Original Article

Effects and Safety of Xylitol on Middle Ear Epithelial Cells

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OBJECTIVE: Xylitol is a natural sugar known to reduce dental decay. Recently, several epidemiology studies have also shown that xylitol can prevent acute otitis media in children. It is assumed that these effects are related to the inhibition of bacterial growth in the nasopharynx. However, the effects and safety of xylitol in middle ear epithelial cells, which play a key role in otitis media, have not been investigated. The present study was performed to investigate whether xylitol shows antiinflammatory or antioxidative effects on human middle ear epithelial cells and to assess its safety profile for clinical use.

MATERIALS and METHODS: We induced inflammation in human middle ear epithelial cells (HMEEC) with lipopolysaccharide (LPS). After xylitol treatment, we measured expression levels of the inflammatory cytokines TNF-α and COX-2 and the mucin gene MUC5AC using semiquantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot analysis. Cell viability and morphological changes following xylitol treatment were investigated by 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MMT) assay, flow cytometry, and Hoechst 33342 staining in human middle ear epithelial cells and mouse inner ear cells.

RESULTS: Inflammatory cytokines and mucin gene expression were unaffected by xylitol. In addition, xylitol showed neither antioxidative effects nor cytotoxicity and did not stimulate apoptosis or necrosis of human middle ear epithelial cells or inner ear cells, even at high doses.

CONCLUSION: Xylitol did not show antiinflammatory or antioxidative effects on HMMEC. It showed no toxicity in middle ear and inner ear cells. Xylitol may therefore be used safely to prevent acute otitis media.

KEY WORDS: Xylitol, human middle ear epithelial cells, otitis media, cytotoxicity

INTRODUCTION

Acute otitis media (AOM) is one of the most common infectious diseases in children, and is the most common cause of paediatric visits to the physician. By the age of 1 year, two-thirds of children will have experienced at least one episode of AOM, and by the age of 3 years, four in five children will have experienced at least one episode [1, 2]. In addition, AOM is the common cause of antibiotic use in children, which is both costly and can increase the likelihood of the emergence of antibiotic-resistant bacterial strains. Surgery is also an expensive and invasive procedure. There are approximately 2.2 million diagnosed episodes of AOM annually, with the cost estimated at $3-5 billion in the USA alone [2, 3]. Due to concerns over antibiotic treatment and surgery, effective and safe prevention of AOM would potentially have a large impact on public health.

However, no effective and safe preventive treatments for AOM are currently available. Xylitol is a five-carbon sugar alcohol that has been used to prevent dental caries. Several studies in children demonstrated the preventive effects of xylitol on AOM [4-8]. In addition, 5% xylitol was shown to inhibit the growth of *Streptococcus pneumoniae* and addition of xylitol to the medium had some inhibitory effects against other bacteria in vitro [9]. To our knowledge, however, there have been no studies regarding the safety of xylitol for the prevention of AOM. In addition, although xylitol is a natural sugar and has been approved for use in foods, its precise mechanism of action in the prevention of AOM is not known [10]. An inhaled form of xylitol has recently been administered to young children who cannot chew, allowing direct exposure to xylitol [11, 12]. This prompted us to investigate the mechanism of action and safety of xylitol in middle ear infections.

The present study was performed to investigate whether xylitol affects inflammation and cell viability in middle ear and inner ear cells. For this purpose, we measured the levels of inflammatory cytokines and mucin expression in human middle ear epithelial cell lines (HMEECs) after induction of inflammation by lipopolysaccharide (LPS). We investigated cytotoxicity, apoptosis, and necrosis in middle ear and inner ear cells. In addition, we measured the antioxidative effects of xylitol, as antioxidants are known to reduce middle ear inflammation.

MATERIALS and METHODS

This study was approved by the Soonchunhyang University Bucheon Hospital Institutional Review Board.

