The Effect of Epigallocatechin-gallate on Inflammatory Protein Production of Odontoblast-like cells

by

Alexander Terry Stavroullakis

A thesis submitted in conformity with the requirements for the degree of Master of Science
Faculty of Dentistry
University of Toronto

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Master of Science
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2018

Abstract

Objective: To evaluate the effect of Epigallocatechin-gallate (EGCG) on the cell viability, cytokinome, and protein production of the MDPC-23 cell line with or without Streptococcus mutans (SM) stimulation, compared with Chlorhexidine diacetate (CHX). Methods: Part 1: Cytotoxicity of CHX and EGCG treatment; cytokine secretion was analyzed using a Cytokine 23-plex Assay. Part 2: Cytotoxicity of SM stimulation with CHX and EGCG treatment; protein production was measured using ELISA and LC-MS/MS. Results: At 24 and 48 hours, cell metabolic activity was stable at 2.5-20 µM (CHX) and 2.5-160 µM (EGCG), respectively. CHX 10 µM (stimulated or not with SM) increased IL-1β levels despite lowering total protein levels compared to control and EGCG at 10 µM with SM stimulation increased peroxiredoxin-1 abundance compared to control. Conclusion: Protein production varied between treatments, with EGCG being viable at higher concentrations than CHX. EGCG may have potential as a treatment in dental caries-related inflammation.
Acknowledgments

Over the course of this project, I have had the privilege to work with many esteemed researchers and scientists. First and foremost, I’d like to thank my supervisor Dr. Anuradha Prakki, who has been the best mentor anyone could ask for. The enthusiasm with which she conducts her teaching and research is truly inspiring.

I am very grateful to the members of my advisory committee Drs. Anil Kishen and Celine Levesque, who have guided me through this work and helped me improve it immensely.

Dr. Delphine Dufour’s kind assistance with the *S. mutans* portion of this project is greatly appreciated.

Finally, I would not have been able to complete this project without the support from my family and friends. Their unconditional love and support have helped me develop into a better student and person.
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<th>Full Form</th>
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<tbody>
<tr>
<td>SM</td>
<td><em>Streptococcus mutans</em></td>
</tr>
<tr>
<td>OBs</td>
<td>Odontoblasts</td>
</tr>
<tr>
<td>MDPC-23</td>
<td>Mouse Dental Papilla Cell-23</td>
</tr>
<tr>
<td>CHX</td>
<td>Chlorhexidine</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>EGCG</td>
<td>Epigallocatechin-gallate</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography-tandem mass spectrometry</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of Infection</td>
</tr>
<tr>
<td>TMT</td>
<td>Tandem mass tags</td>
</tr>
<tr>
<td>DEJ</td>
<td>Dentino-enamel junction</td>
</tr>
<tr>
<td>EPS</td>
<td>Extracellular polysaccharides</td>
</tr>
<tr>
<td>GTFs</td>
<td>Glucosyltransferases</td>
</tr>
<tr>
<td>FTFs</td>
<td>Fructosyltransferases</td>
</tr>
<tr>
<td>NCPs</td>
<td>Non-collagenous proteins</td>
</tr>
<tr>
<td>SIBLINGS</td>
<td>Small Integrin-Binding Ligand N-linked Glycoproteins</td>
</tr>
<tr>
<td>DSP</td>
<td>Dentin sialoprotein</td>
</tr>
<tr>
<td>DPP</td>
<td>Dentin Phospho Protein</td>
</tr>
<tr>
<td>DSPP</td>
<td>Dentin Sialo Phospho Protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>DMP-1</td>
<td>Dentin Matrix Protein – 1</td>
</tr>
<tr>
<td>OPN</td>
<td>Osteopontin</td>
</tr>
<tr>
<td>TIMPs</td>
<td>Tissue inhibitors of matrix metalloproteinases</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharides</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>TLRs</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>LTA</td>
<td>Lipoteichoic acid</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor-kappa light chain enhancer of activated B cells</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/IL-1 receptor</td>
</tr>
<tr>
<td>TRIF</td>
<td>(TIR) – containing adaptor inducing interferon-β</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response gene 88</td>
</tr>
<tr>
<td>TAK1</td>
<td>Transforming growth factor-β-activated kinase-1</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase</td>
</tr>
<tr>
<td>NEMO</td>
<td>NF-κB essential modifier</td>
</tr>
<tr>
<td>DPP-MP</td>
<td>Moderately phosphorylated phosphophoryn</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s medium</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte-colony stimulating factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>KC</td>
<td>Keratinocyte chemoattractant</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>Macrophage inflammatory protein-1 α</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
</tr>
<tr>
<td>LF</td>
<td><em>Ligamentum flavum</em></td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor activator of nuclear factor-kappa B ligand</td>
</tr>
<tr>
<td>THYE</td>
<td>Todd-Hewitt Yeast Extract</td>
</tr>
<tr>
<td>MAPK/ERK</td>
<td>Mitogen-activated protein kinase/extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>PEP</td>
<td>Phosphoenolpyruvate</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
</tbody>
</table>
Chapter 1
Introduction

Dental caries is an extremely common chronic disease, affecting 75% of children by age 15, while by late adolescence 95% of adults will be affected by enamel or root surface caries. It also disproportionately affects minority and low socioeconomic populations [1 – 4].

The risk of caries occurs throughout an individual’s lifetime, and is triggered by a multitude of environmental and lifestyle factors, the most influential of which is invasion by oral bacteria [3, 5, 6]. A diet rich in carbohydrates, most notably sucrose, triggers the growth of acidogenic bacterial communities, and their resulting metabolic acids are able to demineralize tooth enamel and degrade collagen in the dentin layers [7 – 9].

The bacterium identified as a pathogen in the development of caries is Streptococcus mutans (SM) [10]. Though it is not always the dominant bacteria in every stage of caries development, its cariogenic capacity has been proven to be the most out of the other bacteria involved in the disease. As such, it has been found that in the advanced stages of carious lesions, SM comprises approximately 30% of the microbiome [11 – 13].

Odontoblasts (OBs) are localized in the pulp and dentin tubules [14]. Due to their location, OBs are the first response to bacterial stimuli and play a role in host response to bacterial infiltration and subsequent inflammation. This is achieved by formation of new dentin in a carious lesion to inhibit the progress of infection or by production of anti-inflammatory cytokines [10, 15]. For the purposes of this in vitro study, the Mouse Dental Papilla Cell-23 (MDPC-23) odontoblast-like
cell line was chosen to be utilized as it has been identified to possess odontoblast characteristics and the means for tooth mineralization [16, 17].

Chemical plaque-control treatment involves the use of chlorhexidine (CHX). It has been shown to inhibit the growth of SM and the activity of metalloproteinases (MMPs) during caries development [18, 19]. However, CHX at certain concentrations has been shown to be cytotoxic to OBs as well as human gingival fibroblasts in various in vitro studies [20 – 22]. Conversely, recent research has highlighted the benefits of frequent green tea consumption to include anti-inflammatory and anti-oxidant activity, specifically via Epigallocatechin-gallate (EGCG), a catechins in the polyphenol group [23, 24]. In a study with nasal fibroblasts and bronchial epithelial cells, EGCG strongly inhibited the production of the pro-inflammatory cytokine IL-8 [25]. The anti-inflammatory effect of EGCG has also been investigated in SM-inflamed dental pulp fibroblasts, showing the inhibition of inflammatory cytokines [23]. Lastly, it is known that EGCG can inhibit the expression of dentin matrix metalloproteinases, such as MMP-9, which has been found in large concentration in carious lesions and is known to play an important role in the process of caries progression [26, 27].

Two common methods for screening protein and pathway modulations in host cells are Bio-Plex Immunoassays and liquid chromatography-tandem mass spectrometry (LC-MS/MS). Studies using these techniques have found that the introduction of a multiplicity of infection (MOI) by bacterial cells alters the protein expression of host cells [28 – 30]. We utilized a 23-Plex Immunoassay and then tandem mass tags (TMT) in LC-MS/MS in our study in order to investigate, respectively, the cytokinome of un-stimulated OBs, and then differential protein production between SM-stimulated and non-stimulated OBs treated with EGCG and CHX. The aim of this study is to compare the interaction of EGCG on the MDPC-23 intracellular
cytokinome and proteome among groups un-stimulated and stimulated with SM, compared to a CHX treatment. The hypothesis is that in similar, non-cytotoxic concentrations, EGCG will be more effective at inhibiting production of pro-inflammatory proteins than CHX.
Chapter 2
Literature Review

2.1 Caries

Dental caries is a highly prevalent chronic disease, affecting 75% of children by age 15, and disproportionately affecting minority and low socioeconomic populations [1 – 3, 31]. By late adolescence, only about 10% of people in the United States are caries-free, while during adulthood upwards of 95% will experience enamel or root surface caries [4]. In Canada, the percentage of adults who have had a cavity is 96%, with 21% suffering moderate to severe periodontal complications [32]. It can be initiated by a host of factors, including, but not limited to, microbial, environmental, behavioural, immunological, and genetic. Despite the many causes, the most prominent and studied is initiation via the actions of oral bacteria [3, 6].

Typically, carious lesions can be characterized as the physical and chemical demineralization of the hydroxyapatite component of the enamel and dentin structures and subsequent destruction of the collagenous tissue in dentin by various cariogenic and non-cariogenic oral bacteria [3, 5, 33]. Initiation of caries lesions occurs in the dentino-enamel junction (DEJ), which the cariogenic bacteria and their resulting by-products invade, eventually causing a cavitation. This lesion expands in the DEJ, and is followed by degradation of the dentin by host collagenases and gelatinases [34]. The way by which the bacteria behave cariogenically is through fermentation of dietary carbohydrates, most commonly sucrose, allowing them to produce lactic, formic, propionic, and acetic acids [4, 35]. As a result, the pH drops below the 5.0 critical value for demineralization of the tooth structure [4, 36]. The acids then diffuse into the enamel and dentin and begin to dissolve the mineral crystals contained within the hydroxyapatite layers, leading to
a diffusion of calcium and phosphate out of the tooth. This results in a caries lesion in the enamel, while in the dentin collagen degradation is also an integral component of the caries process [5, 35].

*Streptococcus mutans* (SM) is a Gram-positive, facultative anaerobe that is implicated as having the most cariogenic potential compared to other members of the oral microbiome [37–42]. Colonization occurs shortly after tooth eruption, and if there are significant amounts of sucrose or other simple carbohydrates in the environment, SM is able to metabolize them and produce acids as well as extracellular polysaccharides (EPSs). EPSs such as glucans and fructans are produced by means of the Glucosyltransferases (GTF) and Fructosyltransferases (FTFs) respectively, which are known to be crucial components of initial attachment and eventual formation of mixed-species biofilms on the tooth surface [37, 39, 42–45]. As well, numerous bacteria including SM are able to attach to Type I Collagen in the dentin through the use of surface adhesins [46].

Once a biofilm is formed, simple carbohydrates are continuously metabolized and organic acids are produced, furthering the tooth demineralization process. SM in particular is important at this stage due to its rapid acidogenicity and its aciduricity. Compared to non-cariogenic bacteria, SM thrives in low pH environments, and as such it is present in high numbers during early caries lesions [37, 39, 44].

