GINGIVAL CREVICULAR FLUID CONCENTRATIONS OF AZITHROMYCIN IN HEALTH AND GINGIVITIS

A Thesis

Presented in Partial Fulfillment of the Requirements for

The Degree Master of Science in the

Graduate School of The Ohio State University

By

Nidhi Jain, D.M.D.

Graduate Program in Dentistry

*****

The Ohio State University
2011

Master’s Examination Committee

Dr. John Walters, Advisor

Dr. Hua- Hong Chien

Dr. Sarandeep Huja
Azithromycin, a macrolide antibiotic is active against several periodontal pathogens, is taken up and concentrated inside gingival fibroblasts, oral epithelium and polymorphonuclear leucocytes. Interactions with host cells in gingiva could influence the pharmacokinetics of this agent in periodontal applications. This issue was examined in two separate clinical studies. The first study, described in Chapter 2, tested the hypothesis that steady-state concentrations of azithromycin are higher and more sustained in gingival crevicular fluid (GCF) than in serum. Four healthy subjects received an initial dose of 500 mg azithromycin followed by 250 mg doses on each of the next 2 days. Serum and GCF samples were obtained 2 hours after the last dose (day 2) and on days 4 and 7. GCF samples were collected from maxillary posterior interproximal sites with paper strips. The strips were pooled and eluted with high purity water. After extraction with diethyl ether, the azithromycin content of the serum samples and GCF eluates was determined with an agar diffusion bioassay. On days 2, 4 and 7, the concentrations of azithromycin in blood serum were 0.22 ± 0.02, 0.08 ± 0.02 and 0.04 ± 0.01 µg/ml, respectively. The concentrations in GCF were 8.82 ± 1.25, 7.90 ± 1.72 and 7.38 ± 1.15 µg/ml, respectively. Mean GCF levels were consistently higher than mean serum levels (P ≤ 0.02, paired t-test). The findings demonstrate that the pharmacokinetic profiles of azithromycin are different in GCF and serum. At steady state, azithromycin
concentrations in GCF were higher and more sustained than those in serum. Based on previous studies, the levels observed in GCF were above the Minimum Inhibitory Concentration (MIC) for *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Prevotella intermedia*.

Azithromycin preferentially localizes in inflamed tissues, suggesting that its concentration in gingival crevicular fluid (GCF) could be higher at gingivitis sites than in health. The study described in chapter 3 tested this hypothesis. Experimental gingivitis was induced in one maxillary posterior sextant in nine healthy subjects. Contralateral healthy sextants served as controls. Subjects took 500 mg of azithromycin followed by 250 mg 24 hours later. Four hours after the second dose, plaque was removed from the gingivitis sites. GCF was collected from 8 surfaces in both the gingivitis and control sextants and pooled separately. GCF samples were subsequently collected on days 1, 2, 3, 8 and 15 and their azithromycin content was determined with the bioassay procedure described in Chapter 2. On days 2 and 3, the pooled GCF volume at gingivitis sites was significantly higher than at control sites (P <0.01) and the mean azithromycin content in 30 second GCF samples pooled from gingivitis sites was significantly higher than at control sites (P < 0.02). However, there were no significant differences in GCF azithromycin concentration between the gingivitis and control pools at any point. Thus, the amount of azithromycin perfusing gingivitis sites is greater than at healthy sites, but its concentration in GCF is similar due to dilution by GCF.

In combination with previous studies, the findings of the current pharmacokinetic studies provide a rationale for further clinical evaluation of the adjunctive benefits of azithromycin in the treatment of periodontitis.
DEDICATION

This thesis is dedicated to my parents for their love, support, and encouragement throughout my education. It is also dedicated to my siblings, and my friends who were there when I needed them.
ACKNOWLEDGMENTS

My initial thanks go out to Dr. Walters. He has been an invaluable mentor and resource during this research project. He has always been willing to take time out of his busy schedule and answer all the questions I had. I really appreciate all his efforts and his understanding when I needed his support when going through a tough time in my life. I would also like to thank Patrick Lai who has been a great friend and who stood by me throughout this project and gave me the words of encouragement when I needed them. A special thanks also to my program director Dr. Tataki for his invaluable advice and guidance throughout my residency. I would also like to thank my committee members Drs. Chien and Huja for their help and assistance. Last but not the least a special thanks to my fellow residents for their help and participation.

This research was supported by grant R21 DE018804 from the National Institute of Dental and Craniofacial Research (NIDCR).
VITA

