Effect of xylitol and sucrose plaque on release of lysosomal enzymes from bones and macrophages in vitro

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Plaque formed during a 5-day xylitol or sucrose chewing gum diet was used as an irritating agent in bone and macrophage culture. The release of hydrolytic enzymes was monitored. The contents of protein, DNA, and ATP were analyzed, to characterize plaque formed during different dietary periods. The release of glycosidases and phosphatases was lower in the presence of xylitol plaque when compared with sucrose plaque as an immediate reaction in macrophage cultures at 3 h and also in bone cultures at 3 days. The results indicate that xylitol plaque had a less irritating effect on macrophages and bones in vitro than sucrose plaque according to the parameters used in this study. □ *Bone resorption; hydrolytic enzymes; sugar diet*

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Xylitol has an exceptional effect on the physiology of oral microorganisms. It is an inert or inhibitory compound to most bacteria (12). Clinically, xylitol has been shown to decrease the growth of dental plaque by approximately 50% when compared with sucrose and fructose (13).

It has therefore been assumed that xylitol would have an advantageous indirect effect on periodontal tissues because of its ability to reduce plaque growth compared with sucrose and to enhance host defence factors in saliva. However, it is not known to what extent the different nature of xylitol plaque affects the reactions of periodontal tissues. In the Turku sugar studies the consumption of xylitol, as compared with sucrose and fructose, reduced those enzyme activities in gingival exudate which are thought to be involved in inflammation (15). Our earlier findings suggest that xylitol plaque in bone culture is less resorptive than sucrose plaque (21).

The purpose of this study was to elucidate the effect of xylitol plaque on the release of lysosomal enzymes in bone and macrophage culture. Plaque material was characterized by determining the content of protein, DNA, ATP, total sugars, and total phosphates.

Materials and methods

Production of plaque material

Plaque was formed during a xylitol (content: xylitol 52%, gum base, calcium carbonate, natural flavor, gelatine, antioxidant (bht 321)) (Xylitol Inc., Turku, Finland) or sucrose (content: sucrose, gum base, starch syrup, peppermint oil, natural flavor) (Hellas Inc., Turku, Finland) chewing gum diet. Chewing gum between meals has been shown to be an appropriate way to consume xylitol to induce beneficial oral effects (4, 6, 17). Twenty-nine dental students (mean age, 24 ± 5.0 years) with no obvious signs of gingivitis or caries produced plaque during a 10-day period without oral hygiene procedures (Fig. 1). The plaque formed during the first 5 days of a normal diet served as a control plaque (CI, CII). After the collection of the control plaque on day 5 the teeth were cleaned, and the test plaque was

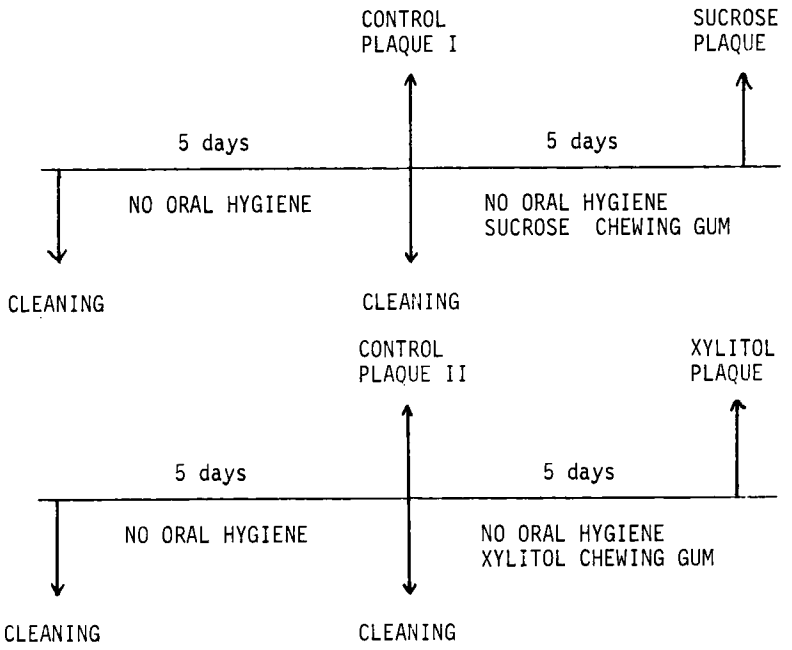

Fig. 1. Experimental arrangements for the production of xylitol, sucrose, and control plaque. There was a 1-week interval between the test periods.

