Anti-inflammatory properties of xylitol in a model of chronic sinus disease

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Abstract

**Background.** Chronic rhinosinusitis is a highly prevalent condition estimated to affect 9% of the adult population in Australia. It is responsible for a significant burden of disease at a population level and imparts considerable morbidity and reduced quality of life to affected individuals. Recent American data has suggested an $8.6 billion annual direct healthcare cost associated with chronic rhinosinusitis. Few medical options for treating chronic rhinosinusitis exist and there is a paucity of evidence in support of a best medical treatment. Currently available treatments confer a modest benefit.

The hallmark of chronic rhinosinusitis is chronic inflammation of the nasal and sinus mucosa lasting in excess of twelve weeks and resulting in typical symptoms. Chronic rhinosinusitis is a multi-factorial disease for which there are many postulated contributory factors. Although the aetiology of the disease is yet to be fully elucidated, current literature suggests the primacy of immune dysfunction in its pathogenesis and treatment recalcitrance. Characteristic inflammatory cell infiltrates and cytokine response have been identified for chronic rhinosinusitis subtypes. Inflammatory mediators with altered activity and/or expression in chronic rhinosinusitis represent potential therapeutic targets.

**Aims and Objectives.** This study sought to investigate the anti-inflammatory and immune modulating activity of xylitol, an agent that has anecdotal utility in CRS and established antibacterial and antibiofilm actions. In order to achieve this aim inflammatory mediator targets were identified and the activity of xylitol against their production was examined relative to that of established anti-inflammatory agents. A further objective in this study was to establish a cell-based *in-vitro* model that best resembled the clinical condition of CRS and considered the effect of xylitol on epithelial and immune cells likely to be encountered *in-vivo*.

**Methods.** An *in-vitro* model was developed to assess the anti-inflammatory and immunomodulatory effects of xylitol and the compounds selected for comparison. The mouse macrophage cell line RAW 264.7 and A549 carcinoma human alveolar basal epithelial cell line were used to assess markers of inflammation and immune response in lipopolysaccharide treated cells. Concentration-dependent experiments were performed in this investigation. To confirm that suppressive effects were not due to cytotoxicity, supernatants were assayed for
the enzyme lactate dehydrogenase using the Cayman LDH Cytotoxicity assay. In addition, cell viability was assessed using the resazurin cell viability assay. Nitric oxide levels were assessed by use of fluorometric assay. Interleukin-6, interleukin-8 levels and tumour necrosis factor alpha concentrations determined by BD Cytometric Bead Array Enhanced Sensitivity Flex Set assay measured by Flow Cytometry. While leukotriene B4 and prostaglandin E$_2$ were measured using Cayman Chemical EIA assay. All assays were performed according to the manufacturer’s instructions.

Experiments were conducted in triplicate and statistical differences between groups confirmed by one-way analysis of variance. A $P < 0.05$ was considered statistically significant.

**Results and Discussion.** Xylitol demonstrated a significant effect against prostaglandin E$_2$ production in each of the individual and co-cultured cell lines. In the respiratory cells it additionally decreased nuclear factor kappa B and increased production of tumour necrosis factor alpha. In the co-culture alone it reduced production of Interleukin-6. These results suggest an immune modulating action unique from that of the drugs tested as comparison agents. However, the nature of effect is beyond the scope of this project. Suppression of prostaglandin E$_2$ in particular is considering the central response.

**Conclusions.** The results obtained with this study are suggestive of an immune modulating effect for xylitol. The positive effects shown in the cell lines tested in this research project are encouraging. An anti-inflammatory action in addition to anti-bacterial and anti-biofilm effect (as demonstrated by other authors) may prove useful in the treatment of chronic rhinosinusitis and other inflammatory disorders. Further research investigating the clinical implications of these findings is warranted.
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5-oxo-ETE  5-oxo-eicosatetraenoic acid
AERD  Aspirin exacerbated respiratory disease
AOM  Acute otitis media
ASL  Airway surface liquid
ATP  Adenosine triphosphate
CAM  Complementary and alternative medicine
cAMP  Cyclic adenosine monophosphate
CF  Cystic fibrosis
COX-1  Cyclo-oxygenase one
COX-2  Cyclo-oxygenase two
CRS  Chronic rhinosinusitis
CRSsNP  Chronic rhinosinusitis without nasal polyposis
CRSwNP  Chronic rhinosinusitis with nasal polyposis
CTL  Cytotoxic T lymphocytes
CysLT  Cysteinyl leukotriene
DC  Dendritic cells
DMEM  Dulbecco’s Modified Eagle’s Medium
DMSO  Dimethylsulfoxide
DNA  Deoxyribonucleic acid
DPB  Diffuse pan bronchiolitis
ECM  Extra cellular matrix
ECP  Eosinophilic cation protein
eNOS  Endothelial nitric oxide synthase
FOXP3  Forkhead box P3 transcription factor
GPCR  G-protein coupled cell receptors
GR-α  Glucocorticoid receptor alpha
IgE  Immunoglobulin E
IL-1  Interleukin one
IL-2  Interleukin two
IL-3  Interleukin three
IL-5  Interleukin five
IL-6  Interleukin six
IL-8  Interleukin eight
IL-10  Interleukin ten
IL-13  Interleukin 13
IL-17  Interleukin 17
IL-22  Interleukin 22
IL-25  Interleukin 25
IL-33  Interleukin 33
ILC  Innate lymphoid cells
IFN-γ  Interferon gamma
iNOS  Inducible nitric oxide synthase
iTreg  Induced Treg
IV  Intravenous
LCK  Lymphocyte-specific protein tyrosine kinase
LDH  Lactate dehydrogenase
LPS  Lipopolysaccharide
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>LTi</td>
<td>Lymphoid tissue inducer</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MHP</td>
<td>Mitochondrial hyperpolarisation</td>
</tr>
<tr>
<td>MMP-7</td>
<td>Matrix metalloproteinase seven</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Matrix metalloproteinase nine</td>
</tr>
<tr>
<td>NCR-22</td>
<td>Natural cytotoxicity receptor 22</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NKR+</td>
<td>NK receptor positive</td>
</tr>
<tr>
<td>NLR</td>
<td>NOD like receptor</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NOD-1</td>
<td>Nucleotide binding oligomerisation domain one</td>
</tr>
<tr>
<td>NOD-2</td>
<td>Nucleotide binding oligomerisation domain two</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
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<tr>
<td>nTreg</td>
<td>Naturally occurring Treg</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>PGI2</td>
<td>Prostacyclin</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated upon activation, normal T-cell expressed, and secreted</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROI</td>
<td>Reactive oxygen+n intermediate</td>
</tr>
<tr>
<td>SAE</td>
<td>Staphylococcus aureus enterotoxin</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>T-bet</td>
<td>T-box transcription factor</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of matrix metalloproteinase</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>Th1</td>
<td>Helper T cell type one</td>
</tr>
<tr>
<td>Th2</td>
<td>Helper T cell type two</td>
</tr>
<tr>
<td>Th17</td>
<td>Helper T cell type 17</td>
</tr>
<tr>
<td>TLR-2</td>
<td>‘Toll-like receptor’ two</td>
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<tr>
<td>TLR-9</td>
<td>‘Toll-like receptor’ nine</td>
</tr>
<tr>
<td>TLR-10</td>
<td>‘Toll-like receptor’ ten</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
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<tr>
<td>TXA2</td>
<td>Thromboxane A2</td>
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</table>
1. Introduction

Chronic rhinosinusitis (CRS) is a common condition, characterised by inflammation of the mucous membranes of the nasal cavity and paranasal sinuses.(1) It is responsible for a significant burden of disease at a population level and considerable morbidity and reduced quality of life for affected individuals.(1, 2) Quality of life surveys have found CRS to have a detrimental impact comparable to that of asthma, angina, and chronic lower back pain.(3) It also carries a substantial economic burden to the healthcare system and across the economy with reduced productivity and days off work and school.(2, 4) Annual work days missed due to CRS in the United States are similar to that of asthma (5.67 and 5.79, respectively).(4)

The exact disease prevalence is difficult to quantify due to the lack of a consistent definition and variability in clinical presentation.(5) It is estimated to have a prevalence of 19.7% in Europe,(6) and 15.5% in America when defined as ‘sinus trouble’ exceeding three months duration.(1) The National Health Interview Survey 2008 showed that rhinosinusitis affected 1 in 7 adults in the USA.(4) A recent study in America reported that at least 5% of the population suffer with CRS and that this diagnosis imparts a $772 annual healthcare cost per person. The same study found there to be an $8.6 billion annual health care cost in America due to CRS.(2)

The Australian Bureau of Statistics conducts three yearly surveys into the health of Australians. It defines chronic sinusitis as inflammation of the sinuses for a duration exceeding eight weeks or ‘sinusitis that recurs frequently or lasts for a prolonged period of time (such as 3 months or more)’. (7) According to data collected through these surveys chronic sinusitis is one of the most prevalent health conditions in Australia.(7, 8)

In 2001 over two million people in Australia (10.5% of the population of the time) were estimated to suffer with CRS. Prevalence was slightly higher among women (12.1%) than males (8.8%). The peak was among people aged 45 to 54 years of age.(8) Results were similar in 2004-5. At this time there were 1.8 million people, representing 9.2% of the population, who had CRS. Prevalence remained higher among women (10.9%) than men (7.5%). The most common age groups affected were people aged 55 to 59 and 75 to 79 years of age.(7)
CRS is a multifactorial disease, the aetiology of which is yet to be fully elucidated.\(^{(1, 5, 9)}\) Diverse host and environmental factors including ostial patency, mucociliary clearance, allergy, immune compromise, bacterial and fungal infection have been implicated in its pathogenesis and recalcitrance to treatment.\(^{(1, 5)}\) Association has also been found with environmental factors including air pollution, smoking, and second-hand smoke exposure.\(^{(4)}\) However, none of these have been conclusively shown to contribute to development of CRS.\(^{(4, 5)}\) There is also evidence for a diversity of immunological mechanisms contributing to the pathophysiology of CRS.\(^{(1)}\)

This uncertainty of causation has ensured that the definition of CRS remains contentious and varies according to the investigative setting. A clinical definition may be based on symptoms, severity, duration, and endoscopic or radiological findings.\(^{(1)}\) Symptoms attributed to CRS include nasal obstruction, rhinorrhoea and facial pressure and pain. Some authors define CRS as the presence of the aforementioned symptoms for a duration of at least 12 weeks.\(^{(6)}\)

Despite the lack of a consensus definition and an accepted aetiology for CRS, the broadly understood concept implies chronic (present for more than 12 weeks without resolution) inflammatory changes in the mucosa of the nasal cavity and the paranasal sinuses (which exist in continuum) without suggestion of a specific underlying pathological causative process.\(^{(1, 5)}\)

CRS is a heterogeneous disease in terms of clinical presentation and histology.\(^{(9)}\) Inflammation is a hallmark of the disease and there is ongoing investigation to further characterise the inflammatory cellular and molecular markers associated with CRS (Figure 1). Recent theories suggest an inappropriate immune response to foreign stimuli underlies its pathogenesis.\(^{(10)}\) An impaired mechanical barrier and innate immune response may be an initiating factor toward an inappropriate adaptive response and the development of persistent inflammation.\(^{(10)}\) Research has shown CRS to be associated with a neutrophil, macrophage and lymphocyte response.\(^{(5)}\) When CRS is associated with nasal polyposis a predominant T helper cell type two (Th2) cytokine response and eosinophilia ensues. When polyposis is absent a T helper one (Th1) response predominates.\(^{(5)}\)
Figure 1. Pathophysiologic pathways of chronic rhinosinusitis. CRSwNP—chronic rhinosinusitis with nasal polyps; CRSsNP—chronic rhinosinusitis without nasal polyps.(5)

CRS, according to current opinion, may be divided into CRS with nasal polyposis (CRSwNP) and CRS without nasal polyposis (CRSsNP).(1, 4, 6) This division is made on the basis of differences in the histological and inflammatory profiles of each subgroup (Figure 2). However, it is not certain whether these groups represent distinct entities from an aetiological and pathological perspective.(1, 6, 11)
1.1 Subtypes of CRS

1.1.1 Chronic rhinosinusitis with nasal polyposis

In CRSwNP tissue is characteristically oedematous with epithelial damage, poor vascularisation and a thickened basement membrane. (1, 6) Loose connective tissue and low collagen content are also demonstrated in affected mucosa. (6) Albumin deposition, pseudocyst formation, and subepithelial and perivascular inflammatory cell infiltration are present as well. (1, 6) The inflammatory infiltrate includes lymphocytes, mast cells,
neutrophils and eosinophils. Eosinophils are the predominant cell type and localise around vessels, glands and beneath the epithelium of the mucosa. (1) Eosinophilic inflammation is characteristic in 80% of cases of nasal polyposis. (1) It is the predominant inflammatory profile for the Caucasian population. (6)

Eosinophils and associated cytokines (Interleukin-5 [IL-5], normal T-cell expressed, and secreted [RANTES], eosinophilic cation protein [ECP], and eotaxin) are found in increased concentrations in patients with CRSwNP. (1, 6) IL5 is increased in people with nasal polyposis when compared to healthy populations and compared to people with CRSsNP regardless of atopic status. (1, 11) It is said to have a significant role in eosinophil recruitment, activation, and inhibition of eosinophil apoptosis. (1, 13) Eosinophilic inflammation is further enhanced when *Staphylococcus aureus* enterotoxins acts as a superantigen resulting in IgE response. (13)

Interestingly, Chinese people with CRSwNP have shown a neutrophil predominant inflammatory pattern with a higher concentration of IL-17. (6, 13) Studies have also shown that in patients with cystic fibrosis or primary ciliary dyskinesia and CRSwNP the primary inflammatory cells are neutrophils and lymphocytes and that the concentration of ECP is significantly lower compared to other caucasians with CRSwNP. (1, 6, 13) The histology of the sinus mucosa in these patient groups shows T cell and plasma cell infiltration with comparable oedema and matrix destruction to Caucasians with CRSwNP. (13)

In all populations with CRSwNP activated T lymphocytes contribute significantly to inflammation. A 2009 study of inflammatory characteristics in Chinese persons with CRS found that in both CRSsNP and CRSwNP that T cells, especially CD8+, were the predominant inflammatory cells. They also found increased numbers of mature dendritic cells in both subtypes, further suggesting T cell response is important in CRS in Chinese populations. (14)

Deficient T regulatory function with down-regulation of the transforming growth factor β (TGF-β) signalling pathway has also been demonstrated in CRSwNP in both Chinese and Caucasian populations. (6) Transforming growth factor-β1 (TGF-β1) is a cytokine with fibrogenic properties (attraction and proliferation of fibroblasts) that stimulates formation of
the extracellular matrix. (1, 13) It additionally inhibits IL5 synthesis and induces eosinophil apoptosis. It is found in low concentrations in CRSwNP. (1) Deficiency of TGF-β in CRSwNP may explain the phenotype of pseudocyst formation (destruction of ECM) and limited fibrosis. (1) TGF-β signalling also affects expression of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs). MMPs are a family of endopeptidases that cause extracellular matrix degradation. (1)

MMPs and TIMPs are expressed differently in CRSwNP and CRSsNP. In CRSwNP MMP7 and MMP9 are increased but inhibitory TIMP-1 is not. This can result in a net destruction of tissue. (13) In CRSsNP MMP-9 is increased but is balanced by increased TIMP-1. This difference in expression may also account for histological differences between the CRS subtypes. (1)

The presence of increased IgE in sinonasal tissue is another hallmark of CRSwNP. This likely represents local production, rather than a manifestation of systemic production, with IgE levels related to eosinophilic inflammation rather than allergy. (1, 6)

1.1.2 Chronic rhinosinusitis without nasal polyposis

CRSsNP accounts for up to 60% of cases of CRS. (15) It is associated with histological changes to the mucosa with fibrosis, thickening of the basement membrane, excess production of thick collagen, goblet cell hyperplasia, subepithelial oedema. Mononuclear cell infiltration is also characteristic. (1, 6) The primary inflammatory cell present in CRSsNP is the neutrophil. Myeloperoxidase, IFNγ, IL-1, IL-8 and IL-3 are all increased in the mucosa of persons with CRSsNP. (1, 6, 15) IL-8 is a chemoattractant for neutrophils, while IL-3 is postulated to contribute to the fibrosis and membrane thickening associated with CRSsNP. (1)

Histamine, cysteinyi leukotrienes and prostagladins have also been shown to increase in concentration in people with CRSsNP. (1) This may indicate an additional mast cell and basophil response. Basophils and mast cells are often present in CRSsNP but to a lesser extent than neutrophils.
Infiltration of eosinophils may occur, but does so to a lesser degree than occurs with CRSwNP. (6) Markers of eosinophilic inflammation are not elevated in CRSsNP as they are with CRSwNP. (1) In one study tissue from patients with untreated CRSsNP showed less than 10% eosinophils. Samples from people with CRSwNP had an overall mean of 50% eosinophils with 90% of samples having greater than 10% eosinophils. (1)

An increase in TGF-β1 and up-regulation of TGF-β signalling has been shown in CRSsNP, contrasting with the down-regulation of CRSwNP. (1, 6) T lymphocyte infiltration in CRSsNP is mainly a Th1 subtype with normal T regulatory cells (Treg). (6, 11, 15) However, a study in Chinese patients found there to be impaired Treg function in both CRSwNP and CRSsNP. The authors concluded that the Th1 response was primarily due to increased IFNγ in patients with CRSsNP. (14) In patients with CRS, with and without polyps, the concentration of leukotriene C4 synthase, 5-lipoxygenase, and cysteinyl leukotrienes were increased in parallel to eosinophil inflammation. COX-2 and prostaglandin E2 (PGE2), however, showed an inverse relationship with the severity of eosinophil inflammation. (6) A summary of inflammatory pathways in CRS are provided in Table 1.

