree of demineralization.

4) Statistical analysis

Student's *t*-test was used to compare differences in the mean value of the degree of enamel demineralization and the amount of artificial biofilm in the control and experimental groups.

Results

1. Effect of XF on artificial biofilm formation by *S. mutans* in culture system

S. mutans MT8148 was cultured in HI broth containing 1% sucrose with 0.5% XF in a glass test tube. The amounts of cells in the total adherent (loose plus firm) fraction were significantly inhibited by XF in a dose-dependent manner. The inhibitory rates for 1%, 2.5%, and 5% XF were 68%, 83%, and 86%, respectively (Fig. 2). Figure 3 shows the amounts of WIG and water-soluble glucan (WSG) in the presence of 1% sucrose and each concentration of XF. The amounts of WIG and WSG in each adherent fraction were significantly inhibited by XF in a dose-dependent manner. XF alone contributed minimally to the development of artificial biofilm formation in terms of bacterial cells and WIG.

2. Effect of XF on artificial biofilm formation, biofilm pH environment, and enamel demineralization in AMS

Two identical artificial mouths were operated simultaneously under the same conditions except for sugar supplementation. Changes in pH values beneath the arti-

cial biofilm are shown in Fig. 4. The pH values of the control (2% sucrose) began to decrease after 8 hr of running the AMS, reaching pH 5.5, which is thought to be a critical pH for enamel demineralization after 13 hr and pH 4.6 after 20 hr. Conversely, the pH values in the experiment (2% XF) remained unchanged for 20 hr. Table 1 shows the changes in Vicker's hardness values (ΔH) of the enamel slabs before and after the experi-

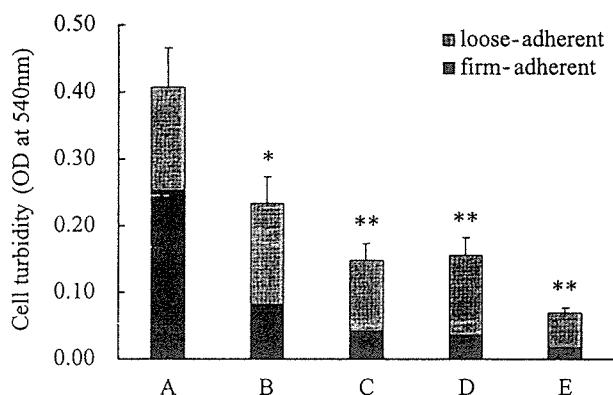


Fig. 2 Amounts of bacterial cells in firm- and loose-adherent fractions in a culture system of *S. mutans* MT8148 in the presence of sugars
 A: 1% sucrose, B: 1% sucrose + 1% XF, C: 1% sucrose + 2.5% XF, D: 1% sucrose + 5% XF, E: 1% XF.
 *: $p < 0.05$, **: $p < 0.01$ (Compared with A)

ments with the amount of artificial biofilm indicated by the turbidity of the bacterial cell suspension and the amount of WIG produced. The amount of artificial biofilm and change of microhardness were considerably lower in the case of XF alone.

Figure 5 shows the change in pH values of AMS in the presence of 1% sucrose (control) and 1% sucrose plus 1% XF (experiment). The decrease in the pH of the experiment was slightly delayed compared to that observed in the control. The pH values beneath the biofilm on the electrode decreased to 5.5 after 14 hr and 17 hr of AMS operation in the control and experiment, respectively. As shown in Table 2, no significant differences were observed in the amount of biofilm and ΔH from the enamel slabs in the control and experiment.

Figure 6 shows the changes in the pH values underneath the artificial biofilm in the AMS in the presence of 1% sucrose (control) and 1% sucrose plus 2.5% XF (experiment). In the control, pH values began to decrease after 8 hr, reaching 5.5 after 16 hr and 4.7 after 22 hr of operating the AMS. However, the pH values in the experiment did not exhibit any apparent decreases, even after 22 hr. The amount of biofilm produced in terms of bacterial cell turbidity in the experiment was significantly less than that of the control (Table 3). Similarly, no WIG could be detected on the enamel slabs in the ex-

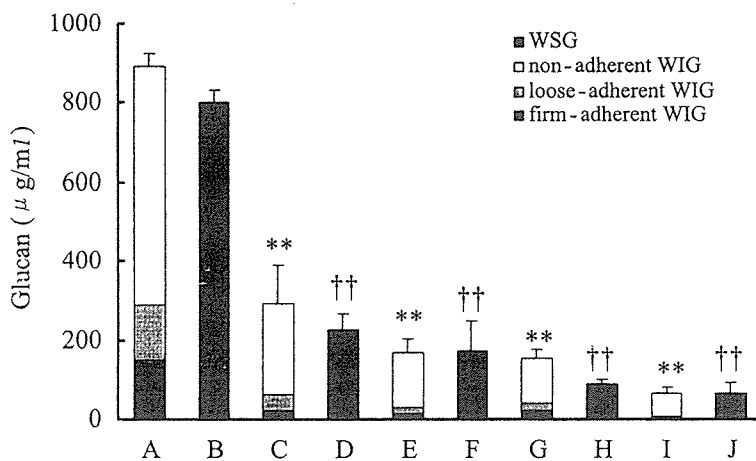


Fig. 3 Amounts of glucans in each adherent fraction in a culture system of *S. mutans* MT8148 in the presence of sugars
 A and B: 1% sucrose, C and D: 1% sucrose + 1% XF, E and F: 1% sucrose + 2.5% XF, G and H: 1% sucrose + 5% XF, I and J: 1% XF
 **: $p < 0.01$ (Compared with A), ††: $p < 0.01$ (Compared with B)

