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A Preliminary Study of Exogenous Dextranase and NaF Directly Influence *Streptococcus mutans* Glucosyltransferase Activity

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Streptococcus mutans **(SM) is one of the most important bacterial pathogen for dental caries. Glucosyltransferases (GTF) are a group of enzymes play as key factors for its virulence. The combination of extranase (Dex) and sodium fluoride (NaF) is reported could reduce the biofilm of SM. This study is to investigate and compare the different inhibitory effects of exogenous Dex alone or with NaF on the activity of glucosyltransferase in solution or adsorbed to a surface. GTF produced by *Streptococcus mutants* ATCC 25175 was purified and the purity and concentration were determined with SDS-PAGE and BCA protein quantitation respectively. GTF was either dissolved or adsorbed to the surface of saliva-coated hydroxyapatite beads and incubated with ^{14}C-labelled sucrose. Exogenous Dex and NaF at various combined concentrations was added. The quantity of sucrose transformed per minute was determined with a scintillation counting technique. Exogenous Dex inhibited GTF activity both in solution and adsorbed state ($p < 0.05$). The inhibitory effect on GTF in solution was stronger (65%) than in adsorption (45%). The addition of NaF did not increase the inhibitory action of Dex ($p > 0.05$). The direct inhibition of GTF activity by Dex is a possible mechanism underlying the management of SM biofilms with exogenous Dex.**

Key words: Dextranase; glucosyltransferase; NaF; enzymatic activity; Biofilm.

Streptococcus mutans (SM) is generally considered as one of the most important bacterial contributors to dental caries. The occurrence of caries is associated with biofilms attached on tooth surface, named "dental plaque". Dental plaque is mainly formed by bacterial cells and their extracellular matrix, which provide bacteria with a stable environment for their survival and proliferation, shelter from outside stimulation/challenge, and a nutrient substrate to support their metabolism. The extracellular matrix also mediates cell adhesion to the tooth surface. Extracellular polysaccharides (EPs) are the main constituents of the extracellular matrix in dental biofilms[1,2].

The production of glucosyltransferase (GTF) is a key factor in the cariogenic capacity of SM. GTF produces water-soluble and water-insoluble *a*-glucans from sugar, which are the major components of the extracellular matrix in dental plaque[3].

Glucans maintain the structural integrity of dental plaque and promote bacterial adhesion to the tooth surface. There are few inorganic components of the dental plaque in glucan-rich biofilms. There are mainly three types of GTFs that SM produces: GTF B mainly produces water-insoluble glucans with *a*-1,3 glycosidic bonds;

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GTF D produces water-soluble glucans with *a*-1,6 glycosidic bonds[4]; and GTF C can produce both water-soluble and water-insoluble glucans[5]. A previous study has shown that GTFs B and C are more closely associated with dental caries than GTF D[5], while Khalikova, Susi, and Korpela.[6] argued that to maximize the cariogenic capacity of S. mutans all three enzymes are required. Active GTFs can either dissolve in human saliva or specifically attach to dental plaque through binding with the acquired membrane of dental plaque[3, 7]. According to Vacca-Smith and Bowen [8], GTFs in solution or adsorbed to the dental surface manifest different characteristic bioactivity and dynamics. The glucans produced by GTFs in solution are mainly found in the biofilm matrix, providing binding sites for SM and thus promoting the formation of dental plaque[9,10] . In contrast, GTFs adsorb to the surface of acquired membranes react with the sucrose and starch ingested as food, their products mediate the bacterial attachment to the dental surface. Therefore, GTFs are considered to be contributing factors to cariogenic bacterial attachment[11].

The inhibition or elimination of GTFs has long been considered an important factor in reducing the cariogenic capacity of SM. Many studies have shown that GTFs can be inhibited to different degrees by a number of natural extracts[12-15], medicines [16], and human saliva[17].

Dextranase (Dex) has received much attention in studies of EP reduction in biofilms. Dex is a kind of glucanase synthesized by bacteria, which express a variety of enzymes, including dextranase and mutanase [6, 18]. The former cleaves *a*-1,6 glycosidic bonds, whereas the latter cleaves *a*-1,3 glycosidic bonds [18, 19], so both can be used in caries control. According to Khalikova, Susi, and Korpela . [6], SM metabolizes dextran by synthesizing Dex. Dex can be used to digest the glucan formed by SM, thus inhibiting the formation of the dental biofilm matrix and dental plaque[20]. Hayacibara *et al*. [18] have demonstrated that the amount of polysaccharides produced by SM decreased in the presence of high concentrations of Dex. The numbers of *a*-1,6 glycosidic bonds are low in insoluble glucan, but the numbers of *a*-1,4 and *a*-1,3 glycosidic bonds are high. Dex increases the number of *a*-1,3 linkages in water-soluble polysaccharides [20,21] . It has been reported that 50 U of Dex can reduce the amount of glucan synthesized by GTFs by 52%–68% [18]. However, it is still unclear whether low concentrations of Dex can as effectively reduce the glucans synthesized by GTFs or whether Dex has a direct effect on the activity of GTFs.