### 2.2 Dentin – Structure

Compared to the approximately 96% inorganic hydroxyapatite mineral content by weight in the enamel, the dentin is composed of approximately 70% mineral, more than cementum or bone
The remainder of the dentin is between 18-20% organic matrix and 10-12% water [47, 49, 50]. The organic matrix is comprised of mainly Type I collagen (90%), which is considered to be the backbone of the dentin, and non-collagenous proteins (NCPs). Both collagen and some of the NCPs are produced by OBs and secreted into the predentin during early dentinogenesis [34, 47, 49].

Type I collagen is a triple helix structure composed of three alpha polypeptide chains and is 300 nm and 1.5 nm in length and diameter, respectively. The collagen molecules self-arrange themselves into fibril networks and are stabilized by means of both reducible and non-reducible cross-linking, and this network houses the crystals of the dentinal mineral content. The collagen network in the dentin is able to sustain its structure even if half of the mineral is lost, but eventual exposure of the network via the caries process irreversibly destroys the reducible cross-links of the collagen fibrils [48, 49, 51]. Within the organic matrix of the dentin lie NCPs, which can be further divided into phosphorylated proteins and non-phosphorylated proteins. The large group of phosphorylated proteins includes the Small Integrin-Binding Ligand N-linked Glycoproteins (SIBLINGs) which are heavily involved in dentinogenesis [50]. Markers of dentinogenesis include Dentin sialoprotein (DSP) and Dentin Phospho Protein (DPP), which are cleavage products of Dentin Sialo Phospho Protein (DSPP), and Dentin Matrix Protein – 1 (DMP-1). DPP and DSP together constitute more than half of the total NCPs contained within the dentin matrix [50, 52, 53]. DMP-1 is produced by ameloblasts as well as odontoblasts, and plays a role in dentinogenesis through the formulation of mineral by use of its ability to bind calcium. Osteopontin (OPN), on the other hand, has a potential role in the inhibition of the mineralization process [50]. Of the non-phosphorylated proteins, cysteine cathepsins such as cathepsin B and the collagenase cathepsin K, are prevalent during caries progression, and their
activity increases as the caries lesion approaches the dental pulp [54, 55]. MMPs are also a much discussed topic due to their contribution to the caries process [48, 56].

MMPs are zinc and calcium-dependent endogenous enzymes, with the ability to degrade many extracellular matrix proteins. They contain pro and catalytic domains, and are inactive until the two domains are dissociated from one another. The acidity generated during the caries process allows the prodomain to cleave and results in activation of the various MMPs sequestered in the dentin. Their prevalence along the DEJ is also important, as increased MMP activity has been observed in carious dentin when compared to normal dentin, and this has been found to be attributed to the enlarging of the lesion in the DEJ. [54, 57, 58]. Though they are produced by OBs and have been linked to the formation of dentin, host MMPs are responsible for the majority of collagen degradation [48, 56, 59]. Most notably, the gelatinases MMP-2 and -9, the collagenases MMP-1 and -8, and MMP-3 and -20 are considered to be the most active in terms of proteolytic capacity during caries [48, 54]. Other proteins sequestered in the dentin include tissue inhibitors of matrix metalloproteinases (TIMPs), and growth factors such as insulin-like growth factors 1 and 2, fibroblast growth factor-2, and transforming growth factor-β (TGF-β) members [55]. The cytokines tumour necrosis factor-α (TNF-α) and interleukin-1 β (IL-1β) have also been indentified within in vivo dentin matrix, released as a response to demineralization via acids, while IL-8 has been found in pulp tissue after stimulation with bacterial lipopolysaccharides (LPS) [55].

As the carious lesion progresses through the dentin and towards the pulp, bacterial external proteins and other by-products are able to travel through the dentinal tubules. The area between the dentin and the pulp of the tooth contains the OBs and pre-odontoblasts, as well as progenitor and other cells yet to undergo differentiation. The OBs form a palisade of cells in the dentin-pulp
complex which acts as a barrier between the dental hard tissues (dentin, enamel) and the dental soft tissue. This palisade is the first line of defense against the bacterial products reaching the highly vascularized and innervated deep layer of the pulp [54, 60, 61].

2.3 **Odontoblasts — background**

OBs are differentiated dental papilla cells, deriving from cells of the neural crest. They are post-mitotic and not replaced in the tooth, existing for the duration of its life. Localized in the dentin-pulp interface, they extend cytoplasmic processes through the dentinal tubules. These processes allow them to deposit pre-dentin (primary) during tooth development and dentin (secondary) thereafter. As well, OBs synthesize type I collagen and various other NCPs which further contribute to the integrity of the dentin layer [14, 17, 62 – 65].

However, OBs can secrete more types of dentin depending on the extent of the tooth injury during caries. Reactionary dentin, a type of tertiary dentin, is secreted as a response to minor injury, in which the OBs survive. This third dentin type is consistent in composition with the primary and secondary types, and serves as the initial healing process. When the injury is such that the primary OBs begin to die, reparative dentin is formed as a means to impede the progression of bacterial products through the dentinal tubules [54, 61, 66].

The OBs function extends past providing support to the structure of the tooth. Various studies have shown that they also produce antimicrobial agents such as nitric oxide and beta defensins. The latter of these is a cationic, potent antimicrobial peptide that acts on the cell membrane of the bacteria and causes leakage. OBs have also been shown to be immunocompetent cells, channeling the innate and adaptive immune responses once challenged by invading bacteria. OBs
sense pathogen-associated molecular patterns (PAMPs) from bacteria and are able to secrete inflammatory cytokines and chemokines in response [60, 61, 67].

One way by which OBs can identify various PAMPs from invading bacteria is by utilizing the toll-like receptors (TLRs). Consisting of 10 members in humans, this family of receptors is able to recognize various microbial products and initiate the inflammatory response. TLR 2 and 4 are the most associated with the oral microenvironment of dental caries. Gram positive bacteria such as SM can be recognized through lipoprotein, peptidoglycan, or lipoteichoic acid (LTA), located on their cell wall, via TLR 2, either by itself or after forming a heterodimer with TLR 1 or 6 [68]. The recognition of LTA by OBs generally occurs during early stage caries, when the lesions are shallow and the oral microbiome consists of higher numbers of Gram positive bacteria. During late-stage caries leading to pulpal exposure and inflammation, the anaerobic Gram negative bacteria are more prevalent [67, 68]. The cell wall component unique to Gram negative bacteria is LPS, which is recognized by the OBs through TLR 4. The signaling cascade initiated by TLR 2 and 4 activation will be discussed in more detail shortly [67 – 74].

Once the microbial components are sensed by the OBs, the innate immune response begins. This involves the expression of pro and anti-inflammatory cytokines and growth factors such as the ILs 1β, 2, 4, 6, 10, 11, TNF-α, chemokine ligands 8 and 10, and various other growth factors that are involved in the inflammatory cascade [75, 76]. These cytokines, chemokines, and growth factors are polypeptides produced by many different types of cells in order to initiate and control the inflammatory processes, and can be divided into pro and anti-inflammatory groups. For example, pro-inflammatory cytokines include IL-1β, -2, -6, -8, and TNF-α, while IL-4, -10 comprise cytokines that serve to mitigate the inflammatory response [75, 76]. IL-1β is the most studied of the IL-1 family, and has been implicated as an important factor in the host response to
PAMPs. After PAMP recognition, the pro-IL-1β molecule is cleaved by caspase-1, allowing the active molecule to be secreted. IL-1 β has been studied both as a result of Nuclear Factor-kappa light chain enhancer of activated B cells (NF-κB) activation and as a cause of it, with many studies utilizing IL-1β as a stimulus [77, 78]. In this way, IL-1β may play a central role in the pro-inflammatory response. Many cytokines and growth factors have been identified in caries lesions within the dental pulp and odontoblast layers, and have been found to be produced by odontoblasts and pulp fibroblasts in response to bacterial challenges, while others such as TGF-β have been found to be sequestered within the dentin [71, 76, 79 – 81].

Recognition of PAMPs by the OBs via the TLRs also influences the NF-κB pathway. NF-κB refers to a family of regulatory molecules that are at the centre of numerous immune and stress responses, and are modulators of cell apoptotic and proliferative processes. In mammals, this family contains five proteins: RelA (p65), RelB, C-Rel, p105 (NF-κB1, the p50 precursor), and p100 (NF-κB2, the p52 precursor). These members are able to form dimers with each other, and the most commonly identified one in TLR signaling pathways is the RelA-p50 heterodimer [78, 82 – 84].

Without a stimulus present, the NF-κB molecule exists in an inactivated form within the cytoplasm by means of one of the several inhibitory (IκB) proteins. In the ‘Canonical’ NF-κB pathway, once an external stimulus is sensed by the TLRs, a signaling cascade occurs. Specifically, TLR2 can signal through either the Toll/IL-1 receptor (TIR) – containing adaptor inducing interferon-β (TRIF) – dependent pathway or the myeloid differentiation primary response gene 88 (MyD88) pathway. These two pathways culminate in the activation of transforming growth factor-β-activated kinase-1 (TAK1) by means of K63-linked ubiquitination. Upon activation, the TAK1 itself activates the IκB kinase (IKK) complex, which consists of
IKKα, IKKβ, and IKKγ, more commonly known as NF-κB essential modifier (NEMO). The IKK complex is responsible for initiating the phosphorylation of IkB serine residues, which leads to ubiquitination of the inhibitory proteins by the 26S proteasome [78]. This allows the NF-κB molecule to translocate from the cytoplasm to nucleus and bind to various κB DNA sequences, resulting in production of any number of inflammatory mediators, including IL-1α/β, IL-6 and TNF-α [78, 82 – 85]. Pathways other than NF-κB have been linked to caries-related inflammation, as seen in a study by Dommisch et al. 2008 and Simon et al. 2010 who introduced bacterial stimulation by SM to identify the upregulation of pro-inflammatory cytokine gene expression and the activation of the p38 MAP kinase pathway in the MDPC-23 line [17, 79]. In carious lesions, inflammation can become chronic if not treated and may result in fibrosis of the collagen and pulp tissue necrosis [75, 86].

2.4 MDPC-23 cell line (Mouse Dental Papilla Cell-23)

This cell line was named after the 23rd clone during culturing by the Hanks group from the University of Michigan. Having exhibited odontoblastic phenotypes, this cell line has been identified as one of the true ‘odontoblast-like’ cell lines. Briefly, this cell line derives from the dental papilla mesenchymal tissue of 18-19 day-old fetal mice, subsequently immortalized by subculturing every third day, using a density of 6 x 10^5 cells in a 500mm plate, also known as the 3T6 method. After subculturing and cloning by dilution, the cells were analyzed for odontoblast-specific proteins [16, 46, 62, 87, 88].