February 4, 1981............................... Born – Bathinda, Punjab, India

1998-2003................................. B.D.S, Govt Dental College, India

2005-2007................................. D.M.D, Tufts University, Boston

PUBLICATIONS


FIELDS OF STUDY

Major Field: Dentistry

Specialization: Periodontology
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td></td>
<td>Dedication</td>
<td>v</td>
</tr>
<tr>
<td></td>
<td>Acknowledgments</td>
<td>vi</td>
</tr>
<tr>
<td></td>
<td>Vita</td>
<td>vii</td>
</tr>
<tr>
<td></td>
<td>List of Figures</td>
<td>x</td>
</tr>
<tr>
<td></td>
<td>List of Tables</td>
<td>xi</td>
</tr>
<tr>
<td></td>
<td>Chapters:</td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>2.</td>
<td>AZITHROMYCIN CONCENTRATIONS IN BLOOD AND GINGIVAL Crevicular Fluid After Systemic</td>
<td>3</td>
</tr>
<tr>
<td>2.1</td>
<td>Abstract</td>
<td>3</td>
</tr>
<tr>
<td>2.2</td>
<td>Introduction</td>
<td>4</td>
</tr>
<tr>
<td>2.3</td>
<td>Materials and Methods</td>
<td>5</td>
</tr>
<tr>
<td>2.4</td>
<td>Results</td>
<td>8</td>
</tr>
<tr>
<td>2.5</td>
<td>Discussion</td>
<td>9</td>
</tr>
<tr>
<td>2.6</td>
<td>Acknowledgments</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>List of References</td>
<td>17</td>
</tr>
<tr>
<td>3.</td>
<td>EFFECT OF GINGIVITIS ON THE AZITHROMYCIN CONCENTRATION OF GINGIVAL CREVICULAR</td>
<td>21</td>
</tr>
<tr>
<td>3.1</td>
<td>Abstract</td>
<td>21</td>
</tr>
<tr>
<td>3.2</td>
<td>Introduction</td>
<td>22</td>
</tr>
<tr>
<td>3.3</td>
<td>Materials and Methods</td>
<td>23</td>
</tr>
<tr>
<td>3.4</td>
<td>Results</td>
<td>26</td>
</tr>
<tr>
<td>3.5</td>
<td>Discussion</td>
<td>28</td>
</tr>
<tr>
<td>3.6</td>
<td>Acknowledgments</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>List of References</td>
<td>36</td>
</tr>
</tbody>
</table>
4. CONCLUSIONS .............................................................................................................39

BIBLIOGRAPHY ............................................................................................................41
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Changes in GCF volume and azithromycin concentrations (A) and Azithromycin concentrations in GCF and blood serum (B).</td>
<td>14</td>
</tr>
<tr>
<td>2.2</td>
<td>Agar plates with zones of inhibition.</td>
<td>15</td>
</tr>
<tr>
<td>2.3</td>
<td>Calibration plot for unknown samples.</td>
<td>16</td>
</tr>
<tr>
<td>3.1</td>
<td>Changes in Plaque Index (A) and Gingival Index (B) during the study.</td>
<td>32</td>
</tr>
<tr>
<td>3.2</td>
<td>Changes in pooled GCF volume from control and experimental sites.</td>
<td>33</td>
</tr>
<tr>
<td>3.3</td>
<td>Azithromycin content at control and experimental sites.</td>
<td>34</td>
</tr>
<tr>
<td>3.4</td>
<td>Azithromycin concentrations in blood serum and GCF (control and experimental).</td>
<td>35</td>
</tr>
</tbody>
</table>
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 Clinical and Pharmacological Observations during the Study</td>
<td>13</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

Periodontitis is a destructive inflammatory disease of the tooth supporting tissues that is initiated by subgingival bacterial pathogens. The initial treatment of this disorder almost always involves scaling and root planing to eliminate subgingival bacteria and remove bacterial products from root surfaces. In recent years, clinical studies have suggested that systemic antimicrobials, when used as adjuntively with scaling and root planing, can enhance pocket depth reduction and clinical attachment gain. The most widely used antibiotic regimen in periodontology is a combination of amoxicillin and metronidazole. Unfortunately, these agents produce some undesirable side effects and challenge patient compliance, since the regimen requires patients to take 6 tablets per day for more than one week.

While it is not in widely used by periodontists, azithromycin is a good alternative to the combination of amoxicillin and metronidazole. Azithromycin is a macrolide which has an enhanced potency and has a wide antimicrobial spectrum against aerobic and anaerobic gram negative microorganisms. It produces fewer adverse effects and is administered in a regimen that encourages patient compliance (one dose per day for five
days). Clinical studies suggest that it can enhance pocket reduction and/or clinical attachment gains in the non-surgical treatment of patients with chronic and aggressive forms of periodontitis. Previous studies have shown that azithromycin can attain higher steady-state levels in gingival tissues than in serum. To the best of our knowledge, there have been no previous studies to assess the levels of azithromycin in gingival crevicular fluid. In the first part of this thesis (Chapter 2) we conducted an exploratory clinical study to determine whether it is feasible to measure the concentration of azithromycin in GCF obtained from healthy volunteers. A bioassay that had previously been used to measure azithromycin levels in tissues was successfully adapted to measure azithromycin contents in very small volumes of GCF.

Gingival fibroblasts, oral epithelial cells and polymorphonuclear leukocytes (PMNs) are capable of accumulating higher amounts of azithromycin. By acting as reservoirs for azithromycin, these cells can potentially sequester azithromycin at the inflamed sites. Since the volumetric density of gingival fibroblasts and PMNs increases in the presence of gingivitis, this led us to hypothesize that systemically-administered azithromycin attains higher levels in GCF at inflamed sites than at clinically healthy sites. The prospective clinical study described in Chapter 3 was conducted to assess the effects of inflammation on the pharmacokinetics of azithromycin in GCF.
CHAPTER 2

AZITHROMYCIN CONCENTRATIONS IN BLOOD AND GINGIVAL CREVICULAR FLUID AFTER SYSTEMIC ADMINISTRATION

2.1 Abstract

Azithromycin is a macrolide antibiotic that is active against several periodontal pathogens. Macrolides are a family of antibiotics including Erythromycin, Clarithromycin and Azithromycin are taken up and concentrated inside gingival fibroblasts, which could influence their pharmacokinetics. This study tested the hypothesis that steady-state levels of azithromycin are higher and more sustained in gingival crevicular fluid (GCF) than in serum. Four healthy subjects received an initial dose of 500 mg azithromycin followed by a single dose of 250 mg on each of the next 2 days. Serum and GCF samples were obtained 2 hours after the last dose (day 2) and on days 4 and 7. GCF samples were collected from maxillary posterior sites with paper strips. The strips were pooled and eluted with high purity water. After extraction, the azithromycin content of the serum samples and GCF eluates was determined with an agar diffusion bioassay. On days 2, 4 and 7, the concentrations of azithromycin in blood serum were $0.22 \pm 0.02$, $0.08 \pm 0.02$ and $0.04 \pm 0.01 \mu g/ml$, respectively. The
concentrations in GCF were 8.82 ± 1.25, 7.90 ± 1.72 and 7.38 ± 1.15 µg/ml, respectively. Mean GCF levels were significantly higher than mean serum levels (P ≤ 0.02, paired t-test). Thus, the findings demonstrate that the pharmacokinetic profiles of azithromycin are different in GCF and serum. At steady state, azithromycin concentrations in GCF were higher and more sustained than those in serum. Based on previous studies, the levels observed in GCF were above the MIC for *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, and Prevotella intermedia*.

