Caries Research

Original Paper

Synergistic Inhibition by Combination of Fluoride and Xylitol on Glycolysis by Mutans Streptococci and Its Biochemical Mechanism

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Abstract
The purpose of this study was to evaluate the combined inhibitory effect of fluoride and xylitol on acid production by mutans streptococci, *Streptococcus mutans* NCTC10449 and *Streptococcus sobrinus* 6715, from glucose under strictly anaerobic conditions at fixed pH 5.5 and 7.0. The bacteria were grown in a tryptone-yeast extract broth under strictly anaerobic conditions (N_2: 80%; H_2: 10%; CO_2: 10%). Reaction mixtures for acid production from glucose contained bacterial cells with fluoride (0–6.4 mM) and/or xylitol (60 mM). Acidic end products of glucose fermentation and intracellular glycolytic intermediates were assayed. The combination of fluoride and xylitol inhibited acid production more effectively than fluoride or xylitol alone. In the presence of fluoride and xylitol, the proportion of lactic acid in the total amount of acidic end products decreased, while the proportion of formic and acetic acids increased. Analyses of intracellular glycolytic intermediates revealed that xylitol inhibited the upper part of the glycolytic pathway, while fluoride inhibited the lower part. This study indicates that fluoride and xylitol together have synergistic inhibitory effects on the acid production of mutans streptococci and suggests that xylitol has the potential to enhance inhibitory effects of low concentrations of fluoride.

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Both fluoride and xylitol have been widely used as cariostatic agents. Fluoride inhibits the acid production and growth of oral bacteria such as the cariogenic bacteria mutans streptococci [Kashket et al., 1977; Hamilton and Ellwood, 1978; van Houte, 1980; Okuda and Frostell, 1982; Marsh et al., 1985; Zameck and Tinanoff, 1987], particularly under anaerobic conditions [Hata et al., 1990]. The effects on streptococcal cells of fluoride are partly ascribed to the inhibition of enolase, one of the series of glycolytic enzymes [Jenkins, 1999]. This inhibition decreases the intracellular level of phosphoenolpyruvate (PEP), and thus decreases bacterial sugar uptake via PEP-dependent phosphotransferase system (PEP-PTS) [Jenkins, 1999]. In addition, fluoride can directly inhibit bacterial proton-translocating ATPase (H^+-ATPase) that is considered to partly contribute to the excretion of proton out of the cells, leading to the acidification of intracellular pH. The dissociation of unionized hydrofluoric acid into H^+ and F^- in the cells also promotes intracellular acidification [Jenkins, 1999]. This intracellular acidification can further reduce bacterial metabolic activity.

Xylitol is a valuable sugar alcohol for caries prevention [Scheinin et al., 1976; Trahan, 1995] because the majority of oral bacteria, including mutans streptococci, do not ferment it [Birkhed et al., 1985]. Xylitol is taken into the cells and phosphorylated to xylitol-5-phosphate (X5P) by PEP-PTS [Assev and Rölla, 1984; Trahan et al., 1985; Trahan, 1995]. Since X5P cannot be metabolized further [Hausman et al., 1984; Assev and Rölla, 1986], the intracellularly accumulated X5P inhibits glycolytic enzymes

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such as phosphoglucose isomerase (PGI) and phosphofructokinase (PFK) and subsequently represses sugar metabolism [Assev et al., 1983; Vadeboncoeur et al., 1983; Assev and Rölla, 1986; Bradshaw and Marsh, 1994]. After that, the X5P can be dephosphorylated into xylitol and excreted out of the cells [Trahan et al., 1991]. In this 'futile cycle' [Assev and Rölla, 1986], the bacterial cells are forced to waste energy and their growth is obstructed as a result [Mäkinen and Scheinin, 1976; Assev et al., 1980, 1983; Vadeboncoeur et al., 1983; Loesche, 1984; Assev and Rölla, 1986; Bradshaw and Marsh, 1994].

Since both fluoride and xylitol have inhibitory effects on sugar metabolism of mutans streptococci as stated above, using them together in clinical situations is expected to inhibit acid production of mutans streptococci effectively. Scheie et al. [1988] demonstrated the combined inhibitory effect of fluoride and xylitol on the growth of mutans streptococci and suggested the inhibitory step of glucose metabolism. Moreover, Rogers and Bert [1992] showed the possibility that xylitol enhances the inhibitory effect of fluoride on mutans streptococci growing in continuous culture. However, the biochemical mechanism of the fluoride and xylitol combination on dental plaque bacteria has not been elucidated yet.