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Cell Culture

Human middle ear epithelial cells are immortalised human middle ear epithelial cells produced using a retrovirus containing the E6/E7 genes of human papillomavirus type 16 [13]. HMEECs were kindly provided by Dr. David J. Lim (House Ear Institute, Los Angeles, CA, USA). Cells were maintained in a mixture of Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY, USA) and bronchial epithelial cell basal medium (BEBM) (1:1) supplemented with bovine pituitary extract (52 μg/mL), hydrocortisone (0.5 μg/mL), human epidermal growth factor (hEGF; 0.5 ng/mL), epinephrine 0.5 (mg/mL), transferrin (10 μg/mL), insulin (5 μg/mL), triiodothyronine (6.5 ng/mL), retinoic acid (0.1 ng/mL), gentamycin (50 μg/mL), and amphotericin B (50 ng/mL). Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2.

HEI-OC1s are immortalised mouse inner ear cells produced using transgenic mice harbouring a temperature-sensitive mutant of the SV40 large T antigen under the control of an interferon-γ-inducible promoter [14]. HEI-OC1s were kindly provided by Dr. Federico Kalinec (House Ear Institute). HEI-OC1s were maintained in DMEM containing 10% foetal bovine serum (FBS; JRH Biosciences, Lenexa, KS) and 50 U/mL interferon-γ without antibiotics. HEI-OC1s were maintained in DMEM supplemented with 10% FBS at 33°C under 10% CO_2. Growth medium was changed every third day. After 6 days, the cells were used for the following studies.

Induction of Inflammation Using LPS

HMEECs were treated with 100 ng/mL LPS (Sigma Chemical Co., St. Louis, MO, USA) for 24 h to induce inflammation. Inflammation was confirmed by measuring TNF-α and COX-2 responses by RT-PCR and Western blot analysis. The effects of xylitol on middle ear inflammation were assessed by measurement of TNF-α and COX-2 by RT-PCR and Western blotting, and MUC5AC expression was assessed by RT-PCR.

RT-PCR

Total RNA from HMEECs was extracted using RNeasy® Mini Kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions (RNeasy handbook, June 2001). The primer sequences of the genes used in this study are shown in Table 1. RNA was reverse transcribed by incubation with 10 mM dNTP, 0.1 M DTT, 1 μL oligo(dT) (500 μg/mL), and 1 μL SuperScript® II (200 U/μL; Life Technologies, Grand Island, NY, USA) at 42°C for 50 min, followed by heat inactivation at 70°C for 15 min. Following reverse transcription, aliquots of the cDNA were transferred into tubes containing specific primer pairs for TNF-α, COX-2, MUC5AC, MUC5B, and β-actin genes. PCR amplification was performed in a thermocycler for 35 cycles of 20 s at 94°C, 10 s at 55°C, and 30 s at 72°C, with an initial denaturation at 94°C for 2 min and a final extension at 72°C for 5 min. PCR products were electrophoresed on 1% agarose gels and visualised by ethidium bromide staining alongside a 100-bp DNA ladder (Bioneer Co., Daejon, Korea). The results shown are from three experiments, each performed in triplicate.

For semiquantitative analysis of the RT-PCR results, gel images were scanned and the intensity of the PCR products was measured using a Scion imager (Scion, Frederick, MD, USA). Relative mRNA expression was calculated by determining the ratio to β-actin expression.

Western Blot Analysis

The studied cells were incubated in 0.4 mL ice-cold lysis buffer (150 mmol/L NaCl, 20 mmol/L Tris, pH 7.5, 1 mmol/L EDTA, 0.1% Triton X-100) containing 0.5% protease inhibitor cocktail III (Calbiochem, San Diego, CA). The cells were centrifuged at 13,000×g for 25 min at 4°C, and the supernatants (total cell lysates) were collected and stored at -70°C. The protein concentration was determined using an RC DC Protein Assay kit according to the manufacturer's protocol (Bio-Rad, Hercules, CA, USA).