### 2.2 Introduction

Elimination of bacterial plaque from root surfaces is a major objective of periodontal therapy. Although this typically halts progression of attachment loss, this is a challenging goal in patients who are infected by invasive subgingival bacteria. *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, and Prevotella intermedia*, can invade pocket epithelium (Christersson et al., 1987, Duncan et al., 1993, Lamont et al., 1995, Dorn et al., 1998). These pathogens are particularly difficult to eliminate by scaling and root planing. Use of systemic adjunctive antibiotics is a logical approach for eradicating these pathogens and enhancing the response to scaling and root planing (Haffajee et al., 2003). Azithromycin is a macrolide antibiotic that inhibits a variety of subgingival bacteria (Pajukanta et al., 1992, Pajukanta et al., 1993, Sefton et al., 1996, Goldstein et al., 1999). Clarithromycin, a closely related macrolide, is actively transported and concentrated inside oral epithelial cells and gingival fibroblasts (Chou and Walters, 2008). Antibiotic uptake by host cells could provide several benefits in the
treatment of periodontitis. Elevated macrolide concentrations inside oral epithelial cells could facilitate the killing of invasive pathogens. Since gingival fibroblasts are a relatively large cellular compartment of the gingiva, (Schroeder and Listgarten, 1997) macrolide accumulation by these cells could allow them to function as drug reservoirs that enhance and sustain therapeutic concentrations at that site. Previous studies have shown that azithromycin and clarithromycin can attain higher steady-state levels in gingival tissue than in serum and suggest that tissue levels are increased in the presence of inflammation (Blandizzi et al., 1999; Burrell and Walters, 2008). Azithromycin concentrations in gingiva reportedly persist for up to 14 days after systemic administration (Gomi et al., 2007). Gingival crevicular fluid (GCF) originates from the vessels of the gingival plexus, within the gingival connective tissue. It seeps through gingival connective tissue and passes through the junctional epithelium prior to entering the gingival crevice (Schroeder and Listgarten, 1997). The rationale of our project was to measure the concentration of Azithromycin in GCF.

Following systemic administration, we hypothesize that steady-state azithromycin concentrations in GCF are higher and more sustained than the corresponding concentrations in blood. The present study tested this hypothesis, using methods adapted to work with small samples of GCF.

2.3 Material and Methods
Subjects: Four healthy adult volunteers (3 males and one female, with a mean age of 30 years) with no clinical periodontal attachment loss were recruited from the student population of the Ohio State University College of Dentistry. Subjects with a history of drug allergy, recent medication use and pregnant females were excluded. Written informed consent was obtained prior to their participation. The study protocol and subject recruitment procedures were reviewed and approved by the Institutional Review Board at the Ohio State University.

Study design: One week prior to administration of azithromycin, all participants had all their teeth cleaned and received oral hygiene instruction. To obtain steady-state levels of azithromycin in periodontal tissues, participants were given an initial dose of 500 mg with subsequent 250 mg doses at 24 and 48 hours. Using an established protocol (Conway et al., 2000), GCF samples were collected and pooled from twelve maxillary posterior interproximal sites (the mesiofacial and mesiolingual aspects of all maxillary first and second premolars and first molars) immediately before the first dose of azithromycin (day 0), two hours after the last dose on day 2, and on days 4 and 7 after the initial dose. These sites were selected because they were readily accessible and easy to protect from contamination by saliva. Prior to GCF collection, the collection sites were isolated with cotton rolls, supragingival plaque was removed and the site was gently dried with air. GCF was collected with paper strips (Periopaper, Oraflow Inc, Smithtown, NY, USA) positioned at the orifice of the crevice for 30 seconds. GCF volumes were determined with a calibrated gingival fluid measurement device (IDE Interstate, Amityville, NY, USA) and pooled. The Gingival Index (GI, Loe and Silness, 1963) and
Plaque Index (PI, Silness and Loe, 1964) were assessed at the collection sites at every study visit. In addition, blood samples were obtained by venipuncture on days 2, 4 and 7. Blood serum and pooled GCF samples were stored at -20° C in sealed vials.

**Sample analysis:** Prior to analysis, GCF was eluted from the pools of paper strips with 200 µl of ultrapure water, using a method previously described (Conway et al., 2000). The efficiency of elution, as assessed with [³H]-labeled macrolide, (American Radiolabeled Chemicals, St. Louis, MO, USA), was 67.4% ± 2.1% (data not shown). GCF eluates and blood serum samples (200 µl) were treated with 40 µl of 0.5 g/ml Na₂CO₃ and extracted three times with 1 ml diethyl ether. The extracts were dried, reconstituted in acetonitrile, and applied to sterile paper disks (BD Biosciences, Sparks, MD, USA). After complete evaporation of the acetonitrile, the azithromycin content of the disks was determined with an agar diffusion bioassay, using *Kocuria rhizophila* (ATCC 9341, American Type Culture Collection, Manassas, VA, USA) as the indicator organism (Gomi et al., 2007)(Figure 2.2). The assay was calibrated with 2 to 18 ng of authentic azithromycin (US Pharmacopeia, Rockville, MD, USA) (Figure 2.3). For GCF samples, calculations for azithromycin content incorporated a correction for the observed elution efficiency.

