ENDOTHELIAL JUNCTIONS IN THE PERIODONTAL LIGAMENT MICROVASCULATURE OF YOUNG AND AGED MICE

A reseach report submitted in partial fulfilment
of the requirements for the
Degree of Master of Dental Surgery

by

John Cameron, B.D.Sc. (Qld.), F.R.A.C.D.S.

Department of Dentistry
The University of Adelaide
South Australia

July, 1995

This research report is dedicated to my beloved wife,
Dr Tracey Anne Winning

TABLE OF CONTENTS i

LIST OF FIGURES iv
LIST OF TABLES vi
SIGNED STATEMENT vii
ACKNOWLEDGMENTS viii

SUMMARY ix

CHAPTER 1
INTRODUCTION 1

CHAPTER 2
AIMS OF THE INVESTIGATION 4

CHAPTER 3
LITERATURE REVIEW 5
3.1 Ageing Theories and the Cardiovascular System 5
3.2 Microvasculature 6
3.2.1 Classification of Microvasculature 7
3.2.1.1 Perivascular Cells 9
3.3 Periodontal Ligament Vasculature 11
3.3.1 Periodontal Ligament Microvasculature 13
3.3.2 Periodontal Ligament Microvasculature Ultrastructure 14
3.4 Endothelial Junctions-Ultrastructure 15
3.4.1 Cardiovascular System Endothelial Junctions 15
3.4.2 Periodontal Ligament Endothelial Junctions 18
3.5 The Role of Endothelial Junctions in Capillary and Postcapillary Permeability 20
3.5.1 Theoretical Models of Capillary Permeability 23
3.5.1.1 Cylindrical Pore Theory 23
3.5.1.2 Fibre-Matrix Theory 25
3.5.1.3 Three-Dimensional Junction-Pore-Matrix Model 25
3.5.2 Endothelial Junction Structure-Function Correlations 26
3.6 Clinical Aspects and the PDL Microvasculature 28
3.6.1 The Role of the Periodontal Ligament Microvasculature in Function 28
3.6.1.1 Microvasculature and Orthodontic Tooth Movement 28
3.6.1.2 Microvasculature and Tooth Eruption 30
3.6.1.3 Microvasculature and Periodontal Health and Disease 31
3.6.2 Ageing and the Periodontal Microvasculature 31

CHAPTER 4
MATERIALS AND METHODS 34
4.1 Laboratory Procedure, Tissue and Section Preparation 34
4.2 Transmission Electron Microscopy 36
4.2.1 Blood Vessel Classification 36

4.2.2 Sample Size 37
4.2.3 Pilot Study 37
4.2.4 Blood Vessel Location 38
4.2.5 Endothelial Junction Inclusion 39
4.2.6 Goniometer 40
4.2.7 Magnification Calibration 40
4.3 Developing and Printing 40
4.4 Morphometry of Junctional Dimensions 41
4.4.1 Endothelial Junction Classification 41
4.4.2 Random Assignment of the Study Sample 42
4.4.3 Method of Measurement 42
4.4.3.1 Junction Length 42
4.4.3.2 Junction Thickness 43
4.4.3.3 Junction Length to PCV Wall Thickness Ratio - 'Meander' 43
4.4.3.4 Junction Width 44
4.4.3.5 Junction Type and Location 44
4.4.3.6 Junction Region Size 45
4.4.4 Random Assessment of Measurement and Junction Classification Error 45
4.5 Statistical Analysis 46

CHAPTER 5
RESULTS 48
5.1 Blood Vessel Type 48
5.2 Blood Vessel Diameter 51
5.3 PCV Location 52
5.4 Junction Type 56
5.5 Junction Dimensions 66
5.5.1 Junction Length 66
5.5.2 Junction Thickness 66
5.5.3 Length:Thickness Ratio - 'Meander' 66
5.5.4 Junction Width 67
5.5.5 Junction Size 68

CHAPTER 6
DISCUSSION 70
6.1 General Discussion 70
6.1.1 Mouse as an Experimental Model 70
6.1.2 Retention, Participation Aspects 70
6.1.3 Fixing Agents and Endothelial Junctions 70
6.1.4 Sample Size 71
6.2 Accuracy in Measuring Method 72
6.2.1 The Interpretation of Two Dimensional Images from Three Dimensional Structures 72
6.2.2 Section Thickness 73
6.2.3 Stained Sections 73
6.2.4 Goniometer 73
6.2.4.1 Effect of a Goniometer with a Rotation Facility on Tight and Close Region Classification 74
6.2.5 Resolution and Accuracy of the Electron Microscope 76