Aliquots of 25 μg of protein were mixed with sample buffer (Bio-Rad) containing 2% mercaptoethanol, boiled for 5 min, and separated by gel electrophoresis on 12% Tris-HCl gels. The lysates were then transferred onto nitrocellulose membranes (Hybond ECL; Amersham Biosciences, Piscataway, USA), and the membranes were blocked with phosphate buffered saline (PBS), 0.1% Tween-20 containing 5% (w/v) dried milk, and 1% bovine serum albumin for 1 h at room temperature. Membranes were probed with COX-2 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), β-actin (Merck Biosciences, San Diego, USA), or control goat immunoglobulin G, followed by donkey anti-goat immunoglobulin G coupled to horseradish peroxidase (1:10,000; Jackson Immuno Research Laboratories Inc., West Grove, USA). The membranes were developed using an ECL detection kit (Pierce, Rockford, IL) and exposed to X-ray film (XAR5; Kodak, Rochester, USA). Results were obtained from three repeated experiments.

1,1-Diphenyl-2-Picrylhydrazyl (DPPH) Assay

Xylitol was diluted in dimethyl sulphoxide (DMSO) to each experimental concentration (0, 0.1, 1, 5, 10, 50, 100, 500, and 1000 μg/mL). A 1 M stock was made with DPPH powder (Sigma Aldrich, St. Louis, USA) in 95% ethanol. CAPE (diluted in DMSO) was diluted twofold in 50% EtOH, and DPPH was diluted 20-fold in 95% EtOH (DPPH solution). Each well was then incubated with DPPH solution and reaction mixture. The DPPH solution was added to each well and incubated at room temperature for 30 min in the dark. The absorbance was measured using a SpectraMax Plus384 Microplate Reader (Molecular Devices, Sunnyvale, USA) at 540 nm. Five wells were measured for each concentration. Results were obtained from three repeated experiments. Scavenging activity was calculated using the following formula:

Scavenging activity = [1-(Xylitol value-blank)/Control]×100

Table 1. Oligonucleotide primers and expected product sizes

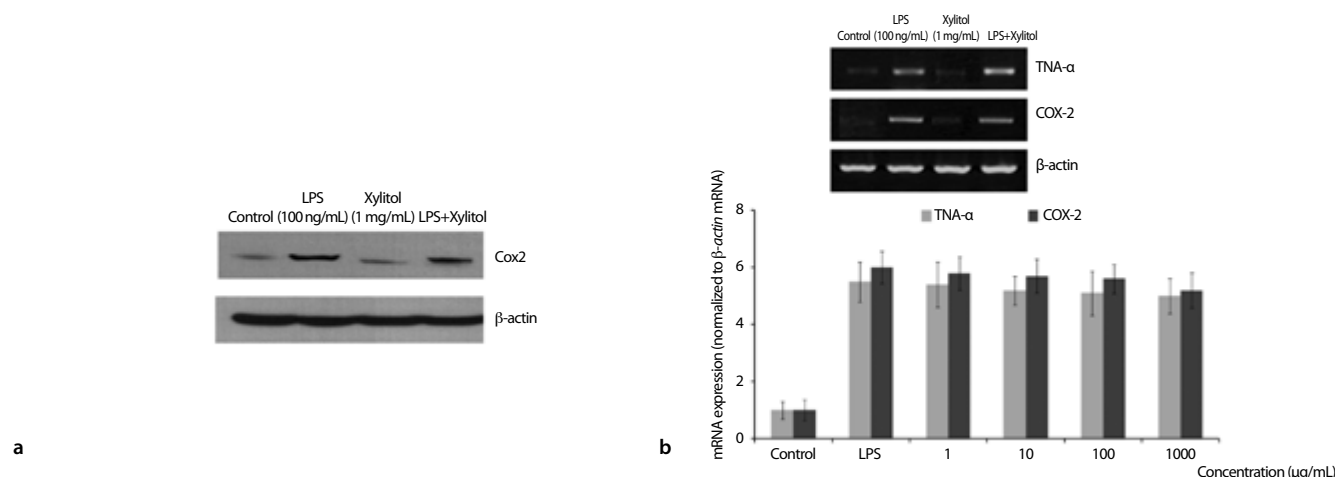| Primer	Forward	Reverse	Product Size (bp)
TNF-α	AAGACCTTCTCCCCATTCTG	TTCCTCTGTGTGTGGATCTAG	269
COX-2	GTCAAAACCGAGGTGTATGT	TGTGATCTGGATGTCAACAC	247
MUC5AC	CCTCTGCAACTACGTGTTCTC	GTGGTTCCACATGAGGACAAG	271
β-actin	GGACTTCGAGCAAGAGATGG	AGCACTGTGTTGGCGTACAG	250

