A T.E.M. INVESTIGATION OF ENDOTHELIAL CELL JUNCTIONS IN MARMOSET INCISOR PERIODONTAL LIGAMENT FOLLOWING ORTHODONTIC EXTRUSION AND RETENTION

A research project presented in partial fulfilment
of the requirements for the
Degree Of Master Of Dental Surgery

by

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Abbreviation	Meaning
TEM	Transmission electron microscope
PDL	Periodontal ligament
PCV	Postcapillary-sized venule
EDTA	Ethylenediaminetetra-acetic acid as di-sodium salt
W1	Junction width measurement one
W2	Junction width measurement two
W3	Junction width measurement three
W4	Junction width measurement four
W5	Junction width measurement five
W6	Junction width measurement six
L1	Length measurement one
L2	Length measurement two
M1	Meander one: calculated using L1
M2	Meander two: calculated using L2
D	Junction depth
CFC	Capillary filtration coefficient
BVD	Blood vessel diameter

SUMMARY

This study was the first to utilize the marmoset as an experimental model to gain morphometric data relating to endothelial cell junctions of upper central incisor periodontal ligament postcapillary-sized venules, following decoronation, root canal obturation, orthodontic extrusion with magnets and retention for 30 weeks.

Material used in this study was sourced from 4 male marmosets aged from 17 to 36 months, originally prepared by Weir (1990). Transverse sections of the periodontal ligament (not including the subapical tooth region) were taken at 150 micron intervals for each of the animals, beginning at the alveolar crest. The contralateral upper central incisor from the same animal was used as a control.

Each 150 micron level provided several sections for staining with uranyl acetate and Reynolds' lead for viewing in a Phillips CM 100 transmission electron microscope. For each PDL level, five postcapillary-sized venules were located, each showing an endothelial cell junction which could be viewed for its entire luminal to abluminal course. These junctions were photographed at 73,000 x, enlarged by a factor of 2 when developed, and used to determine a variety of measures for each junction. Low magnification, 900 x, photomicrographs of each blood vessel used were also enlarged by a factor of 2 when developed. These were used for orientation and determination of blood vessel diameter and type as either apericytic or pericytic. Photographs of a replicating graticule at high and low magnification were taken at the completion of each TEM session to enable accurate determination of the magnifications used. Junctions were defined as close if only close zones were located in that junction. Tight junctions were defined as having any tight zones,

irrespective of the presence of any close zones within that junction. Junctions with no close or tight zones were designated as no tight/close junctions. A goniometer was used to rotate the specimens on the TEM stage to determine whether zones which appeared at first inspection either tight or close were, in fact, tight or close at the new orientation.

Measurements taken for each blood vessel/junction included:

1. Two different measures of junction length.
2. Six measures of junction width.
3. The number of tight or close zones occurring in each of three defined regions; luminal third; middle third; abluminal third.
4. The total length of any tight or close zones per junction.
5. The depth of the junction across the luminal wall.
6. The presence/absence and size of any luminal junction flaps.
7. Whether the junction was straight or convoluted.
8. The internal luminal diameter of the blood vessel.
9. Utilizing the above measurements, capillary filtration coefficients (Casley-Smith, 1975) were calculated.
10. Meander (defined as length over depth) was also calculated.

To assess the error of the method and error involved in junction classification, 28 randomly chosen TEM negatives were re-developed and all measurements and classifications of PCV and junction type re-determined. For each of the measured and calculated variables the coefficient of variability was less than 5%. A Kappa coefficient of 0.8 indicated a reliable determination of junction classification.

A total of 322 junctions was considered adequate for analysis. Descriptive statistics for mean and standard deviation for each of the measured variables were determined for the combined control and combined experimental groups. Correlations between the variables were also performed.

