Comparison of Anti-inflammatory Effects Produced in Gingiva by Metronidazole and Azithromycin

THESIS

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By

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Abstract

Objective: Previous studies suggest that azithromycin (AZM) inhibits subclinical gingival inflammation in individuals with minimal plaque and clinically healthy gingiva. While it is unclear whether other antibiotics produce similar effects in gingiva, metronidazole (MET) has been shown to decrease pro-inflammatory cytokine production by cultured periodontal ligament cells. This randomized, blinded, crossover study compared anti-inflammatory effects produced by AZM and MET.

Methods: Twelve healthy adult subjects with good oral hygiene and clinically healthy gingiva were randomly allocated to receive a blinded regimen of either AZM (500mg initial dose, then 250mg at 24 and 48 hrs) or MET (375mg every 12 hr for 48 hr). At baseline (immediately before starting the regimens) and 2, 4, 7 and 14 days later, gingival crevicular fluid (GCF) samples were collected from twelve maxillary interproximal sites, measured with a calibrated Periotron, pooled, and stored frozen. After a 21 day washout period, each subject received the alternative regimen. A second set of GCF samples were collected at the same time points. GCF samples were assayed for pro- and anti-inflammatory cytokine signatures by multiplex immunoassay.

Results: Both agents induced significant transient decreases in the rate of GCF flow on days 2, 4 and 7. No significant differences in their effects were observed. With both antibiotics, the GCF flow rate increased to approximately 86% of baseline between days 7
and 14. In parallel with their effects on GCF volume, AZM and MET induced transient reductions in the GCF content of several pro-inflammatory cytokines (IL-1β, IL-12, IL-17, G-CSF) as well as IL-8, RANTES, VEGF, and IL-4. The effects of AZM and MET were similar in magnitude and the most significant decreases below baseline levels were apparent on days 4 and 7. MET induced a larger decrease in GCF IL-8 content than AZM. Neither agent produced significant changes in the amounts of the anti-inflammatory cytokines IL-10 and IL-1 receptor antagonist in GCF.

Conclusions: In addition to their known antimicrobial effects, AZM and MET both appear to inhibit production of a similar range of pro-inflammatory cytokines and chemokines in gingiva. This could potentially enhance the efficacy of these agents in the treatment of inflammatory periodontitis.
Dedication

This document is dedicated to my family.
Acknowledgement

I deeply appreciate the guidance and support of my advisor and thesis committee. I want to thank Dr. Eubank and his lab for carrying out the immunoassay on our samples I’m grateful to my fellow periodontal residents, who volunteered to be my subjects. I acknowledge the support of the OSU Center for Clinical and Translational Science (CCTS).
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Fields of Study

Major Field: Dentistry
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CHAPTER 1
INTRODUCTION

Periodontitis is a destructive inflammatory disorder that is caused by a polymicrobial infection. Periodontitis leads to formation of periodontal pockets and loss of alveolar bone and clinical attachment and is one of the main causes of tooth loss in adults. A number of species, including: *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, *Fusobacterium nucleatum*, *Prevotella intermedia*, *Parvimonas micra*, *Campylobacter rectus*, and *Capnocytophaga* sp, have been shown to be more closely related to periodontitis than healthy individual (Paju et al. 2009). One mechanism by which these pathogens initiate the host response is by interaction with toll-like receptors in gingival epithelial cells and leukocytes. Toll-like receptors, which detect highly-conserved bacterial molecular structures, activate the transcription and induction of a broad range of cytokines (Benedetto et al. 2013). Cytokines play crucial roles in modulating the recruitment of leukocytes and the proliferation and migration of mesenchymal cells in the periodontium. In addition, cytokines also mediate periodontal tissue destruction and disease progression. In particular, the pro-inflammatory cytokines IL-1β, IL-4, IL-6, IL-8, TNF-α, IL-12, IL-17, G-CSF, and GM-CSF are present in the diseased periodontal tissue and are associated with disease progression (Okada & Murakami, 1998).
Gingival crevicular fluid is a serum transudate or inflammatory exudate that (GCF) contains serum components, bacterial by-products, inflammatory mediators, host-derived enzymes, and tissue-break-down products. GCF production is induced by inflammation, trauma, or mechanical stimulation that causes an increase in the permeability of capillaries underlying the junctional epithelium and sulcular epithelium (Egelberg, 1966). The rate of GCF flow is high in gingivitis and relatively low in clinically healthy gingiva (Alfano, 1974). The relationship between crevicular fluid production with clinical and histological signs of inflammation has been thoroughly investigated in both cross sectional and longitudinal studies (Hancock et al. 1979, Lamster et al., 1980; Rudin et al., 1979), and GCF flow rate is considered to be a useful indicator of gingival inflammation (Griffiths, 2003).