The MDPC-23 line is positive for the expression of Type I collagen, DSP, moderately phosphorylated phosphophyrm (DPP-MP), while being negative for DMP-1. The presence of
these proteins indicate that the cells are of odontoblastic lineage [16, 46, 87]. In vitro studies utilizing dental materials in order to assess their cytotoxicity on cells commonly utilize the MDPC-23 cell line, despite the lack of a gold standard cell line for such investigations. Light curing for the polymerization of resin-based materials is very commonly used and therefore has been studied for its effects on odontoblast cytotoxicity, metabolism, morphology, several other crucial factors that may negatively affect the integrity of the odontoblast layer of the tooth [88 – 90]. Other studies have stimulated the cell line using ultrasound in order to determine if the resulted vascular endothelial growth factor (VEGF) expression may play a role in the odontoblast response to dentin degradation [91]. CHX gels have also been applied to in vitro studies utilizing the MDPC-23 cells, with the rationale that the compound, when applied on acid-etched dentin, may diffuse through the tubules and, at certain concentrations, exhibit a cytotoxic effect on the odontoblast layer [92]. The MDPC-23 cell line is a commonly utilized and therefore very reliable odontoblast-like cell line for the purposes of testing restorative materials and dentin therapeutic compounds in vitro.

2.5 Chlorhexidine

As discussed, there are various factors that are involved with the progression of dental caries, and as such there have been many different methods advocated to halt the demineralization and eventual degradation of the dental hard tissues. However, the most widely targeted factor of dental caries is the bacterial influence, and for many years a 0.12% chlorhexidine gluconate treatment has been the most utilized and studied measure of controlling dental plaque [18, 20]. CHX is a cationic, amphiphilic bis-biguanide that exhibits many anti-bacterial properties. It has demonstrated bacteriostatic effects on SM, as well as a range of activity against other Gram
positive and negative bacteria. This compound easily binds to the negatively charged bacterial cell wall, and in low concentrations merely inhibits metabolic activity, but at high concentrations it may cause precipitation of cytoplasmic contents. CHX has also been shown to be a potent inhibitor of the cysteine cathepsins B, K, and L as well as the MMPs -2, -8, and -9 via a mechanism related to the cysteines and sulfhydryl groups in their active sites lending more to its efficacy against dental caries [19, 21, 93 – 97].

Despite the positive uses for CHX, there are drawbacks which include such side effects as tooth and restoration staining, and alterations to taste. The cytotoxicity of CHX is also a concern on odontoblasts, fibroblasts, and other various types of cells in and around the tooth. High concentrations and high exposure time to CHX proved to cause necrosis in MDPC-23 and other eukaryotic cells, with no potential for recovery [20]. A lower exposure time of 2 hours was not seen as a viable solution as the cell condition did not improve following incubation in medium containing no CHX for 48 hours [98]. The affinity of CHX for the plasma membrane has been postulated to be the reason for its cytotoxicity. Its positive charge allows it to be an effective antibacterial compound, however the non-specific binding by which it interacts with bacterial cells may be harmful to the plasma membrane of eukaryotic cells [98, 99, 100]. Other studies have revealed that CHX can also disrupt DNA and protein synthesis, which may result in an alteration of the host cell cytokinome and proteome [18, 20, 21]. Furthermore, a study by Wand et al found that certain Gram-negative bacteria are able to develop a resistance to CHX while retaining their virulence [101]. Though CHX is effective in many ways, its drawbacks are concerning and thus there is a constant search for alternative treatments that share its potency without having the impeding cytotoxic effects on host cells.
2.6 *Epigallocatechin-gallate*

Green tea is a widely consumed beverage, and numerous studies have linked its consumption to decreased risks of cardiovascular diseases, oxidative stress, cancer, inflammation, and the attachment of cariogenic bacteria to the tooth surface [23, 102, 103]. The components of green tea that these benefits have been attributed to are the catechin group of polyphenols, 59% of which are comprised of EGCG [102 – 106]. EGCG contains a gallate moiety which allows it to more readily form hydrogen bonds with the phospholipid bilayer when compared to polyphenols lacking the moiety. Owing to its abundancy and biological activity, it has been a heavily investigated part of green tea [106, 107]. EGCG has also been recognized to have anti-microbial properties in studies with cariogenic bacteria. Various studies showed that treatment with the compound resulted in diminished numbers of planktonic SM and *Lactobacillus* strains, as well as stunted biofilm growth [103, 107, 108]. Further research indicated that EGCG inhibits the activity of GTFs produced by various cariogenic bacteria, resulting in less acidic conditions and hence a less viable oral environment for the progression of caries [14, 103, 107]. Finally, EGCG has displayed an ability to inhibit MMPs-2 and -9, which are found in high concentrations within carious lesions [26, 105, 109]. The mechanism of action for this inhibition is through binding of EGCG to the MMPs to create EGCG-MMP complexes which reduce the activity of MMPs. MMP inhibition plays a significant role in slowing caries progression, and is a trait that EGCG shares with CHX [26, 27]. The anti-inflammatory capabilities of EGCG have been greatly documented, where researchers have found that it was able to reduce the release of pro-inflammatory cytokine and growth factors such as IL-1β, IL-6, IL-12 and TNF-α in macrophages, nasal fibroblasts, bronchial epithelial cells, and hepatocytes challenged with LPS [104, 110, 111]. Recently, it has also been linked to the inhibition of NF-κB, both through MMP inhibition and independently of MMPs, leading to the suppression of cytokine release in both
bone marrow macrophages and SM-challenged dental pulp cells [23, 85]. EGCG is also exhibits relatively low cytotoxicity to the MDPC-23 cell line, as reported in findings by Zarella et al (2016) where experiments were performed measuring the cytotoxicity of EGCG extracted from dental copolymers [112].
Chapter 3
Objectives

3.1 General Objective

The general objective of this study is to investigate the effect of EGCG on inflammatory-related protein production of MDPC-23 odontoblast-like cells. This study is divided into two parts. In Part 1, cells treated with EGCG were evaluated for cytotoxicity and viable treatment groups were screened for cytokine secretion.

In Part 2, an MOI using heat-killed S. mutans strains was introduced to the MDPC-23 cells in order to simulate inflammatory conditions. Cytotoxicity experiments were performed with SM stimulation and EGCG treatment. Furthermore, both the production of pro-inflammatory cytokine IL-1β and the differential protein production of whole MDPC-23 cell lysates were measured in cells both stimulated and un-stimulated with SM and treated with ECGC. In parts 1 and 2, Chlorhexidine diacetate was used as a positive control.

3.2 Specific Aims

3.2.1 Specific Aims Part 1

Specific Aim 1: To evaluate the effect of EGCG on the viability of MDPC-23 cells.

Specific Aim 2: To evaluate the effect of a viable concentration of EGCG on the cytokinome profile of treated MDPC-23 cells, compared to CHX and un-treated cells.

Hypothesis: The cytokinome of EGCG-treated odontoblast-like cells would predominantly be anti-inflammatory when compared to un-treated cells, while CHX-treated cells would result in secretion of pro-inflammatory cytokines.
3.2.2 Specific Aims Part 2

Specific Aim 1: To evaluate the effect of *S. mutans* MOI on the viability of MDPC-23 cells.

Specific Aim 2: To evaluate the effect of simultaneous MOI stimulation and EGCG treatment on production of IL-1β by MDPC-23 cells, compared to CHX treatment, to MOI stimulation only, and to un-treated cells.

Specific Aim 3: To evaluate the effect of simultaneous MOI stimulation and EGCG treatment on the differential protein production in whole MDPC-23 cell lysates, compared to CHX treatment, to MOI stimulation only, and to un-treated cells.

**Hypothesis:** In a simulated inflammation model, EGCG treatment will be more effective at inhibiting production of pro-inflammatory proteins, while increasing the production of anti-inflammatory proteins, when compared to CHX treatments.
Chapter 4
Part 1: Manuscript Accepted: February 7, 2018
in Dental Materials

Profiling cytokine levels in Chlorhexidine and EGCG-treated Odontoblast-like cells

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ABSTRACT

Objective: To screen the effect of two compounds, chlorhexidine diacetate (CHX) and epigallocatechin-gallate (EGCG), on the levels of cytokines produced by odontoblast-like cells (MDPC-23). Methods: Cells were seeded at 24h and 48h with serial dilution of the compounds to determine cell metabolic activity by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay \((n=3)\). Cells with no compound treatment were used as control (Ctr). For the highest equal non-cytotoxic compound dilution tested at 48h cell treatment, total protein concentration was measured using a Pierce bicinchoninic acid (BCA) assay \((n=3)\), and expression of 23 cytokines was analyzed using the Bio-Plex cytokine assay \((n=2)\). Data were analyzed by one-way ANOVA and Tukey’s test \((\alpha=5\%)\). Results: The MTT assay revealed that at 24h and 48h, CHX and EGCG did not reduce cell metabolic activity at concentrations of 2.5–20 \(\mu M\) (CHX) and 2.5–160 \(\mu M\) (EGCG), respectively \((p>0.05)\). At 48h, total protein levels were consistent across all groups for 20 \(\mu M\) compound dilution \((Ctr: 1.04 \text{ mg/mL}; \text{CHX: 0.98 mg/mL; and EGCG: 1.06 mg/mL})\). At 20 \(\mu M\) dilution, both CHX and EGCG significantly increased the secretion of IL-1\(\beta\), IL-10, IL-12, KC, MIP-1\(\alpha\), IFN-\(\gamma\) and IL-6 \((p<0.05)\). Treatment with CHX significantly increased secretion of IL-4 and RANTES \((p<0.05)\). Treatment with EGCG significantly increased Eotaxin secretion \((p<0.05)\). Both CHX and EGCG significantly decreased secretion of IL-17 \((p<0.05)\). GM-CSF and TNF-\(\alpha\) did not present significant change in secretion after treatment with either CHX or EGCG \((p>0.05)\). Significance: Both CHX and EGCG modulate secretion of various inflammatory and anti-inflammatory mediators in odontoblastic cells.

Keywords: chlorhexidine, EGCG, cytokines, MTT, MDPC-23, cytokinome
Introduction

Cytokines are produced by different cells of the body and although their grouping into cytokine families is not consensual in the literature, it is accepted that they can be arranged into structural families that include: interleukins, chemokines, interferons, transforming growth and tumor necrosis factor families. Families of cytokines share sequence similarity and exhibit homology and some promiscuity in their reciprocal receptor systems. Despite being structurally related, members belonging to the same cytokine family may exhibit rather diverse functions. Essentially, cytokines are small (~5 to 20 kDa) signaling proteins synthesized by a range of both immune and tissue structural cells in response to different cellular stresses and stimuli. These molecules are able to regulate cellular immune and pro- and anti-inflammatory responses, and further modulate gene expression and biochemical responses in target cells via second messenger signaling mechanisms [1]. The fine-tuned cytokine networks maintain a balance between pro- and anti-inflammatory processes creating the desired environment for tissue repair [2]. While cytokines and chemokines are likely produced in response to bacterial invasion, interesting reports show molecules such as transforming growth factor-β (TGF-β), insulin-like growth factor-1 and -2 (IGF-1 and -2), fibroblast growth factor-2 (FGF-2) sequestrated within dentin [1]. These can be released by acids and restorative materials [3], and may stimulate, for instance, expression of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) by macrophages [4]. Both TNF-α and IL-1β can in turn induce expression of IL-8, a neutrophil recruiter and activator [5]. The interactions and clinical effects of cytokines and chemokines sequestrated within dentin and/or produced by odontoblasts, on tissue remodeling, certainly entail further understanding.

