AZITHROMYCIN SUPPRESSES *P. GINGIVALIS* LPS INDUCED PRO-INFLAMMATORY CYTOKINE AND CHEMOKINE PRODUCTION (IL-6, IL-8, MCP-1 & GRO) BY HUMAN GINGIVAL FIBROBLASTS *IN VITRO*

A thesis submitted to The University of Adelaide in partial fulfilment of the requirements of the Degree of Doctor of Clinical Dentistry (Periodontology)

Dr Catherine Jane Doyle

BDent, BSc (Biomedical Science) (Hons)

THE UNIVERSITY of ADELAIDE

Table of Contents

Table of Figures **4**

Abstract **5**

Declaration **6**

Statement of Authorship **7**

Acknowledgements **9**

Chapter 1. Literature review on the potential role of azithromycin in the mediation of periodontal inflammation **10**

1.1 Introduction 10

1.1.1 Aetiology and current treatments for periodontitis 10

1.2 Inflammatory mediators involved in pathogenesis of periodontitis 11

1.2.1 Specific polypeptide mediators in inflamed periodontal tissues 12

1.2.1.1 IL-1 13

1.2.1.2 IL-6 13

1.2.1.3 IL-8/CXCL-8 14

1.2.1.4 IL-17 (IL-17A) 14

1.2.1.5 MCP-1/CCL-2 14

1.2.1.6 GRO/CXCL-1 15

1.2.1.7 VEGF 15

1.2.2 Th cells in periodontitis 16

1.2.3 The Treg/Th17 axis 16

1.3 Inflammatory cytokine/chemokine biochemical signalling pathways 18

1.4 Antibiotics as an adjunctive treatment for periodontitis 20

1.5 Therapeutic use of azithromycin in periodontitis 22

1.5.1 Chronic periodontitis 23

1.5.1.1 Smokers and effect on chronic periodontitis 24

1.5.2 Aggressive periodontitis 25

1.5.3 Locally delivered azithromycin 26

1.5.4 Microbial effects of azithromycin in periodontal treatment 26

1.5.5 Recent clinical studies 27

1.5.6 Treatment of gingival enlargement 28

1.6 Properties of azithromycin 28

1.6.1 Pharmacokinetic and pharmodynamic properties 29

1.6.2 Antimicrobial properties of azithromycin 31

1.7 Anti-inflammatory/immune modulating effects of azithromycin 32
1.7.1 Effect on cytokines production and transcription factors 33
1.7.2 Effect of azithromycin on the innate immune cells 34
1.7.2.1 Effect of azithromycin and polymorphonuclear leukocytes (neutrophils) 35
1.7.2.2 Effect of azithromycin on monocytes, macrophages, and dendritic cells . 36
1.7.3 Effect of azithromycin on adaptive immune system 38
1.7.4 Effect of azithromycin in periodontal tissues 38
1.8 Common side effects and antibiotic resistance 39
1.9 Compliance 40
1.10 Conclusion 41
1.10.1 Study rationale, hypothesis and aims 41
1.11 References 43
Chapter 2. Azithromycin suppresses *P. gingivalis* LPS induced pro-inflammatory cytokine and chemokine production (IL-6, IL-8, MCP-1 & GRO) by human gingival fibroblasts *in vitro* 62
2.1 Introduction 63
2.2 Materials and methods 67
2.2.1 Sample collection and cell culture 67
2.2.2 *P. gingivalis* LPS and azithromycin treatments 67
2.2.3 WST-1 cell viability assay 68
2.2.4 Multiplex analysis of cytokines and chemokines production by gingival fibroblasts 68
2.2.5 Statistics 69
2.3 Results 69
2.3.1 Effect of *P. gingivalis* LPS, azithromycin, or combination treatments on HGF cell viability 69
2.3.2 Effect of azithromycin on *P. gingivalis* LPS-induced GRO production 70
2.3.3 Effect of azithromycin on *P. gingivalis* LPS-induced IL-6 production 72
2.3.4 Effect of azithromycin on *P. gingivalis* LPS-induced IL-8 production 74
2.3.5 Effect of azithromycin on *P. gingivalis* LPS-induced MCP-1 production 76
2.4 Discussion 78
2.5 References 84

Table of Figures

Figure 1.1 Toll-like receptor signalling 20
Figure 1.2 Structure of azithromycin 29
Figure 2.1 Effect of *P. gingivalis* LPS or azithromycin treatment on HGF cell viability ... 70
Figure 2.2 Combined donor data demonstrating the effect of azithromycin on LPS induced GRO production 71
Figure 2.3 Variability of induced GRO production between donors after treatment with azithromycin 72
Figure 2.4 Combined donor data demonstrating the effect of azithromycin on LPS induced IL-6 production 73
Figure 2.5 Variability of induced IL-6 production between donors after treatment with azithromycin 74
Figure 2.6 Combined donor data demonstrating the effect of azithromycin on LPS induced IL-8 production 75
Figure 2.7 Variability of induced IL-8 production between donors after treatment with azithromycin 76
Figure 2.8 Combined donor data demonstrating the effect of azithromycin on LPS induced MCP-1 production 77
Figure 2.9 Variability of induced MCP-1 production between donors after treatment with azithromycin 78