**Statistical analysis:** The paired t-test was used to evaluate differences in azithromycin concentration in GCF and blood. Based on a projected difference of 4 µg/ml in the mean azithromycin concentrations in GCF and blood serum and a pooled standard deviation of 1.5, the estimated number of subjects required to achieve a power of 0.80 with an alpha
of 0.05 in a paired t-test was 4. Repeated measures analysis of variance (ANOVA) was used to examine the statistical significance in changes in azithromycin concentration, GCF azithromycin content and GCF volume over the course of the study. The Holm-Sidak test was used for post-hoc comparisons. In all statistical analyses, the statistical unit was the subject rather than the site.

2.4 Results

Consistent with maintenance of gingival health, the median PI and GI values were 0 throughout the course of the study (Table 2.1). After administration of azithromycin, there was a persistent and statistically significant reduction in the pooled volume of GCF collected from the study sites (P < 0.001, repeated measures ANOVA, power of test = 0.99, Figure 2.1A). The pooled volumes on days 2, 4 and 7 were significantly lower than the baseline level (P < 0.05, Holm-Sidak test). On day 2, the azithromycin concentrations in GCF and blood serum were 8.82 ± 1.25 and 0.22 ± 0.02 µg/ml, respectively. Over the next five days, these concentrations decreased to 7.38 ± 1.15 and 0.04 ± 0.01 µg/ml, respectively (Figure 2.1B). Although concentrations in GCF did not decrease significantly between day 2 and day 7, there was a significant decrease in serum concentrations on days 4 and 7 (P < 0.05, Holm-Sidak test). Mean GCF levels were significantly higher than mean serum levels on days 2, 4 and 7 (P ≤ 0.02, paired t-test, power of tests ≥ 0.84). The rate of azithromycin infusion into the gingival crevice, as assessed by the amount recovered per 30 second pooled GCF sample, did not change significantly between days 2 and 7 (Table 2.1).
2.5 Discussion

The findings support the hypothesis that steady-state azithromycin concentrations in GCF are significantly higher and more sustained than those in serum. Azithromycin concentrations in GCF were more than 40-fold higher throughout the course of the study, and they decreased at a slower rate than the levels in serum. Azithromycin levels in GCF decreased by approximately 20% between the second and seventh days, while the levels in serum decreased by 80% during the same period. This may be attributed to the low degree of azithromycin binding to plasma proteins in combination with active accumulation of azithromycin by cells in peripheral tissues (Foulds et al., 1990, Gladue et al., 1990). Gingival connective tissue contains a large volume of fibroblasts, which could serve as reservoirs for maintaining high local levels of azithromycin. Oral epithelial cells and polymorphonuclear leukocytes may also accumulate azithromycin at this site (Chou and Walters., 2008; Gladue et al., 1989). Our findings are consistent with a previous report that azithromycin concentrations in gingival tissue are up to 25-fold higher than the corresponding concentrations in blood (Malizia et al., 1997). Macrolides are not the only antimicrobial agents that have a propensity to concentrate in GCF. Tetracyclines (e.g., doxycycline) and fluoroquinolones (e.g., ciprofloxacin) reportedly attain GCF concentrations that are several-fold higher than their concentrations in serum (Conway et al., 2000; Gordon et al., 1981; Pascale et al., 1986; Lavda et al., 2004). The pharmacological properties of all these agents are presumably influenced by their ability to be taken up, sequestered and released by fibroblasts, leukocytes, and other types of cells (Gladue et al., 1989; Chou and Walters, 2008).
Azithromycin concentrations observed in GCF are substantially higher than the minimal inhibitory concentration (MIC) previously reported for several periodontal pathogens, including *Aggregatibacter actinomycetemcomitans* (0.25-2.0 µg/ml, Pajukanta et al., 1992), *Porphyromonas gingivalis* (0.125-1 µg/ml, Pajukanta, 1993; Goldstein et al., 1999), *Prevotella intermedia* (0.03-1 µg/ml), and *Peptostreptococcus micros* (0.5-1 µg/ml, Goldstein et al., 1999).