Figure 1. a, b. Effects of xylitol on LPS-induced inflammation in HMEECs. Xylitol did not affect the expression of TNF-α or COX-2 by LPS-induced inflammation in HMEECs. RT-PCR of TNF-α or COX-2 (a), Western blotting of COX-2. Bands were quantified by densitometry and the expression levels of each gene were calculated relative to the internal control, β-actin. The data shown are means±standard deviations of three repeated experiments with triplicate samples (b)

HMEEC: human middle ear epithelial cells; LPS: lipopolysaccharide RT-PCR: reverse transcriptase-polymerase chain reaction

Cell Viability Assays

Cell viability was measured using the 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) assay (Sigma Aldrich, St. Louis, MO, USA). HMEECs were seeded in 96-well plates, with each well containing 7×10^3 cells. After 24 h, the culture medium was replaced with that containing xylitol at concentrations of 0, 1, 10, 100, or 1000 μg/mL for 24 h. Six wells were used for each concentration, and 40 μL fresh MTT (5 g/L DW) was added to each well. After incubation for 4 h at 37°C, the culture medium was aspirated and formazan crystals, formed from MTT by NADH-generating dehydrogenases in metabolically active cells, were dissolved by the addition of 100 μL DMSO to each well. Plates were then shaken at room temperature for 30 min, and the optical density at 595 nm (A_{595}) was measured using a microplate reader.

Annexin V-FITC/Propidium Iodide Double Staining and Flow Cytometry

Annexin V-FITC/propidium iodide staining was used to quantify the percentages of apoptotic HMEECs and HEI-OC1s. Cells were seeded in six-well culture plates after treatment with xylitol at 1 mg/mL for 24 h. Cells were collected, washed with PBS, and resuspended in 1× binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$). Cells were transferred into FACs tubes and stained using an Annexin V-FITC apoptosis detection kit in accordance with the manufacturer's protocol (Becton Dickinson, San Diego, USA). Cells were then measured using a flow cytometer (Beckman Coulter, Fullerton, USA).

Hoechst 33342 Staining

The nuclei of the HMEECs and HEI-OC1s were stained with the chromatin dye Hoechst 33258. Cells were washed twice with PBS and fixed with 3.7% glutaraldehyde for 10 min at room temperature. After fixation, cells were washed twice with PBS and incubated with 10 μg/mL Hoechst 33258 (Sigma Aldrich, St. Louis, MO, USA) for 10 min at room temperature in the dark. Cells were washed twice in PBS and imaged using an inverted fluorescence microscope (BX61; Olympus, Tokyo, Japan).
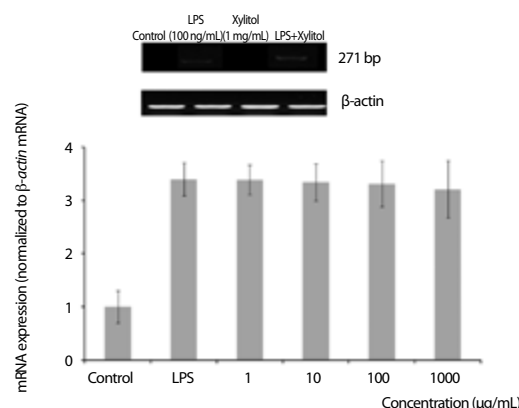