Table 1 Effect of XF on enamel demineralization and the amount of artificial biofilm formed on bovine enamel slabs by *S. mutans* MT8148 in the artificial mouth system

	ΔH ^{a)}	OD ₅₀₀ ^{b)}	WIG ($\mu\text{g}/\text{mm}^2$) ^{c)}
Control (2% sucrose)	252.7 \pm 5.1	0.009 \pm 0.002	2.503 \pm 0.360
Experiment (2% XF)	8.2 \pm 4.2 ^{d)}	ND	0.239 \pm 0.183 ^{d)}

a) : Differences in microhardness of 4 enamel slabs before and after experiments (Mean \pm SD)

b) : Bacterial cell turbidity at 500 nm per square mm of 4 enamel slabs (Mean \pm SD)

c) : Amount of total WIG per square mm of 4 enamel slabs (Mean \pm SD)

d) : p < 0.01 (Compared with the control group)

ND : Not detected

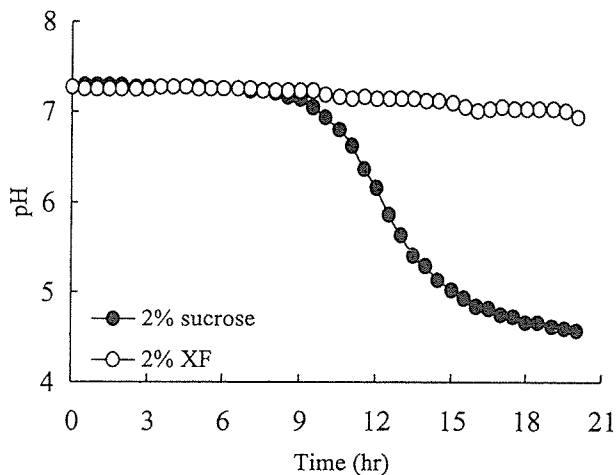


Fig. 4 Changes in pH underneath the artificial biofilm formed by 2% sucrose or 2% XF

periment, and the value for ΔH in the experiment was significantly less than that of the control.

Discussion

Adherence of *S. mutans* MT8148 cells and WIG to a glass surface were inhibited by XF in a dose-dependent manner in this study. WSG production was inhibited by XF in a similar manner. Imai et al.¹¹⁾ reported that 83% of WIG adherence by *Streptococcus sobrinus* 6715 in the presence of 5 mM sucrose was inhibited by 10 mM XF in a culture system. Takeuchi¹⁵⁾ reported that 10 mM XF inhibited both the production and adherence of WIG by crude glucosyltransferase from *S. sobrinus* 6715 in the presence of 5 mM sucrose by 86% and 94%, respectively. Kishi¹⁶⁾ reported that XF effectively inhibited purified GTF from *S. sobrinus* MT3791 and that XF com-

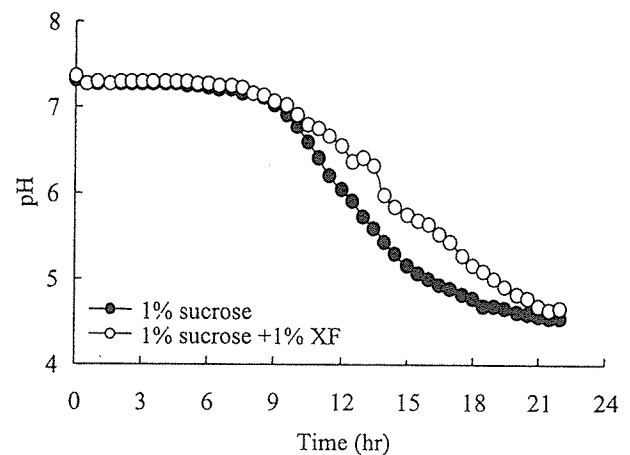


Fig. 5 Changes in pH underneath the artificial biofilm formed by 1% sucrose or 1% sucrose + 1% XF

petitively inhibited the sucrose degrading activity of GTF, inhibiting the activity of glucan production. Togashi¹⁷⁾ reported that purified GTF from *S. mutans* MT8148 was competitively inhibited by XF.

Under the culture conditions used in this study, 43% of *S. mutans* MT8148 cells and 33% of WIG were recovered in the total adherent (loose and firm adherent) fraction. Imai¹⁸⁾ reported that the adherence ability of mutans streptococci to a smooth surface was dependent on the ability to produce WIG from sucrose, and that a positive correlation existed between adherent WIG and adherent bacterial cells. Consequently, bacterial cells and WIG were both suitable for use as adherence parameters of *S. mutans*.