A fundamental goal in the treatment of periodontitis is the elimination of supragingival and subgingival microbial biofilm by scaling and root planing. However, mechanical debridement is not always effective in eliminating periodontal pathogens (Fujise et al., 2002). While this may be due to anatomical limitations, some pathogens can penetrate into gingival tissue to evade debridement. The remaining bacteria can activate the host’s innate immune system and induce recurrent production inflammatory cytokines. This response can contribute to an unsuccessful treatment outcome and ultimately precipitate the progression of periodontitis.
Systemic antibiotics have been used as adjuncts to periodontal scaling and root planing, targeting pathogenic bacterial species. The rationale for use of adjunctive systemic antimicrobials is to further reduce the bacterial load and resolution of the inflammation in the periodontal pocket (Heitz-Mayfield 2009). Pathogens in periodontal biofilm are difficult to kill with antimicrobial agents, so disruption of biofilm by scaling and root planing is needed to obtain the best clinical outcome with adjunctive antibiotics. Systemic antimicrobial chemotherapy has been shown to enhance non-surgical periodontal treatment results (Heitz-Mayfield, 2009 & Haffajee et al., 2007), especially with pockets of 6 mm or deeper (Herrera et al., 2002). Metronidazole (MET) has been widely used in this role (Mestnik et al., 2012; Feres et al., 2012), and azithromycin (AZM) has also been evaluated in several studies (Hirsch et al., 2012; Haas et al., 2008; Mascarenhas et al., 2005; Oteo et al., 2010).

Azithromycin (AZM) is a macrolide antimicrobial compound that inhibits bacterial protein synthesis at the 50S subunit of the bacteria ribosome, thereby inhibiting bacterial mRNA translation (Foulds et al., 1990; Whitman & Tunkel, 1992). AZM produces bacteriostatic effects against a wide range of bacteria, including gram-positive bacteria and particularly strong effect on gram-negative bacteria (Hirsch et al, 2012). Furthermore, AZM appears to be capable of infiltrating biofilm (Tamura et al., 2008) and its concentrations were 15 to 50-fold higher in GCF than in blood serum (Jain et al., 2012). AZM is
an analogue of erythromycin with an additional nitrogen atom which can provide a higher
degree of structure stability, tissue penetration, and long half-life of approximately 68 h
(Hirsch et al., 2012 & Gomi et al., 2007). MET is active against gram negative anaerobic
bacterial infections (Pakhla et al., 2005). It has a serum half-life of 5.2 to 8.6 hours and is
primarily metabolized in the liver. While native MET is relatively inactive against bacte-
ria, it can be reduced to metabolites that inhibit bacterial DNA synthesis. The reduction
process occurs at low oxidation reduction potentials associated with anaerobic conditions,
so the drug is relatively specific for anaerobes (Greenstein 1993). AZM and MET are
both effective in inhibiting pathogens associated with periodontitis (Oteo et al., 2010 &
Greenstein 1993), but AZM has a substantially longer therapeutic half-life than metroni-
dazole.

Traditionally, we select antibiotics according to their antimicrobial spectrum, but there
are other criteria to consider. In addition to inhibiting periodontal bacteria, AZM can
produce certain anti-inflammatory effects, such as decreasing inflammatory cytokines in
GCF and the rate of GCF flow (Ho et al., 2010). Some studies suggest that MET inhibits
inflammatory cytokine production by mononuclear phagocytes in vitro (Rizzo et al.,
2010; Krehmeier et al., 2002). Rizzo et al. also found that MET can modulate the release
of interleukin (IL)-1β, IL-6, IL-8, IL-12 and TNF-α. AZM has been shown to exert anti-
inflammatory effects in the treatment of chronic inflammatory pulmonary diseases
(Giamarellos-Bourboulis, 2008), and there is evidence that it may produce anti-inflammatory effects in gingiva (Ho et al., 2010). AZM is known to inhibit activation of nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), which regulate the production of pro-inflammatory cytokines (Desaki et al., 2000; Kikuchi et al., 2002). These effects may have therapeutic value, since periodontitis is a destructive inflammatory disorder in which bacteria serve as an inflammatory stimulus.