6. 3 Blood Vessels 76
6.3.1 PCV Morphology 76
6.3.1.1 PCV Type 76
6.3.1.2 PCV Diameter 78
6.3.2 Number and Location of PCV 78
6.4 Endothelial Junctions 79
6.4.1 Tight Regions 80
6.4.2 Close Regions 82
6.4.3 Junction Length 82
6.4.4 Junction Thickness 82
6.4.5 Junction Length to PCV Wall Thickness Ratio- 'Meander' 83
6.4.6 Junction Width 83
6.5 Suggestions for Future Research 83

CHAPTER 7
CONCLUSIONS 85

CHAPTER 8
APPENDICES 88
Appendix 1: Data Recording Sheet 88
Appendix 2: Results of the Pilot Study 89
Appendix 3: Tissue Preparation and Electron Microscopy 90
Anaesthetic 90
Anticoagulant 90
Glutaraldehyde Solution 90
Cacodylate Buffer 90
Osmium Tetroxide Solution 90
Tissue Fixing And Staining 91
Tissue Sectioning 92
Perfusate 92
Decalcifying Solution 92
Block Stain 92
Embedding Medium (Lx-112) 93
Light Microscopic Stains 93
Grid Stains 93
Radiographic Equipment 94
Transmission Electron Microscope 94
Appendix 4: Junction Width, Length, Thickness and 'Meander' Data 95

BIBLIOGRAPHY 97

LIST OF FIGURES

		Page
Figure 3.1	Ultrastructural appearance of different endothelial cell junctions.	15
Figure 3.2	A segment of a capillary wall with the junctional region exposed.	17
Figure 3.3	A diagram of capillary endothelium transport pathways.	21
Figure 3.4	Dimensions of interendothelial clefts of the capillary wall.	22
Figure 3.5	Two-dimensional diagrams of a capillary intercellular pathway.	24
Figure 3.6	Two-dimensional representation of a capillary close junction.	24
Figure 3.7	Three-dimensional junction-pore-matrix model of the intercellular cleft.	26
Figure 4.1	Diagram of the region of tissue collected along the root length.	35
Figure 4.2	Quadrat sampling procedure.	38
Figure 4.3	A diagram of the periodontal ligament divided into three regions, tooth, middle, and bone circumferential thirds.	39
Figure 4.4	A postcapillary-sized venule divided into 4 quadrants.	39
Figure 4.5	The method of measuring an endothelial junction.	43
Figure 5.1	An arterial capillary, an apericytic postcapillary-sized venule and a postcapillary-sized venule.	48
Figure 5.2	A pericytic postcapillary-sized venule.	49
Figure 5.3	A fenestrated postcapillary-sized venule.	50
Figure 5.4	Number of all postcapillary-sized venule plotted against the periodontal ligament level (μm) for young and old mice.	53
Figure 5.5	Total number of postcapillary-sized venules for all levels of the periodontal ligament for young and old mice.	55
Figure 5.6	A 'simple junction' from a postcapillary-sized venule. No tight or close regions are present.	56

Figure 5.7	A tortuous postcapillary-sized venule junction.	57
Figure 5.8	A 'simple junction' from a postcapillary-sized venule.	58
Figure 5.9	(A) A section at 0° prior to tilting with a goniometer around the x-axis to classify a 'close junction'.	59
Figure 5.9	(B) The same section illustrated in figure 5.9 (A) tilted at 45° clockwise with a goniometer around the x-axis to classify a 'close junction'.	60
Figure 5.10	(A) A junction from a postcapillary-sized venule with more than one 'close region'.	61
Figure 5.10	(B) The same section illustrated in figure 5.10 (A) tilted with a goniometer.	62
Figure 5.11	Summarised close and tight junction percentages in relation to the total number of junctions against the PDL level (μm) for young and old mice.	65
Figure 5.12	Mean junction length and thickness (nm) against the PDL vertical level (μm) for young and old mice.	66
Figure 6.1	Variation in image width at different angles.	72
Figure 6.2	Electron micrographs of one endothelial junction viewed with a single and a double axis goniometer.	75