Therefore, this study aimed to evaluate the combined inhibitory effect of fluoride and xylitol on the acid production of mutans streptococci from glucose under strictly anaerobic conditions like those of dental plaque with a pH-stat that can exactly keep the constant pH of reaction mixtures. Furthermore, we attempted to elucidate the biochemical mechanism by analyzing the profiles of intracellular glycolytic intermediates.

Materials and Methods

Bacterial Strain
Streptococcus mutans NCTC10449 and *Streptococcus sobrinus* 6715 were used in this study.

Anaerobic Procedure
All the experiments for bacterial cultivation and metabolism were conducted under strictly anaerobic conditions [Yamada and Carlsson, 1975] like those of the deep layers of mature dental plaque [Ritz, 1967; Kenney and Ash, 1969; Katayama et al., 1975], which are closely associated with causation of dental caries. The storage and cultivation of bacteria were performed in an anaerobic chamber (type NHC, gas phase: N₂, 80%; H₂, 10%; CO₂, 10%, Hirasawa Works, Tokyo, Japan). Preparation of bacterial cell suspensions and the bacterial metabolism experiments were carried out in another anaerobic chamber (type NH, gas phase: N₂, 90%; H₂, 10%, Hirasawa Works). During centrifugation outside the anaerobic chamber for harvesting and washing, the bacterial cells were protected from exposure to air by double sealed centrifuge tubes (Kubota Commercial Affairs, Tokyo, Japan) [Miyasawa et al., 2003]. To exclude oxygen, growth media and solutions were placed in the anaerobic chambers for at least 3 days prior to use, while powdered reagents and experimental instruments were placed in the anaerobic chamber for at least 1 day prior to use.

Bacterial Growth Conditions
The bacterial strains, cultured on blood agar plates and stored at 4°C in the anaerobic chamber, were inoculated into 5 ml of tryptone-yeast extract (TYE; pH 7) broth containing 1.7% tryptone (Difco, Detroit, Mich., USA), 0.3% yeast extract (Difco), 85.5 mM NaCl, 14.4 mM dipotassium phosphate and 11.1 mM glucose, and incubated anaerobically at 37°C overnight. The bacterial suspension was subcultured into another 100 ml of TYE broth (inoculum size 5%) for 12 h, and then further cultured into 800 ml of TYE broth (inoculum size 5%). Bacterial growth was monitored by optical density at 660 nm of aliquots from the culture using a spectrophotometer (model UV-160 Shimazu, Ltd., Kyoto, Japan). The cells were harvested at the early exponential phase of growth by centrifugation (10,000 g for 10 min at 4°C). The cells were washed twice with a solution containing 150 mM KCl and 5 mM MgCl₂ (4°C) by centrifugation (13,000 g for 8 min at 4°C) and the washed cells were finally resuspended in the same solution. The cell suspension was adjusted to 2.7 mg dry weight cells per milliliter and stored at 4°C under anaerobic conditions until the following experiments.

Acid Production from Glucose in the Presence of Fluoride and/or Xylitol
The reaction mixture contained bacterial cells (0.9 mg dry weight cells), 150 mM KCl and 5 mM MgCl in 2 mM potassium phosphate buffer (pH 5.5 or 7.0). Potassium fluoride (0–6.4 mM), xylitol (60 mM) or both were added into the reaction mixtures. The control reaction mixture contained no fluoride or xylitol. The reaction mixture was maintained at pH 5.5 or 7.0 by titration with 60 mM KOH using a pH stat (AUTO pH STAT; model AUT-211S, TOA Electronics, Tokyo, Japan) with agitation by a magnetic stirrer, and then preincubated at 35°C for 4 min. The reaction was started by the addition of 10 mM glucose.

Analysis of Carboxylic Acids from Glucose Fermentation
Lactic, formic and acetic acids were assayed following the method of Takahashi et al. [1987]. At 10 min after the addition of glucose, aliquots of the reaction mixture of *S. mutans* NCTC 10449 were sampled and immediately mixed with 0.6 N perchloric acid. The resultant mixture was brought out from the anaerobic chamber, filtered to remove cell debris through a membrane filter (pore size 0.20 μm; Advantec, polypropylene, Toyo Roshi Ltd., Tokyo, Japan), diluted with 0.2 N HCl and assayed with a carboxylic acid analyzer (model Eyela S-3000, Tokyo Rika Co. Ltd., Tokyo, Japan).