Figure 2. Effects of xylitol on mucin gene expression following LPS treatment in HMEECs. Xylitol did not affect the expression of MUC5AC by LPS in HMEECs. Bands were quantified by densitometry and the expression levels of each gene were calculated relative to the internal control, β-actin. The data shown are means±standard deviations of three repeated experiments with triplicate samples

HMEEC: human middle ear epithelial cells; LPS: lipopolysaccharide

Statistical Analysis

All data are expressed as means±standard deviations. One-way analysis of variance was used to determine statistical significance of differences between control and test groups at each time point or dose. Scheffé's F-test was used to correct for multiple comparisons when statistically significant differences were identified by analysis of variance. Statistical analyses were performed using SPSS version 11 (SPSS Inc., Chicago, USA). In all analyses, $p < 0.05$ was taken to indicate statistical significance.

RESULTS

Xylitol Did Not Affect the Expression of Inflammatory Cytokines or Mucin Genes Following LPS Treatment in HMEECs

Inflammatory responses in HMEECs were elicited by incubation with 100 ng/mL LPS for 24 h, and RT-PCR (Figure 1a) and Western blotting (Figure 1b) were performed to assess the upregulation of TNF-α and COX-2. As shown in Figure 1, xylitol (1, 10, 100, and 1000 μg/mL) did not affect TNF-α or COX-2 expression.
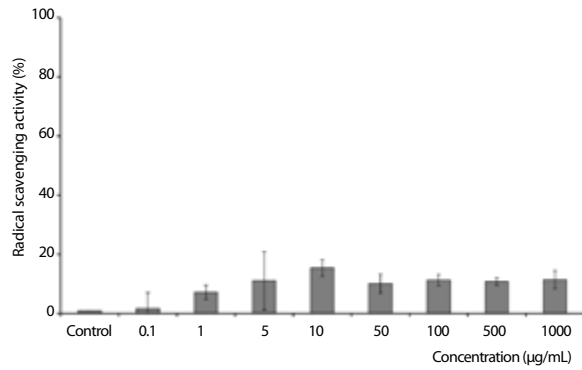
Figure 3. Radical scavenging effects of xylitol. Xylitol did not show any radical scavenging effects in DPPH assays. The data shown are means±standard deviations of three repeated experiments with triplicate samples

DPPH: diphenylpicrylhydrazyl

The expression of MUC5AC in HMEECs was significantly increased by stimulation with LPS for 24 h at a concentration 100 ng/mL. Xylitol at 1, 10, 100, and 1000 µg/mL did not affect the expression of MUC5AC as determined by RT-PCR (Figure 2). In addition, xylitol did not show any antioxidative effects in DPPH assays (Figure 3).