LIST OF TABLES

Table 3.1	Classification of blood vessels in the microcirculation. (Adapted from Rhodin, 1967, 1968).	8
Table 3.2	Summary of studies on the total vascular bed volume as a percentage of the total periodontal ligament volume.	12
Table 3.3	Ultrastructural features of mouse molar microvasculature (Freezer, 1984).	14
Table 3.4	Components of interendothelial junctions (Anderson *et al.*, 1993; Lampugnani *et al.*, 1993).	27
Table 5.1	Total number of postcapillary-sized venules in each periodontal ligament level for young and old mice.	51
Table 5.2	Luminal diameter of postcapillary-sized venules averaged across ages.	52
Table 5.3	Number of postcapillary-sized venules in each of 8 mice classified by periodontal ligament circumferential region.	53
Table 5.4	Number of postcapillary-sized venules for young and old mice classified by periodontal ligament level and circumferential third.	54
Table 5.5	Number and ratios of postcapillary-sized venules for young and old mice classified by periodontal ligament circumferential region and postcapillary-sized venule type.	55
Table 5.6	The mean percentage of tight and close junctions.	63
Table 5.7	Effect of periodontal ligament level on number of close and tight junctions.	64
Table 5.8	Relationship between junction region and junction type.	65
Table 5.9	Summary of changes in junction width 1, junction width 2 and junction thickness with periodontal ligament levels for young and old mice.	67
Table 5.10	Junction size at different periodontal ligament (coronal to apical) levels for young and old mice.	68
Table 5.11	Junction size in each region along the length of the junction for young and old mice.	69
Table 8.4.1	Summary of changes in junction length, junction thickness and 'meander' with periodontal ligament (coronal to apical) levels for young and old mice.	95
Table 8.4.2	Summary of changes in junction widths 1, 2, 3 and 4 with periodontal ligament (coronal to apical) levels for young and old mice.	96

SIGNED STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university and that, to the best of the candidate's knowledge and belief, the thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis. The author consents to the thesis being made available for photocopying and loan if applicable, if accepted for the award of the degree.

John Cameron
31 July, 1995.

ACKNOWLEDGMENTS

I am grateful to my supervisors, Professor M.R. Sims, Discipline of Orthodontics, Faculty of Dentistry, University of Sydney and Dr. W.J. Sampson, Orthodontics Unit, Faculty of Dentistry, The University of Adelaide, for providing the facilities for this work to be carried out, for advice during experimentation and for their comments during the preparation of this manuscript. I also would like to acknowledge other members of the Department of Dentistry, The University of Adelaide, in particular, Mrs. M Leppard and Ms. V. Hargraves, for their helpful assistance.

I am grateful for assistance and advice that I received from outside the Faculty of Dentistry, in particular, Mr. P. Leppard, and the staff at the Electron Microscope Centre, Flinders Medical Centre, for the use of equipment and advice. Dr. H. Rosser and Dr. K. Crocker, from the Centre for Electron Microscopy and Microstructure analysis, The University of Adelaide, for the use of equipment and helpful assistance; and Dr. R. Correll, Biometrics Unit, CSIRO, for his statistical advice and analyses.

I am grateful to the Faculty of Dentistry for providing support through the Oliver Rutherford Turner Scholarship, for the second year of my candidature.

I would like to acknowledge the support of the Australian Society of Orthodontics Foundation for Research and Education for funding the project.

Finally, I would like to thank my family and friends both from within and outside the university, in particular my wife, Dr. Tracey Winning, for her love, support and assistance in the achievement of my goal.

SUMMARY

Populations in industrialised countries are living longer. One aspect of the proposed theories of the human ageing process relates to alterations in the microvasculature. Vascular permeability, which is directly proportional to changes in the ultrastructural dimensions of endothelial junctional complexes (Bundgaard, 1988), decreases with ageing (Hruza, 1977). An increased number of endothelial junction tight regions correlates with a reduction in permeability of microvessels (Rippe and Haraldsson, 1994). Therefore, any changes in the number and distribution of tight junctions in the microvascular bed endothelium may indicate alterations in vascular permeability with ageing and an effect on orthodontic tooth movement. Any such data from animals would be of value for initial extrapolation to humans.

The general aim of the present study was to investigate the effects of ageing on the morphology of periodontal ligament (PDL) endothelial junctions. The null hypothesis to be tested was that no changes occur in proportions of 'tight' and 'close' regions, and the dimensions of endothelial junctions in the microvascular bed of aged mouse PDL.

Tissue specimens used in the present study were from Freezer's (1984) and Sims' (1987) studies and consisted of molar PDL from four young (35 days) and four aged (365 days) ALCA-strain mice. Anaesthetised mice were perfused with 5.6% glutaraldehyde and 0.9% osmium tetroxide W/V solution in cacodylate buffer. The right and left mandibular first molars and their bony sockets were dissected en block. The tissue blocks were demineralised at 4°C with 0.1M EDTA in 2.5% glutaraldehyde and embedded in resin. The mesiobuccal portion of the PDL was sectioned parallel to the occlusal plane from the alveolar crest to the tooth apex. Sections were collected at 160 µm intervals resulting in 7 to 9 levels per root. Sections were stained and processed for transmission electron microscopy (TEM).