In previous study (Ho et al., 2010), subjects with good periodontal health and undetectable levels of dental plaque exhibited a significant decrease in GCF volume when they were treated with systemic AZM. The decrease in GCF volume was accompanied by a transient decrease in the content of IL-1β, IL-8, TNF-α, and VEGF in GCF. Since this study was conducted with clinically healthy subjects with low bacterial load, the impact of the antimicrobial effects of AZM was minimized and direct anti-inflammatory effects were more apparent. Based on previous studies, we hypothesize that AZM produces more pronounced and sustained anti-inflammatory effects in gingiva than MET. This hypothesis will be addressed by directly comparing the anti-inflammatory effects of AZM and MET to determine whether the previously observed effects of AZM are novel. The study will characterize the effects of AZM and MET in subjects with clinically healthy gingiva and focus on changes in the rate of GCF production and changes in the concentration of inflammatory cytokines in GCF.


CHAPTER 2
MATERIAL AND METHODS

Subjects: Twelve healthy volunteer subjects recruited from the population of students in the Ohio State University College of Dentistry. Pregnant or nursing females and patients taking antibiotics or any other type of medication were excluded. The subjects were at least 21 years of age and had no history of drug allergy. Other than localized minor facial gingival recession, these subjects had no clinical periodontal attachment loss. Informed consent was obtained under the protocol approved by the Institution Review Board.

Study Design: This project was based on a prospective, randomized, double-blinded longitudinal study design that included a washout period (Table 1). Each subject received a prophylaxis and oral hygiene instructions (including interproximal flossing and tooth-brushing) seven days prior to administration of the first antibiotic regimen. At baseline (day 0), six subjects were administered a regimen of metronidazole (375 mg on day 0, followed by 375 mg doses at 12, 24, 36 and 48 hr) to establish steady-state antibiotic levels in gingival crevicular fluid. The other six subjects were administered a regimen of azithromycin (a single 500 mg dose on day 0, followed 24 and 48 hr later by 250 mg doses). To blind the subjects and the clinical examiner to the identity of the antibiotic, subjects who received azithromycin took capsules of similar appearance containing corn-
starch 12 and 36 hr after the initial dose. A research pharmacist prepared the masked drug regimens to support the blinded and randomized study design. Subjects were asked to document the times and dates they took the medication.

GCF samples and indices of plaque and gingival inflammation were obtained on study days 0, 2, 4, 7, and 14. The subjects then underwent a 3 week washout period, during which their teeth were cleaned to assure gingival health. Following the washout period, subjects began the alternate antibiotic regimen 5 weeks after initial registration. GCF samples and indices of plaque and gingival inflammation were obtained as previously described on days 0, 2, 4, 7, and 14 days.

At each study visit, GCF samples were obtained from 12 maxillary interproximal sites (corresponding to the MF and ML aspects of the maxillary premolars and first molars) using filter paper strips in conjunction with a procedure previously published by our laboratory (Conway et al., 2000). Briefly, the sites were isolated and the gingival tissues were air dried to avoid contamination. GCF samples were collected at the orifice of the crevice for 30 seconds and measured with a Periotron 6000 gingival fluid meter that has been calibrated by an established method (Preshaw et al., 1996). The paper strips were pooled and stored in microcentrifuge tubes at -20 °C. The clinical team consisted of a clinical operator who obtained the GCF samples and determined the Gingival index (GI).
and Plaque index (PI) scores, and a Periotron operator who determined the volume of the collected samples, stored the samples on ice and recorded all data.

**Sample analysis:** At the time of analysis, the paper strips containing GCF samples were thawed. Samples were eluted from each pool of paper strip samples with a 200 µL volume of phosphate buffered saline as previously described (Conway et al., 2007). A commercially-available multiplex bead-based cytokine immunoassay (BioPlex, BioRad Laboratories Inc., Hercules, CA, USA) was used to measure the content of cytokines according to the manufacturer’s directions. Results were expressed as the total amount (in picograms) recovered from each pool of twelve GCF samples.

**Data analysis procedures and sample size estimates for primary outcomes:** The sample size projection was based on the assumption that azithromycin concentrations in GCF were more sustained over time than those of metronidazole (supported by Jain et al., 2012).

The mixed procedure were used to compare the effect of azithromycin and metronidazole on cytokine content of GCF samples obtained at each sample collection point over the course of the study. Repeated ANOVA or repeated measures ANOVA on ranks were used to compare effect of each antibiotic on different time points to baseline. Based on previous studies of GCF (Ho et al, 2010), we predicted that the GCF samples obtained on day 4 or the azithromycin regimen to contain less amount of cytokines than that obtained
on day 4 of the metronidazole regimen. The projected sample sizes to obtain a statistical power of 0.9 with an alpha value of 0.05.