In vivo test systems such as animal experiments¹⁹⁾, human plaque pH tests²⁰⁾, and human intraoral cariogenicity tests (ICT)²¹⁾ have been used to assess both the cari-

Table 2 Effect of XF on enamel demineralization and the amount of artificial biofilm formed on bovine enamel slabs by *S. mutans* MT8148 in the artificial mouth system

	$\Delta H^a)$	OD ₅₀₀ ^{b)}	WIG ($\mu\text{g}/\text{mm}^2$) ^{c)}
Control (1% sucrose)	245.8 ± 8.4	0.014 ± 0.005	4.527 ± 0.991
Experiment (1% sucrose + 1% XF)	244.2 ± 11.3	0.014 ± 0.002	4.183 ± 0.627

a) : Differences in microhardness of 4 enamel slabs before and after experiments (Mean ± SD)

b) : Bacterial cell turbidity at 500 nm per square mm of 4 enamel slabs (Mean ± SD)

c) : Amount of total WIG per square mm of 4 enamel slabs (Mean ± SD)

Table 3 Effect of XF on enamel demineralization and the amount of artificial biofilm formed on bovine enamel slabs by *S. mutans* MT8148 in the artificial mouth system

	$\Delta H^a)$	OD ₅₀₀ ^{b)}	WIG ($\mu\text{g}/\text{mm}^2$) ^{c)}
Control (1% sucrose)	242.7 ± 5.4	0.009 ± 0.002	1.850 ± 0.692
Experiment (1% sucrose + 2.5% XF)	11.1 ± 3.0 ^{d)}	0.0005 ± 0.0001 ^{d)}	ND

a) : Differences in microhardness of 4 enamel slabs before and after experiments (Mean ± SD)

b) : Bacterial cell turbidity at 500 nm per square mm of 4 enamel slabs (Mean ± SD)

c) : Amount of total WIG per square mm of 4 enamel slabs (Mean ± SD)

d) : p < 0.01 (Compared with the control group)

ND : Not detected

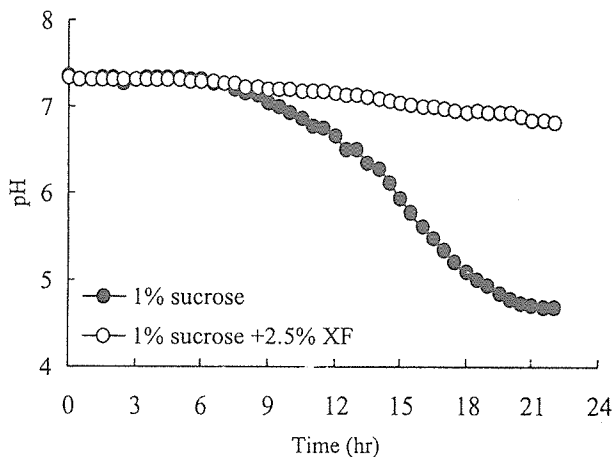


Fig. 6 Changes in pH underneath the artificial biofilm formed by 1% sucrose or 1% sucrose + 2.5% XF

ogenic potential and inhibition of sugar substitutes or enzyme inhibitors. However, *in vivo* test systems have had several associated intrinsic time and economic problems, so that *in vitro* test systems, especially artificial mouth systems, have been developed²²⁻³²⁾. We improved an AMS originally developed by Hinoide et al.³¹⁾. The characteristics of this system made it possible to assess

the important parameters responsible for causing dental caries such as biofilm formation, the pH underneath biofilm, and enamel demineralization.

As mentioned previously, XF was not used as a substrate for GTF and it was found to inhibit GTF activity and the production of WIG. In ICT in human subjects, 2% XF did not inhibit enamel demineralization in the presence of 2% sucrose³³⁾. However, enamel demineralization by 2% XF alone was significantly lower than that observed when exposed to 2% sucrose, indicating that XF appears to be a low-cariogenic sugar. It is not known whether or not XF produces the same results in the AMS as those in ICT. As shown in Fig. 4 and Table 1, 2% sucrose caused marked decreases in pH, the production of large amounts of biofilm, and considerable enamel demineralization. These results indicate that the use of the AMS facilitated the assessment of the cariogenicity of sucrose. Conversely, 2% XF alone did not decrease the pH in the AMS, and the amount of biofilm and enamel demineralization were considerably low, suggesting that XF might be a low- or non-cariogenic sugar. These results corroborated previous findings obtained by ICT. Using *S. sobrinus* 6715, Hinoide et al.³¹⁾ re-

ported that xylitol, a non-cariogenic sugar alcohol, did not decrease the pH of their AMS, the prototype of the system used in our study, over a period of 24 hr.

The capacity of 1% and 2.5% XF to differentially inhibit the cariogenicity of 1% sucrose was interesting. While pH values under conditions of the simultaneous addition of 1% XF and 1% sucrose were slightly delayed compared with 1% sucrose alone in AMS, 1% XF did not suppress the formation of biofilm and enamel demineralization (Fig. 5 and Table 2). There was also a discrepancy between the results obtained in the culture system and those from the AMS. In the culture system, 1% XF significantly inhibited the adherence of *S. mutans* cells and WIG to glass surfaces in the presence of 1% sucrose (Figs. 2 and 3). This discrepancy might be ascribed to the differences in experimental conditions between the static conditions of the culture system and the flowing system of the AMS. However, 2.5% XF significantly inhibited the formation of biofilm on enamel slabs, pH decrease underneath the biofilm, and enamel demineralization (Fig. 5 and Table 3). These results suggested that an appropriate concentration of XF could inhibit, in part, the cariogenicity of sucrose.