To investigate the effects of Dex and extend its use, a comparison with sodium fluoride (NaF) was included in this study. NaF is already widely used in clinical practice and is considered being able to prevent the synthesis of GTFs by bacteria, especially GTFs act on water-insoluble polysaccharides[22]. In 1987, Scheie and Kjeilen[23] reported that fluorides have no obvious effects on GTFs. However, it has been reported that 8.4 mg ml^{-1} NaF inhibits the growth of SM and their expression of GTFs[21]. Others have reported that fluoride inhibits the activity of GTFs only when its concentration is higher than 4.2 mg ml^{-1}, whereas complete inhibition requires a concentration 100 times higher [24]. Unfortunately, such high doses of fluoride inevitably increase the undesirable possibility of fluoride toxicity.

Our previous study had already proved that NaF would not affect the activity of Dex.,and the combination of Dex and NaF could reduce the exopolycsaccharide and the bioflim formation.[25] However, the influence of this combination on GTF is still unclear. In the present study, we used exogenous Dex with and without a sub-inhibitory concentration of NaF, and compared their effects on GTF activity. We analysed the effects of Dex alone and of Dex combined with NaF to explore the possible mechanism by which Dex digests the biofilm matrix, to potentially establish a new method for plaque control.

MATERIALS AND METHODS

Bacterial culture and gross enzyme extraction

The preserved SM strain (ATCC 25175 International Reference Strain (serotype c) , State Key Laboratory of Oral Diseases, SKLOD, P.R. China) was resuscitated and passed down to the second generation, which was then cultured in BHI liquid medium containing 1% Tween 20, 200 mM $MnSO_4$, 1 mg ml^{-1} glucose, 20 mg ml^{-1} maltose, 10 mM Phenylmethanesulfonyl fluoride (PMSF), and 10 mg ml^{-1} erythromycin. The bacteria were cultured under 80% N_2, 20% CO_2 at 37°C for 24 h. The purity of the culture was verified by microscopic

observation. Then the medium was centrifuged at 3,000 ´ g for 20 min at 37°C. The supernatant was collected. The cellular pellicle was mixed with 5 mM KPO_4 containing 0.01% Triton X-100, vortexed for 2 min (250 W), and then centrifuged at 12,000 ´ g for 10 min. The supernatant was collected and adjusted to pH 6.5 with KOH.

The buffer used in these procedures contained 0.1 mM proteinase inhibitor (PMSF) and 0.02% NaN_3.

GTF extraction and purification

The GTFs produced by SM were purified with chromatography on an HA column (Chromatographic column, Kenqiang Instruments, Shanghai, P.R. China)[26]. PMSF was added to the extracted GTFs at a final concentration of 0.1 mM. The samples were freeze-dried to powder, which was weighed and dissolved in imidazole buffer containing 10% glycerin, and stored.

GTF purity

The purity of the extracted GTFs was determined with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Hoefer Mighty Small SE245, Hoefer, Inc., Holliston, MA, USA). The settings were: 10% spacer gel with 10 mA current, 5% separation gel with 20 mA current [18]. The gel was dyed with bromophenol blue, the marker was a protein marker from Sigma, US)

The purified enzyme was quantified with a BCA protein quantification method (BCA Protein Quantification/Quantitation Kit, KeyGEN Biotech, Nanking, P.R. China).

Units of enzymatic activity

The definition of one unit of GTF activity was the amount of GTF required to transform 1 mmol glucose into glucan in 4 hours (unit = mmol substrate transformed per min). The enzymatic activity of GTF was determined by mixing 0.5 ml of GTF with ^{14}C-labelled glucose (0.1 mCi ml^{-1}; NEN Research Products, Boston, MA, USA) at 37°C for 4 h and quantifying the amount of glucose transformed.

Effects of exogenous Dex and NaF on SM GTF activity

Whole saliva collection

Non-stimulated whole saliva was collected from a volunteer into a centrifuge tube. The saliva sample was centrifuged at 17,000 x g at 4°C for 20 min. The supernatant was mixed with 25.0 mM iminazole buffer (pH 6.5) in a volumetric ratio of 1:1. NaN_3 was then added to a final concentration of 0.02% and PMSF to a final concentration of 1.0 mM.