developed during the next 5 days of a chewing gum diet. The same procedure was repeated after a week with the other chewing gum to obtain comparable plaque samples for xylitol (X) and sucrose (S) from the same individuals. The students were asked to chew at least five sticks per day and write down the consumption. There were no significant differences in the amount of chewing gum used or in the frequency of intake between xylitol and sucrose test periods.

Collection and treatment of plaque

Plaque was collected from air-dried tooth surfaces with excavators and immediately immersed in cold sterile 0.9% saline. Plaque was used in two forms as a test agent: 1) fresh plaque suspension and 2) plaque extract. For plaque suspension the pooled material was thoroughly mixed with a glass rod and weighed (wet weight). A part of the material was diluted with saline according to wet weight to make the various plaque suspensions comparable. Immediately after collection, 850 μg of plaque in a volume of 30 μl was used for each bone culture. Heated plaque suspensions (boiled for 1 min) and saline served as controls.

The rest of the collected plaque pool was stored at −20°C for plaque extract. Before use it was thawed, disintegrated, centrifuged, and filtered. The treatment was the same as that described by Tenovuo et al. (20). The clear filtrates were added to the macrophage cultures so that they yielded a final concentration of 280 μg of protein/ml of medium. Heated extract and 0.9% saline served as controls.

The pellet from the treatment described above was used for further analysis. Before use it was stored at −20°C.

Plaque determinations

Either plaque suspension or plaque extract was used for protein, DNA, and ATP determinations. The protein content was determined by the method of Lowry et al. (9). DNA was analyzed in accordance with Burton (1) to quantify cells in plaque samples. The ATP level was assayed by the bioluminescence method, using the luciferase reaction with a Lumac celltester M 1030 in accordance with the manufacturer's instructions (Lumatek, Kauniainen, Finland). The ATP content of the plaque suspension was

used as an indicator of the viability of the plaque cells.

The plaque sediments were freeze-dried and weighed (dry weight). Thereafter, glycerophosphates were isolated by phenol extraction (22). Total sugars were determined by the anthrone method (2, 11) and total phosphates by the malachite green method (7).

Millipore chambers

To make it possible to add fresh plaque suspension to sterile bone cultures, Millipore ring chambers were used. The chamber consisted of a plastic ring (ring chamber, diameter 14 mm, height 2 mm, cat. no. PR0001400) and a Millipore filter (filter type TH, pore size 0.45 μ, cat. no. THWF01400; Millipore Corp., Bedford, Mass.). The filters were glued (MF® cement, formulation # 1, cat. no. XX7000000) to the bottom of the rings and sterilized in ethylene oxide (+69°C, 2 h followed by ventilation for 8 h). The ring chambers were placed onto metal grids in Trowell type organ cultures. Fresh plaque suspensions were pipetted onto the filters, through which the plaque communicated with the medium.

A ring chamber was also placed in the control cultures into which heated plaque or saline was added.

Bone culture and enzyme determinations

Radia and ulnae from 21-day-old embryos of Long Evans rats were prepared under sterile conditions. The culture conditions were the same as those described by Tenovuo et al. (20). Two milliliters of protein-free BGJb medium was changed after 24 h of preincubation. Thereafter the bones were incubated with xylitol, sucrose, or control plaque for 72 h.

Plaque suspensions in Millipore ring chambers were incubated under conditions similar to those of the bone cultures and for the same length of time. The activity present in the medium of plaque incubation was subtracted from the activities measured in the corresponding bone culture medium.