Table 1. Summary of inflammatory pathways in CRS. Modified from Eloy (2011)(16)

<table>
<thead>
<tr>
<th>T cell profile</th>
<th>CRSsNP</th>
<th>CRSwNP</th>
<th>CRSwNP (Chinese)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Th1</td>
<td>Th2</td>
<td>Th17</td>
</tr>
<tr>
<td><strong>Inflammatory cells</strong></td>
<td>Prominent neutrophils,</td>
<td>Prominent eosinophils,</td>
<td>Neutrophils, T cells</td>
</tr>
<tr>
<td></td>
<td>T cells</td>
<td>B cells, T cells</td>
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<tr>
<td><strong>Cytokines and</strong></td>
<td>IFNγ, TGFβ1, IL-1, IL-</td>
<td>IL-4, IL-5, IL-13, ECP,</td>
<td>IL-17, IL-6, IFNγ, IL-</td>
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<td><strong>chemokines</strong></td>
<td>3, IL-6, IL-8</td>
<td>IL-8, RANTES, eotaxin</td>
<td>4, IL-5, IL-10, TGFβ</td>
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<tr>
<td><strong>Transcription factors</strong></td>
<td>FoxP3 upregulated</td>
<td>Tbet and GATA-3 upregulated</td>
<td>Tbet, GATA-3</td>
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<td></td>
<td>Tbet and GATA-3</td>
<td>FoxP3 downregulated</td>
<td></td>
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<tr>
<td><strong>Matrix remodelling</strong></td>
<td>Collagen, MMP-9</td>
<td>MMP-1, MMP-2, MMP-9 and MMP-7 upregulated.</td>
<td></td>
</tr>
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<td><strong>proteins</strong></td>
<td>countered by TIMP-1,</td>
<td>Psuedocyst formation containing albumin</td>
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<td>fibrosis</td>
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<td><strong>Tissue remodelling</strong></td>
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<td>vessels and glands, no</td>
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<td><strong>Co-morbidities</strong></td>
<td>Recurrent URTIs and</td>
<td>Asthma, AERDs</td>
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<td>allergic rhinitis may be</td>
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1.2 Contributory factors to CRS

Bacterial toxins and antigens are postulated as being contributory to the pathogenesis of CRS. *Staphylococcus aureus*, although a colonising organism in 20-30% of people, is frequently found in acute and CRS, especially in patients with CRSwNP.(6) *S. aureus* enterotoxins (SAE) are capable of activating T and B lymphocytes and stimulating an inflammatory reaction.(6, 13) Reports have shown that IgE specific to these enterotoxins (SAE-IgE) may be present in 50% of patients with polyps. These people have higher levels of eosinophils, eotaxin and IL-5.(6)

Nasal polyposis may also be associated with hypersensitivity to aspirin and asthma. This is referred to as aspirin-exacerbated respiratory disease (AERD).(6) Patients with this condition are provoked by aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) resulting in bronchospasm and other symptoms including rhinitis. The mechanism for this response is thought to be cyclooxygenase 1 (COX-1) inhibition, eosinophil and mast cell infiltration, Th2 cell activation, and overproduction of cysteinyl leukotrienes. Increased colonisation rates of *S. aureus*, local IgE, specific IgE to SAE and ECP, and IL-5 have been reported in patients with nasal polyps and aspirin sensitivity.(6)

Fungal infection is another possible contributor to the development of CRS. Fungi, eosinophilic mucin, and eosinophils are present in the nose and sinuses of the majority of patients with CRS. However, trials have failed to demonstrate improvement in symptoms following treatment with topical and systemic antifungals.(17) Also, studies have not shown there to be a significant difference in fungal isolates compared with healthy controls.(6)

Impaired ciliary function within the nasal cavity and sinuses has been suggested as contributory to CRS. Normally functioning mucociliary transport mechanisms are required for aeration of the sinuses and clearance of secretions from the sinuses. Inadequate clearance of secretions and debris can contribute to bacterial stasis and growth with mucosal inflammation. Dysfunction of cilia may be a component of genetic conditions such as primary ciliary dyskinesia.(1) In cystic fibrosis, there is an impairment of mucociliary clearance and a high prevalence of CRS.(9)

In addition to ciliary function, anatomical variation within the nasal cavity and paranasal sinuses can result in impaired drainage. The ostiomeatal complex (comprising
maxillary infundibulum, the frontal recess, the ethmoidal bulla, and the middle meatus) allows for ventilation and drainage of the maxillary sinus, anterior ethmoidal cells, and frontal sinuses. Proliferation of micro organisms, ciliary dysfunction and inflammation may occur if this region is inadequately opened and secretions are retained within the sinuses. Bulla of the concha, Haller cells, enlarged or malaligned middle turbinates, uncinate process abnormalities, and septal deviations have also been implicated in poor mucociliary clearance. However, studies have shown these variations may also be present in healthy persons without CRS.(1)

1.2.1 Bacterial biofilms

There is a lot of interest in the role of biofilms in the pathogenesis and treatment recalcitrance of CRS.(15, 18) Biofilms are organised communities of bacteria or fungi that are contained within a polymeric glycocalyx that is attached to the organisms and hosts.(15) Biofilms allow for local modulation of the bacterial environment, development of virulence factors, and facilitate signalling between bacteria.(18) They also provide a physical barrier that affords protection from immune cells and complement proteins.(15) In their natural state most bacteria exist as biofilms.(18) Of particular relevance to CRS Haemophilus influenzaes, S. aureus and P. aeruginosa form biofilms. (15)

Initiation of biofilm formation occurs when a mucosal population of bacterial colonies reaches sufficient growth to initiate co-ordinated signalling between the cells.(18) Chemical signalling between cells within a biofilm is known as quorum sensing.(18) This results in upregulation of genes, leading to the production of the glycocalyx. The glycocalyx is comprised of polysaccharides, nucleic acids and proteins.(18) It becomes organised and forms channels within the matrix that aid in delivery of nutrients and removal of waste and irreversible attachment.(18) Biofilms, or the glycocalyx thereof, may encase a polymicrobial mix of colonies which may include fungal elements within a primarily bacterial biofilm.(15, 18) The formation of a glycocalyx leads to decreased nutrient and oxygen requirements for the bacteria of the biofilm. It also helps in adhering to mucosal surfaces and protects the bacteria from an unfavourable external environment.(18)

Biofilms have a relative antibiotic resistance. It is theorised that resistance of biofilms to antibiotics comes from increased resistance gene sharing among the microbial community.
(when comprised of varied organisms) and a state of slowed metabolism in the biofilm.(15, 18) Biofilms do not provide a physical barrier to antibiotics, studies have proven full penetration occurs.(18) However, the matrix is negatively charged and this may repel aminoglycoside antibiotics.(18)

Biofilms are associated with treatment resistance and persistent infection with continued stimulation of the mucosal immune system.(18) This may occur due to specific stimuli such as *S. aureus* superantigens that activate ‘toll-like receptors’ (TLR) and can lead to T cell activation.(15) The biofilm itself may also interact with sinus epithelium and elicit an immune response. Biofilms have been found in biopsies of mucosa from patients with CRS but have also been found in healthy controls.(18) More research is probably required to characterise the role of biofilms in inflammatory disease and in health.

### 1.2.2 Impaired immune response

Deficiency in host defence characterised by an impaired mechanical barrier and immune function likely plays a key role in the pathogenesis of CRS.(6, 9, 10) Such impairments may predispose sinonasal mucosa to antigen stimulation and subsequent chronic inflammation.(6) An abnormal immune response to pathogenic micro-organisms and their products, including biofilms, underlies the supposition of an infectious aetiology in this chronic disease process. Impaired immune response constitutes a major area of CRS research.(6, 9)

Evidence in support of an impaired immune response rather than a primary infectious cause for CRS comes with a general lack of disease resolution to anti-infective agents.(6) Numerous studies examining antifungal agents have failed to show an effect.(17) Similarly, there are mixed results reported with antibacterial treatments.(9)

Current research considers the role of both the innate and adaptive immune systems in the pathogenesis of CRS. The adaptive immune response has been extensively investigated; however, the innate immune response is a more recent area of interest. An impaired innate immune response may lead to an inappropriate adaptive immune response.(9, 10)
1.3 The immune system and CRS

1.3.1 Innate immunity

The innate immune system functions without the requirement of prior exposure to pathogens. It provides a chemical and physical barrier and simulates further immune response with activation of the adaptive immune system. Recent research has shown the innate immune system to have the capacity for differentiation between self and pathogens.(10) Current research on the innate immune system has focused on epithelium, receptor function and secretion of immunologically active mediators.(9)

The mucosa of the nose and paranasal sinuses affords an initial defence against pathogens (Figure 3). Pattern recognition receptors (PRRs) are present throughout the mucosa and associated nasopharyngeal lymphoid tissue. These receptors recognise and bind to specific molecular patterns associated with pathogenic organisms, thus triggering an immune response.(9, 19)

**Figure 3.** Innate immunity of the sinonasal tract.(20)
1.3.2 Cell receptors

TLRs are the most extensively studied family of PPR that function in the detection of pathogens. There have so far been 10 TLRs identified.(10) It is thought recognition of pathogen related molecular patterns such as endotoxin, bacterial lipopeptide, flagellin, double stranded RNA and bacterial DNA by TLR results in initiation of an immune response. Factors that are considered important in TLR signal transduction include MyD88, IL-1 receptor associated kinase, TNF receptor associated factor 6, mitogen-activated protein kinases (MAPK) and nuclear factor kappa B (NF-κB).(10) Activation of TLRs ultimately leads to alteration in pro-inflammatory gene expression.(10) Research into CRS has investigated the expression of TLR in healthy and diseased sinonasal mucosa and how TLRs may act as a trigger a further immune response.

A recent study by Zhao et al. (2011) showed increased TLR9 and TLR10 expression in gland, epithelial, and mononuclear cells in non-atopic, non-asthmatic Chinese patients with CRSwNP when compared to healthy controls.(10) TLR9 is a receptor for unmethylated CpG dinucleotides that are found in bacterial and viral DNA and has previously been shown to have increased expression in epithelial cells exposed to bacterial DNA.(10) Activation of TLR9 results in a Th1 type response with activation of type 1 IFN, natural killer cells and CD8 T cell response.(10) TLR9 expression has been demonstrated in airway epithelium, sinonasal mucosa, monocytes/macrophages, B cells, neutrophils and dendritic cells.(10) Other studies on CRSwNP in non-Chinese populations, where there is a predominantly Th2 immune response pattern, have shown decreased TLR9 in sinonasal epithelial cells.(10)

TLR2 is important in mucosal defence and recognition of gram positive organisms.(15) Expression of TLR2 has also been investigated CRS. Variable results have been obtained. Some researchers have shown increased TLR2 in CRS mucosal epithelium when compared to controls.(10) However, other studies have linked reduced expression of TLR2 to CRS or have failed to find a link between TLR2 gene expression and CRS.(9)

Nucleotide binding oligomerisation domain (NOD) like receptors (NLR) are another family of receptor that trigger an immune reaction in response to pathogen associated molecular patterns.(9, 19) These receptors are more recently discovered and research is
ongoing to determine their role in inflammation. There are 23 identified NRL subtypes, NOD1, NOD2 and NALP3 subtypes are currently the best understood. NOD1 and NOD2 trigger an immune response by activating NF-κB, while NALP3 activates caspase1 dependent inflammasome.(19) NOD1, NOD2, and NALP3 have been demonstrated in neutrophils, nasal epithelial cells, tonsillar and adenoidoid lymphoid tissue and in lung tissue. A recent study by Mansson et al. (2011) showed increased expression of NOD1, NOD2 and NALP3 in untreated nasal polyps. This study also found expression of NLR was decreased following topical steroid treatment.(19)

1.3.3 Antimicrobial Peptides

Alteration in expression and/or activity of antimicrobial peptides is also postulated as being contributory to CRS.(9) These peptides are part of the innate immune system and have reported activity against viruses, parasites, bacteria and fungi.(9, 21) Lysozyme and lactoferrin are the most prevalent airway antimicrobial peptides.(21)

Lysozyme is present in nasal secretions and has been suggested as having a possible link with CRS however, studies linking altered expression to disease are lacking.(9) Lactoferrin is mostly expressed in epithelial cells and is secreted into mucosal environments. It has been suggested to have immune regulatory, anti-inflammatory and anti-biofilm activity in addition to general antimicrobial actions.(22) Reduced expression of lactoferrin in sinonasal mucosa of patients with CRS compared to controls has been demonstrated, however more research is necessary determine the significance of this finding.(21)

1.4 Inflammation

Chronic rhinosinusitis is, by definition, an inflammatory condition. Many of the pathological processes that occur in CRS may be understood in the context of alterations and derangements characteristic of an inflammatory state. Inflammation is a complex process that arises secondary to cellular damage and/or the presence of an injurious agent.(23) In the case of CRS an exact inciting factor is unknown but may include irritants and pathogens to which the sino-nasal mucosa is exposed and unable to adequately clear.(20) The inflammatory response involves vascular and cellular elements. These include, but are not limited to fluid, plasma proteins, the extracellular matrix, blood vessels and specialised immune cells.(23)
Inflammation may be broadly categorised as acute or chronic. Acute inflammation occurs rapidly and is of a short duration (minutes to days). It is characterised by oedema with a predominantly neutrophilic leukocyte infiltration.(23) Acute inflammation of the sino-nasal tract as occurs with acute rhinosinusitis (ARS) follows this pattern. ARS usually resolves within five days but may last for up to four weeks. It typically arises after a viral infection and has a characteristic Th1 inflammatory profile with release of TNF, INFγ, IL-1β, IL-6, and IL-8 inflammatory cytokines.(16)

Chronic inflammation has an extended duration ranging from weeks to months. It is primarily associated with lymphocyte and macrophage infiltration and with proliferation of blood vessels, and fibrosis.(23, 24) In both the acute and chronic state inflammation is mediated by compounds that have been produced or activated as a result of exposure to an inflammatory stimuli.(23)

In chronic inflammation there exists a state of active inflammation and tissue destruction along with concurrent repair of damaged tissue.(23) Chronic inflammation may arise following a period of acute inflammation if there remains persistence of the inciting stimuli. Such stimuli include infection with an organism of low toxicity and ongoing exposure to exogenous toxins. Auto immunity is another important cause of chronic inflammation.(23)

1.4.1 Chemical mediators of inflammation

The inflammatory process is controlled by the actions of a variety of substances, or chemical mediators that function to control the events of inflammation. Mediators may be plasma or cell derived. Plasma mediators include complement proteins and kinins and require activation before they are able to enact an effect.(23) Mediators that are released from cells include cytokines, eicosanoids and lysosomal enzymes.(23) The main source of cell-derived inflammatory mediators is platelets, neutrophils, macrophages and mast cells. Epithelium, endothelium and fibroblasts can also be induced to produce these mediators.(23)

In general these mediators are activated or produced directly by the presence of microbial products or damaged tissue, or by circulating endogenous proteins that have themselves been produced as a result of cellular activation by microbes or tissue damage.
Chemical mediators are usually short lived and cause an effect through interaction with specific cell receptors. Effects can include stimulation of release of other mediators with agonist or antagonist activity, and can vary according to the cell type upon which the mediator is acting.(23)

1.4.2 Cytokines

Cytokines are soluble protein mediators of inflammation that are produced by many cell types.(25) Lymphocytes and macrophages are the primary source of cytokines but they are also produced by endothelial, epithelial, and connective tissue cells.(23) Cytokines are involved in acute and chronic inflammation.(23)

TNFα and IL-1 play a major role in inflammation. They are primarily produced by macrophages, with their production stimulated by microbial derivatives, injury, immune complexes and other inflammatory stimuli.(23)

IL-6 is a pro-inflammatory cytokine that has been implicated in the pathogenesis of chronic inflammatory disease. Specifically it may be involved in the development of a chronic adaptive inflammatory state from an initial acute innate response. It inhibits recruitment of neutrophils and enhances granulocyte apoptosis. It is also important in T cell recruitment and survival, and in the differentiation of Th17 cells.(26)

IL-6 mediated inhibition of T cell apoptosis is considered significant in the chronic T cell inflammation of conditions including Crohn’s disease. Impaired T cell apoptosis secondary to IL-6 signalling is also reported in uveitis. IL-6 also acts as a growth factor for B cells. Increased IL-6 in sinus tissue may promote local B cell follicle formation and IgE production. Increased IL-6 may also promote Th2 inflammation and decrease Treg response.(26)