The results showed that both measurements of junction length exhibited the same trends. Therefore, either measurement of junction length, as long as it was clearly defined and used consistently, would be suitable for future studies designed to assess endothelial cell junction length. The mean length L2 of the total number of control junctions was significantly greater than for the experimental junctions but the differences were not statistically significant at each PDL level. Mean junction length for the control group was 1411nm and for the experimental group was 1211nm. The difference was statistically significant ($p<0.001$).

Mean junction width W1 was 66.8 nm for the control group and 63.9 nm for the experimental group. Mean junction width W2 was 11.4 nm for the control group and 11.6 nm for the experimental group. Mean junction width W3 was 11.2 nm for the control group and 11.5 nm for the experimental group. Mean junction width W4 was 11.2 nm for the control and 11.3 nm for the experimental group. Mean junction width W5 was 11.1 nm for the control group and 11.2 nm for the experimental group. Mean junction width W6 was 28.1 nm for the control group and 29.1 nm for the experimental group. The only statistically significant difference noted was for W5 and the difference was only significant at the > 900 micron PDL level.

The mean junction zone size for the control group was 66.7 nm and for the experimental group was 56.7 nm. The difference was not statistically significant. Average width of close zones was 6nm. For the control group, 60.9% of close and tight junction zones occurred in the luminal third. The experimental group exhibited 85.3% of close and tight junction zones in the luminal third.

For the 900 micron and >900 micron PDL levels, there was a significant difference ($p<0.05$) in the number of close junctions for the control group, with the 900 micron level exhibiting fewer, and the >900 micron level exhibiting greater numbers of close junctions. The experimental group showed significant differences ($p<0.05$) in the number of close junctions at the 750 micron and 900 micron PDL levels, with the 750 micron level exhibiting greater, and the 900 micron level exhibiting fewer close junctions. The same group showed a significantly greater number ($p<0.05$) of tight junctions at the >900 micron PDL level.

Meander was found to differ significantly ($p<0.05$) between control and experimental groups but not down PDL levels. The mean meander M1 was 0.7928 for the control group and 0.7503 for the experimental group. This is not unexpected as the calculated variable is dependent upon junction length, which was found to vary in a similar fashion.

Capillary filtration coefficient also differed significantly ($p<0.01$) between the control and experimental groups but not down the PDL levels. The mean CFC was 0.0364 and 0.0482 for the control and experimental groups respectively. As with meander, CFC

calculation was highly dependent upon junction length so the noted difference between the two groups was not unexpected.

In conclusion, the study suggested that following a retention period of thirty weeks, some significant differences were still present in marmoset incisor PDL postcapillary-sized venules. These findings support those of a previous study by Chintavalakorn (1994) who utilized the same material to examine PDL blood vessel and nerve reconstitution. Chintavalakorn (1994) concluded that blood vessel and nerve reconstitution was essentially, but not entirely, complete following the treatment and retention period. These findings imply that either the PDL is altered irrevocably by orthodontic treatment, or that the reconstitution takes longer to finalize than the 30 weeks used with the present animal model.

SIGNED STATEMENT

This research report contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text of the report.

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CHAPTER 1

INTRODUCTION

To the author's knowledge this was the first study to examine the endothelial cell junctions of primate periodontal ligament postcapillary-sized venules. Previous animal models utilized to study endothelial cell junction morphometry included dog hind leg skeletal muscle (Casley-Smith et al., 1975), normal and tensioned rat maxillary molar periodontal ligament (Chintakanon, 1990), and old and young mouse maxillary molar periodontal ligament (Cameron, 1995).

It was hoped that by providing morphometric data related to endothelial cell junctions, utilizing a primate model, further data relevant to orthodontic tooth movement, tooth support, and the required length of retention following treatment would be elucidated.