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人工口腔装置におけるスクロースのう蝕原性に及ぼす 二糖類キシロシルフルクトシドの影響について

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概要 : 甘味二糖類キシロシルフルクトシド (XF) が歯面へのミュータンスレンサ球菌の付着や、非水溶性グルカンの合成を効果的に阻害することが報告されている。本研究は *in vitro* の実験系と、う蝕誘発過程に重要なバイオフィーム形成、バイオフィーム直下の pH, エナメル質脱灰度を同時に検討できる人工口腔装置による評価系を用いて、XF のう蝕誘発性およびスクロースのう蝕誘発性に及ぼす XF の抑制効果を検討した。ガラス試験管に 1% スクロースと 1~5% XF をそれぞれ含む培地に、*S. mutans* MT8148 を植菌し、培養を行った。その結果、XF はガラス管壁附着性の非水溶性グルカン量と菌体量を濃度依存的に抑制した。また人工口腔装置において、1% XF は 1% スクロース存在下における人工バイオフィーム形成やエナメル質脱灰度は抑制しなかったが、2.5% XF は人工バイオフィーム直下の pH をほとんど低下させず、人工バイオフィーム形成量、エナメル質脱灰度を有意に低下させた。以上のことから、XF は濃度によってスクロースのう蝕誘発性を部分的に抑制することが示唆された。

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索引用語 : キシロシルフルクトシド, 人工口腔装置, う蝕誘発性, バイオフィーム形成, ミュータンスレンサ球菌

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原 著

口腔ケアの評価指標と real-time PCR による舌苔中細菌数との関連

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杉浦 剛 相澤 文恵 米満 正美

概要：口腔ケアの評価に用いられることの多い臨床的指標と、舌苔湿重量ならびに舌苔中細菌数との関連を検討し、比較的簡便に得られる臨床的指標が口腔内の細菌量を反映しているかを検討した。調査対象は体系的な口腔ケアが行われているリハビリテーション病院に入院した者 27 名とし、入院時と入院 2 か月後の両時点で同一の口腔内指標を採得した。臨床的指標には口中 VSC 測定値、舌苔スコア、CPI を用い、舌苔中細菌数の測定には real-time PCR を用いた。その結果、入院時と入院 2 か月後の比較において、舌苔スコア、CPI、 CH_3SH 濃度および舌苔湿重量に有意な減少が認められ、 H_2S 濃度と舌苔中総細菌数には減少傾向が認められた。一方、舌苔 1 mg 当たりの細菌数はほぼ同等の値となった。臨床的指標と舌苔中細菌数の関連において、入院時と入院 2 か月後のいずれにおいても、口中 H_2S 濃度と舌苔 1 mg 中細菌数および舌苔中総細菌数の間に有意な関連を認めた。舌苔スコアと VSC 測定値を独立変数、舌苔中総細菌数を目的変数とした重回帰分析では舌苔スコアと H_2S 濃度が有意な変数として選択された。これらのことから、口腔ケアの臨床的指標として舌苔量を評価することに加えて口中気体の H_2S 濃度を測定することで、舌苔中細菌数の変動をより詳細にとらえていると考えられ、それらを口腔ケアの指標とすることの妥当性が確認された。

索引用語：口腔ケア、舌苔、VSC、Real-time PCR

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緒 言

専門的な口腔ケアが要介護高齢者の呼吸器疾患に対する予防効果があることが報告されて以来^{1,5)}、入所者に対する口腔ケアに取り組む医療施設や養護施設が増加し、その効果について数多く報告されている⁶⁻¹²⁾。それら報告において、口腔ケアの効果を評価するアウトカム指標として、舌苔や口臭を用いる例が多くみられる。高齢者や病弱者の誤嚥性肺炎予防のためには口腔内の肺炎起炎菌を減少させることが重要であるが、口腔ケアは肺炎起炎菌を特異的に減少させるのではなく、口腔内の総細菌数を減少させる方法である^{4,13)}。舌苔付着量の変化は口腔内の総細菌数の変動を反映しているものと考えられる。なぜなら舌苔は舌背表面に堆積する細菌性バイオフィルムの一つであり、唾液や歯垢への細菌の受容・供

給相互関係にあると考えられている¹⁴⁻¹⁶⁾こと、さらに付着面積が大きいために舌苔の口腔内総細菌量への影響が少なくないものと考えられるからである。一方、口臭の揮発性硫黄化合物 (VSC) 濃度は舌苔付着量との関連が強いことから¹⁷⁻²²⁾、口臭強度が間接的に口腔内細菌量を反映する可能性がある。舌苔や口臭の評価は非侵襲的であり、無菌顎者や唾液分泌が減少した高齢者に対しても適用が可能である。このような理由から、舌苔や口臭の評価が口腔ケアのアウトカム指標としてこれまで多く用いられてきたものと考えられる。しかし、これまでの報告における評価法は簡便な視診や官能試験によるものがほとんどであり、舌苔や口臭と口腔内細菌量との関連は明瞭とはいえない。