Determination of Glycolytic Intermediates
Glycolytic intermediates were extracted from cells following the method of Iwami et al. [2001]. The cells of *S. mutans* NCTC 10449 were collected by filtering 3.0 ml of reaction mixture through a membrane filter (pore size 0.45 μm; Acrodisc, polyethersulfone, Pall Gelman Laboratory, Ann Arbor, Mich., USA) at 0, 2, 4 and 6 min after the addition of glucose. The glycolytic intermediates
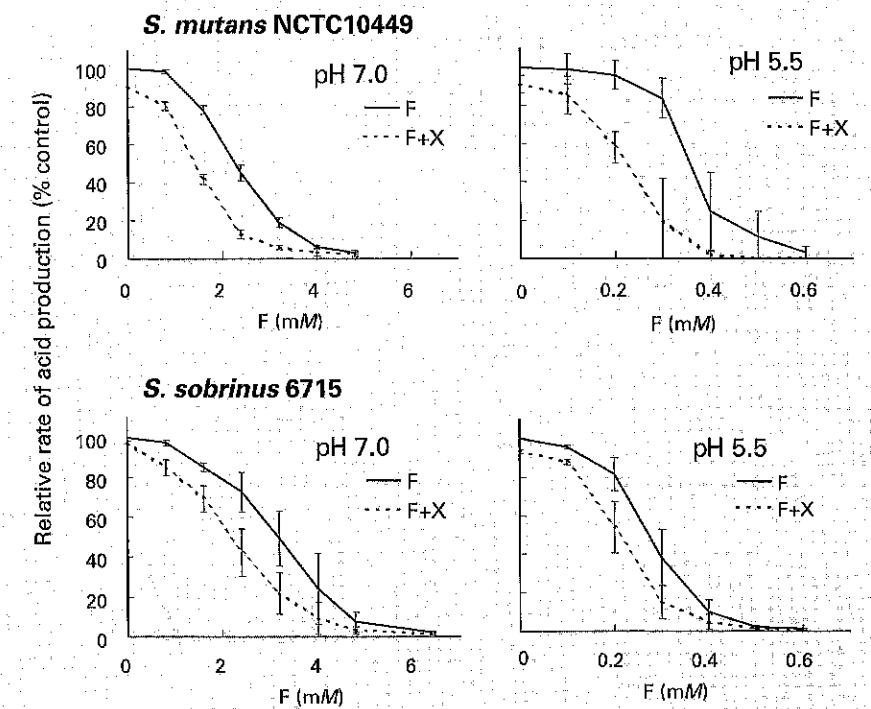
were immediately extracted by passing 2.0 ml of 0.6 N perchloric acid through the filter. The filter was filtered twice besides with the same perchloric acid solution to extract glycolytic intermediates fully. The extracts were neutralized to pH of 6.0 using 1 M triethanolamine-HCl and 5 M K₂CO₃ with chilling at 4°C, and filtered through another membrane filter (pore size 0.20 μm; Advantec, polypropylene, Toyo Roshi Ltd., Tokyo, Japan) to remove sediment. The neutralized extracts were stored at 4°C until assayed.

Glycolytic intermediates in the cell extracts were determined at 35°C by the fluorometric methods of Maitra and Estabrook [1964] and Takahashi et al. [1991] with some modifications. A fluorescence spectrophotometer (model CAF-110, Jasco Corp., Tokyo, Japan) was used with a slit width of 10 nm and excitation/emission wavelengths of 340/470 nm. The assay mixture for glucose 6-phosphate (G6P) and fructose 6-phosphate (F6P) contained 20 mM triethanolamine hydrochloride-NaOH buffer (TA buffer, pH 7.5), 0.06 mM NADP and the cell extract. The amounts of G6P were determined from the increase of NADPH after the addition of 0.6 U/ml glucose 6-phosphate dehydrogenase (EC 1.1.1.49). After NADPH increase reached a plateau, 3.3 U/ml phosphoglucose isomerase (EC 5.3.1.9) was added, and the amount of F6P was then determined from the subsequent increase of NADPH. The assay mixture for dihydroxyacetone phosphate (DHAP), glyceraldehyde 3-phosphate (G3P) and fructose 1,6-bisphosphate (FBP) contained 20 mM TA buffer, 0.5 mM ethylenediamine tetraacetate, 7.5 mM NADH and the cell extract. Glycerol 3-phosphate dehydrogenase (EC 1.1.1.8; 0.85 U/ml), triose phosphate isomerase (EC 5.3.1.1; 25 U/ml) and aldolase (EC 4.1.2.13; 0.23 U/ml) were added sequentially to determine the amounts of DHAP, G3P and FBP from the respective decrease of NADH. The assay mixture for 3-phospho-