Principal collagen fibres were the most obvious features of light microscopic investigations of the PDL. These fibres were considered to traverse the PDL space, uniting alveolar bone and tooth root cementum, in unbroken or continuous strands (Berkovitz, 1990). Arranged in the form of principal bundles, the collagen fibres were later shown to have different orientations in different regions of the PDL but, more recently have been recognised as having multiple branching and anastomosing rather than an intact network of discrete fibres (Berkovitz, 1990). Sicher (1942) suggested the presence of an intermediate zone or plexus to describe the branching and anastomosing region and suggested it was located in the middle of the PDL. Such a three-dimensionsal arrangement, showing

overlapping of fibre bundles in adjacent layers, precluded the tracing of intact fibre bundles across the entire PDL space (Sloan, 1979, 1982).

Oxytalan fibres in the PDL were reported by Sims (1973, 1977, 1983) who proposed a potential role in tooth support due to their close proximity to PDL blood vessels. Being pre-elastin type fibres, and forming three-dimensional meshworks extending from the cementum to the peripheral PDL blood vessels (Sims, 1975,1976), oxytalan fibres were suggested to act as a mechanoreceptive system which modulated the behaviour of PDL blood vessels. Such regulation may have been direct, or via the production of a more generalized neural response.

Ground substance within the PDL has been reported as another important source of tooth support (Berkovitz, 1990). They were found to consist of large molecular weight compounds such as chondroitin sulphate, dermatan sulphate and fibronectin, and possess enormous water binding potential (Ferrier and Dillon, 1983). This was suggested to enable the PDL to act as a hydrodynamic damping system as described by Bien (1966). The viscoelastic properties of the PDL were studied by Picton and Wills (1981) using a monkey animal model. They suggested that the reservoir of blood in the PDL blood vessels, the water binding potential of the ground substance, and also the biochemical turnover of collagen fibre networks, were responsible for tooth support.

Examining rat molar apical PDL, Cooper et al. (1990) demonstrated the presence of tissue channels responsible for the exchange of fluid between the interstitium and the PDL microvascular bed. They advocated that the PDL ground substance can exist in both solid and fluid states which increased the adaptability of the PDL under various stresses. The

interchange of fluid between the interstitium and the PDL microvasculature was suggested as producing a shock absorbing mecahnism for tooth support.

PDL blood vessels were the common link in all of the concepts of tooth support. Therefore, it seemed relevant to investigate the junctional complexes which exist between endothelial cells of PDL blood vessels, in order to add to the array of knowledge relating to the permeability of the barrier between the interstitium and the blood vessel interior.

Calculating capillary filtration coefficients for control and experimental, non-apical periodontal ligament was performed to provide a base line of data for future studies which may lead into research relating to the permeability of blood vessels under normal and pathological conditions, including neoplasia.

CHAPTER 2

AIMS OF THE PRESENT STUDY

This study was designed to provide morphometric data on the endothelial cell junctions of marmoset incisor periodontal ligament postcapillary-sized venules following orthodontic extrusion and retention.

Accordingly, the aims of the present study were:

1. To define normal PDL vascular endothelial cell junctions.
2. Compare endothelial cell junctions, following tooth extrusion and retention, with normal endothelial cell junctions by comparing junction type, distribution down the PDL, and junction dimensions.
3. Compare width, depth, and length of endothelial cell junctions of control and experimental incisors.
4. Calculate capillary filtration coefficients for each junction and compare these values for control and experimental groups and along the length of the PDL.
5. Compare the type and minimum intra-luminal diameter of control and experimental postcapillary-sized venules.
6. To test the null hypothesis that following 30 weeks retention, no significant differences would be found between the nominated dimensions of control and experimental endothelial cell junctions.

CHAPTER 3

REVIEW OF THE LITERATURE

3.1 Periodontal Ligament Vasculature

3.1.1 Gross Anatomy

The PDL vasculature has been implicated in nutrition, tooth eruption , tooth support and tooth movement. Primarily, the source of arterial blood to the PDL has been found to be via the maxillary artery. In particular the superior alveolar and inferior alveolar arteries which supply the maxilla and mandible, respectively. Additional maxillary blood supply has been reported via the greater palatine artery, superior labial branch of the facial artery, and the infra-orbital arteries. Collateral supply has been found to exist in the mandible also, via the lingual artery and branches of the buccal, inferior labial, masseteric and mental arteries. Minor blood supply also has been found via the periosteum (Edwall, 1982).