そこで本研究では、入院中に口腔ケアによる口腔内状態の改善が期待できるリハビリテーション病院におい

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表1 対象者の入院原因となった傷病と入院時食事状況

傷病名	食事形態			計
	常食	刻み食*	経管**	
脳血管疾患	11	7	2	20
脊髄損傷	3	1	0	4
脳炎	0	1	0	1
脳挫傷	1	0	0	1
神経疾患	0	1	0	1
計	15	10	2	27

* 全粥, とろみ食を含む
 ** 経鼻管栄養摂取

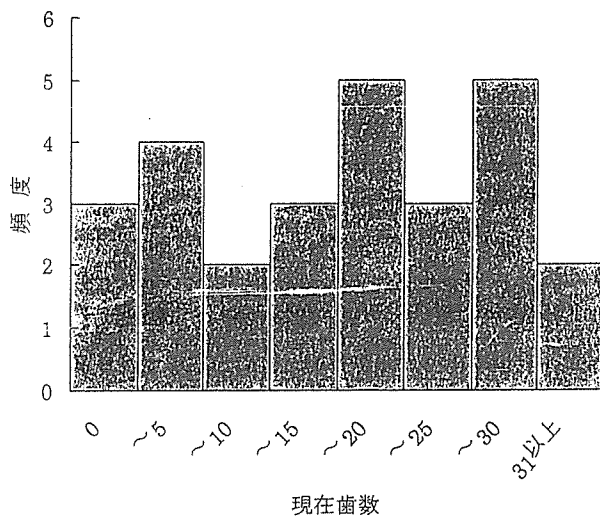


図1 対象者の現在歯数の分布

て、入院時と入院2か月後に、口腔ケアの評価に用いられることの多い臨床的指標と、細菌学的指標を採得した。臨床的指標にはポータブルガスクロマトグラフィによる口臭測定、視診による舌苔スコア、CPIを用い、細菌学的指標として舌苔湿重量の測定ならびに real-time PCR を用いた舌苔中細菌量の定量を行った。それらの関連を分析することにより、比較的簡便に得られる臨床的指標が口腔内の細菌量を反映するかを検討した。

対象および方法

1. 対象

岩手県内某リハビリテーション病院において、2004年8月の1か月間に入院した者27名(男性21名, 女性6名, 平均年齢±標準偏差: 58.7±16.5歳)を対象とした。当該リハビリテーション病院は、傷病のための急性期医

療をほかの病院で行った後の回復期医療を担当する医療機関であり、口腔ケアについても歯科衛生士を中心とした体系的な口腔ケアを行っている。入院時と入院2か月後の同時刻に、口臭測定、口腔内検査(硬組織検査、舌苔スコア評価、歯周組織検査)および舌苔採取を行った。口腔内検査は、十分にキャリブレーションを行った2名の検査者が両検査時点で異なる対象者を調査した。

対象者の入院原因となった傷病と入院時食事形態の分布を表1に示す。両調査時点での食事形態に関する差違は、経管栄養からとろみ食に移行した者が1名、その他刻み食の刻み程度が大きくなった者が2名であった。対象集団の1人平均現在歯数は16.0±10.8であり、無歯顎者は3名であった。対象集団の現在歯数の分布を図1に示す。

2. 口臭測定

ポータブルガスクロマトグラフィ(Oral Chroma[®], ABILIT, 大阪)を用いて口中気体の硫化水素(H₂S)とメチルメルカプタン(CH₃SH)の測定を通法²³⁾で行った。すなわちデイスポーザブルシリンジを被験者の口腔内に挿入し、30秒間鼻呼吸のもと口唇閉鎖させ、口中気体1.0mlを採取し、0.5mlを廃棄、残りの0.5mlをガスクロマトグラフィに注入し測定した。

3. 舌苔スコア

舌苔付着状況を Miyazakiら²¹⁾の視診による基準に従い、4段階で評価した。すなわち、0:舌苔がほとんど認められない、1:舌苔が舌背の1/3未満を覆っている、2:舌背の1/3以上2/3未満を覆っている、3:舌背の2/3以上を覆っている。

4. 歯周組織検査

有歯顎者に対しては歯周組織の状態をすべての残存歯についてCPIの基準²⁴⁾で評価し、記録された最も重度なコードを個人のCPIコードとした。

表2 入院時と入院2か月後における評価指標の比較

アウトカム指標	入院時		入院2か月後
H ₂ S (ppb)	231 ± 76.5		88.5 ± 24.7
CH ₃ SH (ppb)	77.1 ± 30.0	— * —	27.7 ± 10.4
CPI	2.57 ± 0.24	— ** —	1.48 ± 0.31
舌苔スコア	1.96 ± 0.21	— ** —	1.04 ± 0.17
舌苔付着量 (mg)	24.4 ± 5.60	— * —	12.8 ± 1.92
舌苔 1mg 中細菌数 (対数平均値)	1.55 × 10 ¹⁰ (9.71 ± 0.21)		1.77 × 10 ¹⁰ (9.71 ± 0.17)
舌苔中総細菌数 (対数平均値)	5.48 × 10 ¹¹ (10.9 ± 0.29)		3.21 × 10 ¹¹ (10.7 ± 0.21)