Clinical applications of chlorhexidine (CHX) in restorative dentistry have been long advocated. Studies have recommended the cleansing of dentin with CHX prior to pulp capping and the use
of cavity liners. This would eliminate bacteria lodged in dentinal tubules after mechanical caries removal, encouraging pulp tissue repair by controlling bacterial contamination and inflammation [6]. Others have demonstrated that CHX is capable of arresting caries when applied to dentin [7]. It has also been shown that due to anti-proteolytic activity against matrix metalloproteinases (i.e., MMP-2, -8, and -9) [8] and cysteine cathepsins [9], the use of CHX on demineralized dentin prior to bonding agent application prevents the degradation of hybrid layer, maintaining the integrity of adhesive restorations over longer periods of time [10]. However, certain limitations of CHX such as its synthetic nature and relative (dose-dependent) cytotoxicity to odontoblastic cells [11, 12], which may induce cell inflammation, has motivated researchers to investigate alternative compounds.

Some attention has been directed to the use of Epigallocatechin-gallate (EGCG), a natural polyphenol derived from green tea, in dentin treatment. EGCG is an effective antimicrobial agent against Streptococcus mutans and inhibits acid production by caries pathogens in dental biofilm [13, 14]. It has been reported that EGCG inhibits dentinal proteases [15], thus preserving the long-term dentin bond strength with equal effect to chlorhexidine [16]. Once released from restorative copolymers, EGCG retains antibacterial and antiproteolytic activities [17, 18]. Natural polyphenols in general, including EGCG, are recognized to have anti-inflammatory properties and to present low cytotoxicity [19]. EGCG was also shown to attenuate production of the pro-inflammatory IL-12 in murine macrophages [20] and of TNF-α in arthritic joints of green tea polyphenols fed mice [21]. In addition, EGCG demonstrated a suppression of NF-κB activation leading to inhibition of pro-inflammatory cytokine release in bacteria-challenged dental pulp cells [22].

To our knowledge, the possible inflammatory or anti-inflammatory responses of CHX and EGCG on odontoblastic cell lines have never been investigated. Odontoblasts represent the first
layer of cells to respond to dentinal stimuli and therapies, and therefore can initiate immunological responses through cytokine signaling. Given the wide range of use and clinical applications of both compounds, including dentinal regions with close proximity with pulp or with micro-exposure to pulp, it was the purpose of this study to profile the effect of CHX and EGCG treatment on the expression of pro-inflammatory and anti-inflammatory cytokines, chemokines, and growth factors on an odontoblast-like cell model. The hypothesis of this study is that the cytokinome of EGCG-treated odontoblast-like cells would profile in a predominantly anti-inflammatory manner, whereas the chlorhexidine-treated cells, in a pro-inflammatory manner when compared to non-treated cells.
Material and Methods

MDPC-23 Odontoblast-like cell culture

The immortalized mouse dental papilla MDPC-23 cells were maintained in Dulbecco’s Modified Eagle’s medium (DMEM; with high glucose, L-glutamine and sodium pyruvate) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), with 100 IU/mL penicillin, 100 mg/mL streptomycin and 0.25 mg/mL fungizone (Gibco). Cells were sub-cultured every 2 days and allowed to grow in a humidified incubator at 37 ºC with 5% CO₂ (Isotemp; Fisher Scientific, Pittsburgh, PA, USA).

MTT cytotoxicity assay

MDPC-23 odontoblast-like cells were seeded in DMEM in 96-well plate at 2×10⁴ cells/200 µL/well and allowed to grow for 24 h. Afterwards, cells were seeded (n = 3) with serial dilution (from 2.5 µM to 160 µM) of either chlorhexidine diacetate (CHX) or epigallocatechin-gallate (EGCG) (Sigma–Aldrich, St. Louis, MO, USA) in DMEM for 48 h. Cells with no compound treatment were used as control (Ctr). Cells metabolic activity was evaluated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Roche Applied Science, Indianapolis, IN, USA) as previously described [18]. Data were analyzed by one-way ANOVA and Tukey post hoc test (α = 0.05). For the following assays, the highest and equal dilution of compounds that did not induce cytotoxicity was used.
**Total Protein Concentration**

At 48 h of cells treatment with 20 µM dilution of CHX and EGCG, total protein concentration was evaluated ($n = 3$). Briefly, cells were washed with phosphate-buffered saline (PBS) and lysed with protein lysis buffer (90 mM trisodium citrate, 10 mM NaCl, 0.1% Triton X-100, and pH 4.8). Total protein concentrations were determined using the Pierce bicinchoninic acid (BCA) assay (Thermo Scientific 23225, Waltham, MA, USA) at 562 nm. Data were analyzed by one-way ANOVA ($\alpha=0.05$).

**Bio-Plex cytokine/chemokine analysis**

At 48 h of cells treatment [23] with 20 µM dilution of CHX and EGCG, supernatants were collected and the cytokine/chemokine array Bio-Plex Cytokine 23-Plex (Bio-Rad, CA, USA) was used to detect the following molecules: Interleukin-1α (IL-1α), IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17, Eotaxin, Granulocyte-colony stimulating factor (G-CSF), Granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon gamma (IFN-γ), keratinocyte chemoattractant (KC), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 α (MIP-1α), MIP-1β, RANTES (Regulated on Activation, Normal T cell Expressed and Secreted), and tumor necrosis factor-α (TNF-α). Table 1 summarizes the main function of each evaluated cytokine. Cytokines were quantified using an automated microsphere-based reader (BioPlex™ 200, Bio-Rad, CA, USA). For each cytokine analyzed, mean values of duplicate samples were analyzed by one-way ANOVA followed by Tukey post hoc test ($\alpha=0.05$) [24].
Results

The MTT assay (Figure 1) revealed that cells with EGCG treatments from 10 µM to 160 µM concentrations had significantly increased cell metabolism when compared to Ctr (p<0.05). No statistical differences were shown for EGCG at 48 h cell treatments. Also compared to Ctr, cytotoxicity was observed for cells treated with CHX at concentrations ranging from 40 µM to 160 µM, both at 24 h and 48 h time points, with a stronger effect observed at 24 h cell treatment (p<0.05). Neither CHX nor EGCG have significantly reduced cell metabolic activity at the concentration of 20 µM (p>0.05). The highest and equal compound dilution tested that did not induce cytotoxicity at 48 h cell treatment (20 µM compound dilution) was used for the BCA and Bio-Plex cytokine/chemokine assays. Total protein levels were consistent and showed no statistical difference (p>0.05) across all groups at 20 µM compound dilution: 1) cells cultured without the compounds: 1.04 (±0.050) mg/mL; 2) cells cultured with CHX: 0.98 (±0.047) mg/mL; and 3) cells cultured with EGCG: 1.06 (±0.046) mg/mL (p>0.05).

At 20 µM dilution, both CHX and EGCG significantly increased the secretion of IL-1β, IL-10, IL-12, KC, MIP-1α, IFN-γ and IL-6 (p<0.05). IL-1β and MIP-1α were significantly higher for CHX treatment compared to EGCG treatment (p>0.05). Treatment with CHX significantly increased secretion of IL-4 and RANTES (p<0.05). Treatment with EGCG significantly increased Eotaxin secretion (p<0.05). CHX significantly decreased secretion of IL-17 whereas EGCG suppressed IL-17 expression (p<0.05). Finally, GM-CSF and TNF-α did not present significant change in secretion levels after treatment with either CHX or EGCG (p>0.05) (Table 2). All remaining cytokines were either not expressed or below the detection threshold.
Discussion

A method to profile the effect of CHX or EGCG on multiple cytokine secretion levels of Odontoblast-like cells (MDPC-23) was used in this study. In order to induce relative high cellular stimulation, the highest and equal CHX and EGCG non-cytotoxic dilutions of 20 µM (Figure 1) at 48 h treatment [23] were tested. The results also confirmed possible chemopreventive effect of catechins on cells [25] at 24 h treatment, as an increase in cellular metabolic activity was noted for EGCG at concentrations of 10 µM to 160 µM. Moreover, the more moderate cytotoxic values observed for chlorhexidine at 48 h are likely due to cell population regrowth from 24 h to 48 h [26]. The solubility properties of chlorhexidine diacetate (salt form) may have allowed for the cellular recovery to happen. Chlorhexidine diacetate has been shown to undergo self-association [27] with the formation of aggregates that influence resulting concentration and therefore activity of chlorhexidine in solution. In fact, the molar critical micellar concentration of chlorhexidine diacetate in aqueous solution (25 °C) has been reported to be of 0.010 - 0.011 [28], which are much lower concentrations than the ones used in our study. At 20 µM and 48 h cell treatment, no statistical difference on total protein levels measured among all evaluated groups was verified, indicating that CHX and EGCG groups had uniform and similar cell growth compared to Ctr group (non-treated cells). The present study showed that direct treatment of Odontoblast-like cells with either compound can lead to a significant interplay in the levels of cytokines and chemokines (Table 2).

The majority of cytokines that had increased secretions caused by both CHX and EGCG (the anti-inflammatory IL-10 and the pro-inflammatory IL-12, KC, INF-γ and IL-6) did not present significant differences between treatments. The exceptions were IL-1β and MIP-1α where significantly higher levels were detected for CHX in comparison to EGCG treatment. Balloni et
al., 2016 [29] recently reported similar results, in which IL-1β was secreted into medium by fibroblasts or keratinocytes washed or unwashed with CHX. After washing cells with CHX a significant increase in IL-1β secretion was also observed. IL-1β has been studied as a toxicity biomarker due to its early involvement in the overall inflammatory cascade [30]. Moreover, the expression of the pro-inflammatory MIP-1α has also been shown to increase in human pulp cells with reversible pulpitis and even more with irreversible pulpitis when compared to normal pulps [31]. This is likely because MIP-1α is a potent macrophage and lymphocyte chemoattractant, and as such may play a role in more chronic inflammatory processes [32]. Previous research has reported that IL-1β enhances expression of β-chemokines such as MIP-1α in neural cells. The intracellular signaling pathway by which it happens is not completely clarified, but there is evidence that it is through nuclear factor kappa B (NF-κB) activation [33]. Increased secretions of IL-1β and MIP-1α might relate to the cytotoxicity of CHX towards Odontoblast-like cells, in comparison to Ctr and EGCG groups, in higher than 20 µM dilutions of CHX observed in this study. If such assumption is confirmed in future experiments, IL-1β and MIP-1α may be used as cytotoxicity biomarkers in studies involving dentinal therapies.

