Ultrastructure of *Streptococcus pneumoniae* after exposure to xylitol

Terhi Tapiainen¹, Raija Sormunen², Tarja Kaijalainen³, Tero Kontiokari¹, Irma Ikäheimo⁴ and Matti Uhari¹*

¹Department of Pediatrics, University of Oulu, PO Box 5000, FIN-90014, Oulu; ²Department of Pathology, Biocenter Oulu, Oulu; ³National Public Health Institute, Oulu; ⁴Clinical Microbiology Laboratory, Oulu University Hospital, Oulu, Finland

**Objectives**: Xylitol is a sugar alcohol which reduces the growth of *Streptococcus pneumoniae* and the adherence of pneumococci and *Haemophilus influenzae* to nasopharyngeal cells. Xylitol prevents acute otitis media but does not decrease nasopharyngeal carriage of pneumococci. We hypothesized that xylitol could affect the surface structures of viable pneumococci, which would further explain the mechanism of action of xylitol in preventing acute otitis media.

**Methods**: We exposed five strains of pneumococci to 0.5%–5% xylitol, 5% glucose, 5% fructose and 5% sorbitol or control medium (brain heart infusion) for 0.5–2 h and examined the ultrastructure of bacteria by electron microscopy.

**Results**: The cell wall of pneumococci became more diffuse, the polysaccharide capsule became ragged and the proportion of damaged pneumococci increased after exposure to xylitol for 2 h, but not after exposure to other sugars or control medium. The phenotype of all pneumococcal strains was opaque before xylitol exposure and became almost transparent both in xylitol and in control medium during the experiment.

**Conclusions**: This study demonstrates further that xylitol has a harmful effect on pneumococci. The observed changes in the polysaccharide capsule and the cell wall of pneumococci could affect the adherence and virulence of pneumococci, explaining the good clinical efficacy of xylitol in the prevention of acute otitis media.

Keywords: electron microscopy, bacterial polysaccharides, otitis media, pathogenicity

**Introduction**

The natural five-carbon sugar alcohol xylitol decreases the pathogenicity of *Streptococcus pneumoniae* and mutans streptococci, reducing the growth of these bacteria. Xylitol also prevents the adherence of pneumococci and *Haemophilus influenzae* to nasopharyngeal cells. Regular use of xylitol lessens the occurrence of acute otitis media in children by 30%–40%. It did not reduce nasopharyngeal carriage, however, either in an animal model or in children during a clinical trial and does not kill pneumococci in vitro in the presence of another carbon source.

In caries prevention, xylitol not only reduces the growth of mutans streptococci but also disturbs the protein synthesis and causes ultrastructural changes in this species. Since xylitol is clinically more effective in preventing acute otitis media than could be expected, as nasopharyngeal colonization of pneumococci is unaffected, we hypothesized that electron microscopic visualization of the pneumococcal surface structure after xylitol exposure could further explain the mechanism of action of xylitol in preventing acute otitis media.

**Materials and methods**

Pneumococcal strains ATCC 49619 (serotype 19F) and four isolates from routine middle-ear effusion samples (serotypes 6B, 9V, 14 and 23F), identified by their colony morphologies and optochin sensitivities, were used. The strains were serotyped in the National Reference Laboratory for Pneumococcus, National Public Health Institute (KTL), Oulu, Finland, and kept in skimmed milk broth at −20°C until used.

The pneumococci were cultured in brain heart infusion (BHI) medium (Difco Laboratories, Detroit, MI, USA) containing 0.2% glucose supplemented with 10% (v/v) heat-inactivated fetal calf serum (PAA Laboratories, Linz, Austria). They were grown...
aerobically at 35°C in a 5% CO₂ atmosphere up to an optical density of 0.3–0.4 at 650 nm (5 h). Three hundred millilitres of the suspension was transferred into 3 mL of test medium containing 0.5% xylitol, 5% xylitol, 5% glucose, 5% fructose, 5% sorbitol or control medium (BHI) for 0.5–2 h. The test media were prepared by adding xylitol (Sigma Chemical Co., St Louis, MO, USA), d-(-)sorbitol (Merck, Darmstadt, Germany), d-fructose (BDH Laboratory Supplies, Poole, UK) or d-(+)glucose (anhydrous; Fluka Biochemika, Switzerland) to basic medium, which was sterilized by filtration (Ministart 0.2 μm pore-size filter; Millipore Corp., Bedford, MA, USA). The number of viable bacteria was estimated after the experiments by plating dilutions. A reduction in growth of pneumococci after exposure to xylitol has previously been reported.1 The pneumococcal strains were evaluated for their colony morphology by viewing colonies using oblique, transmitted light.8

The bacteria were harvested by centrifugation from a volume of 1.5 mL (3000 rpm, 10 min). The bacterial pellet was fixed overnight at +7°C with 1 mL of 2.5% glutaraldehyde in 0.1 M phosphate buffer containing 100 μL of ruthenium red (Sigma Chemical Co.) for staining. It was then washed three times in phosphate buffer, post-fixed in 1% osmium tetroxide, dehydrated in acetone and embedded in Epon LX112. Thin sections were cut with a Reichert Ultracut ultramicrotome and examined with a Philips CM100 transmission electron microscope.

The proportions of totally damaged bacteria, dividing bacteria and bacteria forming diplococci were calculated based on a minimum of 100 bacteria in each sample. The diameter of a whole bacterium and the thickness of the polysaccharide capsule and bacterial cell wall of a sample of 30 bacteria in ATCC 49619 were measured.