A study by Peters et al. (2010) demonstrated increased IL-6 levels in mucosal tissue from patients with CRSwNP when compared to those with CRSsNP and controls.(26) However, baseline nasal epithelial cell secretion of IL-6 in patients with CRS and controls was not appreciably different when tested *in vitro*. Receptor mediated phosphorylation of STAT3 transcription factor is a major pathway for IL-6 inflammatory response activation.
Analysis of STAT3 and phosphorylated STAT3 (p-STAT3) in this study showed similar levels of STAT3 in the groups but lower p-STAT3 in CRSwNP compared to controls. Thus the source of increased IL-6 in CRSwNP remains unknown at this time.(26)

**1.4.3 Eicosanoids**

Eicosanoids are mediators of inflammation that are derived from arachidonic acid.(24, 25) Arachidonic acid is an unsaturated 20 carbon fatty acid which is present in the lipid bilayer of most cells.(27) Upon activation cells can rapidly remodel membrane lipids to generate compounds with biological activity. Arachidonic acid is usually esterified within the membrane but is released by the action of cellular phospholipases.(23) Following release from the cell membrane there are a number of specific pathways through which metabolism of arachidonic acid can occur.(27, 28)

**Prostaglandins**

One such route of arachidonic acid metabolism is the cyclo-oxygenase (COX) pathway. At least two isoforms of the COX enzyme exist. COX-1 is expressed constitutively while COX-2 is inducible by various stimuli including lipopolysaccharide (LPS), IL-1β and IL-6.(23, 28, 29) Metabolism of arachidonic acid via the COX pathway leads to production of prostaglandins (PGs). The initial steps in the production of PGs are formation of prostaglandin G2 and its conversion to prostaglandin H2. PGH2 is then acted on by PG synthase, the specific type of which determines the final type of prostaglandin produced.(27, 28)

There are five primary prostaglandins that are important in inflammation.(23) They are: PGE2, PGF2α, PGD2, prostacyclin (PGI2) and thromboxane A2 (TXA2).(27, 30) Synthesis of each of the prostaglandins is relatively cell specific with one or two types predominant depending on the PG synthases present in that cell type. In macrophages, for example, PGE2 and TXA2 represent the major COX products.(28) Whereas vascular endothelium, which has prostacyclin synthetase, produces PGI2.(23) PGE2 is the most ubiquitous of the PGs in both physiological and pathophysiological states and exerts a variety of actions.(31)
Prostaglandins act in a paracrine or autocrine manner eliciting an action local to their site of production. They are rapidly inactivated and circulate at low levels within the body. The action is elicited through interaction with G-protein coupled cell receptors (GPCR). Each PG interacts with a specific receptor or receptors. PGE\(_2\) has four receptor subtypes: EP1, EP2, EP3, and EP4.

Prostaglandins mediate a variety of physiological functions. These include inflammation, pain, fluid transport and smooth muscle tone. They have a role in acute and chronic inflammation and interact with other immunologically active compounds and cells. PG production may lead to a variety of effects in the target tissue. PGE\(_2\), for example, can cause both smooth muscle relaxation and constriction with the effect determined by the site of the smooth muscle. There is also functional antagonism evident within the class. TXA\(_2\) causes platelet aggregation whereas PGI\(_2\) opposes aggregation. The relative amount of each PG produced, as well as the receptor expression determines effect. It has been hypothesised that TXA\(_2\) produced through COX-2 in macrophages may be responsible for apparent resistance to aspirin inhibition of platelet aggregation.

Prostaglandins have a role in T cell differentiation and expansion. It was initially believed that their role lay in suppression of Th1 selection. However, recent studies have found evidence in support of Th1 and Th17 differentiation and expansion with PGE\(_2\). These seemingly antagonistic effects arise due to the presence of other biologically active molecules and to the particular receptor with which PGE\(_2\) is interacting.

Prostaglandin mediated inhibition of T cells is thought to occur with PGE\(_2\) cAMP activation of lymphocyte-specific protein tyrosine kinase (LCK), an action which is antagonised by T cell receptor (TCR) stimulation. PGE\(_2\) has however also demonstrated enhancement of TCR stimulation.

PGE\(_2\) inhibits Th1 cytokine production and T cell proliferation when acting through EP2 or EP4 receptors. Th1 differentiation is promoted with EP1 receptor interaction and Th17 expansion via EP4 receptors. PGE\(_2\) may also affect the activity of cytokines. It can enhance the effect of IL-1 on MMP-1 and IL-6 synthesis in macrophages.
PGI\textsubscript{2} is also potentially active in Th differentiation and activation. Analogues of prostacycline have shown suppression of Th1 and Th2 cytokine production in cell based experimental models.\textsuperscript{(30)} They have also been shown to inhibit airway DC function and Th2 recruitment in mouse airways and inhibit bronchoconstriction in humans.\textsuperscript{(30)} Agonists for the IP receptor (through which PGI\textsubscript{2} acts) have also been reported to inhibit rhinorrhea in humans although the exact effect of PGI\textsubscript{2} in remains unclear.

**Leukotrienes**

Leukotrienes (LTs) are inflammatory mediators produced following arachidonic acid metabolism via the 5-lipoxygenase pathway.\textsuperscript{(23, 33, 34)} LTA\textsubscript{4} is an initial product of the pathway but is an unstable molecule that is converted to LTC\textsubscript{4} by conjugation to glutathione or to LTB\textsubscript{4} by epoxide hydrolase in neutrophils and other inflammatory cells. LTB\textsubscript{4} is a neutrophil chemoattractant and also can initiate eosinophil chemotaxis.\textsuperscript{(35)}

LTC\textsubscript{4} contains a cysteine residue, it and its cleavage products (LTD\textsubscript{4} and LTE\textsubscript{4}) are referred to as cysteiny1 LTs (cysLTs). CysLTs are abundant in mast cells, eosinophils and alveolar macrophages.\textsuperscript{(35)} They may increase survival of eosinophils, mast cells and basophils. They also may promote Th2 cytokine production.\textsuperscript{(35)} Cysteinyl leukotrienes (LTs) have a potential role in the pathogenesis of CRS.\textsuperscript{(34, 36)}

They have been extensively investigated in the setting of asthma where they are shown to cause bronchoconstriction, mucous hypersecretion and eosinophilic chemotaxis.\textsuperscript{(36)} LTs exert an effect through interaction with cysLT1 and cysLT2 receptors. CysLT2 is associated with bronchoconstriction, mucus secretion and airway oedema. CysLT2 enhances vascular permeability, tissue fibrosis and inflammation.\textsuperscript{(34)}

LT are believed to promote inflammation and contribute to persistent inflammation. Macrophages produce LTB\textsubscript{4} and LTC\textsubscript{4} among their inflammatory mediators. The function of macrophages can also be affected by the action of cysLTs.\textsuperscript{(35)} Activation, priming and reduced apoptosis have been reported. LTD\textsubscript{4} in particular has been shown to induce macrophage release of TNF\textalpha{} and NO when incubated with LPS.\textsuperscript{(35)}
Arachidonic acid can also be metabolised through other pathways to produce immunologically active compounds. Metabolites include 5-oxo-eicosatetraenoic acids (5-oxo-ETEs) which are chemoattractants for eosinophils and neutrophils.(35) Lipoxins cause vasodilation, inhibit neutrophil chemotaxis and can stimulate monocyte adhesion.(23) Their role in inflammation continues to be investigated and it has been established that nasal polyp tissue can produce lipoxins. Perez-Novò et al. (2005) found increased levels in patients with CRS relative to controls and decreased levels in aspirin sensitive compared to aspirin tolerant patients.(37)

1.4.4 Nitric oxide

Nitric oxide (NO) is a diffusible gas with a short half-life. It has a diversity of roles within the body. It is involved in cellular signalling, blood flow regulation, airway tone, mucociliary regulation, inflammation, and neurotransmission.(38, 39) NO also modulates mitochondrial events involved in apoptosis and necrosis and regulates mitochondrial biogenesis in many cell types, including lymphocytes. (38) It also regulates intracellular redox balance and has antibiotic effects against certain organisms. (39) Within the nose and paranasal sinuses NO is said to contribute to environmental sterility, mucociliary beat frequency and have anti-viral and anti-bacterial effects. (40) Although it is an important mediator of many physiological processes over production of NO has been linked to autoimmune disease, chronic inflammation and T cell dysfunction. (38)

NO is produced within the body by the action of nitric oxide synthetase (NOS) enzymes. There are three recognized subtypes of NOS endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). (23, 39, 41) eNOS and nNOS are constitutively expressed and are rapidly activated by increased intracellular calcium. (23, 39) iNOS activity is calcium independent and requires transcription and hence is slower in response than eNOS and nNOS. (39) iNOS gene transcription is activated by cytokines including IL-1 and TNFα and is inhibited by glucocorticoids. iNOS is able to produce much more NO than the other NOS subtypes and is present in inflammatory cells, including macrophages and dendritic cells. (38, 39)
**NO and T cells**

NO has many established and postulated regulatory roles within T lymphocytes. T cells do not express iNOS but may be affected by diffusion of NO produced by iNOS expressing cells (such as macrophages) in close proximity.\(^{38}\) TNFα and NO both modify T cell activation.\(^{38}\) NO has a role in lymphocyte cellular signalling pathways. Overproduction of NO has been suggested to cause alterations in T cell activation, differentiation and effector response.

Altered levels of NO may additionally lead to cell death. NO promotes mitochondrial hyperpolarization (MHP) and relative resistance to apoptotic stimuli. Low levels of NO inhibit cytochrome c oxidase, resulting in ATP depletion and possible necrosis or apoptosis. NO may also have a role in regulation of TCR expression. Cross-linking of TCR results in increased Ca\(^{2+}\) in the cytosol and up regulation of reactive oxygen intermediates (ROI) and NO production.\(^{38}\)

The link between NO production and T cell dysfunction has been investigated in autoimmune disease. In systemic lupus erythematosus (SLE) there is persistent mitochondrial hyperpolarization (MHP) of T cells and an increased production of NO by monocytes compared to patients without SLE.\(^{38}\)

Similarly, in rheumatoid arthritis (RA) there is characteristic chronic joint infiltration with activated T cells, neutrophils, monocytes and B cells. Studies have demonstrated over production of TNFα in RA and increased nitrite levels in synovial fluid from affected joints.\(^{38}\) There is suggestion that NO related oxidative stress is important in T cell activation and persistence in inflammatory diseases such as RA.\(^{38}\) It is also theorised that NO-induced ATP depletion may favour a necrotic rather than apoptotic response with subsequent stimulation of an immune response and chronic inflammatory state. iNOS polymorphism has been found to be associated with RA susceptibility.\(^{38}\)

**NO within the sinonasal cavity**

There has been interest in recent years in the measurement of nasal NO as a measure of sinus disease and treatment impact.\(^{39, 41}\) NO is continuously secreted into the upper airways. The source of NO is however controversial with some authors suggesting it arises
primarily from ciliary epithelium iNOS within the paranasal sinuses while others assert that NO measures represent production by the nasal mucosa.(39-42) There is also suggestion that nasal mucosa contributes to NO levels minimally but may increase excretion when iNOS is upregulated during states of inflammation.(41) Alternatively, during inflammation it may be that inflammatory cell iNOS becomes the major contributor to NO in the nasal cavity with down regulation of sinus iNOS expression.(40, 42) The significance of the secreted NO remains unclear but it has been proposed to act as an antimicrobial and mucocilliary modulating agent in this context.(39-41) A pro-inflammatory action has also been postulated. (40)

Levels of nasal NO are reported as being decreased in acute sinusitis and in nasal polyposis.(41) However, studies into nasal NO levels in CRS have been equivocal with some researchers finding levels to be lower than in healthy controls, while others have found no significant difference.(39, 41) Levels of nasal NO in patients with CRS may increase following treatment, however variability in pre-treatment levels limits the utility of nasal NO measurement as a marker of treatment success.(39, 41) Also, some studies have failed to demonstrate a significant difference in post treatment nasal NO between patients successfully treated and those who remain symptomatic.(39) A recent literature review has concluded that the role of nasal NO in the health of the sinuses remains to be clarified and that there is no evidence that low levels of nasal NO is harmful.(39)

Metabolites of NO (nitrate and nitrite) have been found in increased concentrations in animal models of CRS when compared with healthy controls. Naraghi et al. (2007) also found that sinus lavage fluid from people with CRS contains higher levels of NO metabolites than controls.(40) The significance of this finding is unclear at this stage. The authors suppose that this may reflect a state of impaired gas diffusion in CRS with decreased exhaled NO and entrapment of metabolites. They have further hypothesised that nitrate and nitrate may be further metabolised within the sino-nasal environment to form cytotoxic agents and contribute to an ongoing inflammatory state.(40)
1.5 Immune cell mediators of inflammation

1.5.1 Macrophages

Macrophages are the predominant cell type present with chronic inflammation.(23) They are part of the reticulo-endothelial system of cells derived from bone marrow and are differentiated from blood based monocytes. Upon entering extra vascular tissue monocytes are transformed into macrophages through the action of cytokines released from other immune cells and from non-immune mediators including endotoxin and fibronectin.(23) Once transformed and activated macrophages produce and release a number of biologically active substances. These substances are designed to eliminate injurious agents and initiate repair but when secretion occurs inappropriately they are also the cause of much of the tissue injury and fibrosis characteristic of chronic inflammation.(23)

Macrophages are long lived and are capable of proliferation within target tissue. Macrophage activation enhances their pathogen killing ability. Activation occurs with stimulation from INF-γ plus endotoxin (or other stimuli).(24)

1.5.2 Lymphocytes

*T* Cells

*T* cells comprise an important component of the adaptive immune response. *T* cells are lymphocytes that arise from stem cells in bone marrow and mature in the thymus.(24) They are responsible for a diverse range of actions including the production of cytokines necessary for activation, differentiation and proliferation of immunologically active cells.(25) Types of *T* cells include CD4+ helper *T* cells (Th), regulatory *T* cells (Treg), and CD8+ cytotoxic *T* cells (CTL).(25, 30) Each of these also has further unique subsets of cells.

*Th* cells are further divided into Th1, Th2 and Th17 subsets. These subsets are characterised according to cytokine secretion and the primary pathogen against which their activity is directed. Th1 cells are primarily involved in cell mediated immunity and against intracellular pathogens whereas Th2 are more frequently encountered in instances of extra cellular pathogens and in conditions of allergy.(30) Th17 cells are likewise mostly involved in extracellular immunity.(30)
Transcription factor expression determines whether naïve T cells will undertake Th1, Th2 or Th17 differentiation. T-box transcription factor (T-bet) is associated with IFN-γ production and favours Th1 differentiation, while GATA-3 results in a Th2 subtype. (11, 13) GATA-3 also controls the expression of IL-4 and IL-5 and can directly inhibit IFN-γ promoter activity contributing to a Th2 differentiation. (11) Other cells, including eosinophils and mast cells may contribute to GATA-3 expression. (11) Chinese patients with CRSwNP show T-bet expression and IFN-γ production consistent with a predominant Th1 response. (13)

T regulatory cells also contribute to the balance between Th1 and Th2 differentiation. (11) Studies have suggested that Treg cells have inhibitory capability against Th2 response and may be important in allergy and in diseases with Th2 predominance. (11, 13) Two main subtypes of Treg cells have been identified, these are naturally occurring Treg (nTreg) and induced Treg (iTreg). Their development is controlled by forkhead box P3 (FOXP3) transcription factor. (11) nTreg cells mature in the thymus and can migrate to mucosal inflammatory sites. iTreg cells are derived from naïve peripheral T cells. Studies have shown TGF-β1 induces FOXP3 in naïve T cells leading to transition to iTreg cells. (11)

Subtypes of iTreg (Tr1 and Th3) are believed to have particular importance in control of inflammation via secretion of the suppressor cytokines IL-10 and TGF-β1. (11) Tr1, via IL-10, suppresses proliferation and cytokine production in Th1 and Th2 cells. T cells that are activated in the presence of IL-10 are induced into a long lasting state of anergy. (11)

Treg cells are also said to have a role in allergic disease. Impaired nTreg or iTreg function is touted as potentially important in the development of allergy and asthma. (11) In asthmatic patients corticosteroid use up-regulates FOXP3 expression and hence Treg cells. In allergic rhinitis there are decreased numbers of FOXP3 positive cells than in healthy controls. (11) CRSwNP is associated with a predominantly Th2 response and may likewise be linked to impaired Treg function and subsequent Th2 driven inflammation. (11)

The T cell response profile in CRS appears characteristic according to the presence or absence of polyps, and on the ethnicity of the patient. (13, 14) CRSsNP is associated primarily with a Th1 response and adequate Treg function. (11) FOXP3 expression and Th1 and Th2
transcription factor expression in the mucosa of people with CRSsNP and that of control subjects has been demonstrated to be comparable.\(^{(11)}\)