The largest projection for the sample size required to attain a statistical power of 0.9 in any of the above outcomes is 8 subjects. Assuming that some subjects may drop out, a target sample size of 12 subjects is a reasonable estimate for this study.
CHAPTER 3

RESULTS

Subject information: Twelve subjects fulfilled the inclusion criteria and completed the study. The subject’s demographic information is presented in Table 1. Overall, subjects in the two groups were well balanced by gender and had a similar mean age. The median Gingival Index and Plaque Index values in both groups were 0 at baseline (Table 1) and throughout the study period (not shown).

Effect of azithromycin (AZM) and metronidazole (MET) on GCF volume: At baseline (day 0), pooled GCF volume per 30 seconds was similar in the AZM and MET groups (Figure 2). Following administration of AZM, there was a progressive decrease in volume until day 4, followed by a graduate increase in volume between days 4 and 14. In response to MET, GCF volume decreased initially, remained in a stable range between days 2 and 7, and gradually increased between days 7 and 14. In both groups, GCF volumes were significantly below baseline on days 2, 4 and 7 (P < 0.05, repeated measures ANOVA with Holm-Sidak post hoc test), but not on day 14. No significant differences between the AZM and MET groups were noted.

Analysis of biological mediators in GCF samples: Twenty different cytokines, chemokines and growth factors were analyzed in the samples acquired in this study. Although
all were detected in GCF (Table 2), the content of some mediators, including IL-6, GM-CSF (CSF2), MIP-1α (CCL3), MIP-1β (CCL4), eotaxin (CCL11), IP-10 (CXCL10), PDGF, basic FGF, IL-10, IL-1 receptor antagonist and interferon-γ exhibited no significant changes from baseline over the 14 day course of the study (P>0.05, repeated measures ANOVA or repeated measures ANOVA on ranks. See Appendix). Data related to these mediators were not subjected to further analysis. Unless noted, no significant differences between the AZM and MET groups were observed at any time point with respect to changes from baseline values.

Changes in pro-inflammatory cytokine content over time: In subjects taking AZM, the IL-1β content of pooled GCF samples decreased from baseline to day 4, then increased from day 4 to day 7. IL-1β remained relatively constant between days 7 and 14 (Figure 3, upper panel). The decrease on day 4 was statistically significant compared to baseline (P<0.05, Holm-Sidak test). In subjects taking MET, there was a significant decrease below baseline IL-1β content on days 4 and 7 (P<0.05, Dunn’s test), followed by a progressive increase toward baseline levels on day 14.

AZM and MET induced a similar pattern of changes in the G-CSF content of GCF (Figure 3, lower panel). G-CSF content decreased progressively from baseline to day 4, then increased toward baseline levels between days 4 and 14. The decrease observed on day 4
was significant in subjects taking AZM, while the decreases observed on days 2 through 7 were significant in subjects taking MET (P<0.05, Holm-Sidak test).

With respect to IL-12 (p70) and IL-17, there was a decrease in the content of both mediators from baseline to day 4 in subjects taking AZM, followed by an increase to levels near baseline between days 4 and 14 (Figure 4). The decreases in IL-12 and IL-17 observed on day 4 were significant (P<0.05, Holm-Sidak test). In subjects taking MET, there was a gradual decrease in the content of both IL-12 and IL-17 between baseline and day 7, followed by an increase toward baseline levels on day 14. With both mediators, the decrease observed on day 7 was significant (P<0.05).

**Changes in chemokine content:** The baseline IL-8 content of GCF was somewhat higher in the MET group (Figure 5, upper panel). AZM and MET both induced a significant decrease in IL-8 baseline to day 4 (P<0.05). After day 4, there was a progressive increase in IL-8 content in both groups. Overall, the MET group exhibited a larger decrease from baseline than the AZM group (P=0.05, repeated measures ANOVA).

RANTES content in GCF gradually decreased between baseline and day 7 in subjects taking AZM, and then gradually returned to baseline between days 7 and 14 (Figure 5, lower panel). The decreases observed on days 4 and 7 were statistically significant (P<0.05, Dunn’s test). Subjects taking MET exhibited significant decreases in RANTES
content on days 2 and 7 (P<0.05, Dunn’s test), followed by an increase towards baseline levels between days 7 and 14.