Moreover, azithromycin concentrations remained above the MICs for these pathogens over the entire five day observation period. Many antibiotics can be classified as having a concentration dependent killing effect or a time dependent killing effect. However, bacterial killing by azithromycin is not solely dependent on either model (Jain and Danzinger, 2008). The duration that target organisms are exposed to azithromycin concentrations above the MIC appears to be the best index of efficacy (Van Bambeke and Tulkens, 2001). Azithromycin also exhibits a prolonged post-antibiotic effect on inhibition of bacterial regrowth (Van Bambeke and Tulkens, 2001). Thus, pathogens found within periodontal tissues or in periodontal pockets that have been treated to disrupt subgingival biofilm should be vulnerable to inhibition by azithromycin. Pathogens living in native subgingival biofilm could be more difficult to inhibit at the azithromycin concentrations observed in this study. However, studies with an in vitro periodontal biofilm model suggest that azithromycin can penetrate the biofilm surface and partially dissolve the biofilm (Tamura et al., 2008). Azithromycin also appears to dissolve biofilm associated with diffuse panbronchiolitis (Nagino and Kobayashi, 1997).
A limited number of randomized, placebo-controlled clinical trials have suggested that azithromycin is a useful adjunct to scaling and root planing (SRP) in the treatment of periodontitis. In a study of patients with aggressive periodontitis, the combination of SRP plus azithromycin resulted in a higher percentage of teeth with attachment gain ≥ 1 mm and a greater reduction in probing depths than SRP alone (Haas et al., 2008). In patients with chronic periodontitis, the combination of SRP plus azithromycin yielded a significantly greater reduction of probing depths for pockets initially ≥4 mm than SRP alone (Smith et al., 2002). A study of non-surgical treatment of chronic periodontitis in smokers also demonstrated that adjunctive use of azithromycin with SRP resulted in enhanced pocket depth reduction and clinical attachment gain at moderate (4 to 6 mm) and deep (>6 mm) periodontal sites when compared to SRP alone (Mascarenhas et al., 2004). Moreover, a recent study of subjects with *Porphyromonas gingivalis*-associated chronic periodontitis demonstrated that, when compared with SRP alone, SRP combined with azithromycin yielded significantly enhanced pocket depth reduction and attachment gain and a significant decrease in the detection of *Porphyromonas gingivalis* (Oteo et al., 2011). Due to their anti-inflammatory activity, macrolides have been used in immunomodulatory therapy for chronic inflammatory lung diseases (Lopez-Boado and Rubin, 2008). Azithromycin also appears to produce anti-inflammatory effects in gingiva, as evidenced by its ability to reduce GCF volume and the GCF content of pro-inflammatory cytokines IL-1β, IL-8 and TNF-α (Ho et al., 2010). The mechanism for these effects appears to involve modulation of nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) (Cigana et al., 2006). Since GCF volume is strongly correlated with
histological signs of gingival inflammation (Griffiths, 2003), the significant reduction of GCF volume observed between days 2 and 7 in this study is consistent with the previous report. The apparent anti-inflammatory effects of azithromycin could represent an additional benefit when applied to treatment of inflammatory periodontal diseases. In conclusion, the results demonstrate that systemic administration of azithromycin produces relatively high and sustained levels in GCF and provide a rationale for further clinical evaluation of its adjunctive benefits in the treatment of periodontitis.

2.6 Acknowledgments

This study was supported by USPHS research grant R21 DE018804 from the National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, USA. I am grateful for the contributions of Pin-Chuang Lai, Weiting Ho and John Walters, who co-authored this manuscript. This work has been accepted as a short communication by Journal of Periodontology. The authors have no conflicts of interest to declare.
<table>
<thead>
<tr>
<th>Days After Initial Azithromycin Dose</th>
<th>Gingival Index*</th>
<th>Plaque Index*</th>
<th>GCF Azithromycin Content (ng per 30 second pooled sample)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0 (0 to 0)</td>
<td>0 (0 to 0)</td>
<td>Not determined</td>
</tr>
<tr>
<td>2</td>
<td>0 (0 to 0)</td>
<td>0 (0 to 0)</td>
<td>3.63 ± 0.39</td>
</tr>
<tr>
<td>4</td>
<td>0 (0 to 0)</td>
<td>0 (0 to 0)</td>
<td>3.41 ± 0.53</td>
</tr>
<tr>
<td>7</td>
<td>0 (0 to 0)</td>
<td>0 (0 to 0)</td>
<td>3.26 ± 0.56</td>
</tr>
</tbody>
</table>

**TABLE 2.1:** Clinical and Pharmacological Observations during the Study

Data are presented as *median and range observed in 4 subjects or †mean ± SEM. Within each column, there were no statistically significant differences between the observed values.
FIGURE: 2.1 Changes in GCF volume and azithromycin concentrations observed during the study. Vertical arrows indicate times when azithromycin was administered. A: Gingival crevicular fluid volumes collected during the study. The data represent the mean (± SEM) pooled GCF volume collected for 30 seconds from 12 maxillary premolar and first molar sites. B: Azithromycin concentrations in GCF and blood serum. The mean concentrations observed in GCF were significantly higher than those in serum on days 2, 4 and 7 (P ≤ 0.02, paired t-test).
FIGURE: 2.2 Representative agar diffusion bioassay plate with known amounts of azithromycin standard.
FIGURE: 2.3 Representative calibration plot for the agar diffusion bioassay for azithromycin. The data were derived from the bioassay plate portrayed in Figure 2.2.
REFERENCES


CHAPTER 3

EFFECT OF GINGIVITIS ON THE AZITHROMYCIN CONCENTRATIONS IN GINGIVAL CREVICULAR FLUID

3.1 Abstract
Azithromycin preferentially distributes to inflamed tissues, suggesting that its concentration in gingival crevicular fluid (GCF) could be higher at gingivitis sites than in health. To test this hypothesis, experimental gingivitis was induced in one maxillary posterior sextant in nine healthy subjects. Contralateral sextants served as controls. Subjects ingested 500 mg of azithromycin followed by 250 mg 24 hours later. Four hours after the second dose, plaque was removed from the gingivitis sites by light scaling. GCF was collected from 8 surfaces in both the gingivitis and control sextants and pooled separately. GCF samples were subsequently collected on the 2nd, 3rd, 8th and 15th day and their azithromycin content determined by agar diffusion bioassay. On days 2 and 3, the pooled GCF volume at gingivitis sites was significantly higher than at control sites (P <0.01) and the mean azithromycin content in 30 second GCF samples pooled from gingivitis sites was significantly higher than at control sites (P < 0.02). However, there were no significant differences in GCF azithromycin concentration between the gingivitis and control pools at any point. Thus, the amount of azithromycin perfusing gingivitis
sites is greater than at healthy sites, but its concentration in GCF is similar due to dilution by GCF.