glycerate (3PG) and 2-phosphoglycerate (2PG) contained 20 mM TA buffer, 2 mM cysteine, 1.5 mM MgCl₂, 1.5 mM ATP, 0.01 mM NADH and the cell extract. Glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12; 2.5 U/ml), 3-phosphoglycerate kinase (EC 2.7.2.3; 8.4 U/ml) and phosphoglycerate mutase (EC 5.4.2.1; 4.5 U/ml) were added sequentially to determine the amounts of 3PG and 2PG from the respective decrease of NADH. The assay mixture for PEP and pyruvate (PYR) contained 20 mM TA buffer, 10 mM KCl, 5 mM MgCl₂, 0.5 mM ADP, 5 μM NADH and the cell extract. Lactate dehydrogenase (EC 2.7.1.40; 3.5 U/ml) and pyruvate kinase (EC 1.1.1.27; 2.4 U/ml) were added sequentially to determine the amounts of PYR and PEP from the respective decrease of NADH.

Statistical Analysis
All numerical data were given as means with standard deviations. Statistical significance was assessed using ANOVA and Dunnette test. Differences were considered significant at the level p < 0.05.

Results

Acid Production from Glucose in the Presence of Fluoride and/or Xylitol
In the presence of fluoride, the acid production by *S. mutans* NCTC10449 from glucose for 10 min decreased at both pH 7.0 and 5.5. The decrease of acid production
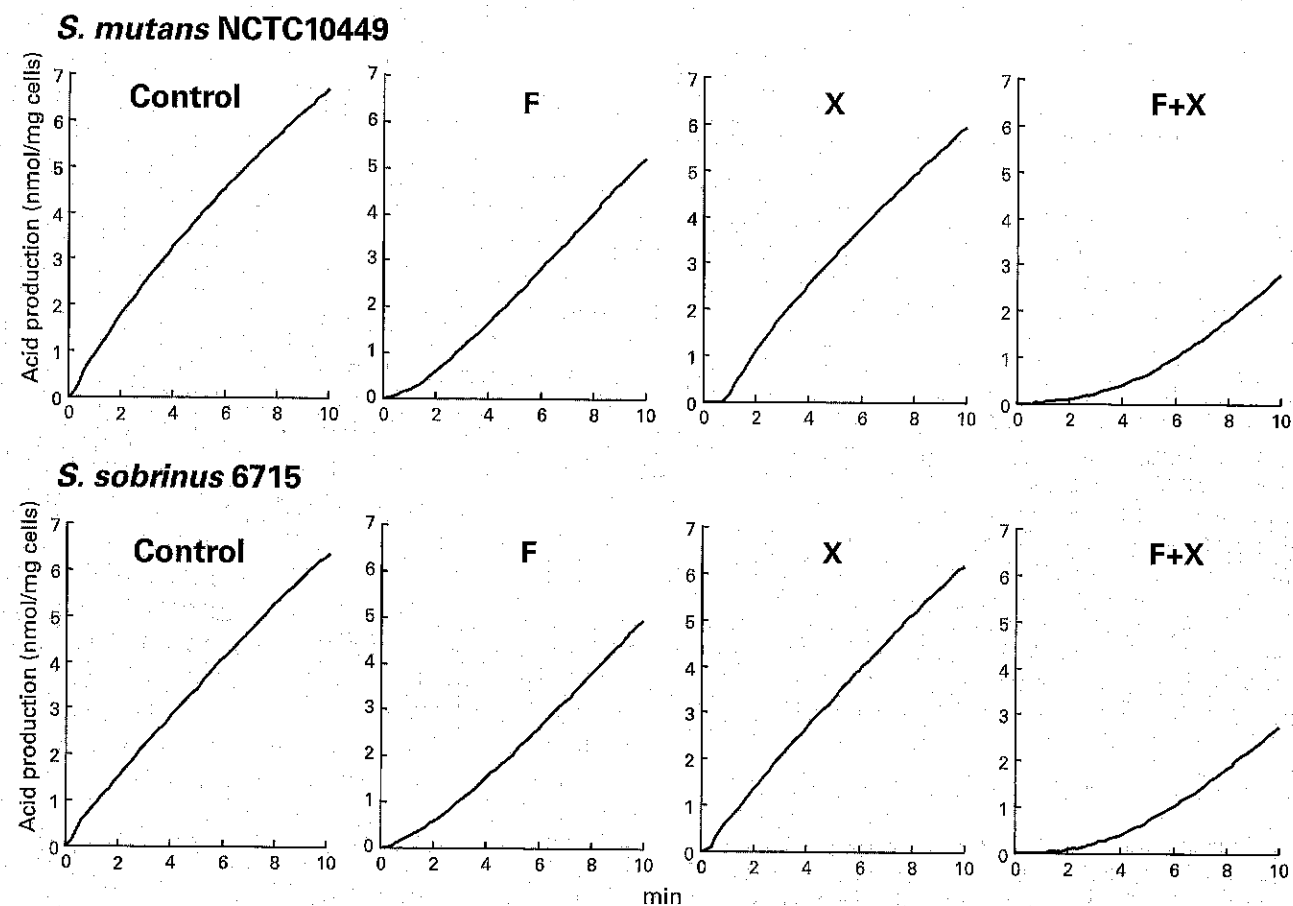