**Changes in growth factor content:** Neither AZM nor MET produced significant treatment effects on PDGF-BB or basic FGF content in GCF, but both agents significantly altered VEGF content (Figure 6, upper panel). In subjects taking AZM, VEGF content gradually decreased from baseline to day 4, and then gradually increased toward baseline levels between days 4 and 14. The decrease in VEGF observed on day 4 was statistically significant (P<0.05, Holm-Sidak test). In subjects taking MET, there was a significant decrease from baseline VEGF content on days 4 and 7 (P<0.05, Holm-Sidak test), followed by an increase toward baseline.

**Changes in anti-inflammatory and T helper cytokines:** Neither agent altered the content of IL-10 or IL-1 receptor antagonist in GCF, but there was evidence they both affected the T\(\text{H}2\) cytokine IL-4 (Figure 6, lower panel). In subjects taking AZM, the IL-4 content remained essentially unchanged on day 2, but exhibited a significant decrease relative to baseline on days 4 and 7 (P<0.05, Dunn’s test). In subjects taking MET, there was a statistically significant decrease in IL-4 content on day 2 (P<0.05, Holm-Sidak test), followed by a gradual increase between days 2 and 14.
<table>
<thead>
<tr>
<th></th>
<th>Group 1 (MTZ first)</th>
<th>Group 2 (AZM first)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>4/2</td>
<td>3/3</td>
</tr>
<tr>
<td>Baseline PI (median &amp; range)</td>
<td>0 (0 – 1)</td>
<td>0 (0 – 1)</td>
</tr>
<tr>
<td>Baseline GI (median &amp; range)</td>
<td>0 (0 – 1)</td>
<td>0 (0 – 1)</td>
</tr>
<tr>
<td>Age (mean ±SEM)</td>
<td>29.0 ± 0.97</td>
<td>30.7 ± 0.67</td>
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</table>

Table 1: Composition of Subject Groups
<table>
<thead>
<tr>
<th>Pro-infl cytokines</th>
<th>Changes induced by AZM (compared to baseline)</th>
<th>Changes induced by MET (compared to baseline)</th>
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<tbody>
<tr>
<td>IL-1β</td>
<td>Post hoc test: significant ↓ on day 4</td>
<td>Post hoc test: significant ↓ on days 4 &amp; 7</td>
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<tr>
<td>IL-6</td>
<td>P=0.7, RM ANOVA on ranks</td>
<td>P=0.12, RM ANOVA on ranks</td>
</tr>
<tr>
<td>IL-12 (p70)</td>
<td>Post hoc test: significant ↓ on day 4</td>
<td>Post hoc test: significant ↓ on day 7</td>
</tr>
<tr>
<td>IL-17</td>
<td>Post hoc test: significant ↓ on day 4</td>
<td>Post hoc test: significant ↓ on day 7</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Post hoc test: no significant effect</td>
<td>Post hoc test: no significant effect</td>
</tr>
<tr>
<td>G-CSF (CSF3)</td>
<td>Post hoc test: significant ↓ on day 4</td>
<td>Post hoc test: signif ↓ on days 2, 4 &amp; 7</td>
</tr>
<tr>
<td>GM-CSF(CSF2)</td>
<td>P=0.42, RM ANOVA on ranks</td>
<td>P=0.7, RM ANOVA</td>
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<tr>
<td><strong>Chemokines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-8(CXCL8)</td>
<td>Post hoc test: significant ↓ on day 4</td>
<td>Post hoc test: significant ↓ on day 4</td>
</tr>
<tr>
<td>MIP-1α (CCL3)</td>
<td>P=0.13, RM ANOVA on ranks</td>
<td>Post hoc test: no significant effect</td>
</tr>
<tr>
<td>MIP-1β (CCL4)</td>
<td>Post hoc test: no significant effect</td>
<td>Post hoc test: no significant effect</td>
</tr>
<tr>
<td>RANTES(CCL5)</td>
<td>Post hoc test: signif ↓ on days 4 &amp; 7</td>
<td>Post hoc test: significant ↓ on days 2 &amp; 7</td>
</tr>
<tr>
<td>Eotaxin(CCL11)</td>
<td>Post hoc test: no significant effect</td>
<td>P=0.06, RM ANOVA</td>
</tr>
<tr>
<td>IP-10 (CXCL10)</td>
<td>Post hoc test: no significant effect</td>
<td>P=0.15, RM ANOVA on ranks</td>
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Table 2: Changes in GCF Biological Mediator Content Induced by AZM and MET

Abbreviations: RM ANOVA: one way repeated measures analysis of variance; RM ANOVA on ranks: Friedman one way repeated analysis of variance on ranks
Table 2 continued