The media and bones were stored at −20°C until enzyme determinations were made. Plaque from the Millipore ring chambers was rinsed with 1 ml of 0.5% Triton X-100 solution and then treated in the same way as the bones. The extraction of enzyme activities from the bones and the details of β-glucuronidase and acid and alkaline phosphatase assays have been described earlier (20). The substrates used were *p*-nitrophenyl-β-D-glucuronide and *p*-nitrophenyl phosphate. The enzyme activities were expressed in terms of μmoles substrate hydrolyzed/min × ml. Lactate dehydrogenase was determined in accordance with Wroblewski & LaDue (23). The protein content was analyzed by the method of Lowry et al. (9), using bovine serum albumin as a standard.

Because the cultures with fresh sucrose, xylitol, and control plaque were performed at different times, the results obtained with plaque were calculated comparing them with the results obtained with saline in the contralateral bones of the same embryo.

Macrophage culture and enzyme determinations

Peritoneal macrophages were harvested from 3-month-old female Long Evans rats with 10 ml of sterile saline containing 20 IU heparin (Medica, Helsinki, Finland) as an anticoagulant. The cells were washed twice (500 *g*, 10 min, +20°C) with the incubation medium, which was made up of Eagle's medium with Earle's salts (Gibco Bio-Cult Ltd., Paisley, Scotland), 20 mM Hepes, 20 mM $NaHCO_3$, 50 μg/ml of streptomycin sulphate, and 100 U/ml of penicillin (Hoechst AG, Frankfurt, FRG). The cells (5×10^6/ml) were suspended in 2 ml of medium, and after 2 h of incubation at +37°C the adherent cells on the bottom of the petri dishes (35 × 10 mm, Falcon) were taken and washed once more with the incubation medium.

The incubations were started by adding 2 ml of medium and 100 μl of plaque extract (280 μg protein) and stopped by removing the media after 3 h. The media were centrifuged (500 *g*, 10 min, +4°C) and stored at +4°C. The cells were scraped from the bot-

tom of the petri dishes and suspended in 2 ml of cold 0.001 M β,β-dimethylglutarate buffer, pH 7.2. The cells were sonicated (MSE Ultrasonic Disintegrator, 2 sec, +4°C, amplitude 3 μ, end diameter of the probe 3 mm) and centrifuged (50,000 *g*, 10 min, +4°C).

The cell supernatants were used for enzyme determinations in addition to the media. The activities of β-glucuronidase, *N*-acetyl-β-glucosaminidase (*p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide as the substrate) and acid phosphatase were assayed as described earlier (20). Neutral proteolytic activity was determined by casein digestion on the basis of the method of Laskowski (8). The enzyme activities released from the macrophages were calculated by subtracting the activities of the added plaque extracts from the total enzyme activity found in the media.

Results

Quantification and characterization of plaque material

The wet weight of sucrose plaque was greater than that of xylitol or control plaque (Table 1). The dry weights of plaque sediments correlated well with the wet weights of plaque suspensions. There were, however, no great differences in the protein content of either plaque suspension or plaque extract among sucrose, xylitol, or control plaque. Sucrose plaque had the lowest DNA and ATP content, suggesting a low cell to extracellular matrix ratio. There was no clear trend in total sugar and total phosphate contents. In the aerobic cultures the proportion of 50% of streptococci in the first control sample (CI) was again found in the second control sample (CII) after the sucrose chewing gum period, during which the corresponding proportion was 90%.

Table 1. Parameters of 5-day-old pooled sucrose, xylitol, and control plaque samples determined from plaque suspension, sediment, or extract

Plaque	Wet weight, mg/ml, suspension	Dry weight, mg × 10^{-2}, sediment	Protein, mg/ml		DNA, μg/ml, extract	ATP, nmol/ml × 10^{-2}, suspension	Total sugars, mg % glucose/mg, sediment	Total phosphate, μg P/mg, sediment
			suspension	extract				
Control I	119.8	5.6	19.8	5.25	71.2	4.9	25.36	30.5
Sucrose	146.5	6.7	22.9	5.40	54.9	2.1	21.04	42.3
Control II	70.1	5.0	21.4	5.99	74.9	5.9	17.00	34.2
Xylitol	72.5	4.3	19.6	5.69	71.2	3.2	21.39	40.4

Bone culture

β-Glucuronidase and acid and alkaline phosphatase activities could be found in the bones and in the media when control plaque was used. When the plaque was modified