3.2 Introduction

*Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* are periodontitis-associated bacteria that are capable of invading cells in the soft tissue wall of periodontal pockets (Christersson *et al*., 1987; Lamont *et al*., 1995). These microorganisms are difficult to eliminate by mechanical debridement alone (Chen and Slots, 1993), but systemic antimicrobial chemotherapy has been used as an adjunct to scaling and root planing to help eradicate these pathogens and enhance gains in clinical attachment level (van Winkelhoff *et al*., 1996; Haffajee *et al*., 2003). Azithromycin is a macrolide antibiotic that is active against *A. actinomycetemcomitans*, *P. gingivalis*, and other Gram-negative anaerobes (Pajukanta *et al*., 1992; Pajukanta, 1993; Goldstein *et al*., 1999). Randomized clinical trials have shown that azithromycin, when used adjunctively with scaling and root planing, enhances clinical attachment gain and probing depth reduction in patients with aggressive and chronic forms of periodontitis (Haas *et al*., 2008; Smith *et al*., 2002; Mascarenhas *et al*., 2005, Oteo *et al*., 2010). Azithromycin also enhances the elimination of *P. gingivalis* from patients with chronic periodontitis (Oteo *et al*., 2010).

The pharmacokinetic properties of macrolides are influenced by their tendency to be taken up, accumulated and released by many types of host cells. Azithromycin accumulates at high levels inside phagocytes (Gladue *et al*., 1989). Clarithromycin, which has a similar structure, is actively accumulated by gingival fibroblasts and oral epithelial cells (Chou and Walters, 2008). These cells function as macrolide reservoirs in
the gingiva. This could explain why azithromycin and clarithromycin can attain higher steady-state levels in gingival connective tissue than in serum and azithromycin persists in gingiva for 14 days (Blandizzi et al., 1999; Burrell and Walters, 2008; Gomi et al., 2007). A recent study demonstrated that steady-state azithromycin concentrations in GCF are significantly higher and more sustained than those in blood serum (Lai et al., 2011).

While previous studies have shown that the azithromycin and clarithromycin content of periodontal tissues tends to increase in the presence of inflammation (Blandizzi et al., 1999; Burrell and Walters, 2008), it is unclear whether gingivitis increases azithromycin levels in GCF. Gingivitis is associated with an increase in the volumetric density of fibroblasts and phagocytes in gingival connective tissue (Schroeder et al., 1973), effectively increasing its local capacity for accumulating azithromycin. It is reasonable to hypothesize that this contributes to an increased concentration in GCF. As gingivitis resolves, the concentration of azithromycin in GCF should approximate that found at healthy sites. These hypotheses were tested in the present study.

3.3 Materials and Methods

Subjects: Healthy adult volunteers (3 males/6 females, mean age 30.4 years) in good systemic and periodontal health were recruited from the student population of the Ohio State University. The subjects exhibited no evidence of clinical attachment loss, aside from localized minor facial gingival recession. Subjects with a history of drug allergy or recent medication use were excluded, as were pregnant females. Written informed consent was obtained under a protocol approved by the Institutional Review Board.
**Study Design:** In this split mouth longitudinal study, each subject received a prophylaxis one week prior to a baseline periodontal examination. A thermoplastic stent was fabricated to cover the maxillary premolars and the molars on one randomly selected side. At the baseline examination, gingival health was assessed bilaterally on the mesiobuccal and mesiolingual aspects of the maxillary first and second premolars and maxillary first and second molars with the Gingival Index (GI, Loe and Silness, 1963), Plaque Index (PI, Silness and Loe, 1964). GCF samples were collected and pooled from the same eight sites on each side as previously described (Conway et al., 2000). Briefly, sites were gently scaled to remove supragingival plaque, isolated with cotton rolls and air dried to avoid contamination. GCF samples were then collected by positioning a paper strip (Periopaper, Oraflow Inc, Smithtown, NY, USA) at the orifice of the sulcus for 30 seconds. GCF volume was determined with a calibrated Periotron 6000 (IDE Interstate, Amityville, NY, USA). The eight samples from each side were pooled and stored at -20°C.

At the conclusion of the examination, a prefabricated thermoplastic stent was delivered to facilitate induction of experimental gingivitis in one posterior region of the maxilla in accordance with the model described by Loe et al. (1965). Subjects were instructed to wear the stent during routine oral hygiene procedures to prevent plaque removal from the experimental side. Subjects were expected to maintain good oral hygiene procedures at all sites except the ones covered by the stent. Contralateral posterior sites not covered by the stent served as healthy controls.

The subjects returned every 7 days for the next three weeks for assessment of GI and PI at the control and experimental sites. At the three week visit, GCF samples were
collected from the control sites. On the following day (study day 0), the subjects ingested an initial dose of 500 mg azithromycin followed 24 hours later by 250 mg. Four to six hours later after the second dose, GI and PI were assessed at the control and experimental sites. Plaque was gently removed from the control teeth. GCF samples were collected, measured and pooled from the control and experimental sites as previously described in chapter 2. A 4 ml sample of peripheral blood was obtained by venipuncture. The subjects were instructed to immediately resume normal oral hygiene procedures. GI and PI assessments and GCF and blood serum samples were obtained in the same manner on days 2, 3, 8 and 15 after the initial dose of azithromycin.

To enhance objectivity and reduce delays that could contribute to sample contamination or evaporation, GCF samples were collected by a team that included a clinical operator, a Periotron operator and a data recorder. The clinical operator assessed the PI and GI scores, isolated the GCF collection sites and collected GCF samples. The samples were quickly passed to the Periotron operator, who measured sample volume and stored the samples in a cooled microtube. The Periotron readout was not visible to the clinical operator during sample collection and the site of origin of the GCF sample was not visible to the Periotron operator. Only the data recorder had direct access to this information.