The results of a pilot study showed that within the available PDL samples there were only sufficient numbers of postcapillary-sized venules (PCV) for analysis. Therefore, five PCV with one complete endothelial junction were selected from each level. These junctions were assessed and photographed using a TEM goniometer to allow identification of the junction type, i.e., tight or close junctions. Measurements of widths and lengths along the junctions

were completed on standardised micrographs magnified x150K, using a Manual Optical Picture Analyser (MOP-3) and digital callipers. The junction type and junction dimensions were analysed with a chi-square analysis and a multiple regression technique, respectively, using Genstat^{TM} 5, Release 3 (AFRC Institute of Arable Crops Research, Clarendon Press, Oxford, UK). A value of $p < 0.05$ was taken as significant.

Analysis of the measurement error, using a paired t-test or Wilcoxon signed rank test, indicated there was no significant difference between the measurement at different time intervals. The coefficient of variation for the measurements ranged from 1.8% to 4.8%. The kappa coefficient was used to test the precision in classification of tight and close regions between first and second observations. This calculation yielded a measure of 1.00, indicating that no significant differences were found between the first and second classifications.

The types of junction found were: (1) junctions with tight regions, (2) junctions with close regions, (3) junctions with tight and close regions, and (4) junctions with no tight or close regions. No open or gap junctions were found. A chi-square analysis showed that the junction types changed significantly with age ($p < 0.001$). The percentage of tight regions was 14.1% ± 3.5% higher in the old mice. The percentages of close regions for young and old mice were 88.8% and 74.7%, and for tight regions 11.2% and 25.3%, respectively. The aged mice had an increased proportion of tight /close regions and greater numbers of tight regions at every PDL level ($p < 0.01$). With respect to PDL level (coronal to apical) effects, significantly ($p < 0.05$) higher numbers of tight regions were found at the alveolar crest by comparison with the apex for each age group. The majority of tight junctions (86.1% in young and 90.0% in old mice) were located at the luminal third of the PCV endothelial wall ($p < 0.05$). Close regions also were more common at the luminal third (66.7% in young mice and 65.5% in old mice).

There was no effect of age on endothelial junction length, thickness, or size. For both groups, the junction length at level 160 μm was higher than other PDL levels, but overall this effect was not significant. There was, however, a significant ($p < 0.05$) effect of PDL level for young and old mice, on the thickness of the PCV wall at the location of the endothelial junction. An increased wall thickness occurred from slightly above average at the alveolar

crest, rising to a maximum at 160 μm and then steadily declining towards the apex.

Junction width changed with age. The junction width a third of the distance along the intercellular cleft from the luminal side of the PCV, at the apex of the PDL, was (1) 3.6 nm ± 0.88 nm wider ($p < 0.05$) in old mice, and (2) increased significantly ($p < 0.05$) for young and old mice, from the 960 μm PDL level to the apex. The junction width at the luminal entrance increased significantly ($p < 0.05$) at the apex by comparison with the alveolar crest in each age group. Age had no effect on the location of the junction region between luminal and abluminal limits of the PCV endothelial wall. Junction size did not change with PDL level (coronal to apical). Tight regions were 2.8 ± 2.4 nm shorter than close regions, but this difference was not statistically significant.

There was no effect of age on either pericytic or apericytic PCV or PCV diameter. A smaller (by 2.5 μm) PCV diameter was found in the old mice compared with the young mice, however, this difference was not significant. In young and old mice, the major proportion of randomly assessed PCV were apericytic. The number of pericytic PCV in each age group increased significantly ($p < 0.05$), relative to the total number of PCV, at the alveolar crest by comparison with the apex.

Significantly more ($p < 0.05$) PCV were found for each group in the PDL circumferential bone third, with fewer in the middle third, and a minimum number in the tooth third. In aged mice, there was a significant increase ($p < 0.01$) in the number of PCV located in the tooth third of the PDL, most of which were apericytic PCV ($p < 0.001$). In the PDL middle circumferential third halfway down the young mice PDL, the number of PCV decreased significantly ($p < 0.001$).

In the present study, the null hypothesis was rejected. The demonstration of significant changes in the proportion of tight and close regions found may lead to decreased permeability of the aged PDL microvasculature. Endothelial junction morphology and structural alterations of PCV in the PDL of mice may represent functional modification of PDL microvasculature during ageing. Ionic tracer studies can assess permeability in aged PDL to confirm this hypothesis. Assessment of the clinical significance of these changes is required.