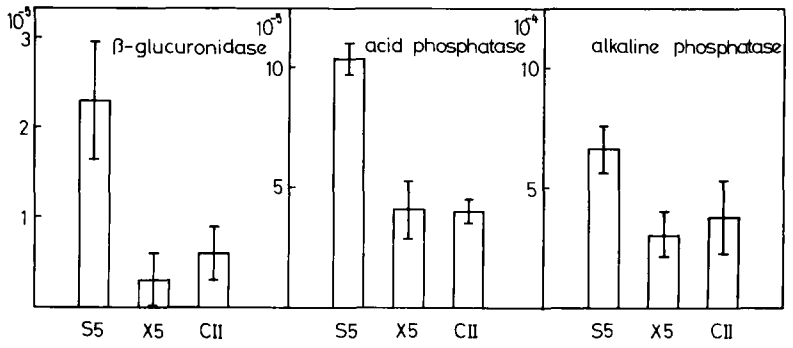

Fig. 2. The effect of fresh suspensions of 5-day-old sucrose (S5), xylitol (X5), and control (CII) plaque in the media of bone cultures incubated for 72 h as compared with saline-affected cultures. Activities of β-glucuronidase, acid phosphatase, and alkaline phosphatase (μmol/min × ml) are expressed as means (± range). Depending on the experiment the number of plaque samples varied from 2 to 5.

with a xylitol chewing gum diet, the activity of β-glucuronidase in the medium decreased 50% from the value of control plaque, and alkaline phosphatase 33% (Fig. 2). Xylitol plaque caused a release of acid phosphatase comparable to that caused by control plaque. On the other hand, when the plaque was modified with a sucrose chewing gum diet, the activity of β-glucuronidase was 3.5-fold, that of acid phosphatase 2.5-fold, and that of alkaline phosphatase 1.4-fold compared with the control. A corresponding increase could also be seen in the bones of cultures affected with sucrose plaque. The activities in the media decreased markedly with heated plaque suspension compared with fresh plaque. The level equaled that of the saline controls.

The activity of LDH stayed at a low level in the media. The type of plaque or the heat treatment of plaque did not have any clear effect.

There were no differences in the protein content of the bones, suggesting that approximately the same bone mass was cultured in each culture. If the results were calculated per plaque protein, plaque DNA, or plaque ATP, a similar pattern of enzyme activities was obtained.

The enzyme activities present in the plaque samples in the Millipore chambers after the bone cultivations were analyzed. Xylitol plaque had the highest activities of β-glucuronidase and acid and alkaline phosphatase and the highest content of protein. Sucrose plaque had as high activity of β-glucuronidase as xylitol plaque; otherwise the activities were at about the same level as in control plaque. Heating destroyed almost all the activities in plaque. The alkaline phosphatase activity of sucrose plaque was the least sensitive to heat treatment. Heating remarkably reduced the protein content of plaque.

Macrophage culture

The presence of sucrose plaque in macrophage cultures resulted in an increase of β-glucuronidase and *N*-acetyl-β-glucosaminidase activity in the media and the corresponding cells when compared with the control plaque (Fig. 3). Xylitol plaque was comparable to control plaque. All four heat-treated plaque extracts caused a similar release of β-glucuronidase and *N*-acetyl-β-glucosaminidase, which was less than the release with the unheated extracts. Thus, the lysosomal enzyme release-promoting factor in the sucrose plaque seems to be heat-labile. The experiment was repeated, with the same results. Similar results were obtained by calculating the results per volume, protein, or ATP of plaque extract.

The percentage proportion of enzyme release was calculated from the enzyme activities (expressed per volume) and compared with those of saline controls (Fig. 4). All four plaque extracts caused an increased enzyme release. The release percentage of β-glucuronidase was similar for all plaque extracts, but in *N*-acetyl-β-glucosaminidase the highest percentage of release was observed when the macrophages were treated with sucrose plaque.