**Sample preparation and analysis:** Prior to analysis, GCF was eluted from each pool of paper strips with 200 µl of ultrapure water, using a method previously described (Conway et al., 2000). The efficiency of elution, as assessed with [³H]-labeled macrolide (American Radiolabeled Chemicals, St. Louis, MO, USA), was 67.4%. GCF eluates and blood serum samples (200 µl) were treated with 40 µl of 0.5 g/ml Na₂CO₃ and extracted
three times with 1 ml diethyl ether. The extracts were dried under streaming nitrogen, reconstituted in acetonitrile, and applied to sterile paper disks (BD Biosciences, Sparks, MD, USA). After evaporation of acetonitrile, the azithromycin content of the disks was determined with an agar diffusion bioassay, using *Kocuria rhizophila* (ATCC 9341, American Type Culture Collection, Manassas, VA, USA) as the indicator organism (Gomi *et al.*, 2007). The assay was calibrated with 2 to 24 ng of azithromycin standard (US Pharmacopeia, Rockville, MD, USA). The reported azithromycin content of GCF samples incorporated a correction for the efficiency of elution from paper strips.

### 3.4 Results

At baseline (22 days prior to the first dose of azithromycin), median PI and GI values at the control and experimental sites were 0 (Figure 3.1), and the mean volumes of pooled GCF samples from these sites were 0.98 µl and 0.88 µl, respectively (Figure 3.2). There were no significant differences between control and experimental sites.

During induction of experimental gingivitis, PI and GI values at the control sites did not change. At experimental sites, both indices increased to a median value of 1.5 within 21 days. The differences in PI and GI values between control and experimental sites were significant at every study visit during this period (P ≤ 0.016, Wilcoxin signed-rank test).

After initiation of the azithromycin regimen, PI and GI values decreased at experimental sites on day 1 (Figure 3.1). The decrease in PI was statistically significant (P = 0.019, Mann-Whitney rank sum test). After plaque removal from the experimental sites, GI decreased to baseline within 7 days.
At the control sites, there were no significant changes in pooled GCF volume during induction of experimental gingivitis (Figure 3.2). In contrast, the GCF volume at experimental sites increased by more than two-fold. GCF volumes at experimental sites were significantly higher than control on days 1 through 3 after the initial azithromycin dose (P ≤ 0.01, paired t-test), although the volume at experimental sites decreased abruptly on day 3. GCF volumes at control and experimental sites converged after day 3 and were not significantly different from day 8 through day 15. Interestingly, there was a transient decrease in mean GCF volume at control sites after administration of azithromycin (P = 0.006, Friedman repeated measures ANOVA on ranks). The observed volumes were significantly lower than baseline on days 3 and 8 (P < 0.05, Dunn’s test), but increased to nearly 90% of baseline on day 15.

The mean azithromycin content of 30 second GCF samples pooled from the experimental sites was consistently higher than control from day 1 to day 3 (Figure 3.3). The differences were significant on days 2 and 3 (P ≤ 0.02, paired t-test). Thereafter, the azithromycin content of experimental and control samples were not significantly different. Between day 1 and day 15, the azithromycin content of the experimental samples was closely correlated with GCF volume at the same sites (r² = 0.93, P = 0.007, ANOVA), but was not significantly correlated with serum azithromycin concentration (r² = 0.41). Over the same time course, the azithromycin content of control samples was closely correlated with serum azithromycin concentration (r² = 0.78, P = 0.048), but not with GCF volume (r² = 0.36).

On day 1, the azithromycin concentration in GCF from control sites was higher than at experimental sites (Figure 3.4). Thereafter, the concentrations at control and
experimental sites converged and were nearly identical on days 8 and 15. There were no significant concentration differences between control and experimental sites between day 1 and day 15, but azithromycin levels in GCF was consistently higher than in blood (P < 0.011, repeated measures ANOVA, P < 0.05, Holm-Sidak test). The azithromycin concentration in control GCF was approximately 26-fold higher than in serum on day 1 and 560-fold higher on day 15. Between days 1 and 15, GCF azithromycin concentration at experimental sites did not exhibit a strong relationship with GCF volume or serum azithromycin concentration (r² = 0.57 and 0.167, respectively). At control sites, GCF azithromycin concentration was not well correlated with GCF volume (r² = 0.24) but exhibited a borderline correlation with serum azithromycin concentration (r² = 0.70, P = 0.076).

3.5 Discussion

The results of the present study refute our hypothesis that GCF azithromycin concentrations are higher in the presence of gingivitis, and suggest that they can be influenced by several factors. Undoubtedly, changes in serum azithromycin concentration can affect GCF azithromycin concentration, since GCF originates from the vessels of the gingival plexus (Schroeder, Listgarten. 1997). After azithromycin passes through endothelial pores and enters interstitial fluid, equilibrium is established between its concentration in tissue fluid and stores sequestered in host cells. In gingival tissues, a substantial amount of azithromycin may be taken up and concentrated inside fibroblasts, epithelium and inflammatory cells (Lai et al, 2011). As azithromycin levels decrease in interstitial fluid, the equilibrium favors release from intracellular stores. Ultimately,
interstitial fluid containing azithromycin seeps through gingival connective tissue and crosses the junctional epithelium into the gingival crevice.

It is feasible that gingivitis increases azithromycin movement through gingival connective tissue due to vasodilatation of the gingival plexus and increased vascular and junctional epithelial permeability. Moreover, gingivitis is associated with a 3-fold increase in the volumetric density of fibroblasts and phagocytes in the gingival connective tissue (Schroeder et al., 1973), which increases the intracellular volume available for azithromycin storage. These factors could account for the observed differences in the absolute amount of azithromycin recovered from pooled 30 second control and experimental gingivitis GCF samples. At healthy control sites, the impact of increased intracellular storage and increased vascular and junctional epithelial permeability is less pronounced. Consistent with this, there was a significant correlation between the amount of azithromycin recovered from control GCF and serum azithromycin concentration. In contrast, the amount of azithromycin recovered from experimental GCF was significantly correlated with experimental GCF volume, not serum azithromycin levels. As gingivitis resolved, differences in reservoir volume and vascular and junctional epithelial permeability disappeared and the azithromycin content of control and experimental GCF converged.