数値は平均値 ± 標準誤差

* p < 0.05, ** p < 0.01 : Wilcoxon 順位検定

5. 舌苔湿重量

前報^{16,23)}同様に舌苔を採取・定量した。すなわち舌背の有郭乳頭から舌尖部の付着物を滅菌歯ブラシにて可及的に採取し、5 ml PBS に懸濁した。懸濁液を超音波処理 (20 W, 5 秒, 5 回) により均一化した後、懸濁液中の舌苔湿重量を測定し、舌苔付着量とした。

6. 舌苔中細菌数の定量

一定量 (4 mg) に調整した舌苔試料から Wizard Genomic DNA Purification Kit (Promega, MI, USA) を用いて、ゲノム DNA を精製後、20 μl の Tris-EDTA 緩衝液に溶解し、TaqMan プローブを用いた real-time PCR (ABI PRISM 7700 system, Applied Biosystems, USA) に供した。PCR には、精製したゲノム溶液 0.1 μl, 1 × TaqMan Universal PCR Master Mix (Applied Biosystems), 200 nM の 16 S rRNA ユニバーサルプライマー、250 nM TaqMan プローブからなる 20 μl 溶液を用いた。プライマーとプローブの設計は既報²³⁾に準じた。スタンダードには 16 S rRNA 領域を挿入したプラスミドを使用した。その総塩基長に平均分子量を乗じて重量を求め、重量当たりのゲノムコピー数を算出し、これを細菌数とした。測定は2回行い、平均 Ct 値をスタンダード直線に当てはめて舌苔 1 mg 当たりの細菌数を求めた。また、舌苔 1 mg 中の細菌数に採取した舌苔付着量を乗じ、舌苔中総細菌数とした。

7. 口腔ケア

看護職員による口腔ケア介入は調査期間中、対象者の自立度に応じて通常と同様に以下に行われた。1. 声かけ・実地指導：処置を要する歯科疾患がなく、適切なオーラルセルフケアが行える者に対して実施。2. 看護職員による口腔清掃：適切なセルフケアが不可能な者に対して歯科衛生士、看護師による定期的口腔清掃を実施。

3. 歯科治療：明らかな菌疾、義歯不調がある場合に実施。

8. 統計解析

入院時と入院2か月後における評価指標の比較には、Wilcoxon 順位検定を用いた。両時点での指標相互の関連分析には Spearman の順位相関分析を行った。臨床的指標と舌苔細菌数の関連の検討には重回帰分析を行った。対象者の入院原因となった傷病については、そのほとんどが脳血管疾患であり、その他も中枢神経を傷害して機能障害をきたす傷病であることから、傷病別の分析は行わなかった。すべての統計解析には SPSS 11.5 J (SPSS Japan, 東京) を使用した。

9. 研究倫理

研究に先立ち、岩手医科大学歯学部倫理委員会 (受付番号 01025) および調査対象施設研究倫理委員会の承諾を得た。調査現場においては、対象者とその主たる近親者から調査に関する説明を行った後、文書による同意を得て行った。

結 果

1. 口腔ケア前後における口腔内状況の比較

入院時と入院2か月後の口腔内状況の比較を、表2に示す。臨床的指標では舌苔スコア、CPI、CH₃SH 濃度に有意な減少が認められ、H₂S 濃度は統計学的に有意な差ではなかった (p = 0.156 : Wilcoxon 順位検定) が減少傾向にあった。また、採取した舌苔の分析で得られた細菌学的指標では、舌苔付着量の平均値が有意に減少していた。舌苔中総細菌数は統計学的に有意な差ではなかった (p = 0.144 : Wilcoxon 順位検定) が減少傾向にあった。また、舌苔 1 mg 当たりの細菌数の対数平均値は口腔ケア前後でほぼ同等の値であった (p = 0.990 : 対応のある t 検定)。また、入院時の食事形態別を、常食とそれ以外

表 3 口腔ケアの指標および現在歯数相互の関連

	H ₂ S	CH ₃ SH	CPI	現在歯数	舌苔スコア	舌苔湿重量 (mg)	舌苔 1mg 中細菌数	舌苔中総細菌数
H ₂ S	1							
	1							
CH ₃ SH	0.725 **	1						
	0.756 **	1						
CPI	- 0.307	- 0.118	1					
	- 0.138	- 0.134	1					
現在歯数	0.101	- 0.148	- 0.541 **	1				
	- 0.115	- 0.091	0.179	1				
舌苔スコア	0.365	0.225	0.080	- 0.128	1			
	0.297	0.046	0.310	- 0.357	1			
舌苔湿重量 (mg)	0.133	0.270	- 0.079	0.029	0.671 **	1		
	0.400	0.306	0.080	- 0.120	0.703 **	1		
舌苔 1mg 中細菌数	0.444 *	0.278	- 0.265	0.112	0.416 *	0.186	1	
	0.456 *	0.110	0.000	0.226	0.228	0.308	1	
舌苔中総細菌数	0.458 *	0.290	- 0.190	0.151	0.684 **	0.620 **	0.783 **	1
	0.557 **	0.212	- 0.070	0.131	0.403 *	0.619 **	0.892 **	1

表中の数値は Spearman の順位相関係数、セル上段に入院時、下段に入院 2 か月後を示す (* p < 0.05, ** p < 0.01). CPI については有歯顎者 (24 名) についての分析結果を示す。

