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Xylitol inhibits J774A.1 macrophage adhesion *in vitro*

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ABSTRACT

The aim of this work was to evaluate the effect of xylitol on J774A.1 macrophage adhesion. Adhesion consisted of a three-hour interval, at room temperature, followed by washing and cell incubation at 37°C/5% CO_2/ 48h. Xylitol was used to treat the cells either before (for 24h) or after the cell incubation (for 48h) at 5% as final concentration in both the situations. It was found that xylitol was effective in preventing the adhesion in both the conditions in spite of the former being 100-fold greater and significant ($p < 0.001$). The results pointed to an important xylitol action on macrophage adhesion, which should be further investigated as an inflammatory control.

Key words: adhesion, J774A.1 macrophages; xylitol

INTRODUCTION

Macrophages are essential cellular components of the body´s host defense system, having critical functions in both native and acquired immunity. The detection of pathogens is first carried out by these sentinel cells of the innate immune system located in the tissues that are in contact with the host's natural environment. As participants in innate immunity, macrophages protect the host during the early phase of an infection (Tiwari et al., 2008). Hence, it is believed that these cells can be involved in steady-state tissue homeostasis via the clearance of apoptotic cells and the production of growth factors. In the macrophages surface, there is a broad range of pathogen-recognition receptors that make them efficient in phagocytosis and induce the production of inflammatory cytokines (reviewed in Geissmann et al., 2010).

Macrophages have presentation antigen functions with antigen uptake and processing capabilities, expression of MHC class II and co-stimulatory molecules. Upon encounter with an antigen, these can release a variety of reactive oxygen species (superoxide anions, hydrogen peroxide and nitric oxide) as well as pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6) (reviewed by Mosser, 2003). Macrophages are adherent cells, seem to be obligatory for T cell activation induced by the concanavalin A (a mannose-binding lectin) and for B cell activation induced by the dextran-sulphate (Persson et al., 1978). Furthermore, macrophages need to adhere

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to tissue surface to play their role. It could be a desired situation, e.g., in wound healing and remodeling of tissue. However, if an inflammatory process, like atherogenesis, occurs, macrophage adhesion to a tissue leads to a deleterious action (Lasser, 1983).

Xylitol is a non-immunogenic and safe carbohydrate (Zopf and Roth, 1996). There are few works reporting its action on the immune system. Takahashi et al. (2005) found that dietary xylitol was a more useful carbohydrate source than glucose to alleviate the growth during the immunological stress. This occurred because the metabolic energy used in the test diets (glucose or xylitol, 150 g/ kg) was 13.1 MJ/ kg and the effect of xylitol on the growth was not caused by an increase in the dietary energy density. Han et al. (2005) demonstrated that xylitol was capable of inhibiting *Porphyromonas gingivalis* lipopolysaccharide (LPS)-induced gene expression and protein synthesis of TNF-α and IL-1 beta in the immunological cells. Xylitol also inhibited the LPS-induced nuclear factor kappa B activation in RAW 264.7 cells.

Another useful ability attributed to this compound (Sajjan et al., 2004, Masako et al., 2005, Ferreira et al., 2008), investigated in the present work also, concerns xylitol inhibition of cell adhesion to a surface, which is an effective and safe form of infection control (Zopf and Roth, 1996).This study is a first phase of an investigation about xylitol influence on J774A.1 macrophage adhesion with promising and innovative results, and considering the importance of this cell adhesion on the first steps of inflammation processes (Foxwell et al., 2001; Hidemura et al., 2003, Kadl and Leitinger, 2005).

MATERIAL AND METHODS

Macrophages from the J774A.1 cell line (purchased from American Type Culture Collection, Rockville, MD, USA) were cultured in the complete RPMI-1640 medium (Gibco BRL, Scotland, UK), supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, and 100 $U.mL^{-1}$ penicillin and 100 $\mu g.mL^{-1}$ streptomycin in a humidified chamber (Thermo Fisher Scientific Inc., Ohio, USA) at 37 ^{o}C and 5% CO_2 atmosphere. Initial macrophage concentration was 10^6 $cells.mL^{-1}$ of medium.

The cell viability under different conditions was determined by a modified Mosmann assay that employed mitochondrial-dependent reduction of MTT to formazan as previously described (Mosmann, 1983). The cells were incubated with 5% xylitol for 48 h. Afterwards, they were pulsed with 10 µL of MTT reagent (5 $mg.\ mL^{-1}$) for 3 h at 37 ^{o}C, followed by 10 minutes incubation with 100 µL of stop-solution (isopropanol and sodium duodecil sulfate, 1: 2, v/v). After this period, absolute optical densities were read at 570 nm in a microplate reader (Molecular Devices, USA). As a control, the cells were treated with 10% formaldehyde during 10 minutes. Cells only in RPMI medium was cultivated and used as the control of 100% viability. This assay was carried out in triplicate.