The pro-inflammatory TNF-α is also known to play an important role in pulp response to infection by recruiting neutrophils. This might indirectly lead to higher levels of the neutrophil collagenase MMP-8 as shown in plasma and pleural fluid of tuberculosis patients [34]. Additionally, studies have reported that TNF-α induces MMP-9 expression [35] via the regulation of NF-κB pathway and activator protein-1 (AP-1) transcription factor [36] in human vascular smooth muscle cells. Apart from TNF-α, IL-1β and INF-γ are known to participate in the secretion of several MMPs, including MMP-8 and -9 [34, 37]. In the study by Kim et al., 2016 [38], an interesting correlation between MMP-2 and -9 with TNF-α and IL-1β on human ligamentum flavum (LF) cells was reported. The IL-1β treated LF cells showed increased
expression of proMMP-2, -9, and active MMP-9, whereas LF cells treated with TNF-α showed elevated expression of proMMP-9. Suggested mechanisms would also involve NF-κB pathway and AP-1 transcription factor [39]. Our results showed no significant change in secretion levels of TNF-α after CHX or EGCG cell treatments. This is in line with Crouvezier et al., 2001 [40] that reported similar results for human blood culture also treated with 20 µM EGCG. These results may be due to the dose of the compound in use as studies that reported ~50% inhibition of TNF-α secretion in RAW 264.7 macrophages, for instance, used 100 µM EGCG [41]. Likewise, no significant changes for GM-CSM secretion compared to Ctr were observed for treated cells. Studies with transgenic mice have demonstrated that whenever GM-CSF is overexpressed pathological changes such as macrophage accumulation, blindness, and severe damages to various tissues were reported [42]. Interestingly, Akhtar and Haqqi, 2011 [43] reported that EGCG is able to reduce IL-1β-induced GM-CSF production in chondrocytes. Both IL-4 and CCL5/RANTES had significantly increased production after CHX cell treatment, but no significant changes were observed between Ctr and EGCG groups. IL-4, IL-10 as well as INF-γ were previously detected in human dental pulp with carious lesions [44]. Although the following are interactions among bacteria colonizing carious tissue and pulp cells, a higher prevalence of IFN-γ (67%) than IL-4 (19%) or IL-10 (29%) was obtained in pulps with shallow carious lesions, suggesting a type 1 (Th1) cytokine mechanism in early pulpitis. In contrast, a trend of higher prevalence of IL-4 was noted in deep carious lesions [44] i.e., in more severe pulp pathogenesis. It is worth mentioning that in the latter study a two-fold increase in IL-4 level was observed for pulp with shallow carious lesions and three-fold to pulp with deep carious lesions in comparison to normal pulp. In the present study, although statistically significant, increase in IL-4 level caused by CHX treatment was of ~19% in comparison to Ctr and EGCG groups. Among other roles, IL-4 is implicated in several inflammatory diseases and directly
enhances the development of Th2 cells from naive T cells [45]. Furthermore, CCL5/RANTES is a member of the CC chemokine family with significant activity toward monocytes and eosinophils [46], shown to be expressed in healthy human pulp [47]. This chemokine is known to upregulate IL-12 [48] and INF-γ [49], both shown here in significantly higher levels after cell treatments in comparison to Ctr group. In Dentistry, the mineral trioxide aggregate was shown to downregulate expression of some cytokines in mouse pulp tissue, including IFN-γ and CCL5/RANTES [50].

Also from the CC chemokine family, CCL11/Eotaxin is implicated in several systemic diseases and known to potently chemo attract cells such as eosinophils and basophils. Previous studies with human keratinocytes have shown that levels of Eotaxin are increased with combination of increased levels of IL-4 & IL-1β or IL-4 & TNF-α [51]. Although our results show increased levels of IL-4 & IL-1β for CHX treatment, no significant increase in Eotaxin level was observed for CHX group. Conversely, we observed significantly higher production of Eotaxin for the EGCG cell treatment. This again disagrees with Hosokawa et al., 2013 [52] where EGCG prevented IL-4/IL-1β and IL-4/TNF-α mediated Eotaxin production in human gingival fibroblasts. Inflammatory cytokines can regulate various chemokines, extra cellular matrix degrading proteins and growth factors; however expression patterns may differ in different types of cells [38]. Therefore, specific mechanisms by which EGCG induces increased levels of Eotaxin in Odontoblast-like cells warrant further investigation. Also in this study, CHX cell treatment significantly decreased IL-17 levels whereas EGCG suppressed its production. Such results may consolidate use of CHX and stimulate use and investigation of EGCG in dental clinical applications such as in root canal treatments. IL-17 is known to induce production of receptor activator of nuclear factor-kappa B ligand (RANKL) by osteoblasts [53], and therefore
its down regulation may ameliorate bone related dental conditions such as periapical lesions and periodontitis.

This study showed that modulation of levels of cytokines by CHX and EGCG is complex, and does not necessarily happen in predominantly pro- or anti-inflammatory manners. For instance, a given compound could directly down-regulate the release of pro-inflammatory IL-1β and up-regulate other end-products that, in turn, stimulate release of IL-1β. As these end-products collect in the cell culture media, the in vitro result may be seen as an overall increase in IL-1β levels. This may explain the significant increase in IL-1β levels, in the present study, for cells treated with EGCG [54]. A variety of extracellular signals increase the rate of IL-1β production, including molecules such as GM-CSF [55], TNF-α [56], and INF-γ [57]. In different degrees, all of these had increased levels in the EGCG-treated cells, and may have accounted to an overall IL-1β increase. Moreover, Saraiva & O’Garra, 2010 [58] describe that production of IL-10 for instance, may be induced together with pro-inflammatory cytokines. Pro-inflammatory immune responses are critical in the mechanisms of cellular defense. Yet, excessive inflammation may potentially damage the host. Thus, different immunoregulatory pathways for CHX and EGCG, controlling inflammatory cytokine production, such as production of negative regulator of inflammatory responses (i.e., IL-10) [59], may have happened to ensure a balanced immune response [60].

A clearer understanding of how these molecules act in dentin and interact with cells, as well as their roles in inflammatory and tissue remodeling and repair cascades requires understanding. Profiling and posteriorly monitoring the effects of dentinal compounds or restoratives on cytokine levels of Odontoblasts may determine ways of detecting early signs of inflammatory conditions that may not be clinically obvious, or else to the development of immunomodulatory restorative materials and therapies.
Acknowledgements

This study was supported by DRI (Dental Research Institute, University of Toronto – Faculty of Dentistry) funds to the principal investigator (AP). We would like to thank Dr. Jacques E. Nor (Department of Cariology, Restorative Sciences and Endodontics, School of Dentistry, University of Michigan, Ann Arbor, MI, USA) for kindly supplying the MDPC-23 cell line to our study.
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Table 1. Main functionality of the cytokines investigated with the Bio-Plex Cytokine 23-Plex

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Principal Function</th>
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<tbody>
<tr>
<td>IL-1α</td>
<td>Various immune responses, (pro)inflammatory processes, hematopoiesis</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Pro-inflammatory (cell proliferation, differentiation and apoptosis)</td>
</tr>
<tr>
<td>IL-2</td>
<td>Immune response (on cells T - tolerance and immunity)</td>
</tr>
<tr>
<td>IL-3</td>
<td>Immune response (proliferation and differentiation of hematopoietic cells)</td>
</tr>
<tr>
<td>IL-4</td>
<td>Anti-inflammatory (suppression of pro-inflammatory cytokines)</td>
</tr>
<tr>
<td>IL-5</td>
<td>Immune response (growth and differentiation factor for both B cells and eosinophils)</td>
</tr>
<tr>
<td>IL-6</td>
<td>Pro-inflammatory and immune response (maturation of B cells, stimulates endogenous pyrogen)</td>
</tr>
<tr>
<td>IL-9</td>
<td>Regulates a variety of hematopoietic cells (stimulates cell proliferation and prevents apoptosis)</td>
</tr>
<tr>
<td>IL-10</td>
<td>Anti-inflammatory and autoimmunity (impedes pathogen clearance and ameliorate immunopathology)</td>
</tr>
<tr>
<td>IL-12 (p40)</td>
<td>Pro-inflammatory (inducer of Th1 cells and mediate long-term protection to an intracellular pathogen)</td>
</tr>
<tr>
<td>IL-12 (p70)</td>
<td>Pro-inflammatory (differentiation of naive T cells into Th1 cells)</td>
</tr>
<tr>
<td>IL-13</td>
<td>Immune response and Anti-inflammatory (mediator of the physiologic changes induced by allergic inflammation)</td>
</tr>
<tr>
<td>IL-17</td>
<td>Pro-inflammatory (activities of NF-kappaB, mitogen-activated protein kinase pathways and stimulates production of IL-6)</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>Pro-inflammatory and immune response (chemotactic cytokine that regulates eosinophil tissue levels)</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Hematopoiesis (controls production, differentiation and function of granulocytes and steam cells)</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Hematopoiesis (controls production, differentiation and function of monocytes-macrophages)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Immune response (activator of macrophages and inducer of Class II major histocompatibility complex)</td>
</tr>
<tr>
<td>KC</td>
<td>Pro-inflammatory (activated by IL-1, a chemoattractant for neutrophils)</td>
</tr>
<tr>
<td>MPC-1</td>
<td>Pro-inflammatory (chemotactic factor for monocytes)</td>
</tr>
<tr>
<td>MIP-1α (CCL3)</td>
<td>Pro-inflammatory and chemotactic</td>
</tr>
<tr>
<td>MIP-1β (CCL4)</td>
<td>Pro-inflammatory and chemotactic (regulate leukocyte activation and trafficking)</td>
</tr>
<tr>
<td>RANTES (CCL5)</td>
<td>Pro-inflammatory and chemotactic (chemotactic for T cells, eosinophils, and basophils, recruits leukocytes into inflammatory sites)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Pro-inflammatory (regulation of cell proliferation, differentiation, apoptosis, lipid metabolism and coagulation)</td>
</tr>
</tbody>
</table>

*Cytokines functionality has been described according to Alberts et al., 2008 [61] and Borish & Steinke, 2003 [62]*
Table 2: Cytokine/chemokine secretion of Odontoblast-like cells treated with CHX or EGCG

<table>
<thead>
<tr>
<th>Analyte (pg/mL)†</th>
<th>Ctr</th>
<th>CHX</th>
<th>EGCG</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cytokines/Chemokines with significantly increased secretion after CHX and EGCG treatments</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>915 ± 80^a</td>
<td>1582 ± 27^c</td>
<td>1443 ± 49^b</td>
</tr>
<tr>
<td>IL-10</td>
<td>0 ± 0^a</td>
<td>505 ± 34^b</td>
<td>424 ± 42^b</td>
</tr>
<tr>
<td>IL-12</td>
<td>0 ± 0^a</td>
<td>540 ± 40^b</td>
<td>506 ± 88^b</td>
</tr>
<tr>
<td>KC</td>
<td>321 ± 7^a</td>
<td>595 ± 4^b</td>
<td>600 ± 9^b</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>0 ± 0^a</td>
<td>303 ± 0^c</td>
<td>215 ± 63^b</td>
</tr>
<tr>
<td>INF-ɤ</td>
<td>0 ± 0^a</td>
<td>517 ± 0^b</td>
<td>757 ± 475^b</td>
</tr>
<tr>
<td>IL-6</td>
<td>106 ± 0^a</td>
<td>146 ± 0^b</td>
<td>197 ± 70^b</td>
</tr>
<tr>
<td><strong>Cytokines/Chemokines with significant increased secretion after CHX treatment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-4</td>
<td>96 ± 2^a</td>
<td>115 ± 0^b</td>
<td>96 ± 2^a</td>
</tr>
<tr>
<td>RANTES</td>
<td>336 ± 0^a</td>
<td>465 ± 75^b</td>
<td>363 ± 37^ab</td>
</tr>
<tr>
<td><strong>Cytokines/Chemokines with significant increased secretion after EGCG treatment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eotaxin</td>
<td>1086 ± 197^a</td>
<td>1180 ± 201^a</td>
<td>1703 ± 92^b</td>
</tr>
<tr>
<td><strong>Cytokines/Chemokines with significant decreased secretion after CHX and EGCG treatments</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-17</td>
<td>415 ± 27^a</td>
<td>309 ± 38^b</td>
<td>0 ± 0^c</td>
</tr>
<tr>
<td><strong>Cytokines/Chemokines with no significant change in secretion after CHX and EGCG treatments</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GM-CSF</td>
<td>923 ± 138^a</td>
<td>1089 ± 30^a</td>
<td>1253 ± 146^a</td>
</tr>
<tr>
<td>TNF-α</td>
<td>790 ± 58^a</td>
<td>887 ± 78^a</td>
<td>869 ± 53^a</td>
</tr>
</tbody>
</table>