Arterioles tended to approach the PDL through the marrow spaces of the interdental and interradicular portions of the alveolar bone and enter it at various levels (Kindlova and Matena, 1962; Kindlova, 1965; Castelli and Dempster, 1965; Carranza et al., 1966).

Venous drainage of the PDL was found not to follow arterial supply (Berkovitz, 1990). In monkeys and man, veins in the alveolar bone and PDL were found to unite before draining into larger veins in the interalveolar and interradicular septi. These larger veins in turn were connected to a rich venous network surrounding the apex of each alveolus (Castelli and Dempster, 1965).

3.1.2 Microvasculature

In non-dental microvasculature beds the term microvasculature has been used to refer to the terminal ends of the vascular system. The types of vessel involved have been found to include arterioles, precapillary sphincters, arterial and venous capillaries, postcapillary venules and muscular venules. Some arteriovenous anastomoses also existed and allowed shunting of blood directly to venules if necessary (Edwall, 1982).

Arteries were found to enter tissues and their ramifications were numerous. Such ramifications were found to reduce the rate of blood flow in any small vessel and ensured adequate exchange of nutrients and waste products between cells and the blood stream. The site of most exchange was at the capillary wall which consisted of a simple monolayer of squamous epithelium forming an endothelial tube (Palade et al., 1979). Ultrastructural morphology of the microvasculature varied between locations and reflected the functional requirements of the surrounding tissue (Palade et al., 1979).

Classification of microvascular vessels has been performed using lumen diameter, number of cell layers, endothelial cell wall thickness and morphology, and the number of perivascular cells present (Bennett et al., 1959; Majno, 1965; Rhodin, 1967, 1968; Baez, 1977; Simionescu and Simionescu, 1984).

Capillaries have been classified with regard to their different physiological functions by Bennett et al. (1959). These authors studied several vertebrate animals and suggested a classification based on:

1. Presence (type A) or absence (type B) of continuous basement membrane.

2. Endothelial cell type.

Type 1 - without fenestrae or perforations.

Type 2 - with intra-cellular fenestrations.

Type 3 - with inter-cellular fenestrations.

3. Absence (type a) or presence (type b) of a complete investment of pericapillary cells.

Majno (1965) classified capillaries into three types using the completeness of the endothelial wall as the criteria. Majno (1965) did not classify vessels as either arterial or venous, and the classification proposed was:

Type I - continuous capillaries

Type II - fenestrated capillaries which resembled Type I but had thinner endothelial cells and were perforated by intracellular openings which were 0.01 microns in diameter and often bridged by a thin diaphragm.

Type III - discontinuous capillaries or sinusoids.

A classification based on the vessel lumen diameter, the endothelial cell lining, the pattern of vessel branching and anastomosis, and the constituents of the vessel wall was devised by Rhodin (1967,1968). The features of this classification are summarized in Table 1.

Utilizing a modification of the classifications of Rhodin (1967,1968) and Clark (1986), Chintakanon (1990) classified vessels based on their smallest luminal diameter and the structure of the vessel wall. Her classification was as follows:

1. Venous capillaries: 1.5 to 10 microns diameter; the endothelial lining being relatively thin and composed of 1 to 3 endothelial cells, a continuous basement membrane; pericytes and fenestrae occasionally present.

2. Arterial capillaries: 2 to 7 microns diameter; in relation to the venous capillary the ratio of endothelial lining to the luminal diameter being greater; the vessel wall usually composed of 1 to 3 endothelial cells with nuclei which often bulged within the endothelial wall; pericytes and fenestrae were occasionally present.

3. Postcapillary-sized venules: 10 to 30 microns diameter; thin endothelial lining; partial or complete pericytic investment occasionally present.

4. Terminal arterioles: 7 to 15 microns diameter; endothelial nucleus thicker and more lobulated; one layer of smooth muscle present in vessel wall.