Conversely, studies have demonstrated downregulation of FOXP3 expression in mucosa of people with CRSwNP.\(^{(11)}\) This FOXP3 downregulation is consistent for Asian and Caucasian patients with CRSwNP despite their otherwise differing inflammatory profile.\(^{(13)}\) CRSwNP in Caucasians has a Th2 predominant response and impaired Treg. CRSwNP in Chinese patients has a Th1/Th17 response with impaired Treg function.\(^{(13)}\) There is thus suggestion that impaired Treg function may account for ongoing inflammation in CRSwNP.\(^{(13)}\) Low TGF-\(\beta1\) and IL-10 are present in CRSwNP possibly reflecting impaired Treg function and contributing to the prolonged inflammatory state characteristic of the disease.\(^{(11)}\)

The role of Th17 cells in CRS is still largely based on speculation. Th17 cells are implicated in the pathogenesis of autoimmune conditions including Crohns disease and have shown \textit{in vitro} induction of pro inflammatory mediators including IL-1 and TNF-\(\alpha1\).\(^{(13, 26)}\) They may also stimulate NF\(\kappa\)B activity, IL-6, IL-8 and MMPs.\(^{(13)}\) Th17 differentiation from naïve T cells is inhibited by FOXP3 and may be enhanced by IL-6, an inhibitor of FOXP3.\(^{(26)}\) Th17 cellular response may explain the oedema and neutrophilia in Chinese and cystic fibrosis patients with CRSwNP.\(^{(13)}\)

\textit{Innate lymphoid cells}

Innate lymphoid cells (ILCs) are a family of cells that are involved with the innate immune response against micro-organisms.\(^{(9, 43)}\) They may also have an additional role in tissue remodelling following infection or injury and in stromal homeostasis.\(^{(43)}\) The ILC family includes natural killer (NK) cells, lymphoid tissue inducer (LTi) cells, and cells with characteristics of both NK cells and LTi cells.\(^{(9, 43)}\) These cells with combined characteristics are producers of IL22 and are called ILC22 cells. They include natural cytotoxicity receptor 22 (NCR22) cells and NK receptor positive LTi (NKR+ LTi) cells. The ILCs have disparate functions but are developmentally related. It has been postulated that ILCs represent an innate equivalent of helper T cells.\(^{(9, 43)}\)
NK cells were the first ILCs to be described. NK cells affect a response via various mechanisms including use of perforin containing granules and granzymes, IFN-γ, and TNF. They have a diversity of function and phenotype that is dependent on the tissue in which they are present. NK cells can be divided into two main populations based on CD56 expression. Cells with high expression of CD56 secrete large amounts of cytokines including GM-CSF, IFN-γ, and TNF and do not express CD16. NK cells with a low expression of CD56 express CD16 as well and have enhanced killing activity. These cells are also capable of increased cytokine production if stimulated appropriately. NK cells thus have a primarily Th1 cytokine production and may be referred to as ILC1 cells.

LTIi cells have a role in tissue remodelling and immunity. They have also been shown to induce lymph node formation in utero and are involved in formation of gut lymphoid follicles after exposure to pathogens. LTIi cells produce IL-17 and IL22. IL-17 is a pro-inflammatory cytokine that induces epithelial cells to produce cytokines and antimicrobial peptides and also promotes neutrophil recruitment. IL22 also induces epithelial production of anti-microbial peptides and also expression of cellular survival and differentiation genes.

ILC22 cells are primarily located in mucosal tissue. They express CD56 and can produce large amount of IL22. They are non-cytotoxic and do not produce an appreciable quantity of IFN-γ. ILC22 cells from tonsils have shown secretion of numerous cytokines including IL2, IL5, IL-8, IL-13 and TNF. They also secrete B cell activation factor and may have a role in regulation of T cell independent antibody production.

A further subset of ILC, ILC2, has been described in mice. These cells are associated with IL25 and IL-33 both of which activate a Th2 immune response. Non-T and non-B cells also respond to IL25 with proliferation and production of Th2 cytokines including IL5 and IL-13. Human ILC2 cells are yet to be defined, however there is already speculation that they may be contributory to chronic diseases with a Th2 immune profile. Early studies into possible human ILC2 cells have shown higher proportions of ILC cells which respond to IL25 and IL-33 with production of IL-13 and IL5 in patients with CRSwNP than in those with CRSsNP and in healthy controls. These ILC cells are dubbed...
1.6 CRS treatments

The multi-factorial aetiology for CRS is associated with uncertainty regarding the best medical treatment for the disease. There are few studies investigating medical treatments for CRS and a ‘gold standard’ remains elusive.(15, 45) Available treatments generally target either infectious or inflammatory causes and often provide a suboptimal outcome.(46)

Antibiotics are used orally, intravenously (IV) and topically. Overuse of oral and IV agents likely occurs and the choice of agent in clinical practice is frequently inappropriate for activity against bacteria associated with CRS.(45) There is no evidence that short term oral antibiotics are of use in CRS.(15) Topical antibiotic use with aminoglycosides has been investigated. Gentamicin is used in treating paranasal sinus disease with anecdotal efficacy, however further research is required.(45) Topical antibiotic use is controversial and also requires further evidence of safety and efficacy.(45) In treatment resistant CRS with proven \textit{S. aureus} culture, topical treatment with mupirocin has shown improvement in symptom scores and eradication of \textit{S. aureus}.(15)

Steroids are frequently used as anti-inflammatory agents in airway disease. They initially bind to the glucocorticoid receptor alpha (GR\( \alpha \)) in the cytoplasm.(33) After binding to the receptor there is subsequent translocation of the steroid-receptor complex to the nucleus where gene expression may be induced or suppressed via binding to DNA.(47) A major mechanism of anti-inflammatory activity is suppression of NF-\( \kappa \)B and AP-1 transcription. AP-1 and NF-\( \kappa \)B induce the expression of genes encoding for most pro-inflammatory cytokines.(47) Steroids also inhibit expression of genes encoding for COX-2 and iNOS.(47) The complex of steroid and receptor can also interact directly with preformed transcription factors for pro inflammatory mediators.(33)

Corticosteroids are thus frequently used as an anti-inflammatory treatment for CRS. Oral steroids are often used as first-line treatment in CRS however there is scant evidence in support of clinical efficacy.(15) Oral steroids are also associated with systemic side effects and are generally not suitable for long term usage.(45, 48) Studies examining the effect of
topical steroids have found conflicting results in CRS. In recent years antibiotics with anti-inflammatory activity have been investigated and used as an alternate to steroids in treating CRS inflammation.

Macrolide antibiotics have immune modulating effects in addition to their antibacterial action. They have been used for many years in the treatment of diffuse panbronchiolitis (DPB), a condition which is associated with a high incidence of CRS and P. aeruginosa infection. Macrolides significantly reduce DPB mortality (from 90% to 10%) even if the bacterial strains are antibiotic resistant. Macrolides have been studied as anti-inflammatory agents in airway diseases, including CRS, where inflammation is a predominant feature. In most trials in CRS there has been demonstrated a clinical improvement as well as inflammatory cytokine reduction.

The immune modulating effects of macrolides are thought to be due to suppression of inflammatory cytokine production, including production of IL-8, IL-6 and TNFα. Macrolides also affect neutrophil migration and adhesion. Macrolides with a 14 membered lactone ring or a 15 membered ring (azithromycin) structure possess these anti-inflammatory properties.

Tetracyclines are another class of antibiotic agent that has anti-inflammatory activity. They have been shown in vitro to inhibit PGE2, IL-1β, IL-6, IL-8 and TNFα production. Additionally they have been shown to inhibit iNOS expression and MMP production. Anti-apoptotic properties and inhibition of neutrophil and macrophage migration has also been demonstrated. Doxycycline has been used clinically in the treatment of inflammatory disease at doses less than that required for antibiotic activity. It appears effective in treating chronic inflammatory diseases of the airways. A study by Van Zele et al. (2010) demonstrated a reduction in polyp size, mucosal inflammation and tissue destruction with doxycycline treatment in patients with CRSwNP. They also found that treatment with doxycycline led to a prolonged reduction in polyp size compared to systemic steroid treatment.

Interest in complementary and alternative medicines (CAMs) has grown in recent years. There are a number of CAMs that are used in treating CRS. As with conventional
treatments there is a paucity of evidence in support of use. A meta-analysis by Guo et al (2006) investigating herbal treatments for rhinosinusitis found that there is evidence, albeit limited, that suggests Sinupret (a herbal product available in Germany that is comprised of Gentiana lutea root, Primula veris flower, Rumex sp. Herb, Sambucus nigra flower and Verbena officinalis herb) and Bromelain (an extract from pineapple stems and fruits) may improve symptoms of CRS.(46)

1.7 Xylitol

Xylitol is a naturally occurring five carbon sugar alcohol (polyol) that is present in a wide variety of plants, fruits and vegetables.(58-60) It is also produced to a small extent in humans.(59) It is used as an artificial sweetener in foods and medications and extensively in dental products and gums as a sweetener and as an antibacterial agent.(59, 60) It is also used as an ingredient in commercially available topical treatments for CRS (Xlear®, FESS Sinu-Cleanse®). Xylitol is a safe and well tolerated compound, the main adverse effect of use being dose dependent diarrhoea.(58-61)

Xylitol is widely used product and is the subject of investigation for use in an expanding variety of medical applications. Xylitol has been recognised as a safe (GRAS) additive by the Food and Drug Administration (FDA).(62) Likewise, the Joint Expert Committee on Food Additives (an institution linked to the World Health Organisation (WHO) and to the Food and Agricultural Organisation of the United Nations) has classified xylitol in the safest category of food additive. The WHO has also stated that xylitol has tested negative for teratogenicity and embryo toxicity and in vitro and in vivo testing for mutagenicity and clastogenicity have also been negative on conventional testing.(62) It is approved for use as an additive, supplement and pharmaceutical agent in over 50 countries worldwide.(62)

Xylitol has established anti-bacterial and anti-biofilm activity and has also been shown to act as a free radical scavenger in experimental models.(63-66) Much of the research on xylitol has occurred in the context of dental disease.(63, 64) There is also published evidence of clinical and experimental effects in rhinosinusitis, otitis media and chronically infected cutaneous wounds.(50, 65, 67)
Xylitol has also been investigated in treating dermatological disease including atopic dermatitis. Cream containing xylitol was shown to prevent *S. aureus* biofilm formation and inhibit growth.(68) The authors did not investigate whether xylitol exhibited anti-inflammatory properties.

There are a number of publications examining xylitol use in acute otitis media (AOM) in children.(60, 67) A recent systematic review and Cochrane review both supported the use of xylitol as a prophylactic agent in AOM but qualified the finding with a recommendation that further research is needed.(67, 69)

An *in-vitro* study by Kontiokari *et al.* (1998) using oropharyngeal epithelial cells found reduced adherence of *S. pneumoniae* in the presence of xylitol.(70) Studies have also been undertaken investigating xylitol use in Cystic Fibrosis (CF). Xylitol diffuses slowly across respiratory epithelium and can lower the airway surface liquid (ASL) chloride concentration and hence enhance the antibacterial activity of secreted lysozyme and beta defensins.(50, 58, 61)

Xylitol is used commercially as a topical agent for use in CRS. It has demonstrated improvement in symptoms when used intra nasally.(50) It’s mechanism of action is uncertain however may be due to antibacterial, antibiofilm, chloride lowering, or other effects.(50, 61, 71)

Limited research has been undertaken investigating the anti-inflammatory properties of xylitol. Han *et al.* (2005) undertook a study of xylitol and anti-inflammatory activity in periodontitis in a model using RAW 264.7 cells.(72) The authors found suppression of TNFα and IL-1 gene expression and protein synthesis with xylitol treatment.(72) These results suggest a possible anti-inflammatory action of xylitol and additional value as a therapeutic agent in inflammatory disease.

The current interest in natural medicines coupled with the lack of an established ideal medical treatment for CRS suggests that further investigation of compounds such as xylitol is warranted. Features of low cost, favourable safety profile and antibacterial activity make xylitol an ideal target candidate for additional investigation of activity.
1.8 Research hypothesis, aims and significance

The hypothesis of this study is that xylitol possesses anti-inflammatory activity that may prove useful in the treatment of CRS. Xylitol is already being used clinically as a treatment for CRS, however it’s mechanism of activity has not been established. Xylitol is an inexpensive and safe compound that has potential utility in a broad variety of diseases especially those characterised by inflammation and the presence of biofilm producing bacteria.

As indicated by the preceding chapter, current literature supports the primacy of immune dysfunction in the pathogenesis and treatment recalcitrance of CRS. Recent research has focused on immune derangements and has identified a number of inflammatory mediators with altered activity and/or expression in sinonasal tissue affected by CRS. These represent potential therapeutic targets whose inhibition may result in disease alleviation.

NF-κB, NO, LTB₄, PGE₂, TNFα, IL-6 and IL-8 were selected as targets appropriate for investigation. These inflammatory markers are widely used in inflammatory research, including research specifically investigating inflammation in the airways.

1.8.1 Aims

This study specifically aims to investigate the anti-inflammatory activity of xylitol as measured by inhibition of the aforementioned markers. Xylitol has established antibacterial activity and postulated immune modulating activity.(65, 72, 73) Azithromycin and doxycycline are antibiotic agents with anti-inflammatory activity.(48) They have been selected as comparator agents due to their possession of activity that it is hypothesised xylitol may also exhibit. In addition, the non-steroidal anti-inflammatory agent diclofenac has been used as an agent against, which any effect on inflammation due to xylitol can be compared.

1.8.2 Objectives

The objectives of this study are to:
• Confirm production of NF-κB, NO, LTB₄, PGE₂, TNFα, IL-6 and IL-8 in macrophage and epithelial cell lines following stimulation with pseudomonas derived LPS using simple, reproducible and high sensitivity assays for each target;

• Establish a co-culture of respiratory epithelial cells and macrophages and assess inflammatory mediator response of these co-cultured cells following stimulation as above; and

• Determine the anti-inflammatory activity of xylitol by assessing the presence and concentration of selected inflammatory markers in each cell line following exposure to xylitol. Compare these anti-inflammatory responses to those of the other antibiotic and anti-inflammatory compounds assessed.

1.8.3 Significance

CRS is a highly prevalent and morbid condition associated with a significantly impaired quality of life. It imparts a substantial economic burden to affected individuals and to the healthcare system. There is currently no satisfactory medical treatment. Development of a safe, accessible, effective treatment is important.

Xylitol is an inexpensive, widely used natural compound devoid of potential contribution to the burden of antibiotic resistance. It is considered safe and is used as an additive and active ingredient in a multitude of products available to adults and children.(62) It is used anecdotally in the treatment of CRS without a body of evidence in support of its use. Establishing the effects of xylitol in CRS will enable guidance of future clinical trials and development of evidence based treatments. This study is focused on the potential immune modulating and anti-inflammatory effects.
2. Materials and Methods

2.1 Chemicals and Reagents

Lipopolysaccharides (LPS) from *Pseudomonas aeruginosa*, resazurin, azithromycin, diclofenac and doxycycline were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). Xylitol was obtained from ENT Technologies (VIC, Australia). All other reagents were of the highest purity available.

2.2 In Vitro cell culture model

RAW 264.7 cells are a murine derived immortal macrophage cell line that is frequently used as a surrogate for immune cells. A459 cells are a human respiratory epithelial cell line and frequently used in airway inflammation studies, including CRS, as a surrogate for human alveolar epithelial cells.

2.3 Routine cell culture

RAW 264.7 cells and A549 cells were obtained from the American Type Culture Collection (ATCC: Manassas VA, USA). Cells were cultured and maintained in tissue culture flasks as a monolayer in Dulbecco’s Modified Eagle’s Medium + 4.5g/L D-Glucose, + L-Glutamine, + 110 mg/L Sodium Pyruvate (DMEM) (Invitrogen, Victoria, Australia) supplemented with 10% foetal bovine serum (FBS) and 50 mcg/ml gentamicin (Invitrogen). Cells were maintained at 37°C in a 5% CO₂ environment.

2.4 Establishment of a co-culture

In addition to performing experiments on each of the RAW 264.7 and A549 cell lines individually, a co-culture of both lines was also assessed. For co-cultures, each cell line was maintained and passaged as above. For experiments both cell lines were plated and mixed in the wells. Cell numbers for each line in the co-culture were the same as that used for the individual cell line.
2.5 Trypan Blue Exclusion Assay

The dye exclusion test is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue. Trypan blue staining of cells was undertaken with each passaging and plating of cells. All cell counts were conducted using 0.4% trypan blue to determine number of viable cells. Cell suspension was thoroughly mixed 1:1 with trypan blue and counted using an automated haemocytometer (Countess®). To avoid trypan blue cytotoxicity cells were counted within 3 min of mixing. Unstained cells were counted as viable and blue stained cells were counted as dead. Only cells with > 90% viability were used in subsequent experiments.

2.6 Drug stock and working solutions

For these experiments LPS from Pseudomonas aeruginosa was used to stimulate an inflammatory response in each of the cell lines. The LPS powder was reconstituted as per the manufacturer’s instructions to produce a 1 mg/mL solution in sterile cell culture medium. Prior to exposure LPS was diluted to final concentrations (10 µg/mL to 1 ng/mL) in sterile culture medium.