<table>
<thead>
<tr>
<th>Growth Factors</th>
<th>( p=0.11, \text{ RM ANOVA} )</th>
<th>( p=0.33, \text{ RM ANOVA} )</th>
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<tr>
<td>PDGF-BB</td>
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<tr>
<td>VEGF</td>
<td>Post hoc test: significant ↓ on day 4</td>
<td>Post hoc test: significant ↓ on days 4 &amp; 7</td>
</tr>
<tr>
<td>Basic FGF</td>
<td>( p=0.12, \text{ RM ANOVA on ranks} )</td>
<td>( p=0.16, \text{ RM ANOVA} )</td>
</tr>
<tr>
<td>Anti-infl cytokines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>( p=0.53, \text{ RM ANOVA on ranks} )</td>
<td>( p=0.53, \text{ RM ANOVA} )</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>( p=0.74, \text{ RM ANOVA on ranks} )</td>
<td>( p=0.44, \text{ RM ANOVA on ranks} )</td>
</tr>
<tr>
<td>T(_h)1 cytokine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-( \gamma )</td>
<td>( p=0.06, \text{ RM ANOVA} )</td>
<td>Post hoc test: significant ↓ on day 7</td>
</tr>
<tr>
<td>T(_h)2 cytokine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-4</td>
<td>Post hoc test: significant ↓, days 4 &amp; 7</td>
<td>Post hoc test: significant ↓ on day 2</td>
</tr>
</tbody>
</table>
Figure 1: Flow chart of subject enrollment and allocation.
Figure 2: Effects of systemic AZM and MET on pooled GCF volume at maxillary interproximal sites. Volumes were derived by pooling samples from twelve maxillary interproximal sites, each collected for 30 seconds. Data represent the mean + SEM of 12 subjects. Legend: # indicates significant from baseline (day 0) of the MET group and * denotes significant change from baseline in the AZM group (repeated measures ANOVA with Holm-Sidak post hoc test).
Figure 3: Effects of systemic AZM and MET on the GCF content of IL-1α and G-CSF. Data are presented as mean ± SEM. AZM and MET produced significant treatment effects on both cytokines (P < 0.05, repeated measures ANOVA). Legend: * denotes significant differences from baseline (day 0) in the AZM group, while # indicates significant differences from baseline in the MET group (Holm-Sidak post hoc test).
Figure 4: Effects of AZM and MET on the GCF content of IL-12 (p70) and IL-17. Data are presented as mean + SEM. AZM and MET produced significant treatment effects on both cytokines (P < 0.05, repeated measures ANOVA). Legend: * denotes significant differences from baseline (day 0) in the AZM group, while # indicates significant differences from baseline in the MET group (Holm-Sidak post hoc test).
Figure 5: Effects of AZM and MET on GCF IL-8 and RANTES content. Data are presented as mean + SEM. AZM and MET produced significant treatment effects on both chemokines (P < 0.05, repeated measures ANOVA). Legend: * denotes significant differences from baseline (day 0) in the AZM group, while # indicates significant differences from baseline in the MET group (Holm-Sidak post hoc test). With regard to IL-8, the MET group exhibited a larger decrease from baseline than the AZM group (P=0.05, repeated measures ANOVA).
Figure 6: Effects of systemic AZM and MET on the GCF content of VEGF and IL-4. Data are presented as mean ± SEM. AZM and MET produced significant treatment effects on both cytokines (P < 0.05, repeated measures ANOVA). Legend: * denotes significant differences from baseline (day 0) in the AZM group, while # indicates significant differences from baseline in the MET group (Holm-Sidak post hoc test).
CHAPTER 4
DISCUSSION

AZM and MET both produce anti-inflammatory effects in healthy subjects with very low levels of bacterial plaque, suggesting that their effects were not strongly dependent on the antimicrobial activity of AZM and MET. The decrease in GCF volume observed in this study showed similar patterns in both groups. Despite the more pronounced reduction on day 4 in the AZM group, both groups showed significant decreases from baseline to day 7. As AZM and MET were metabolized and eliminated, GCF volume increased to approximately 86% of baseline on day 14. There is a strong positive correlation between histological signs of inflammation and GCF volume (Griffiths, 2003). The results suggest that both agents produce anti-inflammatory effects in gingiva for at least one week. The similarity of the effects refutes our hypothesis that AZM produces anti-inflammatory effects of longer duration.