Fig. 1. Effect of fluoride on the acid production for 10 min from 10 mM glucose in the presence and absence of 60 mM xylitol by the cells of *S. mutans* NCTC 10449 and *S. sobrinus* 6715 at pH 7.0 and 5.5. F = Fluoride alone; F + X = fluoride plus xylitol. The data are the means of three independent experiments. Vertical bars indicate standard deviations from three independent experiments.

S. mutans NCTC10449

S. sobrinus 6715

Fig. 2. Effect of fluoride and/or xylitol on the acid production curves of the cells of S. mutans NCTC 10449 and S. sobrinus 6715 after addition of 10 mM glucose at pH 7.0. F = 1.6 and 2.4 mM fluoride alone for S. mutans and S. sobrinus, respectively; X = 60 mM xylitol alone; F + X = fluoride plus xylitol. The data represent three independent experiments. The results were similar among three independent experiments.

Fig. 3. Effect of fluoride and/or xylitol on the production of acidic end products from 10 mM glucose for 10 min by the cells of S. mutans NCTC 10449 at pH 7.0. Left = Amounts of acidic end products; right = profiles of acidic end products. F = 1.6 mM fluoride alone; X = 60 mM xylitol alone; F + X = fluoride plus xylitol. The data are the means of three independent experiments. Error bars indicate standard deviations from three independent experiments. * Significant difference from control (p < 0.05).

was dose-dependent (fig. 1). At pH 7.0, 2.4 mM fluoride was required to reduce acid production by 50% or more, while only 0.35 mM fluoride had a similar effect at pH 5.5. Similar results were obtained in S. sobrinus 6715 (fig. 1).

The inhibition of acid production by fluoride was enhanced in the presence of xylitol. At pH 7.0, the acid production by S. mutans decreased by 21.7% in the presence of 1.6 mM fluoride alone and by 8.9% in the presence of 60 mM xylitol alone, while the acid production was decreased by 58.4% in the presence of a combination of 1.6 mM fluoride and 60 mM xylitol. The enhanced inhibition caused by the addition of xylitol was also observed at pH 5.5, with 16.3% inhibition in the presence of 0.3 mM fluoride alone and 80.1% inhibition by the combination of 0.3 mM fluoride plus 60 mM xylitol. A similar inhibition by the combination of fluoride and xylitol was observed in S. sobrinus 6715.