The concentration of azithromycin in GCF depends not only on the amount of azithromycin routed to gingival connective tissue and its partitioning between interstitial fluid and intracellular storage, but also on the degree of dilution by gingival fluid flow. This could explain why GCF azithromycin concentration was not significantly correlated with either serum azithromycin concentration or GCF volume. No significant differences
in azithromycin concentration were observed between control and gingivitis GCF. Although the concentration was initially higher in control GCF, the concentrations began to converge by day 2 and were essentially identical on days 8 and 15. The concentrations and pharmacokinetics of azithromycin were consistent with those reported in a previous study (Lai et al, 2011).

Although the clinical changes observed at the control and experimental sites were consistent with expectations for this well-characterized model of disease, two observations are worthy of comment. As in previous studies (Ho et al, 2010; Lai et al, 2011), azithromycin induced a transient decrease in control GCF volume. In parallel with this effect, azithromycin induces decreases in the GCF content of IL-8, IL-1β and TNF-α. These anti-inflammatory effects have been attributed to modulation of cytokine gene expression via nuclear factor-κB and activator protein-1 (Cigana et al, 2006). Moreover, azithromycin appeared to induce a decrease in PI and GI at experimental sites on day 1. There was a biological basis for this observation, since GCF azithromycin levels were high and plaque was in close proximity to the orifice of the crevice. Azithromycin reportedly penetrates and dissolves periodontal biofilm (Tamura et al, 2008) as well as biofilm associated with diffuse panbranchiolitis (Nagino and Kobayashi, 1997).

To reinforce the clinical relevance of our findings, two doses of azithromycin produced sustained and relatively high antimicrobial concentrations in GCF. Two weeks after the final dose, GCF azithomycin concentrations were above the minimal inhibitory concentration for *Aggregatibacter actinomycetemcomitans* (0.25-2.0 µg/ml, Pajukanta et al, 1992), *Porphyromonas gingivalis* (0.125-1 µg/ml, Pajukanta, 1993; Goldstein et al, 1993).
1999) and *Prevotella intermedia* (0.03-1 µg/ml; Goldstein et al, 1999), suggesting that azithromycin could produce beneficial antimicrobial activity even in patients who fail to complete the standard five dose regimen. Azithromycin was preferentially distributed to gingivitis sites, although its concentration in GCF was similar to that at control sites. It is likely that its concentration in experimental GCF was diluted by the increased volume of GCF associated with gingivitis. The pharmacokinetic properties of azithromycin, in conjunction with its antimicrobial and anti-inflammatory activities, suggest that it is well suited for treating inflammatory periodontitis and provide a convincing rationale for conducting definitive clinical trials to evaluate its adjunctive effects in periodontal therapy.

### 3.6 Acknowledgments

This investigation was supported by USPHS research grant R21 DE018804 from the National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD 20892, USA. I am grateful for the contributions of Pin-Chuang Lai and John Walters, who co-authored this manuscript. The authors have no conflicts of interest to declare.
Figure 3.1: Changes in clinical indices observed during the study. Arrows indicate the beginning and end of the plaque accumulation during the experimental gingivitis phase.

A: Plaque Index at control and experimental sites. Differences between the control and experimental sites were statistically significant at all time points from day –15 to day 1 (P < 0.05, Wilcoxon signed rank test). B: Gingival Index at control and experimental sites. Differences between control and experimental sites were statistically significant from day –15 to day 4 (P < 0.05).
Figure 3.2: Changes in GCF volume pooled from control and experimental sites during the study. Each pool was derived from eight paper strip samples, each collected for 30 seconds. The data are presented as mean + SEM. Arrows indicate the beginning and end of the experimental gingivitis phase. Asterisks denote significant differences between control and experimental pools (p<0.05, paired t-test).
Figure 3.3: Azithromycin content of GCF samples pooled from control and experimental sites. Data are presented as mean ± SEM, and the arrow indicates the end of experimental gingivitis phase. Asterisks denote significant differences between control and experimental pools (p<0.05, paired t-test).
Figure 3.4: Azithromycin concentrations in blood serum and pooled GCF samples. Data are presented as mean $\pm$ SEM. The arrow denotes time of plaque removal at the end of the experimental gingivitis phase. The concentrations measured in the control GCF pools were significantly higher than those found in blood at all observation points ($p<0.05$, Repeated measures ANOVA with post-hoc Holm-Sidak test). However, azithromycin concentrations in samples from control and experimental sites were not significantly different.
REFERENCES


CHAPTER 4

CONCLUSIONS

There have been no previous studies of the pharmacokinetics of azithromycin in GCF. The results indicate that azithromycin has properties that are well-suited for periodontal antimicrobial chemotherapy. As described in chapters 2 and 3, azithromycin produces steady-state concentration in GCF that are considerably higher and more sustained than its levels in blood serum. There is evidence that the absolute amount of azithromycin perfusing an inflamed gingival crevice is higher than in healthy crevices, although its concentrations in GCF are similar at inflamed and healthy sites. The studies also confirm that azithromycin produces anti-inflammatory effects in gingiva, resulting in a transient decrease in GCF volume at healthy gingival sites.

In conjunction with previous studies, our findings provide a convincing rationale for conducting definitive clinical studies to assess the effectiveness of adjunctive macrolide therapy in treating aggressive and recurrent forms of periodontitis. As an example, it would be reasonable to conduct a triple arm randomized clinical trial to compare the efficacy of a) scaling/root planing alone b) scaling/root planing with adjunctive amoxicillin and metronidazole and c) scaling/root planing with adjunctive azithromycin.
Ultimately, these studies could lead to improvements in non-surgical periodontal therapy that could reduce the cost burden of treating periodontitis.
BIBLIOGRAPHY