Preprocessing/Pretreatment of GTF

Pretreatment of GTF in solution

GTF (1.0–1.5 U) was mixed with the different treatment factors and adjusted to a final solution volume of 1000 ml, which was dispensed equally into the wells of a 12-well plate.

Pretreatment of GTF in the adsorption state

Hydroxyapatite (HA) beads (Integrated Separation Systems, Hyde Park, MA, USA) (10 mg) were placed in a 1.5 ml microcentrifuge tube and incubated overnight at room temperature in buffer containing 50.0 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM KPO_4, and 0.1 mM $MgCl_2$ (pH 6.5). The tube was then centrifuged at 18 x g and the supernatant was discarded. The beads were covered with processed whole human saliva for 30 min at 37°C, and were then washed three times with 1.0 ml of 25 mM iminazole buffer (pH 6.5). To each tube was added 0.5 ml of iminazole buffer containing 0.5 mg of GTF, and the tubes were shaken for 30 min at 37°C to ensure that GTF adsorbed to the HA beads. The tubes were then washed three times with iminazole buffer. The HA beads were incubated in 1.0 ml of iminazole buffer at 37°C for 2 h and washed again in iminazole buffer[18].

Preparation of standard sucrose substrates

Sucrose and dextran 9000 were dissolved in 25 mM iminazole buffer at final concentrations of 100 mM sucrose and 20 mM glucan. To the solution was added 1 ml of ^{14}C-labelled sucrose and 0.02% NaN_3 in a volumetric ratio of 1:500. The concentration of the radioactive marker added to the sucrose substrate was 300,000 cpm ml^{-1} (0.2 mCi ml^{-1} or 7.4 KBq ml^{-1}). The different test groups received different treatment factors, as shown in Table 1.

Enzymatic reaction

Enzymatic reaction in solution

The pre-treated GTF (500 ml) was incubated with 500 ml of standard sucrose substrate at 37°C for 4 h, after which ethanol was added to a final concentration of 70% to terminate the enzymatic reaction. The solution was filtered through a 1.2 mm glass microfibre filter. The amount of glucose transformed per minute was determined with a liquid scintillation counting technique[18].

Enzymatic reaction in adsorption state

The pre-treated HA beads were incubated with 1.0 ml of standard sucrose substrate at 37°C for 4 h, after which ice-cold ethanol was added to a final concentration of 70% to terminate the enzymatic reaction. The deposition was collected, washed twice with iminazole buffer, and then filtered through a 1.2 mm glass microfibre filter. The radioactivity of the filter was measured with a liquid scintillation counting technique.

Statistical analysis

The data acquired were analysed with SPSS version 13.0. The differences between the various treatment groups and the blank control groups were compared with one-way analysis of variance (ANOVA) and Dunnett's two-tailed *t* test. Pearson's correlation analysis was performed for the various groups. The level of statistical significance was set at $\alpha = 0.05$.

RESULTS AND DISCUSSION

The weight of the extracted GTF enzyme was 590 ng. The molecular weight of the extracted GTF was established with chromatography. And the BCA protein quantitation showed the concentration of the purified GTF was 0.259 mg ml^{-1}(Figure. 1). The enzymatic activity of GTF in the dissolved and adsorption states was 65 $m^{U}m g^{-1}$ (Table. 2). No difference was observed between these two groups (P > 0.05).

Influence of Dex on the enzymatic activity of GTF in solution and adsorption: In solution, Dex reduced GTF activity by 60%–65% ([CPM control – CPM enzyme treatment]/CPM control) (P < 0.05). This reduction was not greatly influenced by the concentration of Dex added (P > 0.05). When adsorbed, the GTF activity was as little as 1 U (10 mg ml^{-1}) Dex significantly inhibited its activity by 60%–65% ($p < 0.05$). At Dex concentrations of 2 U and 4 U, the percentage reductions were only slightly but not significantly increased (60.3% and 63.8%, respectively; ANOVA, $p > 0.05$). The inhibition of the adsorbed GTF activity was lower (35%–45%), and the differences among the three groups treated with different concentrations of Dex were not statistically significant ($p > 0.05$).