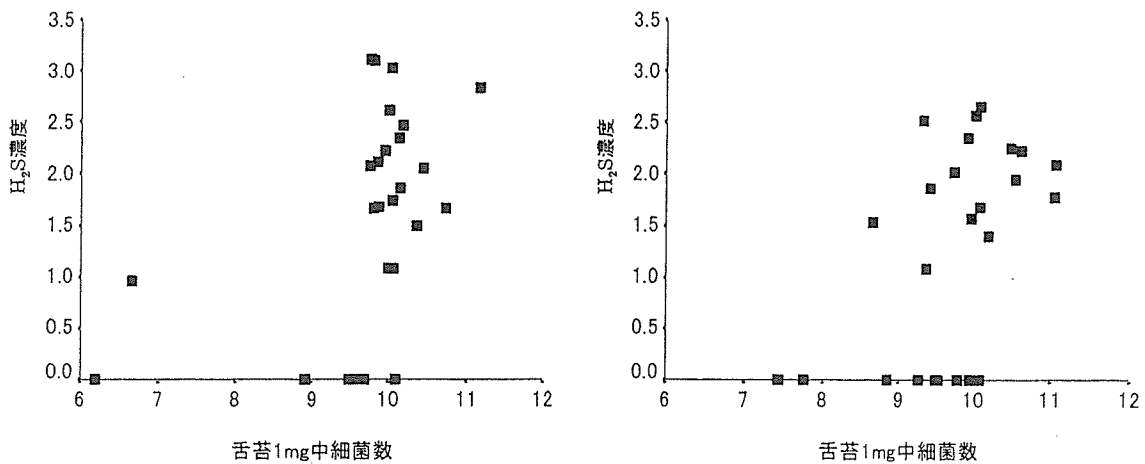


図 2 入院時と入院 2 か月後における口中 H₂S 濃度と舌苔 1 mg 中細菌数の関連*
*口中気体の H₂S 濃度ならびに舌苔 1 mg 中細菌数はいずれも対数変換値 = log₁₀ (実測値 + 1) で表した。

の摂取形態に分類して比較したところ、2 調査時点のいずれの評価項目についても差は認められなかった。

2. 臨床的指標と細菌学的指標の関連

入院時と入院 2 か月後の両時点における臨床的指標と細菌学的指標の関連を、表 3 に示す。口中気体の H₂S 濃度と舌苔 1 mg 中細菌数および舌苔中総細菌数の間に有意な相関を認めた。両時点におけるそれらの関連を図

2, 3 に示す。CH₃SH 濃度は H₂S 濃度と高い相関を呈したが、ほかの評価項目との関連は有意ではなかった (表 3)。さらに、口中気体の VSC 濃度と舌苔スコアを説明変数とし、舌苔中総細菌数を目的変数とした重回帰分析を行ったところ、入院時点では舌苔スコアのみが有意な説明変数であったが、入院 2 か月後の時点では舌苔スコアと H₂S が有意となり、偏回帰係数は H₂S のほうが大

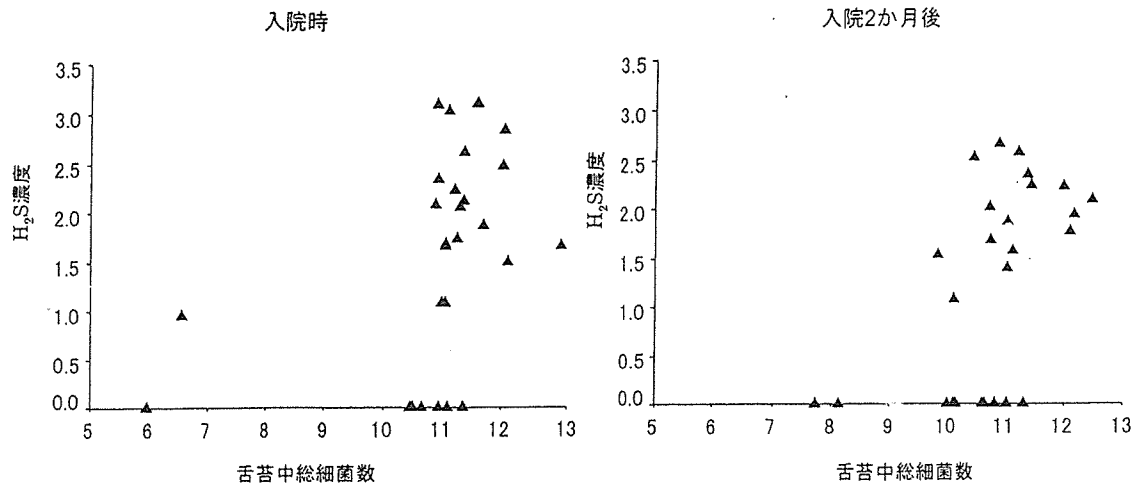


図3 入院時と入院2か月後における口中H₂S濃度と舌苔中総細菌数の関連*

* 口中気体のH₂S濃度ならびに舌苔総細菌数はいずれも対数変換値 = log₁₀(実測値 + 1) で表した。

表4 口中VSC濃度および舌苔スコアと舌苔総細菌数の関連*

入院時			
選択された変数**	非標準化係数(標準誤差)	標準化係数	有意確率
舌苔スコア	0.884 (0.204)	0.638	0.0004
R ² =0.407			
入院2か月後			
選択された変数**	非標準化係数(標準誤差)	標準化係数	有意確率
H ₂ S濃度	0.493 (0.178)	0.458	0.011
舌苔スコア	0.487 (0.215)	0.374	0.034
R ² =0.434			