Azithromycin, diclofenac, doxycycline and xylitol stock solutions (1 mM) were prepared in sterile DMSO and then diluted to final concentrations (1 µM and 10 µM) in sterile culture medium. Vehicle controls were included in all experiments.

2.7 Resazurin Assay

The metabolic reduction of non-fluorescent resazurin to fluorescent resorufin was used as an indicator of cell viability, as described previously. Resazurin is non-toxic and is stable in cell culture medium. It is commonly used as an indicator of cell proliferation and cytotoxicity in cell cultures. Viable cells have the capacity to reduce resazurin to resorufin, which is highly fluorescent. A549 and Raw 264.7 cells were seeded at a density of 5x10⁴ trypan blue-excluding cells/mL in 96-well microtiter plates and incubated for 24 h for adhesion. The co-culture consisted of 5x10⁴ trypan blue-excluding cells/mL of each cell line. Cells were pre-treated with xylitol, azithromycin, diclofenac and doxycycline (1 µM and 10 µM) for 30 min prior to the addition of LPS (10 µg/mL to 1 ng/mL). After a further 24 h,
the medium above the cells was removed and replaced with fresh medium containing 44 μM resazurin. After a 3 h incubation, the reduction of resazurin to resorufin was determined by fluorescence (excitation: 530 nm, emission: 590 nm) and read using a Fluroskan Ascent microplate fluorometer (Thermoscientific, Victoria, Australia).

2.8 Lactate Dehydrogenase Release Assay

A549 and Raw 264.7 cells were seeded at a density of 5x10^4 trypan blue-excluding cells/mL in 96-well microtiter plates and incubated for 24 h for adhesion. Cells were pretreated with xylitol, azithromycin, diclofenac and doxycycline (1μM and 10 μM) for 30 min prior to the addition of LPS (10 ng/mL). Lactate dehydrogenase (LDH) release into culture supernatants was then assessed using the LDH assay kit from Cayman Chemicals (Ann Arbor, Michigan USA). This kit involves addition of NAD^+ to the previously collected supernatant. NAD^+ is then catalysed by LDH to NADH. NADH is then used to catalyse the reduction of a tetrazolium salt to formazan. Formazan is highly coloured and has strong absorbance at 490-520 nm. The production of formazan is proportional to the amount of LDH present in the cell supernatant.

2.9 NF-κB

Subconfluent A549, RAW 264.7 cells and the co-culture cells were pretreated with xylitol, azithromycin, diclofenac and doxycycline for 30 min prior to the addition of LPS (10 ng/mL) for 24 h. Following incubation cells were harvested by scraping into ice-cold PBS. Nuclear extracts were prepared using a nuclear extraction buffer (Cayman, Michigan, USA) supplemented with protease and phosphatase inhibitors and then assayed for protein using the Bradford protein assay (Bio-Rad, Munich, Germany). NF-κB concentration was determined by use of a NF-κB (human p50/p65) Combo Transcription Factor Assay Kit (Cayman Chemicals, Ann Arbor, Michigan USA). This kit is an enzyme linked immunosorbant assay (ELISA). It works by binding NF-κB containing nuclear extracts to a NF-κB response elements contained within 96-well plates. Primary and secondary NF-κB antibodies are then added and colorimetric absorbance read at 450 nm. Absorbance correlates with NF-κB protein concentration. The steps involved in the assay are depicted in the Figure 4 below.
Figure 4. Schematic of the transcription factor binding assay.

Briefly, 10 µL of nuclear extract/positive control was diluted 10-fold in assay buffer and 100 µL of this diluted extract was added to a 96-well microtitre plate. The plate was then incubated overnight at 4 °C. After the overnight incubation the wells were emptied and washed five times with wash buffer. The NF-κB primary antibody was then added to each well and the plate incubated for 1 hour at room temperature. After incubation, wells were emptied and washed five times using wash buffer. The secondary antibody (Goat anti-rabbit HRP conjugate) was then added to each well. After 1 h incubation at room temperature, the plates were washed as before. The developing solution (100 µL) was then added and the reaction was stopped after 15 min using 100 µL of stop solution. Absorbance (405nm) was then measured using a Tecan Sunrise plate reader and X-read software ((Tecan, Grödig, Austria).

2.10 Nitric oxide (NO) determination

Subconfluent A549, RAW 264.7 cells and the co-culture cells were pretreated with xylitol, azithromycin, diclofenac and doxycycline for 30 minutes prior to the addition of LPS (10 ng/ml) for 24 h. NO production was determined using the Nitrate/Nitrite Fluorometric Assay Kit (Cayman Chemicals, Ann Arbor, Michigan USA). NO is rapidly
degraded, the final products in vivo are nitrate (NO₃⁻) and nitrite (NO₂⁻). The proportion of each is variable and cannot be accurately predicted. Thus, both NO₃⁻ and NO₂⁻ levels should be measured to most accurately represent the total amount of NO produced. This kit firstly converts any nitrite to nitrate using nitrate reductase. The addition of 2,3-diaminonaphthalene (DAN) and NaOH then leads to an enhanced fluorescent product (1(H)-naphthotriazole), which represents the NO₂⁻ concentration present (Figure 5). (82-84)

Figure 5. Chemistry of nitrate/nitrite chemistry.

Briefly, 100 µL of either sample or standard in a 96-well microtitre plate was incubated with 10 µL of enzyme co-factor and 10 µL nitrate reductase for 30 min. Following incubation, 10 µL of DAN was added to each well. After 10 min at room temperature 10 µL of NaOH was added to each well to stop the reaction. Fluorescence (excitation: 360 nm, emission: 430 nm) was then read using a Flurosakan Ascent microplate fluorometer (Thermoscientific, Victoria, Australia).

2.11 Cytometric Bead Array (CBA) analysis of IL-6, IL-8, TNF-α

Cells were seeded at 5x10⁵ cells per well in 6-well plates. After 24 h, cells were pre-treated with xylitol, azithromycin, diclofenac and doxycycline for 30 min prior to the addition of LPS (10 ng/mL). After 24 h, cytokine (IL-6, IL-8, TNF-α) release in cell culture supernatants was determined using a BD Cytometric Bead Array (CBA) Human Inflammatory Cytokines Kit according to the manufacturer's instructions (Beckton Dickinson, NSW, Australia). CBA is a flow cytometry application that allows users to quantify multiple proteins simultaneously. The BD CBA system uses the broad dynamic range of fluorescence detection offered by flow cytometry and antibody-coated beads to
efficiently capture analytes. Each bead in the array has unique fluorescence intensity so that beads can be mixed and run simultaneously in a single tube.

Briefly, 50 μL of mixed capture beads was added to either sample or standards. Following addition of the cytokine PE detection reagent the intensity of fluorescence was measured using a BD LSR Fortessa™ flow cytometer (Beckton Dickinson, NSW, Australia). A minimum of 10,000 cells per sample was analysed after gating to exclude debris. Data was processed using FACSdiva software (Beckton Dickinson, NSW, Australia).

2.12 PGE\textsubscript{2} and LTB\textsubscript{4} measurement

Cells were seeded at 5x10\textsuperscript{5} cells per well in 6-well plates. After 24 h, cells were pre-treated with xylitol, azithromycin, diclofenac and doxycycline for 30 min prior to the addition of LPS (10 ng/mL) for a further 24 h. PGE\textsubscript{2} and LTB\textsubscript{4} release in culture supernatant was then measured by EIA (Cayman Chemicals, Ann Arbor, Michigan USA) according the manufacturers recommendation. The assay is based on the competition between PGE\textsubscript{2}/LTB\textsubscript{4} and PGE\textsubscript{2}/LTB\textsubscript{4}-acetylcholinesterase conjugate (PGE\textsubscript{2}/LTB\textsubscript{4} tracer) for a limited amount of monoclonal antibody. The tracer concentration is held constant while the concentration of PGE\textsubscript{2}/LTB\textsubscript{4} varies. Therefore, the amount of tracer that is able to bind the monoclonal antibody is inversely proportional to the concentration of PGE\textsubscript{2}/LTB\textsubscript{4} in the well. The antibody- PGE\textsubscript{2}/LTB\textsubscript{4} complex then binds to the capture antibody (goat anti-mouse IgG) in the well. The reaction is developed with Ellman’s reagent and absorbance is read at 412 nm. The steps involved in the assay are depicted in the Figure 6 below.

Briefly, 50 μL of either sample or standard were added to a 96-well microtitre plate. The tracer (50 μL) and monoclonal antibody (50 μL) were then added to each well and the plate was incubated overnight at 4°C. Following incubation, the plate was washed 3 times with wash buffer. Ellman’s reagent was then added to each well and absorbance (405 nm) was read after 15 min using a Tecan Sunrise plate reader and X-read software (Tecan, Grödig, Austria).
Figure 6. Schematic of the EIA.

2.13 Statistical analysis

The results are expressed as mean ± SD from the indicated number of experiments. Significance of difference was determined by one-way ANOVA and post-hoc Tukey-Kramer multiple comparisons test using Graphpad Instat software (version 3.06). Statistical significance was defined as $p < 0.05(*)$. Graphs were drawn using GraphPad Prism software (version 4.01).
3. Results

3.1 In Vitro Model

CRS is associated with an epithelial and immune cell cytokine response. In this study cultures of epithelial and immune cells were established to simulate the inflammatory environment of CRS and to determine any anti-inflammatory response following exposure to xylitol and control agents in this environment.

Macrophages are an integral part of the innate immune system and are found within the mucosa of the sinuses. Previous studies have confirmed that macrophage infiltration occurs in CRS. In the presence of antigens macrophages are stimulated to secrete cytokines and chemokines that subsequently react with other cells resulting in a state of inflammation. Prominent cytokines associated with macrophage activation include IL-1, L-6, IL-8, IL-12 and TNFα.

RAW 264.7 cells are a murine derived immortal macrophage cell line. This cell line is frequently used in research models investigating inflammatory response or states of immune dysregulation including cancer research. A459 cells are a human respiratory epithelial cell line. This cell line is frequently used in research in airway inflammation, including the investigation of CRS. Cultures of A459 and RAW 264.7 cells were established for demonstration of inflammatory response (Figure 7).

Figure 7. Co-cultured cells stained with crystal violet.
3.2 DMSO toxicity

To establish the limit of DMSO suitable for inclusion as a vehicle for drug solubilisation, concentration-dependent toxicity was studied in the model. Resazurin reduction to resorufin was used to determine toxicity. Resazurin is reduced to resorufin in live, viable cells. The extent of reduction is proportional to the number of viable cells. This assay is thus used as a measure of cell proliferation and cytotoxicity in cell cultures. A549, RAW 264.7 and co-cultures were exposed to DMSO concentrations up to 10% v/v. As shown in Figure 8 A DMSO was only toxic at a concentration of 10% v/v in A549 cells (P < 0.05). In RAW cells (Figure 8 B concentrations greater than 1% v/v were shown to be toxic (P < 0.05). Co-culture (Figure 8 C) viability was significantly affected by 10% v/v DMSO (P< 0.05). Subsequent to these findings DMSO concentrations for solubilisation of investigation compounds was kept below 0.1% v/v.

Figure 8. DMSO Toxicity. Cells were treated with DMSO (0-10% v/v) for 3h and viability was measured using resazurin reduction to resorufin. (A) A549 cells; (B) RAW 264.7 cells; and (C) Co-culture. Data is represented as mean ± SEM of 3 independent experiments. Comparisons DMSO vs control; * P < 0.05; ** P < 0.01; *** P < 0.001
3.3 LPS toxicity

In this experiment LPS derived from *P. aeruginosa* was investigated for cytotoxicity using the resazurin assay. LPS is frequently used in experimental research to induce an inflammatory response. This investigation was undertaken to establish a non-toxicity concentration of LPS at which the immunological and inflammatory effects could be studied. A549, RAW 264.7 and co-cultures were exposed to LPS between the concentration range of 0-10 µg/mL and viability measured using resazurin reduction to resorufin. LPS concentrations up to 10 µg/mL were shown to be non-toxic in A549 cells and co-culture (Figures 9 A and 9 C) (P > 0.05 when compared to vehicle control). Viability of RAW cells was significantly affected at LPS concentrations greater than 0.1 µg/mL. Subsequent to these findings LPS concentrations were kept below 0.1 µg/mL to investigate immunological and inflammatory effects.

![Graph A](image1)

**A**

![Graph B](image2)

**B**

![Graph C](image3)

**C**

*Figure 9. LPS Toxicity.* Cells were treated with LPS (0-10µg/mL) for 3 h and viability was measured using resazurin reduction to resorufin. (A) A549 cells; (B) RAW 264.7 cells; and (C) Co-culture. Data is represented as mean ± SEM of 3 independent experiments. Comparisons LPS vs control; * P < 0.05; ** P < 0.01; *** P < 0.001
3.4 Toxicity of investigation drugs in the presence of LPS

The cumulative toxicity of azithromycin (1 µM and 10 µM), diclofenac (1 µM and 10 µM), doxycycline (1 µM and 10 µM), xylitol (1 µM and 10 µM) and LPS were investigated using both resazurin reduction to resorufin and LDH leakage as markers. LDH is a cytosolic enzyme and leakage into the supernatant indicates that cell membrane damage has occurred. As shown in Figure 10 A A549 cells remained unaffected by any investigational drugs at the concentrations tested (P > 0.05).

Figure 10. Investigational drug toxicity. Cells were treated with azithromycin (1 µM and 10 µM), diclofenac (1 µM and 10 µM), doxycycline (1 µM and 10 µM) and xylitol (1 µM and 10 µM) and viability was measured using resazurin reduction to resorufin as well as LDH leakage. (A) A549 cells; (B) RAW 264.7 cells; and (C) Co-culture. Data is represented as mean ± SEM of 3 independent experiments. Comparison vs vehicle control: * P < 0.05; ** P < 0.01; *** P < 0.001
Similar findings were obtained for RAW 264.7 cells (Figure 10 B; P > 0.05). LDH leakage in RAW 264.7 cells increased in the presence of 0.01 µg/mL LPS, which followed the trend observed using the marker of resazurin reduction to resorufin (Figure 10 B). Co-culture viability remained unaffected by exposure to any of the investigational drugs (Figure 10 C; P > 0.05).

3.5 Prostaglandin E\(_2\) production

Supernatants from A549, RAW 264.7 and co-cultures were collected following a 3 h exposure to azithromycin (1 µM and 10 µM), diclofenac (1 µM and 10 µM), doxycycline (1 µM and 10 µM), xylitol (1 µM and 10 µM) and controls. PGE\(_2\) concentrations were adjusted according to cell viability determined from earlier resazurin reduction experiments.

In A549 cells basal production of PGE\(_2\) was reduced to a significant extent (P < 0.05 compared to vehicle control) following exposure to each of azithromycin (1 µM and 10 µM), diclofenac (1 µM and 10 µM) and xylitol (1 µM and 10 µM). Doxycycline, however, did not produce a reduction in basal PGE\(_2\) production at both 1 µM and 10 µM concentration. In A549 cells LPS 10 ng/mL did not significantly increase PGE\(_2\) production from baseline (Figure 11 A).

In contrast, exposure of RAW 264.7 cells and the co-culture to LPS 10 ng/mL resulted in a significant (P < 0.05) increase in production of PGE\(_2\) relative to basal (vehicle control) production. Pre-treatment of RAW 264.7 cells with azithromycin 10 µM, diclofenac 1 µM and 10 µM, doxycycline 10 µM, and xylitol 1 µM and 10 µM significantly reduced production of PGE\(_2\) relative to LPS-induced levels (P < 0.05; Figure 11 B).

In the co-cultured cells pre-treatment with azithromycin 10 µM, diclofenac 1 µM, doxycycline 10 µM, xylitol 1 µM and xylitol 10 µM significantly decreased LPS-induced PGE\(_2\) production (P < 0.05 compared to LPS control). Diclofenac 10 µM did not affect LPS-induced PGE\(_2\) production in these cells, whereas doxycycline 1 µM produced an increase in PGE\(_2\) levels relative to LPS-induced controls (Figure 11 C)
Figure 11. Prostaglandin E₂ production. Cell supernatants were collected and PGE₂ concentration determined using ELISA. (A) A549 cells; (B) RAW 264.7 cells; and (C) Co-culture. Data is represented as mean ± SEM of 3 independent experiments. Comparison vs vehicle control; * P < 0.05; ** P < 0.01; *** P < 0.001 Comparison vs LPS stimulated control; # P < 0.05; ## P < 0.01; ### P < 0.001

3.6 Leukotriene B₄ production

A549, RAW 264.7 and co-cultured cells were exposed to azithromycin (1 µM and 10 µM), diclofenac (1 µM and 10 µM), doxycycline (1 µM and 10 µM), xylitol (1 µM and 10 µM) LPS 10 ng/mL and DMSO 0.1% v/v vehicle controls as previously described in the methods. Cell supernatants were collected 3 h after initial exposure. Analysis of LTB₄ levels was undertaken using ELISA. Concentrations were then normalised to resazurin reduction. As shown in Figure 12 LTB₄ was not detectable in any of the models.
Figure 12. Leukotriene B₄ production. Cell supernatants were collected and LTB₄ concentration was determined using ELISA and adjusted per viability. (A) A549 cells; (B) RAW 264.7 cells; and (C) Co-culture. Data is represented as mean ± SEM of 3 independent experiments.