The influence of xylitol (Fluka BioChemika, Switzerland) on the macrophage adhesion was evaluated in two different conditions. In the first one, a standardized number of cells was treated for 24 h prior to adhesion and in the other, xylitol was used to treat the cells for 48 h after the adhesion. In both cases, xylitol was used at 5.0% as final concentration. The adhesion consisted of a three-hour period at room temperature in 24-well culture plates containing a cover slip. Non-adherent cells were removed by washing in RPMI medium and a new medium was substituted. Culture supernatants were harvested after 48 h incubation and macrophages were washed with 1.0 mL PBS pH 7.2, fixed with cold methanol and stained with *Giemsa* for absolute counting (optical microscopy, 1000 X magnification). Two independent assays were carried out in duplicate. TNF-α, IL-1, IL-10, IL-12(p40) in the culture supernatants were measured, individually, by two-site sandwich ELISA (BD Biosciences, CA, USA). The standard curve was obtained with recombinant mouse cytokine. The minimal detectable concentration in the test was 31.2 ng/mL for IL-10 and 15.6 ng/mL for TNF-α, IL-1 and IL-12(p40). The reaction was developed with peroxidase-conjugated streptavidin, followed by the substrate mixture containing tetramethylbenzidine (TMB) as chromogen. Absorbances were determined at 450 nm in an ELISA plate reader (Molecular Devices, USA). Nitric oxide (NO) was determined using the method developed by Green et al. (1982).

Kruskal-Wallis test was performed to detect the significant difference between the control and the effect of xylitol used to treat the cells before and

after the adhesion, followed by Dunn´s multiple comparison test. Differences in inflammatory mediators between the groups were evaluated using the same test. Differences were considered to be significant when $p < 0.05$.

RESULTS

Although 5% xylitol did not interfere with the J774A.1 cells viability when compared to the control (cells on RPMI) ($p = 0.675$), it influenced the cell adhesion under both the assayed conditions. The non-parametric test could not detect a difference between the control group (median = 6750, Q_3-Q_1 = 5899) and the experimental one (median = 622, Q_3-Q_1 = 549) when xylitol was used after the adhesion**.** However, it was observed that on the control cover slips, the number of adhered cells was ten times higher. The difference can be clearly visualized in Figure 1.

Microphotographies, obtained in a representative situation of each experimental condition showed that xylitol was effective when used to treat the cells before the adhesion (Fig. 2B) and after (Fig. 2A) when compared to the control (Fig. 2C). However, in the latter situation, a greater action was achieved. Xylitol was still effective in inhibiting the cell surface adhesion when used after the adhesion.

Interleukins and NO levels were presented in Table 1.

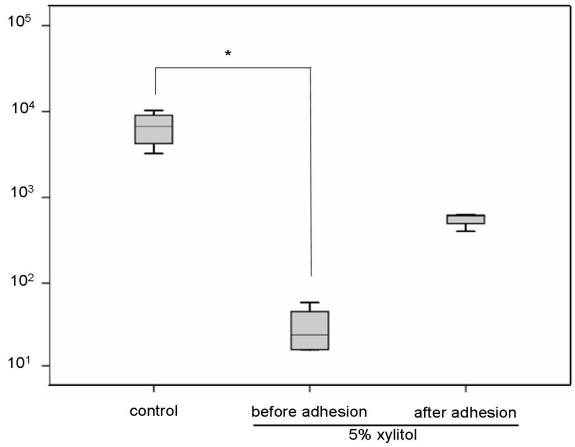

Figure 1 - Effect of 5% xylitol on macrophage J774A.1 adhesion *in vitro*. Xylitol was effective on inhibiting cell adhesion both when used to treat cells before and after adhesion. Each point represents the median and the bars, interquartile range. Kruskal Wallis followed by Dunn´s test was performed (*$p < 0.001$). Data are representative of two independent assays carried out in duplicate.

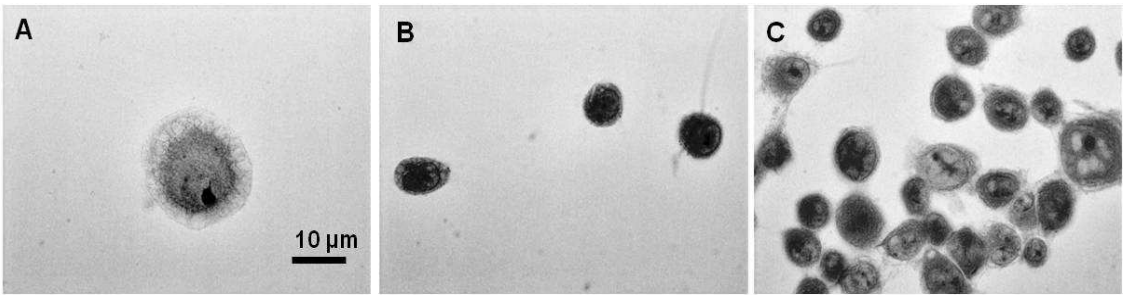

Figure 2 - Effect of 5% xylitol on macrophage J774A.1 adhesion *in vitro*. Period of adhesion consisted of one three-hour interval, at room temperature, followed by washing and medium replacement, and cell incubation for 48 hours, 37°C, CO_2 5%. A) Xylitol used to treat cells for 24 hours before adhesion; B) Xylitol added in the medium after adhesion and C) Control, without xylitol. Microphotographies were representative for each tested condition. Bar scale: 10 µm.