In the absence of fluoride or xylitol, the acid production by S. mutans NCTC 10449 started immediately after the addition of glucose and gradually slowed down (fig. 2, control). In the presence of xylitol alone (fig. 2, X), no acid production was detected for about 1 min after the addition of glucose. Once it started, the acid production pattern was similar to the control, though the acid production rate was slower. In the presence of fluoride (fig. 2, F) and the combination of fluoride and xylitol (fig. 2, F + X), the acid production was very low right after the addition
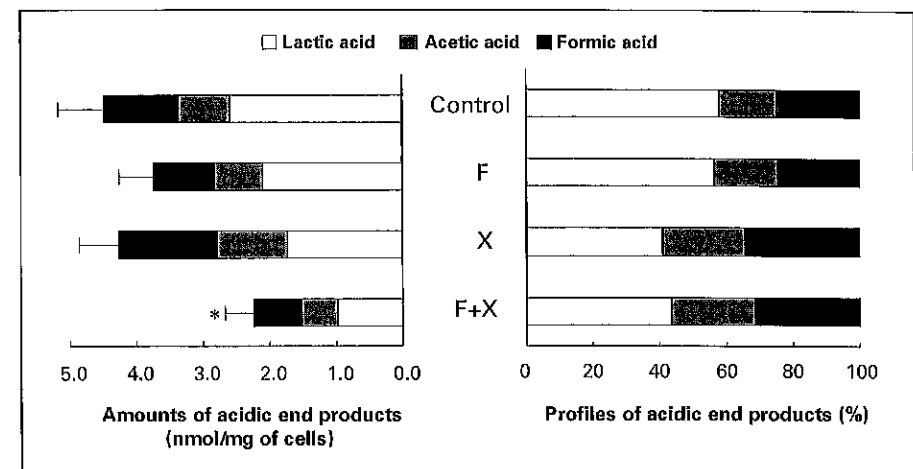
of glucose and increased gradually and became nearly constant by 10 min. Similar inhibitory effects of fluoride and xylitol were observed in S. sobrinus 6715.

Production of Carboxylic Acids from Glucose in the Presence of Fluoride and/or Xylitol

The effects of fluoride and xylitol on the formation of acidic end products from glucose and the profile of intracellular glycolytic intermediates were evaluated at a fluoride concentration of 1.6 mM and pH 7.0 using S. mutans NCTC 10449, where the combined effect of fluoride and xylitol was evident. The total amount of carboxylic acids (sum of lactic, formic and acetic acids) produced by S. mutans NCTC10449 at pH 7.0 for 10 min was 4.49 nmol/mg of cells in the control, while those in the presence of fluoride alone and xylitol alone were 3.75 and 4.27 nmol/mg of cells, respectively. In the presence of fluoride and xylitol combined, the total amount decreased to 2.23 nmol/mg of cells (fig. 3). These results showed that the combination of fluoride and xylitol was more effective in inhibiting the acid production than fluoride or xylitol alone, similar to the results of acid production rate obtained by pH stat (fig. 1). In addition, lactic acid as a proportion of the total amount of carboxylic acids decreased, while the proportion of formic and acetic acids increased in the presence of xylitol alone. A similar result was observed in the presence of a combination of fluoride and xylitol (fig. 3, right).

Profile of Intracellular Glycolytic Intermediates in the Presence of Fluoride and/or Xylitol

In the presence of fluoride alone, the levels of FBP and PYR were lower and the levels of 3PG and 2PG were

higher than those in the control during glucose fermentation (fig. 4). In contrast, in the presence of xylitol alone, the levels of glycolytic intermediates except PYR were mostly lower. Moreover, in the presence of the combination of fluoride and xylitol, the levels of G6P through G3P were similar to those in the presence of xylitol alone, while the levels of 3PG through PYR were similar to those in the presence of fluoride alone (fig. 4).

At 0 min (just before glucose addition), the control cells had low levels of glycolytic intermediates, except 3PG, 2PG and PEP (the intermediates which comprise the 'PEP potential' for sugar uptake into cells [Thompson, 1979]). The levels of these 'PEP potential' intermediates (3PG, 2PG and PEP) at 0 min in the presence of fluoride alone were twice as high as those of the control, while the levels of those were negligible in the presence of xylitol alone. The level of 'PEP potential' in the combination of fluoride and xylitol was close to the control at 0 min, though PEP was not detected (fig. 4).