Same superscript letters indicate no statistical differences within same row (α = 0.05); † all excluded cytokines were below the detection threshold or out of standard concentration range.
Figures

Figure 1: MTT assay results of serial dilutions of CHX (left) and EGCG (right) on Odontoblast-like cells at 24 h and 48 h time points.
Chapter 5
Part 2: Manuscript in Preparation

Interaction of Epigallocatechin-gallate with Proteins in S. mutans contaminated Odontoblast-like Cells: cytotoxicity, IL-1β, and co-species proteomic analyses

ABSTRACT
Objectives: To evaluate the effect of S. mutans (SM) stimulation and epigallocatechin gallate (EGCG) treatment on the viability, IL-1β production, and inflammatory protein production on MDPC-23 odontoblast-like cells. Methods: Cells were stimulated with SM multiplicity of infection (MOI) concentrations of 100 to 1000 and subsequently treated with serial dilutions of 100 to 1 µM of EGCG. No cell treatment and chlorhexidine (CHX) were used as controls. Combined stimulation/treatment of cells were tested for cytotoxicity (Alamar Blue, n=3), total protein levels (Pierce bicinchoninic acid assay, n=3), IL-1β levels (ELISA, n=3), and mass spectroscopy (LC-MS/MS, n=2). Results: MOI stimulation in conjunction with EGCG and CHX treatment at 10 µM did not negatively affect the metabolic activity of the cells. IL-1β production was significantly increased in both un-stimulated and stimulated CHX 10 µM groups when compared to un-treated control (p<0.05). The MOI 100 CHX 10 µM group significantly increased IL-1β levels compared to the un-stimulated CHX 10 µM and EGCG 10 µM groups, as well as the MOI 100 EGCG 10 µM group (p<0.05). LC-MS/MS revealed proteins that are found in both SM and mammalian, with tooth-specific proteins exhibiting different abundance levels depending on stimulation or treatment. For instance, the relative abundance of peroxiredoxin-1 (anti-inflammatory protein) increased up to 1.5 fold in the MOI 100 EGCG 10 µM when compared to other stimulation, treatment, and control groups. Conclusions: SM MOI did not cause cytotoxic effects on the MDPC-23 cells. MOI combined EGCG treatment did not cause
cytotoxicity, however MOI combined CHX 100 µM treatment decreased cell viability. Treatment with CHX 10 µM significantly increased IL-1β levels. For the majority of proteins, no change in protein production was observed with cellular MOI stimulation in conjunction with EGCG and CHX treatments. Clinical Significance: Within the proposed model, EGCG did not exert cytotoxic or inflammatory effects on OB and SM stimulated OB cells.

**Keywords:** Chlorhexidine, EGCG, *Streptococcus mutans*, MDPC-23, IL-1β, LC-MS/MS
Introduction

Dental caries dramatically affects the majority of the populations of Canada and the United States, with 96% of Canadian adults having had a cavity, and 95% of Americans having experienced either enamel or root surface caries [1, 2]. While the cause of dental caries is multifactorial, the most common etiologic factor is via the metabolic acid production of oral bacteria [3, 4]. *Streptococcus mutans* (SM), a highly acidogenic and aciduric organism, is the most implicated bacterium in the initiation and progression of early caries [5 – 7]. In late stage caries, SM amounts to roughly 30% of the oral microbiome [8 – 10]. As SM invades the demineralized tooth, the odontoblasts (OBs) represent the first layer of cells to respond to dentinal stimuli and contamination, initiating immunological responses through the production of different anti- and pro-inflammatory proteins [11 – 13]. Interleukin-1β (IL-1β) for instance, has a central role in the inflammatory process, as once its inactive molecule is cleaved, it activates inflammatory pathways such as the nuclear factor kappa B (NF-κB) [14]. Furthermore, in comparison to caries-free tissue, carious tissue has increased levels of IL-1β [15].

Chlorhexidine (CHX) is a commonly used dentin therapeutic agent. This is due to its ability to inhibit the growth of bacteria, encouraging pulp tissue repair by controlling contamination and inflammation, as well as the action of host proteases which may further contribute to degradation of the tooth structure during caries [16 – 18]. Though useful in its inhibition of bacteria, CHX at certain concentrations exhibits negative effects on cells such as gingival fibroblasts and odontoblasts [19 – 21]. As such, alternatives to CHX are being investigated for their efficacy and viability. Epigallocatechin-gallate (EGCG) is a very commonly studied catechin due to its abundance (approximately 60% of all catechins in green tea) and its efficacy in the inhibition of cariogenic bacteria growth. Furthermore, it has been shown to inhibit the actions of proteases such as MMP-2 and -9, which are found in high concentrations within carious lesions and are
heavily involved in caries progression [22 – 27]. EGCG has generally been studied for its anti-inflammatory capabilities, having displayed inhibitory properties on pro-inflammatory cytokines such as IL-1β, IL-12, TNF-α in a variety of cell types that have been challenged with bacterial lipopolysaccharides [28 – 30].

Common methods for simultaneous screening and/or quantification of multiple proteins in host cells are the bead-based immunoassays or liquid chromatography-tandem mass spectrometry (LC-MS/MS). Studies using the latter of these techniques have found that the introduction of a multiplicity of infection (MOI) by bacterial cells does alter the protein expression of host cells [31, 32]. It was the purpose of this study to evaluate the effect of EGCG on the cytotoxicity, IL-1β levels, and inflammatory protein production by LC-MS/MS using an in vitro SM-stimulated OB model. The two null hypotheses tested were that there will be no difference on the (1) production of IL-1β and (2) inflammatory proteins detected by LC-MS/MS on SM-stimulated OB cells treated with EGCG or CHX.
Materials and Methods

MDPC-23 Odontoblast-like cell culture

Mouse odontoblast-like cells (MDPC-23) were cultured in Dulbecco’s Modified Eagle Medium (Gibco #11995-065) supplemented with 10% Fetal Bovine Serum (Gibco #12484-028), 1% penicillin/streptomycin (Sigma #P4333-100ML). Cells were cultured in a humidified incubator with a 5% carbon dioxide atmosphere at 37°C until 70-80% confluence. Cells used for assays were between passages 6 and 10.

Bacterial growth

For the purposes of cell stimulation with SM, the UA159 strain was used. Overnight cultures of SM incubated at 37°C and 5% CO₂ in Todd-Hewitt Yeast Extract (THYE) broth were diluted 1:20 for counting and absorbance measurement each hour for seven hours. Using a previously determined growth curve, the desired growth time was between three and four hours for a CFU/mL of 10⁹ [33]. In order to prevent growth during experiments, SM was sonicated to separate the cells at a setting of 6 for 8 seconds and heat-killed for 30 minutes at 56°C. At this point, bacterial cultures were resuspended in fully supplemented cell culture media and diluted according to the multiplicity of infection (MOI) required for MDPC-23 stimulation [15].

Cell stimulation

MDPC-23 cells were seeded in a 96-well plate at a density of 25,000 cells per well and left to attach for two hours. At this point, heat-killed SM MOIs from 1000 – 1 were added to the cells, with a control group of THYE with no bacteria added to cells [15]. MOI is defined as the ratio of bacteria to cell targets. It utilizes the Poisson distribution to calculate the probability of a cell being infected [34]:
\[ P(n) = \frac{m^n \cdot e^{-m}}{n!} \]

Where \( m \) = the multiplicity of infection, \( n \) = the number of infections a particular target will receive, \( P(n) \) = the probability that a target will be infected, and \( e \) = Euler’s constant.

Cells were then incubated for 6, 24, and 48 hours before subsequent cell viability analysis.

**Cell treatment and stimulation**

Epigallocatechin-gallate (Cayman Chemical Company #70935) was dissolved in media and a serial dilution was performed between 100 - 1 μM. This was compared to a chlorhexidine diacetate (CHX) (MP Biomedicals #191361) positive control group at the same concentrations. MDPC-23 cells were seeded onto a 96-well plate and allowed to attach for 2 hours. Treatments were then added and cells were incubated for 24, 48, and 72 hours. For combined stimulation and treatment assays, cells were seeded onto a 96-well plate and allowed to attach for 2 hours, at which point EGCG, CHX, and MOIs 100 and 1000 were added. An untreated, un-stimulated control group was used. The chosen MOIs ensured that each cell would experience at least a single infection [15]. Cells were incubated for 24, 48, and 72 hours.

**Cell toxicity and total protein assays**

To determine the effect of bacterial stimulation, EGCG and CHX on the MDPC-23 cell line, an Alamar Blue viability assay (Invitrogen, Carlsbad, CA) was performed after each of the treatment and stimulation time points \( (n=3) \). This assay indicates the oxidation-reduction in cell viability assays for mammalian and bacterial cells and measures the aerobic respiration. Once the time point was reached, the media was aspirated and new media containing no treatment was added. Then, 10% Alamar Blue was added to each well and the cells were incubated for 4 hours.
The Optical Density was then measured using the Cytation 3 (Bio-Tek) plate reader at wavelengths of 570 to 600nm. The reduction of Alamar Blue was calculated by the manufacturer’s provided formula. A difference in the reduction indicates varying cell metabolic activity. Data were analyzed by one-way ANOVA ($\alpha=0.05$).

Furthermore, a Pierce Bicinchoninic Assay (Thermo Scientific, Waltham, MA) was used to evaluate the cell proliferation ($n=3$). Once the media from the treated and stimulated cells was extracted for the Alamar Blue assay, the remaining cells were washed with Dulbecco’s Phosphate Buffered Saline (PBS) (Sigma #D8537). Cell lysis was then carried out with a 1X RIPA Buffer (Cell Signaling #9806). Samples were then scraped and centrifuged at 13,000 rpm for 10 min and the supernatants were analyzed using the Pierce Bicinchoninic Assay kit at 562nm in the Cytation 3 (Bio-Tek) to determine the protein concentrations. Data were analyzed by one-way ANOVA ($\alpha=0.05$).