Results
Exposure to 5% xylitol for 30 min caused no change in the morphology of the pneumococci, but after 2 h of exposure the structure of the cell wall became diffuse and less well defined than in the control medium (Figure 1). The normal trilaminated cell wall structure became irregular. The morphological changes were seen in all the serotypes tested, but were most prominent in the 19F serotype (ATCC 49619). Exposure to 0.5% xylitol for 2 h resulted in similar changes in the bacteria to those seen after exposure to 5% xylitol.

Exposure of the pneumococci to 5% xylitol for 30 min caused no difference in the morphology of the polysaccharide capsule, which was thin in both control and exposed bacteria, but after exposure for 2 h the mean diameter of the polysaccharide capsule became wider, 119 nm (s.d. 44.8) as compared with 100 nm (31.4), a difference of 19 nm [95% confidence interval (CI) 3.6–34.6, P = 0.008]. Also, the wider polysaccharide capsule was sparse and ragged (Figure 1). After exposure to 0.5% xylitol for 2 h, the absolute diameter of the polysaccharide capsule was smaller than that of the bacteria in the control medium.

The proportion of dividing pneumococci was reduced after exposure to 5% xylitol for 30 min relative to that in the control medium (26% versus 53%; 95% CI of the difference 14%–37%, P < 0.001), and the proportion was likewise smaller after 2 h of exposure (10% versus 26% in the control medium; CI of the difference 12%–19%, P < 0.0001). After exposure to 0.5% xylitol for 2 h, the proportion of dividing pneumococci was smaller (20% versus 32%; CI of the difference 0.03%–21%, P = 0.008).

There was no difference in the chain formation between the pneumococci exposed to 5% xylitol for 30 min and those in the control medium. When pneumococci were exposed to 5% xylitol

Figure 1. Streptococcus pneumoniae (ATCC 49619) after 2 h in BHI (a) or 5% xylitol (b and c).
for 2 h they were less often seen in the form of diplococci than in the control medium (16% versus 23%; CI of the difference 2%–11%, \(P = 0.005\)). They also formed longer chains in the control medium, comprising four to six pneumococci at a time, which was not seen in the xylitol medium.

There was no difference in the proportion of dead bacteria after 30 min of xylitol exposure compared with that in the control medium. After 2 h of 5% xylitol exposure, the proportion of totally damaged or autolysed bacteria was 13% in the xylitol medium compared with 3% in the control medium (CI of the difference 0.07%–12.2%, \(P<0.0001\)) (Figure 1).

The morphology of the pneumococci in 5% glucose remained unchanged and the proportion of dividing bacteria was >60%, i.e. greater than in the control medium (Figure 2). After exposure to 5% fructose, the cell wall structure remained well defined relative to that of the bacteria exposed to xylitol (Figure 2). The polysaccharide capsule was smaller and denser than in the control medium, but it remained unbroken. After sorbitol exposure, the cell wall structure became slightly more diffuse and the polysaccharide capsule was slightly ragged (Figure 2), as in the xylitol medium, although less often than in the bacteria exposed to 5% xylitol. The proportion of totally damaged pneumococci was 6% when exposed to 5% sorbitol compared with 1% when grown in BHI (CI of the difference 1%–10%, \(P = 0.01\)) and 23% in the xylitol medium (CI of the difference 10%–24%, \(P<0.001\)).

As pneumococci exposed to xylitol resembled the electron microscopic morphology of the transparent phenotype of pneumococci,8 the phenotype of each pneumococcal strain was evaluated during xylitol exposure and compared with that seen in control medium. All strains were opaque in the beginning of the experiment and after 0.5 and 2 h of logarithmic growth. After 4 and 8 h of logarithmic growth, the morphology of pneumococcal colonies changed towards a transparent phenotype in all middle ear strains except in ATCC 49619. There was no difference in the transformation towards a transparent phenotype between bacteria cultured in xylitol medium or in control medium.

**Discussion**

Our results demonstrate that xylitol changes the ultrastructure of pneumococci. The polysaccharide capsule became ragged and sparse. The cell wall became diffuse and the clear trilamellar structure disappeared. Despite these changes, the bacteria were viable at the end of the experiment.

The observed alterations in the polysaccharide capsule of pneumococci could be due to changes in polysaccharide metabolism, as has been observed in *Streptococcus mutans*.9 The uptake of xylitol into *S. mutans* and *S. pneumoniae* is a rapid process due to an active fructose phosphotransferase system capable of taking xylitol into the cell and phosphorylating it.1,10 Accumulation of xylitol phosphate is toxic to oral streptococci.10 The phosphotransferase systems regulate the metabolism in oral streptococci depending on the oral sugar environment. After xylitol exposure, the altered polysaccharide metabolism and consequently polysaccharide structure could lead to the observed morphological changes and further explain decreased adherence of pneumococci to nasopharyngeal cells.3
Since dietary use of xylitol does not affect nasopharyngeal colonization of pneumococci, other factors are more likely to be important in the efficacy of xylitol in preventing acute otitis media. Xylitol is detected in saliva of children only for minutes after consuming xylitol chewing gum (a xylitol mixture in doses equal to those used in clinical trials showing xylitol effective in preventing acute otitis media). If xylitol were only a short-term mechanical barrier between bacteria and the host, the efficacy of xylitol could be assumed to be less than the observed 40% decrease in the occurrence of acute otitis media. The altered ultrastructure of pneumococci could affect the adherence and virulence of pneumococci. This effect could persist after short-term xylitol exposure in the oral cavity, explaining the good clinical efficacy of xylitol.

Acknowledgements

This study was financially supported by Maud Kuistila Foundation, Finland and Paediatric Research Foundation, Oulu, Finland.

References