Discussion

Acid production from glucose by mutans streptococci was inhibited in the presence of fluoride or xylitol as previously reported [Kashket et al., 1977; Hamilton and Ellwood, 1978; Assev et al., 1983; Vadeboncoeur et al., 1983; Marsh et al., 1985; Bradshaw and Marsh, 1994]. In addition, this study has demonstrated that the inhibitory effect was enhanced by a combination of fluoride and xylitol. This is consistent with previous results for combined effects of fluoride and xylitol on the growth of mutans streptococci [Scheie et al., 1988; Rogers and Bert, 1992].
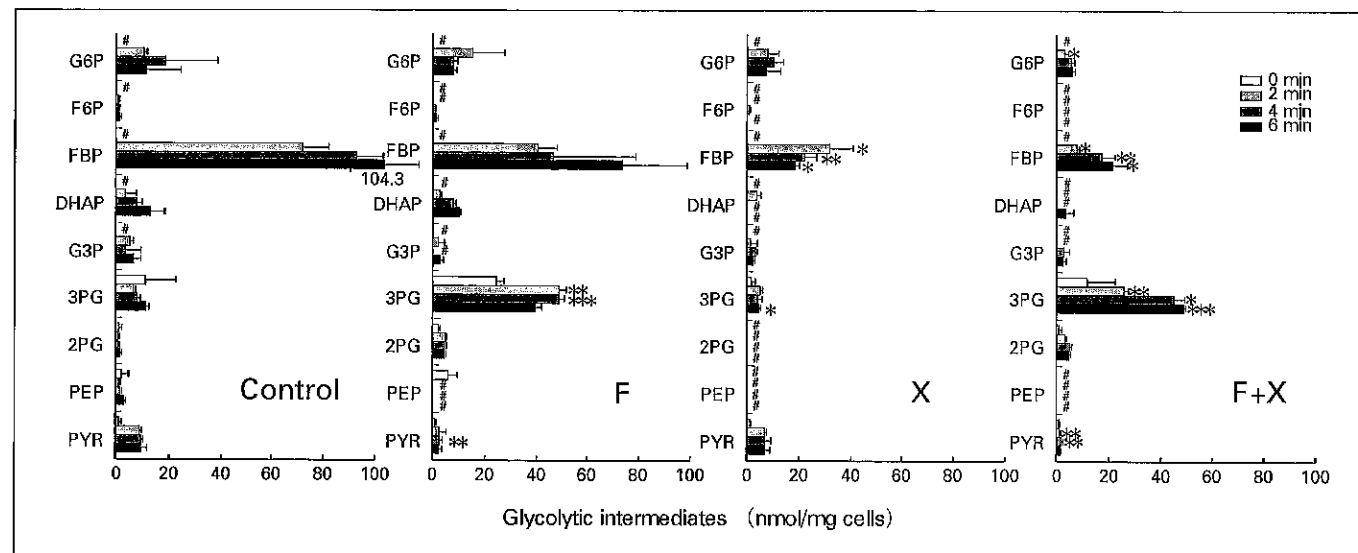
Fig. 4. Effect of fluoride and/or xylitol on the levels of glycolytic intermediates of the cells of *S. mutans* NCTC 10449 during glucose fermentation. The levels of glycolytic intermediates at 0, 2, 4, and 6 min after addition of 10 mM glucose to the cells *S. mutans* NCTC 10449 in the presence of 1.6 mM fluoride and/or 60 mM xylitol. F = 1.6 mM fluoride alone; X = 60 mM xylitol alone; F + X = fluoride plus xylitol. Error bars indicate standard deviations from three independent experiments. Significant difference from control (* p < 0.05; ** p < 0.01; *** p < 0.001). # = Not detected. The data are the means of three independent experiments.

Moreover, percent inhibition by combination of fluoride and xylitol was larger than the sum of percent inhibition by fluoride alone and percent inhibition by xylitol alone, indicating that the combined effect was synergistic (fig. 1, 2).

The observation that fluoride did not change the profile of end products while xylitol decreased the proportion of lactic acid implies a difference in inhibitory mechanism between xylitol and fluoride (fig. 3). This finding led us to the analysis of glycolytic intermediates to understand the biochemical mechanism for the synergistic effects of xylitol and fluoride.

The presence of fluoride alone increased the level of 2PG and 3PG in the lower part of glycolysis as reported previously [Hata et al., 1990]. This indicates that fluoride inhibited enolase (a glycolytic enzyme catalyzing the conversion of 2PG to PEP) and resulted in the accumulation of 3PG and 2PG and the decrease of PEP (fig. 4). In bacterial cells, PEP is a phosphoryl donor for sugar uptake by PEP-PTS [Schachtele and Mayo, 1973; Dills et al., 1980] and builds 'PEP potential' together with 3PG and 2PG, precursors of PEP. Although 3PG and 2PG levels were high in the presence of fluoride, the slow supply of PEP from 3PG and 2PG caused by the fluoride inhibition could decrease glucose uptake via PEP-PTS and slow

down the entire glycolytic process [Kanapka and Hamilton, 1971; Bender et al., 1985] (fig. 2, 4).