Xylitol Did Not Affect Cell Viability or Morphology of HMEECs and HEI-OC1

Xylitol (1000 µg/mL) did not affect cell viability as assessed by MTT cell viability assays in HMEECs and HEI-OC1s (Figure 4a). Flow cytometry showed that xylitol at concentrations of up to 1000 µg/mL did not induce apoptosis or necrosis in HMEECs and HEI-OC1s (Figures 4b, 4c). The morphology of HMEECs and HEI-OC1s showed no changes on exposure to xylitol at concentrations of up to 1000 µg/mL (Figures 4c, 4d).
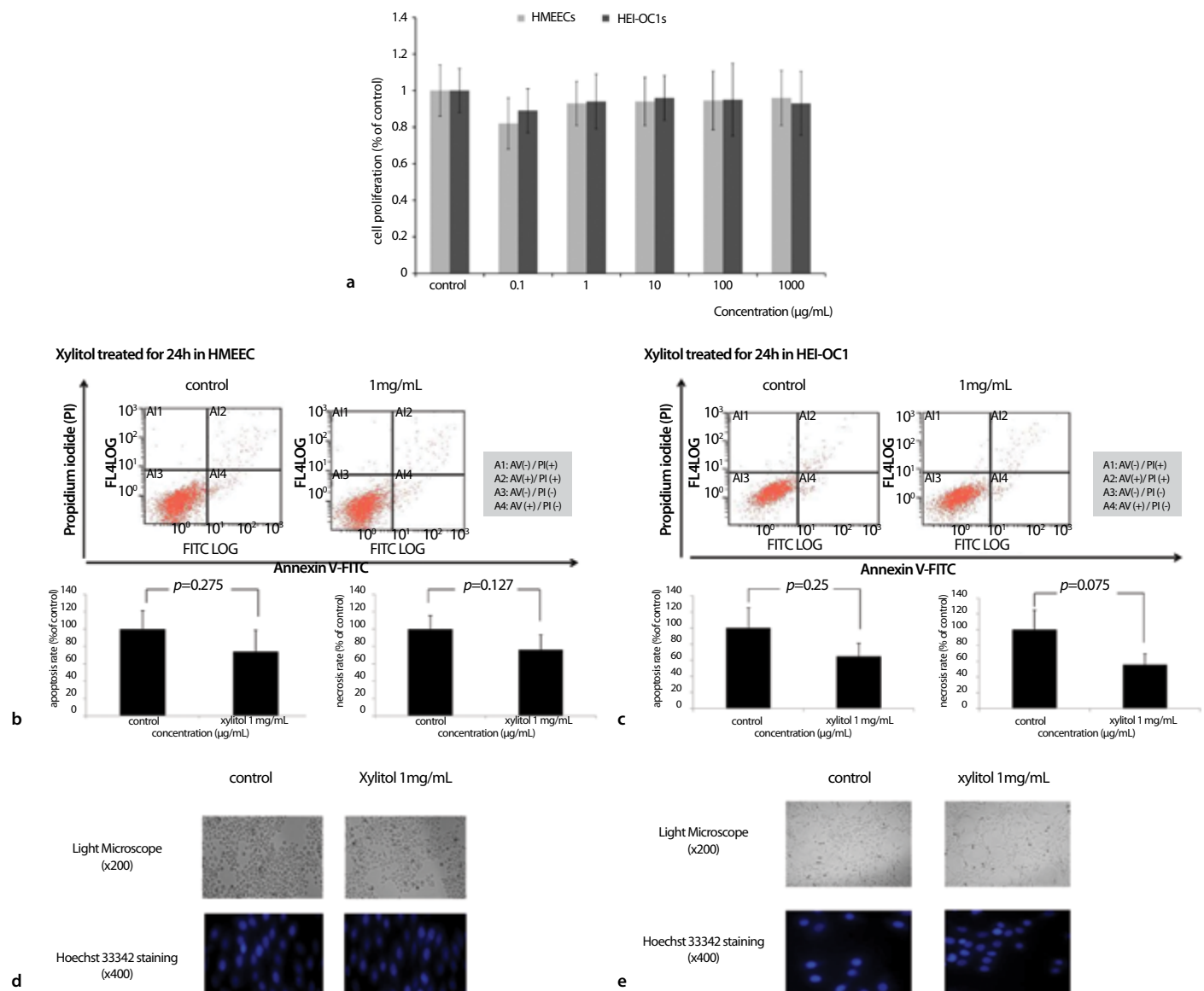

Figure 4. a-e. Effects of xylitol on viability of HMEECs and HEI-OC1s. Xylitol at concentrations of up to 1000 µg/mL did not decrease the viability of HMEECs and HEI-OC1s (a). Xylitol at concentrations of up to 1000 µg/mL did not induce apoptosis or necrosis of HMEECs and HEI-OC1s (b, c). Morphology of HMEECs and HEI-OC1s remained unchanged by xylitol at concentrations of up to 1000 µg/mL (light microscope, x200 and Hoechst 33342 staining, x400). The data shown are means±standard deviations of three repeated experiments from six samples (c, d)

HMEEC: human middle ear epithelial cells

DISCUSSION

In this study, we investigated the effects and safety of xylitol in middle ear epithelium. Xylitol showed no antiinflammatory or antioxidative effects on middle ear epithelium *in vitro*. In addition, xylitol showed no cytotoxicity and did not induce apoptosis or necrosis.