With respect to biological mediators, the results indicate that both agents can reduce the amount of certain pro-inflammatory cytokines (IL-1β, IL-12, IL-17, G-CSF) in GCF, as well as IL-8, RANTES, VEGF and IL-4. Most of the pro-inflammatory cytokines exhibited a tendency toward reduction from baseline through day 7. Only IL-8 exhibited a significant difference in the effects produced by AZM and MET, evidenced by a larger overall decrease from baseline in subjects taking MET. The anti-inflammatory cytokines
IL-10 and IL-1ra were not significantly affected by either antibiotic. The overall similarity of these effects of AZM and MET allows us to reject the hypothesis that AZM produces more pronounced and sustained anti-inflammatory effects in gingiva than MET.

GCF cytokine content reflects the rate of their production in gingiva. Reduction of cytokine content by AZM and MET could significantly alter inflammatory cell recruitment and vascular permeability associated with subclinical gingival inflammation. As an example, IL-1β occurs at high concentration in GCF from inflamed periodontal tissue and stimulates the inflammatory cell infiltration and alveolar bone resorption. IL-1β appears to play an essential role in the pathogenesis of periodontitis (Chiang et al., 1999; Gemmell et al., 1997; Gamonal et al., 2000). IL-8 is a chemokine produced by polymorphonuclear cells, fibroblasts and keratinocytes in response to bacteria and pro-inflammatory mediators like IL-1β and TNF-α. This chemokine induces migration of polymorphonuclear leukocytes, monocytes and macrophages to sites of infection (Cazalis et al., 2009). Reduced production of IL-1β and IL-8 can decrease the polymorphonuclear leukocyte recruitment and reduce the permeability of capillaries and junctional epithelium. These effects can lead to reduction of GCF flow. G-CSF stimulates the survival, proliferation, differentiation, and function of neutrophil precursors and mature neutrophils (Metcalf, 1985). Therefore, reduced G-CSF production can decrease polymorphonuclear cells maturation.
Vascular endothelial growth factor (VEGF), which stimulates angiogenesis and vascular permeability, plays a critical role in inflammation and wound healing (Lal et al., 2001; Booth et al., 1998). By reducing levels of VEGF, AZM and MET could potentially induce a decrease in vascular permeability. Lastly, IL-4 plays an important role in the adaptive immune system. This cytokine can promote Th2 cell differentiation and survival and growth of lymphocytes, mast cells, and endothelial cells. Inhibition of IL-4 production can down regulate the humoral immune response (Ito et al. 2009).

The observed reduction of GCF volume by AZM is consistent with the report of Ho et al. (2010). In the Ho study, a significant decrease in GCF volume was noted on days 2, 4, and 7, followed by an increase to 90% of baseline levels by day 14. As in the present study, this effect was accompanied by a transient decrease of GCF levels of IL-1β, IL-8 and VEGF. The present study extends these findings by demonstrating that MET produces similar anti-inflammatory effects. It is possible that AZM produces its anti-inflammatory effects by inhibiting activation of nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), which regulate the production of pro-inflammatory cytokines (Desaki et al., 2000; Kikuchi et al., 2002). Furthermore, the reduction of pro-inflammatory cytokines may also be caused by down-regulation of neutrophil activation (Hirsch et al., 2012). MET is known to inhibit the production of inflammatory mediators in vitro. It modulates the release of interleukin IL-1β, IL-6, IL-8, IL-12 and TNF-α by cultured peri-
odontal ligament cell (Rizzo et al., 2010) and also down-regulates pro-inflammatory cytokine production by human peripheral blood mononuclear cells (Krehmeier et al., 2002). The mechanism underlying these effects is not fully understood. While most previous studies of antibiotics used in periodontal therapy have examined their anti-microbial effects, this study focused on a direct comparison of anti-inflammatory effects produced by MET and AZM. The human subjects examined in this study had low levels of bacterial plaque and clinically healthy gingiva. At the histological level, clinically healthy gingiva contains a sparse infiltration of inflammatory cells in both the junctional epithelium and the gingival connective tissue (Page & Schroeder, 1976). Lymphocytes, macrophages and neutrophils can be visualized in the gingiva, and neutrophils can also be detected in GCF (Egelberg, 1967). The presence of protective inflammatory cells helps explain why it is possible for anti-inflammatory agents to reduce GCF volume and pro-inflammatory signatures in GCF in individuals with clinically healthy gingiva.