**IL-1β ELISA**

Cytokine levels in cell lysates were determined using commercial ELISA kits for IL-1β (Invitrogen, Vienna, Austria). Treatment concentrations of 10 $\mu$M of EGCG and CHX as well as MOI 100 stimulation were utilized with un-treated and un-stimulated groups used as controls. Using the Cytation 3 (Biotek) plate reader, the intensity of the colour detected at 450 nm (with a background wavelength of 620 nm) was measured after the addition of a substrate solution, and cytokine concentrations were calculated from the standard curve prepared by diluting the standard solution provided in the kit ($n=3$). Data were analyzed by one-way ANOVA ($\alpha=0.05$).
Cell treatments for Mass Spectrometry

In order to determine the effect of EGCG and CHX treatments on un-stimulated and SM-stimulated OBs, cell lysates were prepared for liquid chromatography-tandem mass spectrometry. MDPC-23 cells were seeded in a 6-well plate at 1 x 10^6 cells/per well. After 48 h of cell growth, treatments with 10 µM dilutions of CHX and EGCG as well as SM at MOI 100 stimulation were added to the cells. After 24 h of incubation, cell lysates were collected from each sample. Briefly, cells were washed with ice-cold PBS and lysed with protein lysis buffer (90 mM trisodium citrate, 10 mM NaCl, 0.1% Triton X-100, and pH 4.8). Cells were then scraped on ice, sonicated, and centrifuged for 10 minutes at 4 °C and 13000 x g. Pellets were discarded and supernatants were retained for analysis. Total protein concentrations were determined using the Pierce bicinchoninic acid (BCA) assay (Thermo Scientific 23225, Waltham, MA, USA) at 562 nm. Data were analyzed by one-way ANOVA (α=0.05).

Liquid chromatography-tandem mass spectrometry

Samples analyzed at the SPARC Biocentre were first reduced, alkylated, digested, and labeled with tandem mass tags (TMT) as per the manufacturer’s instructions (Thermo-Fisher TMT 10 Plex, Product 90110). All labeled sample peptides were combined and lyophilized, followed by a cleaning with an SCX column, and a final lyophilization.

Sample analysis was performed on the Thermo-Scientific Orbitrap Fusion-Lumos Tribid Mass Spectrometer (Thermo-Fisher, San Jose, CA) featuring a nanospray source and EASY-nLC 1200 nano-LC system (Thermo-Fisher, San Jose, CA). Sample peptides were dissolved in 0.1% formic acid before being loaded onto a 75 µm x 50 cm PepMax RSLC EASY-Spray column containing 2 µM C18 beads (Thermo-Fisher, San Jose, CA) with an 800 Bar pressure at 60 °C. Elution of
peptides was set to 120 minutes at 250 nL/minute using a 0%-40% gradient of Buffer A (0.1% formic acid) and Buffer B (0.1% formic acid in 80% acetonitrile). Peptides were inserted into the Fusion-Lumons mass spectrometer (Thermo-Fisher) via nano-electrospray. MultiNotch MS3 acquisition with synchronous precursor selection (SPS) was used to acquire the data. MS1 acquisition used a scan range of 550 m/z – 1800 m/z, a resolution of 120,000, 50 ms maximum injection time and AGC target of 4e5. MS2 scan isolation occurred in the quadrupole, with a 0.7 isolation window. MS2 scans in the linear ion trap had a 50 ms maximum injection time and a 35% normalized collision energy. MS3 scans utilized HCD, with a 30% collision energy, and were measured in the orbitrap at a resolution of 50,000. Scan range was 100 m/z – 500 m/z, AGC Target of 3e4, and a 50 ms maximum injection time. Dynamic exclusion utilized a maximum exclusion list of 500 and one exclusion duration of 25 seconds.

Scaffold (version Scaffold_4.7.5, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability. Peptide Probabilities from X! Tandem were assigned by the Scaffold Local FDR algorithm. Peptide Probabilities from PEAKS Studio were assigned by the Peptide Prophet algorithm with Scaffold delta-mass correction [34]. Protein identifications were accepted if they could be established at greater than 99% probability and contained at least 2 identified peptides [36]. Protein probabilities were assigned by the Protein Prophet algorithm [37]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. Log2 Fold Changes of Proteins were utilized to analyze differences in protein production between the Control group and treatment/stimulation groups.
Results

Cytotoxicity and total protein concentrations for SM MOIs from 1 to 1000 stimulated cells are shown in Figures 1, 2, 3, and 4. The Alamar Blue assay showed that MOI stimulation at all concentrations and time points did not reduce cell viability when compared to control groups ($p<0.05$). Total protein levels for all time points except 6 hours showed no statistical difference ($p<0.05$). The 6 hour MOI stimulation with concentrations 1 through 1000 exhibited significantly increased total protein levels in a dose-dependent manner when compared to the control groups ($p<0.05$). Cytotoxicity and protein concentrations for combined MOI 100 and 1000 stimulations and EGCG or CHX (1 to 100 µM) treatments, tested at the 24h time point, are shown in Figures 5 and 6. The results show that there was no difference in cell viability between bacterial stimulation treatments ($p<0.05$). However, the CHX 100 µM significantly reduced cell viability in the control and stimulation groups ($p<0.05$). The total protein concentrations of the combined cell stimulation and treatment are shown in Figure 6. Within the un-stimulated and MOI groups, the CHX 10 µM, 100 µM and EGCG 100 µM treatments significantly reduced total protein concentrations when compared to their respective un-treated control groups ($p<0.05$). Furthermore, in the MOI 1000 group, the EGCG 1 µM caused a slight reduction in total protein when compared to the un-treated control ($p<0.05$). Patterns in cytotoxicity and protein levels were similar in the 48 hour and 72 hour timepoints.

The IL-1β ELISA results in pg/mL and pg/mg of total protein are shown in Figure 7 Parts (A) and (B), respectively. Both the MOI 100 EGCG 10 µM and the MOI 100 CHX 10 µM concentrations significantly reduced IL-1β absolute levels when compared to control ($p<0.05$). When normalized to the total protein of each sample, the amount of IL-1β per mg total protein was significantly increased in both the CTR CHX 10 µM group and the MOI 100 CHX 10 µM group, when compared to the control ($p<0.05$). The MOI 100 CHX 10 µM group also
significantly increased the IL-1β per mg total protein when compared to the CTR EGCG 10 µM, CTR CHX 10 µM, and MOI 100 EGCG 10 µM groups (p<0.05).

Proteomic analysis of whole cell lysates was performed by LC-MS/MS. A total of 2110 mammalian proteins were identified and classified into their most common functions and locations. Information pathways and biological processes are the most prevalent functions of the 1151 differentially regulated proteins, while many of them occur intracellularly, as seen in Figure 8. Four SM-specific proteins were identified, two of which were uncharacterized (\([\text{R}]f\mathrm{AGYDQIVMNk}[C], [\text{K}]d\mathrm{IENEVAIVISGANISGIGk}[D]\)). The remaining two were a fragment of pyruvate kinase, and phosphopantothenoylcysteine decarboxylase. In terms of the mammalian-specific proteins, collagens identified included 1A1, 1A2, 4A3, 5A1, 6A2, and 16A1. The non-collagenous proteins vimentin and biglycan, cathepsins B and D, and MMP-2 were also identified, as were the enamel annexin a1, a2, a5, and a6. Proteins found in cementum included trifunctional enzyme subunit alpha and aconitate hydratase, while proteins identified from dentin were dihydropyrimidase-related protein 3, gelsolin, tubulin alpha-1A and beta-4B chain, and protein disulfide-isomerase. Lastly, identified acid-soluble tooth proteins were vimentin, phosphoglycerate mutase 1, 14-3-3 protein zeta/delta, peptidyl-prolyl cis-trans isomerase B, l-lactate dehydrogenase B chain, triosephosphate isomerase, fructose-bisphosphate aldolase A, filamin-A, moesin, alpha-enolase, and 14-3-3 protein epsilon. For the majority of proteins, no change in protein production was observed with cellular MOI stimulation in conjunction with EGCG and CHX treatments.

Table 1 displays a shortlist of proteins found in the un-stimulated (Control, EGCG 10 µM, CHX 10 µM) groups and the stimulated (MOI 100 Control, MOI 100 EGCG 10 µM, MOI 100 CHX 10 µM) groups. There was no difference in the number of peptide hits, however, the Log₂ Fold Changes of protein abundance among groups are also listed in Table 1. Furthermore, the proteins
in Table 1 that are involved in inflammatory processes are annexin a1 and a2, glyceraldehyde-3-phosphate dehydrogenase, heat shock cognate 71 kDa protein, and peroxiredoxin-1, the last of which was the only one that exhibited a fold change among all groups, having a 1.5 fold increase in the MOI 100 EGCG 10 µM group when compared to all other groups.

**Discussion**

To our knowledge, the present study is the first to utilize EGCG and CHX treatments within an SM-stimulated MDPC-23 cell culture model. The stimulation models were developed and previously published by Dommish et al (2008) and Simon et al (2010), wherein odontoblast-like cells were stimulated with heat-killed *Streptococcus mutans* [15, 33]. The former study investigated the effect of the SM stimulation on the gene expression of the inflammatory mediators IL-6 and IL-8 by human-derived odontoblast-like cells, finding that after 6 hours stimulation, the expression of both cytokines was greater than at the 24 h stimulation. The authors subsequently incorporated a pre-treatment with the phosphatidylinositol-3-kinase inhibitor LY 294002 and found that the mRNA expression levels were significantly reduced (Dommisch 2008). The study by Simon et al (2010) similarly treated the MDPC-23 cell line with heat-killed SM, and investigated the relation of the stimulus to the phosphorylation and nuclear translocation of p38 within the MAPK/ERK pathway [15, 33]. They concluded that the method of using heat-killed SM appeared to have been a useful starting point towards future models where p38 inhibitors may be utilized [33].

The aforementioned studies provided a framework for the objectives of this study by establishing models that elicited the expression of pro-inflammatory cytokines by odontoblast-like cells. The Dommisch et al (2008) study and an EGCG treatment-based study by Hirao et al (2010) utilized
multiplicities of infection (MOI) of 100, that is, 100 bacterial cells to 1 odontoblast cell, in their methodology, and we emulated those while adding MOIs of 1, 10, and 1000 as well in our studies [15, 39]. Furthermore, the cells were stimulated for time periods of up to 72 h as compensation for eventual treatment times.

The primary objectives were to establish a non-cytotoxic model, combining varying amounts of cell stimulation and cell treatment within time points up to 72 h. As there were many variables to consider, this investigation began with the treatment of the MDPC-23 cells with EGCG and CHX in a wide range of concentrations. While there have been studies focusing on the effect of CHX digluconate on dentinal surfaces, the focus here was to investigate those that incorporated CHX diacetate treatment directly onto cells [19]. A study by de Souza et al (2007) concluded that all high concentrations of CHX introduced to MDPC-23 cells were cytotoxic, with their lowest concentrations of 0.0024% and 0.004% displaying less harmful effects [21]. Lessa et al (2010) found that concentrations of CHX ranging from 2% to 0.06% significantly reduced cell viability, even after allowing the cells a 24 h recovery time post-treatment [20]. In this study, the working concentrations were at the lower threshold of those two reports, with the lowest concentrations of 1 and 10 μM amounting to approximately 0.0001% and 0.0006% respectively.