* 分析にはH₂S濃度, CH₃SH濃度の対数変換値(log₁₀(実測値+1))を用いた。

** H₂S濃度, CH₃SH濃度, 舌苔スコアを初期の投入変数としたステップワイズ重回帰分析(変数減少法)で, 有効な説明変数として選択された変数。

きかった(表4)。

考 察

本研究で対象としたリハビリテーション病院では, 全身だけでなく口腔を対象としたリハビリテーションが行われており, 口腔ケアについても一般の急性期医療施設に比べて高い水準のケアがなされているものと考えられる。しかし高水準の体系的な口腔ケアが供給されている

医療施設であっても, 原疾患の重症度や生活自立度, 口腔内状態などによってケアの内容は異なる。また全身のリハビリテーションによって, オーラルセルフケアのレベル, 食事形態, 口腔機能などが変化することから, 純粹に口腔ケアのみのアウトカム評価は困難である。それゆえ, 本研究では, 同一集団の, 異なる口腔内状態にある2時点(表2)での舌苔中細菌数と臨床的指標の関連を分析することを目的とした。

舌苔と口臭強度の関連については、これまでに多くの報告がある¹⁷⁻²³⁾。われわれも、歯周病健全者の舌苔付着量と口中総 VSC 濃度²⁴⁾や、無歯顎高齢者の舌苔スコアと口中 H₂S 濃度に関連のあること²⁵⁾を報告してきた。本研究結果でも統計学的に有意ではなかったが、舌苔スコア、舌苔付着量という舌苔の量的評価項目と口中気体の H₂S 濃度、CH₃SH 濃度にある程度の関連が認められた。この結果に加え、本研究では口腔ケア前後の両時点で、口中気体の H₂S 濃度が舌苔 1 mg 当たりの細菌数および舌苔中総細菌数と強い関連を呈した(表 3, 図 2, 3)。本研究で得られた舌苔中細菌数は 1 mg 当たりのゲノムコピー数として 1.59×10⁹~1.43×10¹¹ に分布しており(平均値=1.55×10¹⁰)、これは舌苔細菌の凝集程度が一様ではなく、ばらつきがあることを示している。すなわち、舌苔を視診や重量で量的に把握するだけでは舌苔の蓄える細菌量を把握するのに十分ではないと考えられる。一方、口中 H₂S 濃度と舌苔中細菌数が正の相関を呈したことは、口腔内の細菌量を評価するうえで、VSC 測定が舌苔の量的評価を補う指標になりうることを示唆するものである。重回帰モデルにおいて、入院時点の舌苔中総細菌数の有意な説明変数は舌苔スコアだけであったが、入院 2 か月後では H₂S が細菌数を推定するうえで舌苔スコアよりも有効な変数となった。両時点での重回帰モデルにおける有効な説明変数の差違は、入院時点では舌苔が多量に付着している者が多く、舌苔付着量が総細菌数に大きく反映されるが、入院 2 か月後には口腔ケアにより舌苔付着量が低下し、舌苔の総量よりも舌苔の単位重量当たりの細菌数が総細菌数に反映されるためと考えられる。それゆえ、ある程度口腔清掃状態が良好な場合には、舌苔スコアだけでなく、H₂S 濃度を測定することによって、舌苔中細菌量をよりよく反映することが示された。

本研究では、舌苔中細菌数を 16 S rRNA を標的とした real-time PCR によって定量した。これまでに舌苔を検体として real-time PCR を行った研究がいくつかなされている²⁶⁻²⁸⁾が、湿重量当たりの細菌数は示されておらず、本研究と細菌数の定量結果を比較することはできない。Lyons ら²⁹⁾は歯垢を試料とした real-time PCR で、歯肉溝内にペーパーポイントを挿入して得た細菌数を 2.10×10⁷~2.15×10¹⁵ と報告している。これも歯垢重量当たりの細菌数の結果ではないが、培養可能総菌数についての従来の知見である 1 g 当たり 10¹¹ 個³⁰⁾に比して多く評価されているものと考えられる。Suzuki ら³¹⁾は 16 S rRNA を標的とした real-time PCR による総菌数の定量では実際の細菌数よりも多く評価される可能性を指摘している。その理由として 16 S rRNA の領域を複数もつ細菌が

多く存在すること、培養法では全細菌に対して同時に至適条件で培養することが不可能であるのに対して PCR では細菌の培養条件にかかわらず検出可能であることなどが挙げられている。本研究で得られた舌苔中細菌数の絶対値としての精度については、培養法との比較検討など、今後さらに研究を進めなければならない。しかし舌苔スコアなどの舌苔の量的評価に加えて口中 H₂S 濃度を用いることにより、少なくとも舌苔中の細菌数の相対的变化を推定できるものと考えられた。以上のことから、舌苔の量的評価と口臭測定を臨床的な評価項目に用いることは、口腔ケアの細菌学的効果の判定にも有効であると考えられた。

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