Xylitol is a natural sugar found in birch, plums, strawberries, raspberries, and cauliflower, and is produced in small quantities during human metabolism [15, 16]. Chemically, xylitol is a pentitol or 5-carbon polyol sugar alcohol. The effects of xylitol on dental caries were first described in the early 1970s. Although xylitol is a sugar alcohol, it is not fermented by cariogenic bacteria in the oral cavity. Thus, it is considered an ideal non-sugar sweetener. Currently, xylitol is widely used in chewing gums, toothpaste, foods, and medicines. In the UK, approximately 1000 tons of xylitol are consumed per year [10, 16]. Xylitol is non-fermentable, so it does not encourage bacterial growth. In addition, exposure to xylitol induces mutant xylitol-resistant strains, which are less virulent in the oral environment. Xylitol acts in a bacteriostatic fashion due to the formation of intracellular vacuoles and the degradation of bacterial cell membranes [10, 16]. Interestingly, it has been reported that when mothers chew xylitol gum, their children display a significantly reduced occurrence of dental caries [17].

Prophylactic administration of xylitol to healthy children in daycare centres reduces the occurrence of AOM by 25-40% [4-7]. Xylitol inhibits the growth of *S. pneumoniae* and reduces the adherence of *S. pneumoniae* and *Haemophilus influenzae* to nasopharyngeal cells [9, 18]. In addition, xylitol can lower the airway surface salt concentration and enhance innate immunity in airway epithelia *in vitro* [19]. Nasal administration of xylitol to nasal mucosa in normal human subjects decreases colony-forming units of coagulase-negative staphylococci [19].

Xylitol has few reported side effects. In adults, xylitol is well tolerated at up to 100 g per day [15, 20]. Oral xylitol solution at doses of 5 g three times per day and 7.5 g once daily is well tolerated by young children [8]. The side effects of xylitol include a dose-dependent osmotic laxative effect [16, 20]. In addition, inhalation of aerosolised xylitol was reported to be well tolerated in naïve and atopic mice and healthy human volunteers [11].

Immortalised human middle ear and mouse inner ear cell lines have been shown to be useful for drug screening and ototoxicity studies [21, 22]. Mucin gene expression is similar in immortalised HMEECs and human middle ear epithelial specimens from in vivo middle ear tissue [22].

Although the preventive use of xylitol in AOM is an attractive option, xylitol is not widely used in this infectious disease. The major limitation of xylitol use in AOM patients is the frequent dosing schedule required; xylitol should be administered 3-5 times per day, which is not convenient for regular use. Gastrointestinal side effects are also a concern, particularly in young children. While chewing is an attractive route of administration in children, it is not applicable in very young infants. Thus, non-oral administration, such as the transnasal route, is required. Attempts to deliver xylitol in an inhaled form for the treatment of lower respiratory tract colonisation in chronic lung diseases, such as cystic fibrosis and dyskinetic cilia syndrome, have been made [11, 12]. In addition, transnasal approaches represent an alternative route for xylitol administration in cases of AOM.

In conclusion, our data indicate that the effects of xylitol on otitis media are related to nasopharyngeal bacterial colony inhibition or the inhibition of bacterial growth rather than any antiinflammatory or antioxidative effects in the middle ear epithelium. Further, in vivo studies and clinical trials are needed to confirm both the effects and safety of more direct use of xylitol in the middle ear.

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