In the presence of xylitol alone, in contrast, most glycolytic intermediates decreased as acid production was inhibited, suggesting the repression of sugar uptake by xylitol. In addition, the marked decrease of FBP as previously reported [Miyasawa et al., 2003] may be due to the inhibition by X5P of PGI and PFK catalyzing the conversion of G6P to FBP [Trahan, 1995]. Since FBP is an activator of lactate dehydrogenase, the decrease in FBP caused inactivation of lactate dehydrogenase and consequent reduction of lactic acid production (fig. 3) as previously reported [Miyasawa et al., 2003].

The combination of fluoride and xylitol increased the intracellular levels of 3PG and 2PG as observed in the presence of fluoride alone, while the other glycolytic intermediates decreased similarly to the presence of xylitol alone. These observations indicate that the simultaneous inhibition, e.g. the inhibition of enolase by fluoride and the inhibition of PGI, PFK and glucose uptake by xylitol, repressed the entire glycolytic pathway more efficiently.

These inhibitory effects seemed more efficient in the initial stage of acid production. During preincubation in the presence of fluoride alone, PEP could not be consumed and 3PG and 2PG were accumulated through flu-

oride inhibition of enolase (fig. 4). After addition of glucose, the 'PEP potential' could be used for glucose uptake, however, the enolase inhibition by fluoride continued to interfere with the conversion of 2PG into PEP and consequently slowed down the entire glycolytic flow (fig. 2). In the presence of xylitol alone, the level of 'PEP potential' was low due to the consumption of PEP (fig. 4) through the phosphorylation of xylitol to X5P by PEP-PTS [Assev and Rölla, 1984; Trahan et al., 1985; Trahan, 1995; Miyasawa et al., 2003]. As a result, the lack of 'PEP potential' delayed acid production from glucose for about 1 min after glucose addition (fig. 2). The combination of fluoride and xylitol was more effective. The combination caused both enolase inhibition and low 'PEP potential' (fig. 4), resulting in the prolonged delay of initiation of acid production (fig. 2).

The inhibitory effect of fluoride on dental plaque acidogenicity in vivo is still unclear since there are numerous clinical observations but very few appropriate methods for evaluating antimicrobial activity of fluoride in vivo [van Loveren, 2001]. Reduction of plaque acidogenicity after sucrose challenge has been reported in plaque from subjects who used fluoridated water or rinsed their mouths daily with 48 mM (912 ppm) sodium fluoride [Edgar et al., 1970; Geddes and McNee, 1982]. Moreover, Neff [1967] reported that when applied simultaneously with sucrose, 0.52 mM (10 ppm) sodium fluoride was sufficient to inhibit acid production of dental plaque. However, Giertsen et al. [1999] argued that mouth rinsing with 12 mM (228 ppm) sodium fluoride had no significant effect on the acidogenic potential of dental plaque. In experiments using a continuous culture of mixed oral bac-

teria [Rogers et al., 1991; Bradshaw and Marsh, 1994] and an artificial biofilm [Bradshaw et al., 2002] that mimic some aspects of the dental plaque micro-ecosystem, fluoride and xylitol as well as a fluoride-xylitol combination were able to reduce the acid production of the micro-ecosystem with a reduction of the number of mutans streptococci. These sophisticated studies support the possibility that the combination of fluoride and xylitol could reduce dental plaque acidogenicity in vivo, though a proper method for clinical application of fluoride and xylitol should be developed as soon as possible.

In conclusion, direct inhibition of glycolytic enzymes, enolase, PGI and PFK and the consequent inhibition on the supply of 'PEP potential' for sugar uptake appears to be the cause of the synergistic inhibition by combination of fluoride and xylitol on glycolysis by mutans streptococci. Attempts have been made to use both fluoride and xylitol together to enhance remineralization of tooth surfaces [Smits and Arends, 1985; Arends et al., 1990; Gaffar et al., 1998; Amaechi et al., 1998]. Besides remineralization, this study suggests that using fluoride and xylitol together can effectively inhibit the sugar metabolism of mutans streptococci and also that xylitol has the potential to enhance the inhibitory effects of low concentrations of fluoride.

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