3.7 NO production

Gross nitrate production (representative of NO production) was determined by analysis of supernatants collected from the A549 cells, RAW 264.7 cells and co-cultures using the Griess assay. Concentrations were then normalised relative to respective cell viabilities calculated from resazurin reduction experiments. Figure 13 A demonstrates that there was no significant alteration in basal nitrate production with exposure to either LPS 10 ng/mL, azithromycin (1 µM and 10 µM), diclofenac (1 µM and 10 µM), doxycycline (1 µM and 10 µM) and xylitol (1 µM and 10 µM) in A549 cells (P > 0.05 relative to vehicle control in all instances). There was however a non-significant trend for an increase in NO production
with LPS relative to the vehicle. LPS stimulated NO levels were reduced with azithromycin 1 µM (P < 0.05) to below basal NO levels.

![Bar charts with cell supernatants collected and nitrate concentration determined using the Griess assay.](Figure 13)

Figure 13. Nitric oxide production. Cell supernatants were collected and nitrate concentration in each cell line was determined using the Griess assay. (A) A549 cells; (B) RAW 264.7 cells; and (C) Co-culture. Data is represented as mean ± SEM of 3 independent experiments. Comparison vs LPS stimulated control; # P < 0.05; ## P < 0.01; ### P < 0.001

Similarly exposure to either LPS 10ng/mL, azithromycin (1 µM and 10 µM), diclofenac (1 µM and 10 µM), doxycycline (1 µM and 10 µM) and xylitol (1 µM and 10 µM) did not significantly affect nitrate production in the co-culture, although azithromycin 1 µM again reduced LPS stimulated production to beneath basal level (P < 0.05 relative to LPS; Figure 13 C). In the RAW 264.7 line nitrate production was not significantly affected by any
of LPS 10 ng/mL, azithromycin (1 µM and 10 µM), diclofenac (1 µM and 10 µM), doxycycline (1 µM and 10 µM) and xylitol (1 µM and 10 µM) (Figure 13 B).

3.8 IL-6 production

Supernatants from A549, RAW 264.7 and co-cultures were collected following exposure to the investigational drugs and controls. IL-6 production was determined using cytometric bead array enhanced sensitivity flow cytometry. IL-6 concentrations were then normalised to cell viabilities calculated from earlier experiments. As shown in Figure 16 A neither LPS 10 ng/mL nor azithromycin (1 µM and 10 µM), doxycycline (1 µM and 10 µM) or xylitol (1 µM and 10 µM) altered basal IL-6 production in A549 cells. In this cell line diclofenac (1 µM and 10 µM) increased production of IL-6 relative to vehicle control and LPS 10 ng/mL exposed cells (Figure 14 A; P < 0.01). In the RAW 264.7 cells there was a similar lack of IL-6 response with exposure to any of LPS 10ng/mL, azithromycin (1 µM and 10 µM), diclofenac (1 µM and 10 µM), doxycycline (1 µM and 10 µM), or xylitol (1 µM and 10 µM). In contrast, exposure of the co-culture to LPS 10ng/mL significantly increased IL-6 production compared to vehicle control (Figure 14 C; P < 0.05). Pre-treatment with xylitol (1 µM and 10 µM) significantly reduced LPS-induced IL-6 production to levels comparable with the vehicle control (Figure 14 C; P < 0.05). Similarly pre-treatment with doxycycline (1 µM and 10 µM) also significantly attenuated the LPS-induced increase in IL-6 (Figure 14 C; P < 0.05). Azithromycin (1 µM and 10 µM) and diclofenac (1 µM and 10 µM) did not affect LPS-induced IL-6 production in this model.
Cell supernatants were collected and IL-6 concentration determined using flow cytometry. (A) A549 cells; (B) RAW 264.7 cells; and (C) Co-culture. Data is represented as mean ± SEM of 2 independent experiments. Comparison vs vehicle control; * P < 0.05; ** P < 0.01; *** P < 0.001. Comparison vs LPS stimulated control; # P < 0.05; ## P < 0.01; ### P < 0.001

3.9 IL-8 production

Analysis of supernatants collected from A549, RAW 264.7 and co-cultured cells exposed to azithromycin (1 μM and 10 μM), diclofenac (1 μM and 10 μM), doxycycline (1 μM and 10 μM), or xylitol (1 μM and 10 μM) and controls were undertaken using cytometric bead array enhanced sensitivity flow cytometry. IL-8 production in A549 cells was significantly (P < 0.05) increased relative to vehicle control following exposure to diclofenac 1 μM (Figure 15 A). Production was unaffected by exposure to azithromycin (1 μM and 10
μM), diclofenac (10 μM), doxycycline (1 μM and 10 μM), or xylitol (1 μM and 10 μM). IL-8 was not significantly altered relative to the vehicle control in either the RAW 264.7 or co-cultured cell lines with azithromycin (1 μM and 10 μM), diclofenac (1 μM and 10 μM), doxycycline (1 μM and 10 μM), or xylitol (1 μM and 10 μM). (Figure 15 B and 15 C; P > 0.05).

**Figure 15.** IL-8 production. Cell supernatants were collected and IL-8 concentration determined using flow cytometry. (A) A549 cells; (B) RAW 264.7 cells; and (C) Co-culture. Data is represented as mean ± SEM of 2 independent experiments. Comparison vs vehicle control; * P < 0.05; ** P < 0.01; *** P < 0.001 Comparison vs LPS stimulated control; # P < 0.05; ## P < 0.01; ### P < 0.001

### 3.10 TNFα production

Production of TNFα in each of the cell lines was determined using enhanced sensitivity cytometric bead array flow cytometry. Concentrations were then adjusted relative
to resazurin reduction. As shown in Figure 16 A exposure of A549 cells to xylitol 10 μM significantly increased TNFα production compared to vehicle control (P < 0.05). There was no effect on TNFα production demonstrated in RAW 264.7 cells with exposure to any of azithromycin (1 μM and 10 μM), diclofenac (1 μM and 10 μM), doxycycline (1 μM and 10 μM) or xylitol (1 μM and 10μM). (Figure 16 B).

![Figure 16. TNFα production. Cell supernatants were collected and TNFα concentration determined using flow cytometry. (A) A549 cells; (B) RAW 264.7 cells; and (C) Co-culture. Data is represented as mean ± SEM of 2 independent experiments. Comparison vs vehicle control; * P < 0.05; ** P < 0.01; *** P < 0.001 Comparison vs LPS stimulated control; # P < 0.05; ## P < 0.01; ### P < 0.001](image)

Although in the co-culture diclofenac 10 μM significantly increased production relative to the vehicle control and LPS 10 ng/mL stimulated control (Figure 16 C; P< 0.001). All other exposures [azithromycin (1 μM and 10 μM), diclofenac (1 μM), doxycycline (1 μM and 10 μM), or xylitol (1 μM and 10μM)] in the co-culture did not significantly alter production.
3.11 NF-κB levels

Nuclear levels of NF-κB were determined using a transcription factor assay kit. Cellular nuclear extracts from each cell line were purified and ELISA used to determine NF-κB. Colorimetric absorbance at a 450 nm wavelength is used for detection. Absorbance was then adjusted according to resazurin reduction from earlier experiments. Production of NF-κB in A549 cells is demonstrated in Figure 17 A.

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

Figure 17. NF-κB production. Nuclear extraction was undertaken and NF-κB levels determined by ELISA. (A) A549 cells; (B) RAW 264.7 cells; and (C) Co-culture. Data is represented as mean ± SEM of 3 independent experiments. Comparison vs vehicle control; * P < 0.05; ** P < 0.01; *** P < 0.001. Comparison vs LPS stimulated control; # P < 0.05; ## P < 0.01; ### P < 0.001

In this cell line azithromycin 10 μM, diclofenac 1 μM and 10 μM, and xylitol 10 μM pre-exposure decreased NF-κB to below basal levels. This reduction was statistically significant (P < 0.05) relative to the LPS 10 ng/mL stimulated control (Figure 17 A).
In RAW 264.7 cells LPS 10 ng/mL, azithromycin 1 µM, diclofenac 1 µM and 10 µM, and doxycycline 10 µM caused a reduction in levels of NF-κB (Figure 17 B; P < 0.05 compared to vehicle control). In contrast, LPS 10 ng/mL, diclofenac (1 µM and 10 µM), doxycycline (1 µM and 10 µM) and xylitol (1 µM and 10 µM) exposure increased NF-κB nuclear levels relative to vehicle control alone in the co-culture. Azithromycin 1 µM and 10 µM reduced LPS stimulated NF-κB levels in the co-culture (Figure 17 C; P < 0.05).
4. Discussion

This study sought to investigate the anti-inflammatory and immunomodulating potential of xylitol in an in vitro model of CRS. CRS, like other chronic inflammatory conditions, involves complex immune interactions. (1) The exact details of the interactions that lead to the clinical condition are yet to be fully clarified and remain the subject of ongoing research. (6) Derangements of inflammatory markers including PGE₂, IL-6, IL-8, and TNFα are known to occur with CRS and were the focus for anti-inflammatory activity in this research model. Other ubiquitous markers of inflammation including NF-κB and NO were also considered in a model where drugs of known anti-inflammatory or immunomodulating action were screened for comparison.

In this study a co-culture of immune and epithelial cells was established in order to more accurately approximate the environment encountered in the upper airway in instances of CRS. In vitro research sometimes fails to produce a result that is readily apparent in clinical experiments. This is especially true for research into inflammation where interactions between immune and other cells are integral for achieving an inflammatory response. Research with other immunomodulating agents including macrolide antibiotics has shown this disparity between in vitro and in vivo effect to occur. (87-89) A cell-based model of inflammation that omits any interaction between different cell types may thus fail to reveal a clinically relevant response. Developing a model that accounts for interactions between cells and considers the effect of secreted markers may better represent a potential in vivo effect.

The cell lines chosen for investigation were RAW 264.7 macrophages and A549 respiratory cells, representing the epithelial and immune cells present in the mucosa of people suffering with CRS. Respiratory epithelial cells are a source of cytokines and are known to modulate innate and adaptive immune response. (90) Their inclusion in a model of CRS investigating immune response is important. Mucosal epithelial cells have, in particular, been implicated in Th2 responses. (90) These Th2 type cytokines are associated with CRSNP. Primary immune cells including macrophages migrate to sites of inflammation and are responsible for many of the clinical evident manifestations of the disease. The results obtained in this study demonstrated that release of inflammatory mediators can be altered by the co-culturing of cells. This response was particularly evident when considering IL-6 production. In each of the single A549 and RAW 264.7 cells stimulation with LPS failed to
produce a significant rise in IL-6. However, when co-cultured stimulation with LPS 10 ng/mL doubled IL-6 production relative to the vehicle control. This result is not entirely unexpected considering the complex signalling that occurs between cells involved in an inflammatory response and their ability to modulate the response of other cells.(23) IL-6 is known to be an important cytokine in macrophage stimulation and interaction with other parts of the immune system.(91)

Nuclear levels of NF-κB also demonstrated a cell line-dependent effect. Exposure to xylitol 10 μM resulted in a decrease in basal NF-κB production in the A549 line, had no effect in the macrophage line, and increased basal production when the cells were co-cultured. The effect of cell specific NF-κB modulation was not unique to xylitol. Diclofenac and doxycycline pre-treatment reduced production of NF-κB to less than basal levels in both the epithelial line and macrophage lines. However, in the co-culture both of these agents stimulated basal production of NF-κB, producing a result seemingly incongruously with the suppression demonstrated in each individual line. Azithromycin was the only agent that produced a consistent effect, reducing NF-κB production in all cell lines (Figure 19). The results obtained in this study demonstrating modulation of single cell line inflammatory response with co-culture are supported by the research of others. Many other research groups have shown that the in vitro immune response is frequently altered in the presence of another cell type and does not reflect the individual response of either cell line.(92-94)

Co-cultures of epithelial, macrophage, and other cell lines have been used in in vitro research by investigators hoping to characterise the effect of interactions between different cell types on inflammatory marker release.(92-97) Wottrich et al. (2004) established a co-cultures of A549 cells with THP-1 macrophages and A549 adenocarcinomic human alveolar basal epithelial cells with Mono Mac 6 macrophages.(94) They investigated the in vitro effect of fine particle exposure on each cell line and in co-culture. Specifically they examined IL-6 and IL-8 supernatant concentrations and established that communication between the epithelial and macrophage cells in the co-culture resulted in increased pro-inflammatory cytokine release compared to either single cell line. In their study the co-culture of A549 and Mono Mac 6 macrophages showed a 10 fold increase in IL-6 release compared to that of the A549 line alone following exposure to certain fine particles.(94) The A549 and THP-1 co-culture similarly showed a statistically significant increase in IL-6 and IL-8 relative to the
A549 cell line with exposure to a number of fine particles. The macrophage cell lines alone did not produce IL-6 or IL-8 to a measurable level. In their study the same cell density was used for the co-culture and the individual cell cultures.(94)

Rubovitch et al. (2007) undertook a study to specifically investigate the effect of lung epithelial cells on the inflammatory response of macrophages.(92) They used A549 and rat RLE-6TN respiratory epithelial cells and investigated the effect on LPS stimulated rat NR 8383 macrophage NO and IL-6 production. They found that relative to macrophages alone, a co-culture with respiratory epithelial cells inhibited NO production. This effect was maximal with the A549 co-culture at 24 h. The inhibitory effect on NO production occurred with the co-culture and also when the cells were in the same media but physically separated with the macrophages cultured in an insert that was suspended above the A549 cells. The extent of suppression was 25% in both instances suggesting modulation of NO by epithelial cells occurs via a soluble factor.(92) LPS stimulated IL-6 modulation was also demonstrated on co-culture. However, surfactant protein A stimulated IL-6 production was not affected by co-culture with epithelial cells.(92)

A co-culture of respiratory epithelial (BEAS 2B) cells and endothelial cells (ECV304) also demonstrated modulation of immune marker release when compared with each single cell line.(96) Mogel et al. (1998) established a co-culture of BEAS 2B and ECV304 cells and measured supernatant concentrations of IL-6 and IL-8 at 1, 4 and 24h following exposure to air or ozone. They found that the co-culture resulted in significantly increased levels of IL-6 and IL-8 relative to each individual cell line at 24h and that this result was more that would be explained by a simple additive effect.(96)

Bodet et al. (2005) investigated the production of IL-1β, IL-6, IL-8, TNF-α and RANTES in a P. gingivalis stimulated co-culture of HeLa epithelial cells and U937 macrophage cells.(95) They found there to be no difference in basal levels of cytokine production in the co-culture and individual cell lines. They co-cultured the cells at ratio of 1:100 and 1:5 (macrophage: epithelial) to reflect the estimated ratios that occur in vivo in health and in with a state of inflammation associated with periodontitis.(95)

Mpga et al. (2006) created a co-culture of He-La cells and THP-1 (monocyte-like) cells in order to mimic in vivo epithelial and immune interactions. They infected their cells
with *Chlamydia trachomatis* and measured the production of a variety of inflammatory markers. They found that in the individual HeLa and THP-1 cell lines pro-inflammatory cytokine production was increased following *C. trachomatis* infection but subsequently reduced. However, in the co-culture secretion of IL-6 and IL-8 was sustained, IL-1β production followed a bell curve and TNFα production decreased.(97) Given the supporting evidence of literature this study investigated the effects of xylitol and other agents in individual lines and co-cultures.

In this study viability testing using a resazurin assay and LDH assay confirmed the non-toxicity of xylitol in all cell lines (Figure 10). The resazurin assay is commonly used as an indicator of cell proliferation and cytotoxicity in cell cultures.(80) This assay works on the premise that viable cells with intact metabolic machinery can reduce resazurin to resorufin, which is fluorescent. The number of viable cells present is thus directly proportional to the extent of fluorescence obtained.(80) This allows for comparison of the number of viable cells in the treatment groups and controls. The LDH assay measures LDH presence in collected supernatant. LDH is usually a cytosolic enzyme and its presence in the supernatant shows that cell membrane leakage or lysis has occurred.(98) The findings of non-toxicity in this study align with those of other investigators. Han *et al.* (2008) conducted in-vitro experiments on xylitol and found no cytotoxic effect using trypan blue exclusion.(72) Ferreira *et al.* (2011) also conducted in-vitro experiments using xylitol and found no alteration in viability in xylitol treated cells relative to control using the modified Mosmann assay.(99) The safety and non-toxicity of xylitol has been extensively tested in clinical and experimental contexts and it appears that the data of this in-vitro study supports these findings.

This research primarily sought to establish whether xylitol has anti-inflammatory activity that will be potentially useful in the clinical context of CRS. After creating an appropriate in-vitro model to represent this condition and establishing the safety of xylitol, pharmacologically active products against which comparison of anti-inflammatory effects could be made were sought. Azithromycin, diclofenac and doxycycline were selected as agents appropriate for comparison.
Azithromycin is a macrolide antibiotic with established immune modulating activity. This effect of is thought to be due to suppression of inflammatory cytokine production, including production of IL-8, IL-6 and TNFα. It has been investigated for use in CRS and is used clinically in the medical management of this condition. It represents an ideal compound for comparison of effect.