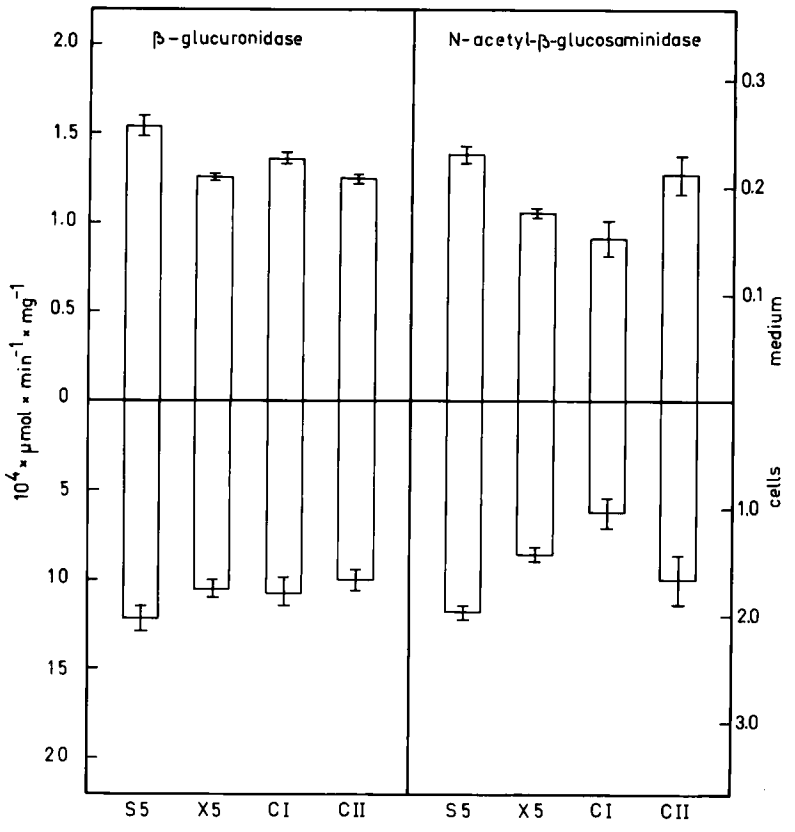

Fig. 3. The effect of the extracts of 5-day-old sucrose (S5), xylitol (X5), and control (CI, CII) plaque on β-glucuronidase and *N*-acetyl-β-glucosaminidase activities (mean ± range) in macrophage cultures incubated for 3 h. N = 3.

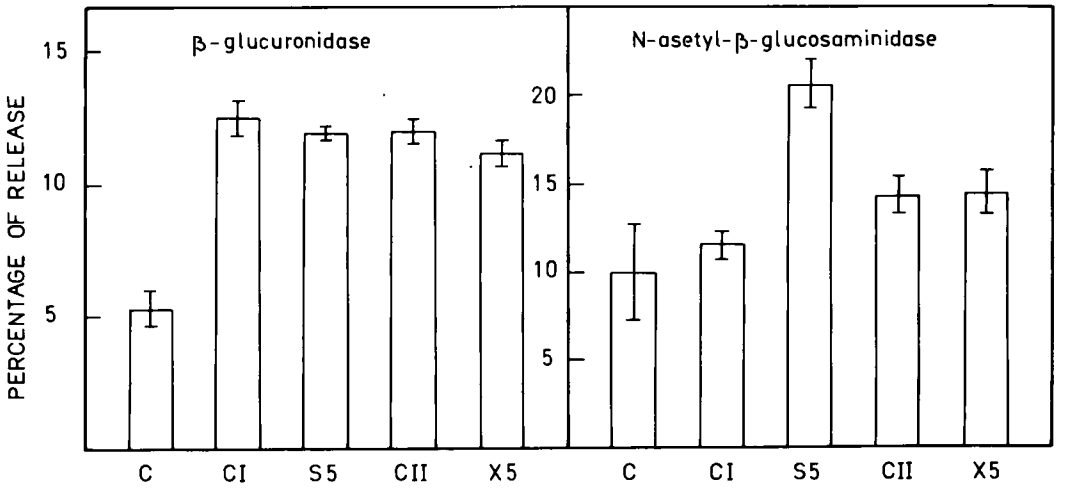

Fig. 4 The activities of β-glucuronidase and *N*-acetyl-β-glucosaminidase in macrophage cultures (see Fig. 3) expressed as percentage release. C refers to cultures treated with physiologic saline.