Table 1. Different treatment factors added to the various test groups

0	Experimental groups			Control
Dex (1 U = 10 mg ml^{-1})	1 U	2 U	4 U	–
NaF (mg ml^{-1})	0	0	0	0
	40	40	40	40
	80	80	80	80

Table 2. GTF activity in solution and adsorption and effect of Dex on the enzymatic activity of GTF in solution and adsorption (n = 5). In solution, Dex reduced GTF activity by 60%–65% ($p < 0.05$). This reduction was not greatly influenced by the concentration of Dex added ($p > 0.05$). The GTF activity was reduced by Dex by 35%–45% when adsorbed($p < 0.05$)

	CPM	
	In Solution	Adsorption
GTF (ml)	8903 ± 2.12	8290 ± 2.20
Dex (1 U = 10 mg ml^{-1})	0	0
0 U	8903 ± 2.12	8290 ± 2.20
1 U	3392 ± 2.24	5579 ± 2.17
2 U	3534 ± 2.65	5181 ± 2.06
4 U	3223 ± 1.77	5316 ± 2.34

reduced by Dex by 35%–45%, and the reduction was not influenced by the concentration of Dex used (Table. 2, Figure. 2).

The data reported is the mean of five repetitions. When GTF was in the dissolved state,

Effects of combined Dex+NaF on GTF activity in solution and adsorption

Effects of Dex+NaF on GTF activity in solution

Compared with the blank control groups, the GTF activity in the Dex+NaF groups was invariably reduced by about 64% ($p < 0.05$). However, the difference of reduction showed no statistically significance within the Dex+NaF group or compared with the Dex group ($p > 0.05$; Table. 3, Figure. 3).

Table 3. Effects of Dex+NaF on the activity of GTF in solution and absorption. The GTF activity in the Dex+NaF groups was invariably reduced by about 64% compared with the blank control groups ($p < 0.05$). This reduction did not differ statistically significantly within the Dex+NaF group or compared with the Dex group ($p > 0.05$)

	NaF (1U=10 mg ml^{-1})	CPM	
		Blank control	Dex group
In solution	0 U	8903 ±2.12	2840 ±2.30
	40 U	7977 ±2.87	3329 ±2.77
	80 U	7737 ±2.40	3552 ±2.96
Adsorption	0 U	8290 ±2.20	5171 ±1.24
	40 U	7817 ±2.23	4526 ±2.89
	80 U	7884 ±2.87	4750 ±2.45

Effects of Dex+NaF on GTF activity in adsorption

Compared with the blank controls, the GTF activity in the Dex+NaF groups was invariably reduced by about 46% ($p < 0.05$). However, the Dex+NaF groups failed to achieve greater reduction rates than the Dex groups ($p > 0.05$; Table.3, Fig. 3).

Dental plaque is generally considered to be a key factor contributing to dental caries. Therefore, the inhibition of dental plaque has long been an important objective of scientific research. The intervention of dental plaque biofilm can stop caries in early stage, avoiding dental decay or tooth loss.[27] The matrix of dental plaque mainly consists of EPs secreted by bacteria resident in the plaque, which provides a safe environment for them and binding sites on the tooth surface. Many researchers have focused to achieve the inhibition of the dental plaque biofilm through the control of bacterial activity.[28] Streptococcus mutans is generally considered as a prominent cariogenic bacteria among the oral flora. The secretion of GTF is an important characteristic of SM, as GTF is a major virulence factor of SM, and is capable of producing glucan, an important component of EPs. It is also an important to research the reduction the biofilm matrix by inhibiting the activity of GTF, which allowing us to control the development of dental plaque and caries progression.

Previous studies have already demonstrated that Dex inhibits the formation of EPs by controlling the amounts and proportions of EPs by cleaving *a*-1,6 glycosidic bonds [25]. In the present study, we focused on whether Dex has a direct impact on the enzymatic activity of GTF, and we used NaF as the control. We also examined

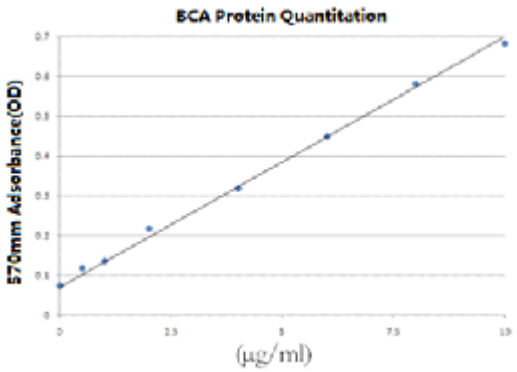

Fig. 1. Standard curve for BCA protein quantitation

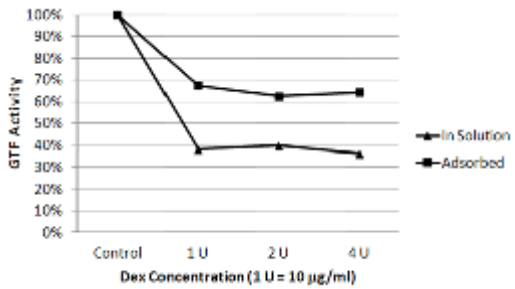

Fig. 2. Reduction of GTF activity induced with various concentrations of exogenous Dex by percentage

whether the combined use of Dex and NaF synergistically affects GTF activity.