Diclofenac is a member of the non-steroidal anti-inflammatory drug class and is effective in treating a wide variety of inflammatory conditions. Its best established mechanism of anti-inflammatory action is inhibition of PG synthesis by inhibition of COX enzymes. It may additionally inhibit thromboxane-prostanoid receptors and inhibit LO enzymes. Eicosanoid production is an important focus of this research thus an inhibitor of production is a useful agent to compare an effect against.

Doxycycline belongs to the tetracycline class of antibiotics. As with azithromycin it has pleotropic effects that include an anti-inflammatory action. It has demonstrated clinical effects in a variety of inflammatory airway disorders including CRS. In vitro tetracyclines have demonstrated inhibition of PGE2, IL-1β, IL-6, IL-8 and TNFα production. Doxycycline may additionally modulate NO production.

According to the findings of this research each of xylitol and the control drugs exhibited an anti-inflammatory action against certain of the targeted markers. The first group of inflammatory mediators considered were the eicosanoids LTB4 and PGE2. Xylitol significantly reduced LPS stimulated production of PGE2 in A549 cells, RAW 264.7 macrophages and the co-culture at both 1 µM and 10 µM concentrations (Figure 11). LTB4 production was not detectable in any of the cell lines in this study (Figure 12). The effect of xylitol on production of LTB4 is thus uncertain and cannot be ascertained from these experiments. Eicosanoids have been hypothesised as having a significant role in the pathogenesis of CRS. The finding that xylitol suppresses PGE2 production in primary respiratory and immune cell lines as well as in the co-culture suggests that further research investigating this effect is indicated.

Studies investigating the effect of xylitol on PGE2 are currently lacking. However, Han et al. (2005) have examined the in vitro anti-inflammatory properties of xylitol.
Among the markers they looked at was IL-1β. They found that xylitol (4% and 8%) significantly decreased IL-1β production in RAW 264.7 cells. This cytokine is recognized as a pleiotropic pro-inflammatory mediator with a significant role in immune response and inflammation.(102) It is produced by a variety of cell types including macrophages.(103) IL-1β induces PGE₂ production via activation of COX-2 and cJun-N-terminal kinase.(103, 104) Other regulators of COX-2 include TNFα, TGF-β1, and IL-6.(104) The suppression in all cell lines of PGE₂ seen with xylitol exposure was not mirrored in effect on IL-6 or TNFα production. Likewise, NF-κB, which controls COX-2 expression, was not uniformly affected by xylitol exposure in this study. Extrapolation of the mechanism of PGE₂ inhibition shown with xylitol exposure is beyond the scope of this research, but worthy of further investigation.

LTB₄ levels were not demonstrably affected by xylitol in this study. However, as mentioned, it is difficult to conclude anything from this result. LTB₄ concentrations produced by each of the cell lines were minimal and were mostly below the accurate level of detection for the ELISA kit used. Thus, it’s not possible to determine whether or not xylitol has an effect on LT production based upon these experiments.

The finding of an anti-inflammatory action for xylitol that involves suppression of PGE₂ in epithelial and immune cells is interesting and may suggest future clinical utility. The important role for eicosanoids in inflammation is well established, including evidence for a significant role in inflammatory disorders of the upper airways such as CRS. Eicosanoid modulation may be particularly important in CRSwNP where studies have demonstrated altered expression of COX and LO enzymes in nasal polyps in comparison to nasal mucosa.(32) The expression of COX and LO have also been shown to differ between patients with polyps and control patients. Some studies have found that differences exist depending on aspirin sensitivity status of the patient while others have failed to relate this response to aspirin sensitivity or tolerance.(105-107) There have been a number of clinical studies undertaken investigating COX expression in CRS with many, often opposing, conclusions having been made.(107)

Liu et al. (2002) found up-regulation of COX-2 in nasal polyp fibroblasts compared to turbinate mucosa and concluded that increased COX-2 contributes to nasal polyp development.(32) In contrast Pujols et al. (2004) found there to be down-regulation of COX-2 and up-regulation of COX-1 in nasal polyps compared to nasal mucosa and that this altered
COX expression, and a presumed inability to up-regulate COX-2 in response to inflammatory stimuli, may contribute to the pathogenesis of CRSwNP. (105) Perez-Novó et al. (2005) did not investigate COX-1 levels but found down-regulation of COX-2 and reduced production of PGE₂ in people with CRSwNP when compared to controls and those with CRSsNP. (108) They also found that 15-LO and lipoxin A₄ levels were increased in CRSwNP and CRSsNP compared to controls. In the Perez-Novó et al. (2005) study IL-5 and ECP were also increased in nasal polyps when compared to people with CRSsNP and normal mucosa. The increases in IL-5 and ECP corresponded with LTC₄, LTD₄ and LTE₄ concentrations and inversely with PGE₂ concentrations. The authors also found that LT levels increased with disease severity.

The effects of xylitol on further markers of inflammation, including production of NO were also assessed. NO production showed no change following exposure to any of the drugs used in the study whether in the presence or absence of LPS stimulation (Figure 15. Results of unstimulated experiments are not shown). In fact, exposure to LPS 10 ng/mL failed to increase production in any of the cell lines. Ferreira et al. (2008) also examined the effect of xylitol on production of NO in an in vitro model. (109) They found that xylitol might affect NO production at a later time point than considered in this research (72 h) even when there is an apparent lack of effect at earlier time points. They also found that the effect of xylitol on NO production (stimulation or inhibition) differed according to the strength of xylitol when tested at 72 h. Thus it is conceivable that xylitol may have an effect on NO production that was not detected in this model which only considered an early effect at 3h following exposure. Future research examining the effect of xylitol on NO production in a time controlled manner may be useful in more fully describing the effect.

In Ferreira’s work the effects of xylitol on NO production were considered in a murine J774A.1 macrophage model. (109) They investigated the effect of xylitol on unstimulated J774A.1 cells as well as macrophages that were infected with the macrophage parasite *Leishmania amazonensis*. They found no effect on cell or pathogen viability with exposure to xylitol. They found that xylitol 2.5% stimulated NO production in macrophages infected by *L. amazonensis* at 24 h and 72 h, but that production was unaffected at 48 h. They also examined the effect of xylitol in unstimulated J774A.1 macrophages and found a statistically significant reduction in NO production at 72 h with 2.5% xylitol. They found a significant increase in NO production at 72 h with 5% xylitol. There were no significant
effects with either strength of xylitol at earlier time points in the unstimulated macrophages.(109)

Ferreira et al. (2011) also considered the effect of xylitol on NO in an in vitro model using J774A.1 macrophages. (99) They measured supernatant levels of NO relative to controls following xylitol exposure. They found that xylitol 5% exposure either before or after a 3 h cell adhesion period reduced NO production. Their research also looked at macrophage adhesion and they found that xylitol exposed cells were less adherent. They have thus qualified their finding regarding NO levels and have suggested that this effect may be due to a lower macrophage concentration rather than represent a direct effect of xylitol. Xylitol’s effect on NO thus remains uncertain and a clear relationship is yet to be determined.

Animal studies investigating the effect of xylitol on NO have also been performed. Takahashi et al. (2000) examined the effect of dietary xylitol on production of plasma NO concentration in chickens. In their experiments the chickens were injected with E. coli LPS and Sephadex-G50 to stimulate an immune response. They found no difference in NO production with a xylitol diet. (110) This group has also undertaken other studies with xylitol and found that dietary xylitol (again in an experimental model using chickens) may affect B cell maturation and proliferation and enhance antibody production. They also found that IL-1, IL-6 and TNFα concentrations did not differ in chickens fed xylitol compared to glucose fed controls. (111) These results suggest a lack of modulation of NO production with xylitol but should be interpreted cautiously as the same agent may have various effects on NO production depending on the cell type or body system considered.

NO production, although acknowledged as important in the process of inflammation, has an uncertain place in the pathogenesis of CRS. Many studies have been undertaken to characterise the role of NO in the upper airways especially in the context of CRS. Due to the variability of results in published studies the clinical utility of exhaled NO measurements and the role of NO in CRS is yet to be determined. (39) Likewise, NO production in the sino-nasal region may arise primarily from epithelial cells or may represent mostly immune cell production. The source of NO may also alter in health compared to when inflammation is present. (39, 40, 42)
Modulation of the production of interleukins 6 and 8 by xylitol was also examined in this research. IL-6 and IL-8 each have a broad role as an immune mediator. This study found there to be no significant reduction in IL-8 production with any of the drugs tested, including xylitol (Figure 15). IL-6 production was similarly unaffected by xylitol exposure in A549 and RAW 264.7 cells (Figure 14). In the RAW 264.7 macrophages there was no significant change in production with any of the drugs. For most of the exposures including the vehicle control and LPS control IL-6 levels were undetectable in these cells. By contrast, in the co-culture, LPS 10 ng/mL exposure significantly increased IL-6 production relative to basal levels. LPS stimulated IL-6 production was reduced by exposure to xylitol in the co-culture (Figure 14). This study showed no significant action from xylitol exposure against the production of IL-6 or IL-8 in the individual epithelial or macrophage cells. However, when the cells were co-cultured IL-6 production was decreased. This result highlights the complexity of the immune response and the interactions that occur between cells and alter the outcome subsequent to immune stimulation.

IL-6 is a pleotrophic cytokine with pro-and anti-inflammatory actions. (100, 112) IL-8 is a pro-inflammatory cytokine, which acts as a chemo-attractant and activator for neutrophils, basophils and T cells. (103) There is increasing evidence for a pathological role for both in chronic inflammatory disorders, including CRS, and in auto-immune diseases. (113) Release of IL-6 is regulated by prostaglandins via an increase in cAMP. (112) Expression of IL-6 is down-regulated by IL-10. (100) Production of IL-6 may also be regulated through modulation of NFκB. (112) Thus, similar to other markers of inflammation alteration in concentration of IL-6 may reflect modulation occurring at a variety of levels.

TNFα is another inflammatory mediator that has a broad range of activity and an established role in the innate immune response, immune cell activation and immune cell differentiation. It is produced by various cell types and has an established role in chronic inflammatory disorders. (103) TNFα production was not reduced by any of the drugs in this study. Interestingly though, xylitol 10 μM increased basal production significantly in the epithelial cells. It exhibited no effect on TNFα production in the other cell lines (Figure 16).

This result may be considered along with those of Han et al. (2005). (72) As earlier mentioned, they tested xylitol in strengths of 1%, 2%, 4% and 8% against a number of inflammatory markers in RAW 264.7 cells. In the Han study xylitol (4% and 8%) produced a
statistically significant reduction in LPS induced TNFα without affecting cell viability. They, however, found no effect on the production of TNFα with concentrations of xylitol less than 4%. This finding of no response with lower concentrations supports the findings of this study.

Ferreira et al. (2011) have also shown a significant reduction in TNFα production with exposure to 5% xylitol. (99) This study is the same one in which they looked at NO production and this result must be interpreted in a similarly guarded manner considering the effect on macrophage adhesion.

NF-κB and the effect of xylitol on its nuclear levels were also considered in this research. Increased nuclear levels of NF-κB (as indicated by increased absorbance representing increased protein concentration) relative to baseline occurred with LPS 10 ng/mL exposure in the epithelial cell line and the co-culture (Figure 19). Conversely in the macrophage experiments NF-κB levels decreased from baseline following LPS exposure. NF-kB activation is one of the early events in the process of inflammation in most cells and stimulation with LPS is predicted. (114) Xylitol 10 μM pre-treatment resulted in a decrease in NF-κB in the A549 cells relative to baseline levels. Xylitol did not affect NF-κB levels in the macrophage cell line. It, however, increased basal NF-κB nuclear levels in the co-culture. This increase in NF-κB nuclear levels was also observed with diclofenac and doxycycline.

NF-κB is a transcription factor that is responsible for regulating the expression of a wide variety of genes including those responsible for many cytokines and other immune modulators. (72) NF-κB is usually found in the cytoplasm but moves into the nucleus following exposure to appropriate stimuli.

The effect of xylitol on NF-κB activation in RAW 264.7 cells was also examined by Han et al. (2005). (72) They assessed activation using an electrophoresis gel shift assay. They found nuclear extract NF-κB activation was decreased with xylitol exposure and that this decrease in activation had an inverse relationship with xylitol concentration (xylitol 1% to 8% was used in their experiments). (72) In contrast to Han’s findings NF-κB levels in macrophages were not significantly affected by xylitol at either concentration in this study. Their work suggested a trend for diminishing effect with lower concentrations of xylitol. Han et al. (2005) did not examine the effect of xylitol at concentrations as low as were used in this study.
study and also used a higher concentration (1 µg) of LPS as a stimulating agent. It is thus not possible to state, based upon their results, whether an effect on NF-κB levels due to xylitol would be anticipated at the concentrations used in this study.

Each of the CRS relevant inflammatory markers that the effect of xylitol was tested against were also measured following exposure to azithromycin. As previously mentioned, azithromycin is a pleotropic antibiotic with known immune modulating properties. Azithromycin, similar to xylitol, demonstrated a suppressive effect against PGE₂ production. In all of the cell lines production of PGE₂ was reduced to a greater extent with 10 µM azithromycin than 1 µM azithromycin although the difference between the strengths did not reach statistical significance. In the A549 cells both 1 µM and 10 µM azithromycin significantly reduced production of PGE₂ relative to baseline levels. In the macrophage cells LPS 10 ng/mL exposure resulted in a significant (P< 0.05) increase in production of PGE₂. In these cells 10 µM azithromycin reduced production of LPS stimulated PGE₂ while azithromycin 1 µM did not. Similarly, in the co-culture azithromycin 10 µM reduced production of PGE₂ relative to the LPS stimulated level, while azithromycin 1 µM did not have an effect (Figure 11). These results suggest that azithromycin has a dose dependent effect on PGE₂ production in macrophages and in cultures affected by macrophage production.

Macrolides, including azithromycin, have demonstrated activity against macrophage derived PGE₂. Munic et al. (2011) have investigated the in vitro anti-inflammatory activity of azithromycin in J774A.1 murine macrophages. They exposed their cells to azithromycin for 2h prior to LPS exposure and collected supernatants for analysis after 6h. They found that the effect of azithromycin on PGE₂ is dose-dependent. All strengths tested in their study reduced production but the higher strengths did so to a greater extent (0.51 of control with 12.5 µM; 0.17 with 50µM azithromycin).(87) The results of Munic et al. (2011) support the validity of this study’s finding of azithromycin suppression of PGE₂ in a concentration-dependent manner.

Azithromycin suppressed production of NO in this study. In the A549 cell line LPS 10 ng/mL stimulated NO production was significantly reduced with azithromycin 1 µM pre-treatment to below basal level production. Azithromycin was the only agent which
demonstrated an effect against NO production in the A549 cells. Azithromycin 1 µM also reduced LPS stimulated production to beneath basal level in the co-culture and again, was the only agent to do so. In the RAW 264.7 line strength of azithromycin had any effect on NO production (Figure 15).

Azithromycin did not demonstrate any significant effect on IL-6 production in the A549 respiratory epithelial cells, macrophages, or co-culture (Figure 14). Munic et al. (2011) also considered the effect of azithromycin on IL-6 production in J774A.1 macrophages. They found that there was a dose-dependent effect but that the effect was less than the effect on PGE2. They also looked at cellular accumulation of macrolides and the link with IL-6 suppression. They found that cellular accumulation correlated with IL-6 inhibition and have hypothesised that the anti-inflammatory effect of macrolides seen in in vitro models arises due to their cellular binding mechanism and intracellular accumulation (which may be substantial with azithromycin). This research group has also undertaken preliminary work suggesting a suppressive effect of azithromycin on IL-6 in RAW macrophages and in human bronchial epithelial cells.(87)

In the macrophage cell line IL-8 production was undetectable. If production had been significant enough to observe an effect with exposure the effect due to azithromycin may have been interesting. Macrolides (erythromycin and clarithromycin) have demonstrated a significant decrease in IL-8 production in nasal lavage of people with CRSwNP. Macrolides have also shown inhibition of LPS stimulated IL-8 production in human nasal epithelial cells. It has been suggested that inhibition of NFkB and AP-1 activation may account for reduction in IL-8 production.(89)

TNFα production was not affected by exposure to azithromycin in any of the cell lines considered in this research (Figure 16). This lack of suppressive effect aligns with the findings of Munic et al. (2011).(87) They found that azithromycin did not reduce production of TNFα in murine macrophages. Instead they found production was stimulated to a non-significant extent. They also considered TNFα production with other macrolides and found results to be variable ranging from 4% inhibition to 20% stimulation at concentrations of 25 to 50 µM.(87) Azithromycin has however, shown reduction in TNFα production in animal (mouse) models of lung inflammation.(89)
Azithromycin 10 µM led to a decrease in NF-κB in the A549 cells relative to baseline levels, while the lower 1 µM strength did not have a demonstrable effect. In the RAW 264.7 cells azithromycin 1 µM reduced basal NF-κB. In the co-culture azithromycin was the only drug that reduced LPS stimulated nuclear NF-κB levels and did so with both strengths (Figure 19). Studies have shown that clarithromycin and erythromycin may inhibit NF-κB. However, this effect requires prolonged exposure (>72 h) to the macrolide before an effect on NF-κB was noted.(89)

Diclofenac is a potent NSAID in wide clinical usage that was selected as a further agent for comparison of xylitol’s effect in this study. It has an anti-inflammatory action primarily mediated through COX-1 and COX-2 inhibition.(100) The finding in this study that diclofenac demonstrated an anti-inflammatory action against basal PGE₂ production is thus a confirmation of the finding of other investigators. This effect was evident in all of the cell lines (Figure 11).