The determination of neutral proteolytic activity and acid phosphatase was disturbed by the high corresponding enzyme activities in the plaque extracts. The macrophages released some acid phosphatase as a response to the plaque extracts, but there was no release of neutral proteinase.

There were no differences in the LDH activity between xylitol, sucrose and control plaque measured from the cells and media.

Discussion

Because of the relatively good intraoral status of the students at the beginning of the study, the clinical signs of gingivitis found after 10 test days were few, mild, and localized. One may ask whether the control and the test plaque were produced under different conditions, considering subclinical gingivitis. In any case, both test plaque samples were formed during the latter half of the collection period and are comparable to each other. The proportion of aerobically cultivable streptococci confirms that the flora had recovered from the earlier test period.

An attempt was made to counteract possible effects of chewing by instructing the subjects to chew at least five sticks of chewing gum per day. There were no statistically significant differences between xylitol and sucrose either in the amount of chewing gum consumed or in the frequency of intake.

Wet weight was chosen for the quantification of fresh plaque after collection because of the short time available before bone culture. Since wet weight is not an exact parameter for plaque, determinations of protein, DNA, and ATP were performed afterwards, and the results were calculated by standardizing plaque in accordance with these parameters. Differences in the effects of sucrose, xylitol, and control plaque on bone were consistent in spite of the various quantification parameters used. We conclude that the effect of the xylitol plaque on bone and macrophages is different from that of sucrose plaque according to the parameters used.

In bone culture, fresh plaque was used to avoid unnatural treatment of plaque, which takes place, for instance, in the process of making plaque extract. In any case, a change in the ecology of dental plaque when transferred to aerobic conditions in bone culture was likely to occur. Whether the new conditions were more suitable for one of the plaque samples than for the others is not known. However, the treatment was the same for all of the plaque samples.

Results obtained in this study confirm the earlier findings that xylitol plaque, having higher lysosomal enzyme activities in itself, induces a less notable release of lysosomal enzymes than sucrose plaque in bone culture. The possibility that we measured plaque-derived activities from the medium was eliminated by subtracting the activities present in similar plaque incubations but without bones. The fact that the activities of the enzymes determined in cultures affected by sucrose plaque were higher both in media and in bones than in cultures affected by xylitol or control plaque shows a greater irritating potency of sucrose plaque. As an indication of this, a high level of LDH was also found in the media and the bones of cultures treated with sucrose plaque. In addition, both the enzymes connected with bone resorption (β-glucuronidase and acid phosphatase) and the enzyme connected with mineralization (alkaline phosphatase) increased in bone cultures affected by sucrose plaque.

Plaque-induced production and release of lysosomal hydrolases from macrophages have been reported by Page et al. (14). Rölla et al. (16) found that sucrose plaque contains large amounts of lipoteichoic acid compared with xylitol plaque. Lipoteichoic acid has been reported to stimulate bone resorption (3) and the release of lysosomal enzymes from macrophages (5). None of the earlier studies has dealt with the effect of xylitol plaque on macrophages. In this study xylitol plaque stimulated macrophages to release less lysosomal enzymes than sucrose plaque, a pattern of action similar to that in bone culture.

Xylitol may affect the inflammatory potential of the gingival exudate, since the exudate collected after a 1-year xylitol diet decreased the microcirculation velocity in the hamster

cheek pouch, whereas the exudate obtained after sucrose diet increased the velocity (10).

Interaction of plaque bacteria and inflammatory cells has been proposed to be an important phenomenon in peridontal disease (19). In vitro incubation of PMN leukocytes with supra- and sub-gingival plaque results in PMN degranulation and release of lysosomal hydrolases (18). This capacity of bacteria to activate PMNs in vitro seems to correlate with their ability to cause experimental periodontal disease in vivo (19). Accordingly, the enhanced release of lysosomal hydrolases from bones and macrophages with sucrose plaque when compared with xylitol plaque may reflect comparable differences in the inflammatory potential of these plaques in vivo.

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