To minimize bias, the study utilized a randomized, blinded design with a crossover. However, the results may have been affected by several factors not related to bias, including minor contamination of GCF samples with saliva, the differences in immune status of the individual subjects, and inadvertent trauma to the experimental gingival sites from food or daily oral hygiene measures. Collectively, these factors could have contributed to
artifacts in determination of GCF volume or inflammatory biomarkers, which could mask the true effects of treatment with antibiotics. Diurnal variations in GCF volume can also contribute to artifacts in GCF sampling, but the magnitude of these intraday variations may not be significant (Suppipat et al, 1977). The design of the present study did not include a placebo arm to assess the potential contribution from a Hawthorne effect, which occurs when human subjects change their normal behavior while under observation as part of an experiment. Experimental artifacts of these types can affect a broad spectrum of outcomes. In the present study, however, the changes induced by AZM and MET were relatively selective. Neither antibiotic caused any significant changes in the amounts of IL-6, TNF-α, GM-CSF, MIP-1α, MIP-1β, PDGF, FGF, IL-10, or IL-1ra in GCF.

When selecting an antibiotic for adjunctive use in the treatment of plaque-induced inflammatory periodontitis, it is rational to select an agent that provides therapeutic levels that are capable of inhibiting subgingival bacterial pathogens over a sustained period of time. It is also reasonable to consider using an agent that can help control the inflammatory response, since inflammation must be resolved to diminish the impact of cytokine-mediated host destructive processes and arrest the progression of inflammatory periodontitis. Consistent with this logic, Ng and Bissada (1998) demonstrated that the combination of a non-steroid anti-inflammatory drug (ibuprofen) with an adjunctive systemic an-
tibiotic produced a modest enhancement in the clinical outcomes of non-surgical periodontal therapy relative to either agent alone. Thus, the present study’s finding that both AZM and MET produce anti-inflammatory effects in addition to their documented antimicrobial effects is clinically relevant. Both agents have been shown to enhance the clinical outcomes of nonsurgical periodontal therapy, especially in treatment of aggressive periodontitis (Haas et al., 2008; Slots & Ting., 2002). While it is likely that most of the therapeutic benefit comes from their antimicrobial effects, additional benefits may accrue from suppression of inflammation. These benefits may be difficult to detect with traditional clinical outcome measurements like attachment gain and periodontal pocket reduction, but the results of present study suggest they could potentially be detected at the molecular or histological level.

As previously mentioned, the study’s subjects had minimal bacterial plaque to minimize the impact of the antimicrobial effects of AZM and MET and make it was easier to detect anti-inflammatory effects. It would be prudent to use this approach to examine other widely-used antibiotics (including doxycycline and amoxicillin, which has no significant direct anti-inflammatory effects) to determine whether they produce similar changes in GCF volume and GCF cytokines. If they don’t, this would validate the study design and provide evidence that MET and AZM are somewhat unique in their ability to induce anti-inflammatory effects. If they do, this would suggest the possibility that the observed re-
ductions in GCF volume and cytokine content are indirect effects related to inhibition of barely detectable levels of pro-inflammatory supragingival bacterial plaque. Since antibiotic levels in GCF are rapidly diluted by saliva as they leave the gingival crevice and enter the oral cavity, it seems unlikely that antibiotic levels near the gingival margin would be high enough to significantly inhibit supragingival plaque.
References


Kikuchi, T., Hagiwara, K., Honda, Y., Gomi, K., Kobayashi, T. & Takahashi, H., (2002), Clarithromycin suppresses lipopolysaccharide-induced interleukin-8 production by hu-


Appendix A

The attached data, which were not discussed in detail in the thesis, portray the effects of systemic AZM and MET on the concentrations of IL-6, TNF-α, GM-CSF, MIP-1α, MIP-1β, eotaxin, IP-10, PDGF-BB, basis FGF, IL-10, IL-1 receptor antagonist, and interferon-γ in GCF. In contrast to the figures in the thesis, data are presented as mean + SEM of the observed concentration in the sample. Unless noted, there were no significant treatment effects (P > 0.05, repeated measures ANOVA or Friedman repeated measures ANOVA on ranks). Legend: * denotes significant differences from baseline (day 0) in the AZM group, while # indicates significant differences from baseline in the MET group (Dunn’s post hoc test).
GM-CSF raw data

Days since start of antibiotic regimen

Observed concentration (pg/ml)

AZM
MET
IP-10 raw data

Days since start of antibiotic regimen

Observed concentration (pg/ml)

AZM
MET
IL-1ra raw data

Days since start of antibiotic regimen

0 2 4 6 8 10 12 14

Observed concentration (pg/ml)

0.0
2.0e+4
4.0e+4
6.0e+4
8.0e+4
1.0e+5
1.2e+5
1.4e+5
1.6e+5
1.8e+5

AZM
MET
IFN-gamma raw data

Days since start of antibiotic regimen

Observed concentration (pg/ml)

AZM
MET
#