As mentioned, the first objective results indicated that within the 1000 to 1 μM range of concentrations, 1 and 10 μM resulted in cell viability that was not statistically different from our control (Figures 1 [Part 1], 1, and 3). The total protein measurements showed that the 10 μM CHX concentration significantly decreased total protein levels compared to the control (p < 0.001) (Figure 3). Various studies have reported that through a mechanism of action that may be related to the denaturation and release of intracellular enzyme proteins within the cell, CHX is able to inhibit protein synthesis in fibroblast cell cultures in a dose dependent manner, with
concentrations of 0.2% inhibiting total protein production by up to 97%, and concentrations that were not harmful to cells significantly reduced production of both collagenous and non-collagenous proteins [20, 42–45]. Lower, viable concentrations of CHX appeared to affect protein synthesis less, but the mechanism by which CHX inhibits protein synthesis could account for the significant reduction of total protein levels in the MDPC-23 cells at the 10 μM concentration, as well as reductions in total protein levels seen in the combined stimulation/treatment cytotoxicity experiments (Figure 6).

EGCG-based cell treatments were performed alongside the CHX treatments, in order to compare the effect of both compounds on the cytotoxicity of the MDPC-23 cells. As we found in Part 1, there is a slight cytotoxic effect at 48 h using CHX 40 μM (Figure 1), and the log10 scale of concentrations showed that CHX 100 μM was highly toxic to the cells (Figure 6). Our results showed no cytotoxicity at any concentration of EGCG. EGCG has shown extreme cytotoxicity in HuT-102 and C91-PL cell lines only at 400 μM, with further reductions in cell proliferative activity being observed at the 100 μM (35% reduction) in the HuT-102 cells and 25 μM (46% reduction) in the C91-PL cells [26]. However, with the intent to compare the two compounds, this study concluded that utilizing the non-cytotoxic concentrations of 10 μM in EGCG and CHX would be most effective for the investigation. The working concentrations were lower compared to previous studies investigating the inhibition of inflammatory mediators via catechin treatment, which found the ideal dosage was 100 μM in their respective studies [27, 41].

IL-1β is a well-studied pro-inflammatory cytokine that has been linked to the regulation of sleep and body temperature, but when it is overproduced it is associated with diseases such as rheumatoid arthritis, osteoarthritis, and Alzheimer’s disease [46]. Its inactive state can be activated by caspase-1 initiated by pathogen-associated molecular patterns (PAMPs) as well as
by proteases from Gram-positive and negative bacteria [14, 47]. One of the many roles of IL-1β is to stimulate the production of MMPs, resulting in increased protease activity, and inhibition of collagen and proteoglycans, which are necessary to preserve the integrity of the dentin [47]. The results from the IL-1β ELISA shown in Figure 7 (A) indicate that the CHX treatment, with or without MOI 100 stimulation, increases the production of intracellular IL-1β. This increase is seen in Figure 7 (B), where the IL-1β production standardized to the total protein of each sample is significantly increased in both CHX groups, showing even higher increase for MOI stimulated CHX group, which significantly increased the IL-1β concentration per total protein compared to the CTR EGCG 10 μM, CTR CHX 10 μM, and MOI 100 EGCG 10 μM groups. Conversely, the MOI 100 EGCG 10 μM group exhibited lower production of IL-1β compared to the control group, while maintaining similar protein levels. These results indicate that CHX may increase the production of IL-1β regardless of there being a bacterial stimulus present, while EGCG lowers production of IL-1β while having no effect on the total protein levels of treated samples. At 24h, the MOI 100 EGCG 10 μM group may have directly downregulated production of IL-1β, whereas production of IL-1β in the case of CHX has been observed in a study by Balloni et al (2016) after washing fibroblasts and keratinocytes with CHX compared to an amine fluoride/stannis fluoride wash [48]. An increase in IL-1β may also be indicative of a type 1 immune response, which involves production of IL-1, IFN-γ, and TNF-α as a beginning for an inflammatory process necessary for remodeling and healing [49].

LC-MS/MS results indicated that tooth and inflammation-related proteins were present in the MDPC-23 whole cell lysates, with several being shortlisted and analyzed for their relative abundance in Table 1. As mentioned, there were proteins involved in the inflammatory response such as peroxiredoxin-1, a known antioxidant protein, which was found to have a 1.5-fold increase in abundance in the MOI 100 EGCG 10 μM group relative to the other groups [50]. This
protein has displayed protective properties on cells via its peroxidase mechanisms, and the ability to seek out and suppress reactive oxygen species [50, 51]. Furthermore, it has been linked to the STAT3 signaling pathway via the use of a peroxiredoxin-1 knock down cell line, which resulted in the up-regulation of the anti-inflammatory cytokine IL-10 [51]. The ELISA and LC-MS/MS found that while stimulation with MOI only had no significant effect on the expression of IL-1β or the differential protein expression, a significant interaction of MOI with CHX and EGCG for treated cells was noted.

Alkaline phosphatase, an enzyme known to play a role in mineralization and found in dentin extracts as well as other calcifying tissues, was identified within the cell lysates, was found to have a more than two-fold lower abundance in the MOI 100 control group when compared to the non-stimulated control [36, 52]. In a study using rat dental pulp cells, introduction of a high LPS concentration to the cells resulted in a decrease of alkaline phosphatase expression, however within this study, further investigation is needed to elucidate the reason that the other MOI 100 treatment groups did not exhibit a change in alkaline phosphatase abundance [53]. Histone 3 is one of the crucial histones required for DNA folding [54]. The LC-MS/MS indicated a decrease in relative abundance in the control and MOI 100 CHX 10 μM groups (more than 2-fold and 8-fold respectively), and the MOI 100 EGCG 10 μM group resulted in a more than 8-fold decrease in abundance. Conversely, the MOI 100 control group resulted in a 1.5-fold increase in relative abundance of histone 3. Though the identified SM proteins exhibited a low number of peptides, pyruvate kinase is a crucial enzyme in the glycolytic process of SM, being responsible for transferring a phosphate group from phosphoenolpyruvate (PEP) to adenosine diphosphate (ADP) [55, 56]. A recent study has also strongly linked SM biofilm formation and caries severity with pyruvate kinase activity [55]. The two uncharacterized proteins identified have only been
predicted in the UniProt database, however, they may be related to the metabolic activity of SM [57].

During the caries process, odontoblasts represent the first response to bacterial invasion through the dentin, and are responsible for the secretion and production of various cytokines to aid the inflammatory response. Chlorhexidine is a known inhibitor of the growth of oral bacteria, but may present drawbacks in terms of cytotoxicity and inhibition of protein synthesis, while EGCG has been studied to possess similar properties in terms of anti-inflammatory and anti-proteolytic activity, presenting little to no cytotoxicity at even at higher concentrations. This study investigated the use of EGCG beside CHX in SM-stimulated odontoblast-like cells, showing that EGCG can be used at higher concentrations safely on the cells, while having potential anti-inflammatory effects through the inhibition of IL-1β production. Furthermore, certain caries-related proteins were detected by LC-MS/MS, indicating a difference in the abundance of certain proteins between EGCG and CHX treatments. Further investigation into pro and anti-inflammatory mediators as well as quantification of caries and dentin-related proteins is required to elucidate the full effect of EGCG as an alternative to CHX, but it may have potential for use in dental therapeutics and implementation in materials.
References


Figure 1: Alamar Blue results for EGCG (white) and CHX (grey) treatments. Mean values and standard deviations are represented as percentages relative to the control groups (n=3). Samples with $p < 0.05$ were considered statistically different.
Figure 2: Alamar Blue results for various SM MOI stimulations. Mean values and standard deviations are represented as percentages relative to the control groups (n=3). Samples with $p < 0.05$ were considered statistically different.
Figure 3: Protein concentrations for EGCG (white) and CHX (grey) treatments. Mean values and standard deviations are represented as percentages relative to the control groups ($n=3$). Samples with $p < 0.05$ were considered statistically different.
Figure 4: Protein concentrations for various SM MOI stimulations. Mean values and standard deviations are represented as percentages relative to the control groups ($n=3$). Samples with $p < 0.05$ were considered statistically different.
Figure 5: Alamar Blue data for MDPC-23 cells stimulated with MOI 1000 and MOI 100, and treated with various concentrations of EGCG (E)/CHX (C) ($n=3$).

Figure 6: Total protein concentrations (mg/mL) for MDPC-23 cells stimulated with MOI 1000 and MOI 100, and treated with various concentrations of EGCG (E)/CHX (C) ($n=3$).
Figure 7: (A): IL-1β concentration (pg/mL); (B): IL-1β concentration normalized to total protein concentration (pg IL-1β/mg protein) ($n=3$). EGCG (E) and CHX (C).
Figure 8: Function and location for differentially expressed proteins (1151/2114 total proteins).
Table 1: (A) Shortlist of analyzed mammalian proteins. Parameters reported: Symbol, molecular weight, % coverage, number of peptides identified, and Log2 Fold Change Results for treatment/stimulation groups relative to Control group. (B) Fold Change Legend.
Chapter 6

Conclusion and Future Directions

6.1 Conclusion

The objectives of this study were to first determine the viability of EGCG compared to CHX when introduced to an odontoblast-like cell line, while also ensuring that a viable bacterial stimulation model using heat-killed SM could be used concurrently with the treatments. Secondly, to determine the interaction of EGCG on protein production of odontoblast-like cells within an MOI stimulus model. It was seen that EGCG treatment could reduce the production of the pro-inflammatory cytokine IL-1β by the cells, and through LC-MS/MS analysis, the relative protein abundance of various proteins found during contamination of odontoblast-like cells when compared to CHX treatments and un-treated groups.

Part 1 of this study concluded that EGCG is safer to use on cells at a wider range of concentrations than CHX, and the two compounds affected the cytokinome of the MDPC-23 cells in a way that was not strictly pro or anti-inflammatory. Part 2 of this study found that an introduction of an MOI stimulation to the MDPC-23 cells with a simultaneous EGCG and CHX treatment did not result in cytotoxicity within the viable concentrations of the treatment compounds. As well, the differential protein production by the odontoblast-like cells was altered between the two treatments, as well as between un-stimulated and stimulated groups when treating with EGCG or CHX. Though EGCG and CHX share many anti-proteolytic properties, the added benefit of EGCG use may be that it inhibits both the activity and production of MMPs via the inhibition of cytokine production.
6.2 Future directions

Future directions for this study will require the establishment of a model that dramatically increases the pro-inflammatory response of the odontoblast-like cells. From there, ELISAs quantifying the secretion of other key mediators of inflammation such as IL-6, IL-10, and TNF-α can be performed in order to compare the efficacy of EGCG and CHX treatments. Cytokines that cause downstream production of MMPs can be targeted to gauge the effect of MMP inhibition via cytokine inhibition. As well, Western blotting can be implemented to determine the activation of the NF-κB pathway through phosphorylation of its inhibitory complex. This study provides a foundation for further investigation into the use of EGCG in inflammatory-related *in vitro* models.
References for Chapters 1 and 2


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