Our results show that Dex inhibits the activity of GTF either in solution or in adsorption. This inhibitory effect was greater in solution, when 1 U of Dex reduced GTF activity by 60%–70%. However, the same amount of Dex only reduced GTF activity by 35%–45% when it was in adsorption. This difference might be attributable to: **a,** the reduced access of Dex to GTF when the GTF was adsorbed to the HA bead surface; and **b,** the enhanced activity of adsorbed GTF compared with that in the dissolved state[3]. It is also possible that the reaction site between GTF and Dex differs in the adsorption state from that in the dissolved state.

In the work of Hayacibara *et al*., with SDS–PAGE and an enzyme-linked immunoassay, they found that: a, Dex did not digest GTF; b, Dex bound to GTF and affected the amount of glucan produced by GTF[18]. However, because Dex can also digest glucan, it was still unclear whether the reduction is a result of Dex action on glucan, or the binding of Dex to GTF explained the reduced glucan production. And It cannot exclude the possibility that Dex had a direct impact on GTF activity. In the present study, we first pre-treated GTF with Dex with/without NaF, and then assessed the specific enzymatic reactivity of GTF, calculating the percentage reduction in GTF activity relative to that in the corresponding blank controls. In this procedure, the Dex action on GTF occurred before the digestion of glucan. Thus we eliminated the influence of glucan digestion by Dex. We found that Dex invariably reduced the activity of GTF in both the dissolved and adsorption states, and that this reduction was not significantly influenced by the concentration of Dex used. Therefore, we inferred that Dex has a direct effect on the activity of GTF.

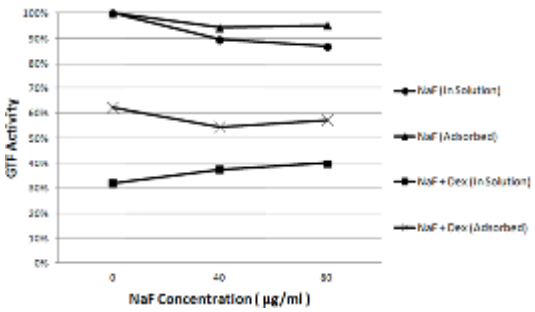

Fig. 3. Effects of combined Dex+NaF on GTF activity

Conversely, 80 mg ml^{-1} NaF had no obvious effect on the GTF activity in either state, or on Dex activity or glucan synthesis. Similar findings have been reported by other researchers [29]. In other studies, the NaF concentration that directly inhibited GTF activity was higher than that used in our study [18, 24], indicating that the direct inhibition of GTF activity requires a high dose of NaF.

In the present study, higher Dex concentrations did not produce stronger GTF inhibition, indicating that there is no dose–effect relationship. Still, we cannot exclude the possibility that GTF is further affected by Dex. Our finding is consistent with those of previous studies[18] and indicates that the ability of Dex to digest glucan should not interfere with our study results. There might be possible explanation for the binding action of Dex on GTFs: Hayacibara *et al*. [18] have demonstrated that Dex can bind to GTF, indicating that the reduction in GTF activity might result from the binding of its bioactive sites by Dex; Another possible explanation is that Dex, as an enzyme, is active even at low concentrations, so it can inhibit GTF activity at concentrations as low as 10 mg ml^{-1}.

This work presents It is possible to control dental plaque by eliminating the synthesis of the biofilm matrix with Dex and other bio-agents, so that less antimicrobial medication is required for plaque control. The use of Dex and other enzymes might also greatly reduce the possible risks inherent in high doses of fluoride. However, the molecular mechanism behind the effect of Dex on GTF activity remains to be explored. It needs further study to determine the influence was specificly on GTF B, GTF C or GTF D. More studies are also required to clarify the threshold concentration of Dex required to achieve the maximum inhibition of GTF activity.

According to the results of this study, Dex can directly digest the glucans synthesized by GTF, and directly impairs the activity of GTF, regardless of the concentration of Dex used. The digestive and inhibitory effects of Dex described above are two ways in which it modulates the amounts and proportions of water-soluble EPs produced by SM. NaF at low concentrations had no effect on GTF activity, and the combined use of

Dex and NaF reduced the activity of GTF in a non-synergistic manner. The extended use of Dex may introduce a new factor into the prevention of dental caries through the inhibition of GTF, a key virulence factor of SM in the process of cariogenesis.

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