Diclofenac induced inhibition PGE₂ and also TXA₂ has been demonstrated in numerous in vivo and in vitro experiments.(91, 100, 115) In addition to the COX mediated mechanism of anti-inflammatory effect it has also been suggested that diclofenac may reduce leukotriene synthesis and modulate AA levels. Ku et al. (1986) found a reduction in 5-HETE and LTC₄ production in purified rat macrophages and PMN in vitro when exposed to supra-therapeutic concentrations of diclofenac.(115) However, Samir et al. (2009) found that COX-2 was induced by diclofenac 500 µM in J774.2 macrophages.(116) Also, Wiesenber-Boettcher et al. (1991) established that diclofenac 0.004 µM reduced PGE₂ in mouse macrophages yet concentrations up to 1 µM of diclofenac did not suppress LTC₄ production.(117) This study was unable to determine an effect of diclofenac on LT production as production was below the level of detection in each of the cell lines (Figure 12).

It has also been postulated that in vivo COX inhibition by diclofenac can increase the concentrations of other pro-inflammatory markers. By sparing AA from COX metabolism other enzyme pathways may up-regulate and from alternative inflammatory entities.(100) There are also other emerging mechanisms for diclofenac activity including modulation of
peroxisome proliferator activated receptor gamma (PPARγ). PPARγ is a nuclear receptor involved in the differentiation of macrophages and in inflammatory processes, including induction of COX-2 proteins in some cells. (100)

NSAIDs may reduce cytokine levels independently of their action on PGE2. (118) In this study diclofenac had no effect on IL-6 production in the macrophage cell line or the co-culture. However, diclofenac 1 μM and 10 μM produced an increase in IL-6 concentration in the epithelial cell line (Figure 14). Diclofenac affected IL-8 concentrations in a similar manner. Diclofenac 1 μM resulted in significantly increased production in the A549 cells but had no effect in the macrophages or co-culture (Figure 15). Other investigators have found variable effects on IL-6 cytokine production after exposure to diclofenac. Tsuboi et al. (1995) found that diclofenac had the capacity to down regulate the expression and production of IL-6 in LPS stimulated peripheral blood mononuclear cells and T cells in a manner partly independent of the effect on PGE2 production. (118) They used a concentration range (up to 10 μM) similar to this study but analysed supernatant levels after 24 h of exposure rather than 3 h as used in this study. They showed a dose response curve favouring greater suppression with higher concentration of diclofenac.

Henrotin et al. (2001) also studied the effect of diclofenac exposure on cytokine production in vitro using human chondrocytes. (119) They found no significant effect on IL-8 production but found that at a concentration of 30 μM diclofenac significantly inhibited IL-6 production relative to basal and IL-1β stimulated levels. Their methodology differed from this study in the use of IL-1β as a stimulatory agent and in the duration of treatment (72 h). They also used different cell lines.

In a clinical study Mahdy et al. (2002) found that surgical patients treated with diclofenac had significantly higher circulating levels of IL-6 compared to untreated controls until a time point 12 h following initial exposure, at which time diclofenac produced significantly reduced levels. (112)

These clinical and experimental studies suggest that diclofenac has an inhibitory role on the production of IL-6. This was not demonstrated with any of the results obtained with this study. Each of the previous studies referred to has considered cytokine analysis following
prolonged exposure to diclofenac. They have also used different cell lines to the ones used here. As the mechanism of IL-6 inhibition with diclofenac is not fully established it is possible that an inhibitory effect would have been revealed if further analysis was undertaken at a later time point.

In this study, diclofenac 10 μM exposure resulted in a significant increase in TNFα in the co-culture. There was no effect demonstrated on TNFα in the other cell lines (Figure 16). Tsuboi et al. (1995) have also studied the inflammatory effects of diclofenac in an in vitro setting. They sought to establish the correlation between NSAID suppression of PGE2 and NSAID mediated up-regulation of other cytokines. They used an LPS stimulated peripheral blood mononuclear cell (PBMC) model and found that diclofenac exposure significantly increased TNFα production compared to controls. They found that other NSAIDs including indomethacin and ketoprofen produced a similar increase in TNFα production. This group also examined the effect of indomethacin exposure on T cell clones from a variety of sources including BAL from a patient with sarcoidosis and an NK3.3 IL-2 dependent line. In all of the cell lines they found that indomethacin significantly increased TNFα production. They found that NSAIDs increased the production of TNFα, IFNγ and IL-2 at the level of mRNA and protein.

Diclofenac reduced nuclear levels of NF-κB in the epithelial and macrophage cultures. Conversely, exposure to diclofenac in the co-culture increased NF-κB nuclear levels (Figure 19). Takada et al. (2004) investigated the effect of diclofenac (and other NSAIDs) on NF-κB. In their study KBM-5 leukaemia cells, H1299 lung cancer cells and A293 embryonic kidney cells were used. The cells were pre-treated for 8 h with diclofenac prior to stimulation with TNFα. They found that diclofenac did not affect NF-κB production in unstimulated cells but reduced the response to TNF stimulation in a dose dependent manner. Takada also investigated the mechanism of NF-κB alteration and showed that NSAIDs including diclofenac block the DNA binding step in NF-κB activation and NF-κB -dependent inflammatory gene expression.

Doxycycline was the final agent against which the anti-inflammatory effect of xylitol on CRS relevant inflammatory markers was compared. The effects of doxycycline on PGE2 production in this study were variable. In the respiratory epithelial cells this was the only
drug that did not reduce basal production. In the macrophages doxycycline 10 μM but not
doxycycline 1 μM reduced LPS stimulated production. Similarly, in the co-culture
doxycycline 10 μM but not doxycycline 1 μM reduced LPS-induced PGE₂ production
(Figure 11).

Other investigators have found similarly complex in vitro effects of doxycycline on
PGE₂ production. Attur et al. (1999) investigated the effect of doxycycline and minocycline
on PGE₂ and NO production in a number of cell lines including LPS stimulated RAW 264.7
macrophages.(120) They found an increase in basal PGE₂ production when cells were
exposed to doxycycline in concentrations from 77 μM to 156 μM. They did not find a
significant increase in production with lower concentrations. They also investigated the
mechanism of PGE₂ increase in RAW cells and found that doxycycline up-regulates COX-2
expression at the level of mRNA.

Attur et al. (1999) also considered the effect of doxycycline on other cell lines. They
found that PGE₂ production was augmented to a significant extent in LPS stimulated bovine
chondrocytes following exposure to doxycycline concentrations as small as 2 μM to 10 μM
but that when the concentration reached 20 μM PGE₂ production was unchanged relative to
the LPS stimulated level. In human cartilage taken from osteoarthritic patients and treated in
vitro, lower strengths of doxycycline (10-39 μM) significantly increased PGE₂ production,
mid-range concentrations (40-79 μM) had no significant effect and high concentrations
inhibited production. Their results suggest that the effect of doxycycline on PGE₂ production
is not predictable and does not produce a consistent effect at a particular concentration but
rather alters in its effect according the cell type on which it is acting.

Doxycycline did not affect production of NO in A549 cells, RAW 264.7 cells or the
co-culture (Figure 15). The effect of doxycycline on NO production has shown cell and
concentration dependent effects in other studies. Attur et al. (1999) undertook an
investigation on the effect of doxycycline on a number of inflammatory markers. They found
that NO production in LPS stimulated macrophages was inhibited by doxycycline in a
concentration-dependent manner through the range 39 - 156 μM.(120) However, they found
that in bovine cartilage exposure to low concentrations (2- 4.8 μM) of doxycycline had no
effect on NO, while 9.7 -19.5 μM of doxycycline inhibited NO production.(120)
Doxycycline exhibited no effect on IL-6 production in the individually cultured cell lines of respiratory epithelial cells or macrophages. However, in the co-culture doxycycline significantly attenuated the LPS 10ng/mL induced increase in IL-6 (Figure 14). Production of IL-8 was unaffected by doxycycline exposure in any of the cell lines (Figure 15). Doxycycline has been shown in other studies to exhibit an anti-inflammatory activity in vitro by reducing the production of IL-6 and IL-8.

Bostanci et al. (2011) found that 6h exposure to sub-antimicrobial doses of doxycycline (20 μM and 49 μM) reduced production of IL-6 and IL-8 to a statistically significant level when compared to A. actinomycetemcomitans stimulated controls in myeloid cells and monocytes.(121) Bostanci et al. (2012) also demonstrated a reduction in P. gingivalis LPS stimulated IL-8 production in myeloid cells with doxycycline 10 μM and 20 μM. Lower concentrations failed to suppress production.(122)

Doxycycline exposure did not affect TNFα production in any of the cell lines. This finding may be compared with the research of others. The effect of doxycycline on TNFα production has been examined in other cell based and animal models. Houri-Haddad et al. (2008) used a mouse model and found that P. gingivalis stimulated TNFα production was reduced to a significant extent with exposure to doxycycline.(123) They also found that doxycycline reduced the levels of IL-10 and IFNγ for a period of up to 24h following exposure. A decrease in TNFα has also been demonstrated in P. gingivalis LPS stimulated monocytes exposed to tetracyclines.(123) Bostanci et al. likewise found a significant reduction in TNFα production in A. actinomycetemcomitans stimulated myeloid cells and monocytes with exposure to low dose doxycycline.(121)

This study has shown each of azithromycin diclofenac, doxycycline, and xylitol has an anti-inflammatory action. As discussed the majority of responses achieved with the established immune modulating agents align with known mechanisms of activity for each. The research done to date with xylitol is extremely limited. This study has added to the body of work and suggests an anti-inflammatory effect in both macrophages and epithelial cells.
The most ubiquitous action appears to be against PGE$_2$ production where a significant effect was noted in all of the cell lines. In the A549 cells xylitol also increased TNF$\alpha$ production and decreased NF-$\kappa$B levels. TNF$\alpha$ is a strong stimulant of NF-$\kappa$B and is a modulator of COX as is NF-$\kappa$B.\textsuperscript{104} Thus to have these findings in one cell line is unexpected although not inexplicable considering the complexities of the immune system and the importance of PGE$_2$ in the regulation of inflammation.

PGE$_2$ is the product of COX metabolism. In classical thinking in the presence of inflammatory stimuli COX-2 metabolism is increased by a number of means including increased expression by NF-$\kappa$B. COX and subsequent PGE$_2$ production is also regulated by other pro-inflammatory cytokines including TNF$\alpha$, IL-1$\beta$ and IL-6.\textsuperscript{104} In addition to the well-known role of PGE$_2$ in creation of the inflammatory state an anti-inflammatory role for also is emerging. Studies have shown that PGE$_2$ may suppress production of TNF$\alpha$ and IL-1$\beta$ in macrophages and inhibit IL-8 and LTB$_4$ in PMN.\textsuperscript{124} PGE$_2$ can also inhibit chemotaxis, aggregation and superoxide production in PMN.\textsuperscript{124} Engagement of PGE$_2$ with EP2 and EP4 receptor subtypes is believed to mediate the anti-inflammatory effects. Signalling via these pathways may also contribute to suppression of AP-1 and NF-$\kappa$B.\textsuperscript{124}

The immune system is complex. As these results and those of others have shown the anticipated response to stimulation or treatment with a compound may not necessarily occur in an \textit{in vitro} context. This study has shown that an inflammatory response may be cell specific or may even require the presence of another co-cultured cell line for an effect to manifest. Measured effects may additionally be concentration-dependent. Although not considered in these experiments time-dependency also has the capacity to affect response to exposure. The work of others has shown this to be the case especially when the marker of interest requires \textit{de novo} synthesis following cellular stimulation. Despite the necessary limitations of this study the findings here are significant enough to suggest an anti-inflammatory effect for xylitol.

5. Conclusion

CRS is a common condition for which a satisfactory medical treatment is lacking. Its exact aetiology remains uncertain however dysfunction of host immune response is
considered central to the chronicity and treatment recalcitrance of the condition.(5, 20) The likely primacy of immune dysfunction and the dearth of successful medical treatment options led to the consideration of alternative compounds with immune modulating potential that may prove useful in treating CRS.

Xylitol was revealed as a compound of possible value in the treatment of this condition. Xylitol is established as a safe, in-expensive compound that is derived from natural sources and has significant appeal to modern consumers. Xylitol has been used in various medical and quasi-medical contexts including diabetes, osteoporosis, haemolytic anaemia, oral health, dermatology, respiratory disease and infectious and inflammatory disorders for many years where anecdotal benefit has been suggested.(62) Of relevance to CRS it is used in the upper airway as a preventative agent for OM and has undergone investigation for use in lower airway disease including CF. It is used as an ingredient in commercially available products used in the treatment of CRS. Research examining the effect of xylitol on inflammation has been limited and has, in some instances, produced equivocal results.

The primary hypothesis of this study was thus that xylitol has an anti-inflammatory action that will be of benefit in the treatment of CRS. In order to investigate the validity of this hypothesis an in vitro model was developed. This model sought to capture alterations in inflammatory markers of relevance in CRS in respiratory and immune cells. An in vitro model is appropriate for initial investigation of drug effects and indeed this one successfully demonstrated a significant immune modulating effect due to xylitol exposure that seems to align with symptom amelioration shown in clinical studies of xylitol use in CRS.

There are however inherent limitations with in vitro models such as this one that aim to establish an effect of clinical relevance. Most obviously, there may be effects shown in cell culture that do not manifest in a clinical scenario and there remains the risk that a cell based model will fail to capture effects that require the interaction of molecules and markers that are not produced by the cell under investigation. This is an especially important consideration in immune and inflammatory research where soluble mediators and interactions with other cells are frequently required for eliciting an effect.

In order to account for these interactions a co-culture was used in this research. The results obtained suggest that, as predicted, the mediators from different cell lines can affect
production of inflammatory markers in a manner that is not seen in single cell cultures. Although the co-culture afforded the opportunity to better analyse the effects in a multiple stimulated environment it still is a gross oversimplification relative to the in vivo environment. Ideally, further studies to confirm the in vitro findings will be undertaken in an in vivo model.

Although this study established that xylitol does have anti-inflammatory activity the findings may not be a true representation of its full anti-inflammatory capabilities. Experiments conducted with xylitol and the other drugs used for comparison were undertaken using only a single upper and lower concentration for each. These values were selected secondary to viability testing and an anticipated range of activity. Other investigators have successfully used higher concentrations of xylitol in cell based research in macrophage and other cell lines and have established the lack of cytotoxicity with those doses. Thus, it is possible that significant effects may be discovered with higher concentrations of xylitol that were not evident in this study.

Similarly, a single time point for supernatant collection was used in this study. This too represents a potential deficiency in design which could be improved with analysis of inflammatory marker concentrations at multiple time points following exposure. As the literature shows, inflammation is a dynamic process and capturing a single instance in the process cannot fully represent the possible modulating effect of xylitol or any of the other drugs tested for comparison.

Despite the acknowledged limitations of this study the primary aim of revealing whether xylitol has an anti-inflammatory action has been achieved. There are many avenues for future research to build on this finding. In the context of developing a viable treatment for CRS this would include in vivo trials to investigate immune modulation and ensure safety and tolerability for clinical use. Development of a novel vehicle for delivery to the affected mucosa would also be indicated if clinical trials demonstrated an effect. The finding of anti-inflammatory action in a respiratory cell line and a co-culture involving respiratory epithelium suggests that xylitol may be useful in treating inflammatory disorders of the lower airways in addition to CRS.
Future research could also consider related compounds. Mannitol is another naturally occurring sugar alcohol that is widely used in medical practice.\(^{(125)}\) It is used in the treatment of Cystic Fibrosis (CF) as an inhaled powder designed to improve mucous clearance.\(^{(62, 125)}\) The exact mechanism of action is not known although it is proposed to act by altering the viscosity of mucous and thus enhancing the clearance of airway secretions.\(^{(125)}\)

Xylitol has shown significant anti-inflammatory activity in this study. It inhibited PGE\(_2\) production in all of the cell lines examined as well as NF-\(\kappa\)B in respiratory cells and IL-6 in the co-cultured model. These results are encouraging and justify further investigation into the immune modulating capabilities of this versatile compound.
References

5. Lee S, Lane A. Chronic Rhinosinusitis as a Multifactorial Inflammatory Disorder. Current Infectious Disease Reports. 2011;13(2):159-68.
75. Sachse F, Von Eiff C, Becker K, Steinhoff M, Rudack C. Proinflammatory Impact of Staphylococcus epidermidis on the Nasal Epithelium Quantified by IL-8 and GRO-[alpha]
Responses in Primary Human Nasal Epithelial Cells. International Archives of Allergy and Immunology. 2007;145(1):24-32.