Abstract

Azithromycin is a macrolide antibiotic that is well known for its antibacterial properties, as well as possessing potential anti-inflammatory and immune modulating effects. This antibiotic has therefore been widely used in medicine for treating conditions ranging from inflammatory pulmonary diseases to dermatologic skin conditions. It has also been shown to be an effective antibiotic against most common periodontal pathogens and is used as an adjunct to treat periodontitis, a condition with bacterial aetiology and an inflammatory pathogenesis. Furthermore, periodontal case studies report regeneration of alveolar bone accompanied by significant reductions in inflammation have been achieved with azithromycin. The mechanisms however, by which these are achieved in the periodontium are largely unknown. This study aimed to determine the potential anti-inflammatory effect of azithromycin on cytokine and chemokine production by healthy human gingival fibroblasts (HGFs) that were stimulated by *Porphyromonas gingivalis* lipopolysaccharide (*P. gingivalis* LPS). HGFs were isolated from healthy gingiva collected from three donors. The effects of azithromycin at concentrations ranging from 0.1 μg/mL to 10 μg/mL were tested. Cytokine and chemokine protein levels were assessed using the Luminex® multiplex immunoassay. *P. gingivalis* LPS induced cytokine/chemokine (IL-6, IL-8, MCP-1 and GRO) protein production in HGFs was suppressed by azithromycin at all concentrations tested, and in all three donors. Suppression by azithromycin of IL-6, IL-8, MCP-1 and GRO *P. gingivalis* LPS protein induction in HGF was statistically significant when all donor results were collated ($p<0.05$). This study demonstrates that azithromycin suppresses *P. gingivalis* LPS induced cytokine/chemokine protein production in HGFs, which may explain some of the clinical benefits observed with the adjunctive use of azithromycin in the treatment of periodontitis.

Key words: azithromycin (AZM), periodontitis, human gingival fibroblasts (HGF's), *Porphyromonas gingivalis* lipopolysaccharide (*P. gingivalis* LPS), cytokine, chemokine, interleukin-6 (IL-6), interleukin-8 (IL-8/CXCL-8), Monocyte chemoattractant protein 1 (MCP-1/CCL-2), growth-regulated oncogene (GRO).

Declaration

I certify that this work contains no material which has been accepted for the award of any other degreee or diploma in any university or other tertiary institution to Catherine Jane Doyle, and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

The author acknowledges that copyright of possible publishable works contained within this thesis resides with the copyright holder(s) of those works.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Published Works:

Doyle, C.J. & Bartold, P.M. How does stress influence periodontitis? *Journal of the International Academy of Periodontology*, Volume 14, Issue 2, pages 42-49, April 2012.

Declared by: Dr Catherine Doyle	Witnessed by:
Signature:	Signature:
Date:	Date:

Statement of Authorship

Statement of Authorship

Title of Paper	Azithromycin suppresses P. gingivalis LPS induced proinflammatory cytokine and chemokine production (IL-6, IL-8, MCP-1 & GRO) by human gingival fibroblasts in vitro.
Publication Status	○ Published, ○ Accepted for Publication, ● Submitted for Publication, ○ Publication style
Publication Details	Submitted to the Journal of Periodontal Research

Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Catherine Jane Doyle		
Contribution to the Paper	Carried out the majority of the laboratory work, analysed samples, interpreted and analysed data, carried out statistical analysis, and wrote basic manuscript.		
Signature		Date	03/10/13

Name of Co-Author	Dr Tracy Fitzsimmons		
Contribution to the Paper	Helped with development of research project. Instructed and supervised laboratory work, provided direction and guidance, helped with data interpretation and editing of the manuscript.		
Signature		Date	3/10/13

Name of Co-Author	Ceilidh Marchant		
Contribution to the Paper	Instructed and supervised laboratory work, provided direction and guidance, helped with data interpretation and edited manuscript.		
Signature		Date	03/10/13

Name of Co-Author	Dr Kencana Dharmapatni		
Contribution to the Paper	Assisted with statistical analysis, and reviewed manuscript.		
Signature		Date	03/10/2013

Name of Co-Author	Dr Robert Hirsch		
Contribution to the Paper	Reviewed manuscript.		
Signature		Date	3/10/13

Name of Co-Author	Professor Mark Bartold		
Contribution to the Paper	Edited manuscript and provided direction and guidance as head research supervisor.		
Signature		Date	3/10/13

Acknowledgements

I would like to acknowledge my principle supervisor Professor Mark Bartold for the development of the research topic, as well as providing guidance and direction theoretically and clinically throughout my Doctor of Clinical Dentistry (Periodontology) candidature. I would also like to acknowledge my external supervisors, Dr Bryon Kardachi and Dr Robert Hirsch for their invaluable knowledge and guidance as clinical tutors and research supervisors throughout the last three years.

I am truly appreciative to my laboratory research supervisors, Dr Tracy Fitzsimmons for her expertise and guidance in helping me design and establish my research project, as well as Ceilidh Marchant for continuing to guide me through the project and imparting her laboratory expertise. Both Dr Tracy Fitzsimmons and Ceilidh Marchant have been invaluable in helping me complete my research project.

I would also like to thank Dr Kencana Dharmapatni who provided assistance and direction with my statistical analysis. Finally, I would also like to thank Catherine Offler from the Colgate Australian Clinical Dental Research Centre for assistance in the final editing of the manuscript.

This study was supported by a grant from the Australian Dental Research Foundation.