Table 1 - Inflammation modulators levels in culture supernatant, TNF- α , IL-12, IL-10 and IL-1 in pg. mL^{-1} and NO in µM. Measures were performed in triplicate using samples obtained in two different assays (n = 6). Results were expressed as mean ± standard deviation.

	NO	TNF-α	IL-12	IL-10	IL-1
Control	62 ± 2^{a}	21 ± 1^{c}	18 ± 3	ND	ND
XOH before	7 ± 2^{b}	9 ± 1^{d}	ND	ND	ND
XOH after	12 ± 2^{b}	9 ± 2^{d}	ND	ND	ND

ND: not detected. XOH: xylitol. Xylitol (at 5 %) was added before or after adhesion. [a,b] NO was different between control group and the both treated with xylitol ($p < 0.001$); [c,d] TNF-α was different between control group and the both treated with xylitol ($p < 0.001$). Test used: Kruskal Wallis followed by Dunn´s multiple comparison test.

DISCUSSION

Xylitol is a GRAS compound widely used in oral products due to its non- and anti-cariogenic properties (Pepper and Olinger, 1988; 'Lon' Jones, 2003). The variety of xylitol applications, such as candies, chewing gums, tooth paste (Steibnerg et al., 1992, Kontiokari et al., 1998; Vernacchio et al., 2007) highlights its potential as a component of such products. Here, as expected (Ferreira et al., 2008), xylitol did not influence the viability of the cultures tested. Other authors (Naaber et al., 1996; Zabner et al., 2000; 'Lon' Jones, 2003; Sajjan et al., 2004; Masako et al., 2005) have found similar results. The un-detected levels of IL-1, IL-10 and IL-12 could be explained due to the lower concentration of macrophages. Since they had not been adhered to the cover slips, they were discarded when the plate was washed. In the control, 4000 cells were estimated in 1.0 mL of RPMI medium, which was really diluted in the supernatant. If xylitol was added before the adhesion, the number of cells was very small (< 40 cells/ well). Hence, it was not possible to conclude if the lower NO and TNF-α levels was due to xylitol or also because of the lower number of macrophages in these situation. These determinations should also be done with a phlogiston agent, such as LPS, added in the culture medium.

The ability of exogenous sugars to block these specific receptors and/or to competitively displace bacteria from their attachment sites on the cells may provide an adjunctive anti-inflammatory and/or antimicrobial treatment (Lloyd et al., 2007). Xylitol used to treat the cells after the infection acted in a non-specific, but satisfactory manner to inhibit the cell adhesion to a surface. Surprisingly, when macrophages were previously treated with 5% xylitol, less than 40 cells adhered per cover slip. This was a preliminary study and only 5% xylitol was tested, since it was the concentration most studied in the literature.

Carbohydrates play a key role in cell adhesion. Complex carbohydrates coat the surfaces of the cells and have the potential to carry the information necessary for the cell-cell recognition. The pretreatment of the isolated lymphocytes with mannose-6-phosphate, or mannose phosphate polysaccharide resulted in binding inhibition. More specifically, mammalian alveolar macrophages adhered to polyacrilamide surfaces derivatized with mannose residues. Adhesion was blocked by the soluble D-mannose, suggesting that a reported cell-surface carbohydrate receptor on macrophages can mediate the cell adhesion (Brandley and Schnaar, 1986).

In the literature, few works describe the effect of carbohydrates (different to xylitol) on the macrophage adhesion. In a recent study (Chen et al., 2010) a possible explanation for macrophage adhesion was addressed. Polysaccharide-rich fractions of *Angelica sinensis* used to treat the peritoneal macrophages improved the immunoreactivity through an increase in the intracellular adhesion molecule-1 (ICAM-1) and Toll-like receptor 4 (TLR 4) gene expressions. ICAM-1 importance for the cell adhesion and inflammation was previously reported (Perreti et al., 1996) and it was demonstrated that dexamethasone attenuated the inflammation acting on ICAM-1 expression.

This was the first study showing the action of the xylitol on an immunological cell adhesion. The adherence is a crucial step in inflammation. Xylitol could be an important adjuvant due to its action on the macrophage cells, which extends its compound action on related microbial growth control. These are promising results, showing the potential biopharmaceutical uses for xylitol in macrophage adhesion cells.

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