5. Collecting venules: luminal diameter larger than 30 microns; pericyte investment usually evident.

Table 1. Classification of blood vessels in the microcirculation (Adapted from Rhodin, 1967, 1968).

Blood vessel type	Lumen calibre	Wall thickness	Endothelial cell morphology	Peri-endothelial cells	Other
Arteriole	100-50 μm	Greater than 6 μm	Cell 0.15 to 2μm in width, few pinocytotic vesicles, upstream cell usually overlaps downstream cell.	2-3 layers of smooth muscle cells, some eosinophils, mast cells and macrophages.	Well developed elastica interna, non-myelinated nerves extending to smooth muscle layer.
Terminal arterioles	Less than 50 μm	Less than 6 μm	Generally as above but with many filaments parallel to the long axis of the blood vessel and with more pinocytotic vesicles.	One layer of smooth muscle cells.	Little elastica interna, nerves closer to vessel wall with more frequent contacts with the smooth muscle layer, some myoendothelial junctions.
Precapillary	7-15 μm	Less than 5 μm	Cell protrudes towards vessel lumen, nucleus shorter, thicker and more lobulated than above, some cytoplasmic filaments, many pinocytotic vesicles.	One layer of smooth muscle cells.	An increased number of unmyelinated nerves associated with a decrease in lumen diameter, frequent neuromuscular and myoendothelial junctions.
Venous capillary	4-7 μm	0.3-1.3 μm	Some rough endoplasmic reticulum, free ribosomes, mitochondria, vesicles, granules and filaments.	Occasional veil cells and pericytes. Some macrophages, leukocytes, lymphocytes and plasma cells	Endothelium may be fenestrated.
Post-capillary venule	8-30 μm	1-5 μm	Cell rarely less than 0.2 μm thick and generally larger than that of venous capillary. Slight overlapping of adjoining cells.	More pericytes and veil cells than above. Some primitive smooth muscle cells around larger vessels.	Endothelium generally lacks fenestrae. Leukocytes may adhere to endothelial wall.
Collecting venulé	30-50 μm	1.7μm	As above.	Continuous layer of pericytes and veil cells around vessel. More primitive smooth muscle than above. Smooth muscle cells around larger vessels.	Single layer of veil cells and some collagenous fibrils surround blood vessels.
Muscular venule	50-100 μm	2.0 μm	As above	1-2 layers of smooth muscle cells.	Veil cells form a complete layer around vessel wall. Myoendothelial junctions present.
Small collecting vein	100-300 μm	2-3 μm	As above but with specific endothelial granules.	2 or more layers of smooth muscle cells.	Unmyelinated nerves situated 5-10 μm from muscular layer.

3.1.3 Vascular And Perivascular Cells

Blood vessel walls have been found to consist of endothelial cells with or without accompanying perivascular cells. Some ultrastructural characteristics of endothelial cells were studied by Avery et al. (1975) using mouse molar PDL as the experimental model. Their study showed that cell nuclei tended to project into the blood vessel lumen as did numerous finger-like projections of the endothelial cells. Cytoplasmic contents included numerous microvesicles, scattered ribosomes and small oval-shaped mitochondria. Cell junctions occured between endothelial cells and occasionally between endothelial and smooth muscle cells, with the intercellular junctions being tortuous in nature (Corpron et al., 1976).

Pericytes were described by Freezer and Sims (1987) as elongated cells with cytoplasmic branching and a basement lamina which was continuous with that of the endothelium. Mouse molar PDL was the model investigated. They suggested that these cells were commonly located around vessels with a luminal diameter of 4 microns, whereas in vessels with a luminal diameter exceeding 20 microns, the pericytic investment was absent or did not appear to completely surround the blood vessel.

Pericytes have been found to have features in common with fibroblasts (Rhodin, 1967,1968), and similarities with smooth muscle cells have been described by Buchanan and Wagner (1990).

The vascular wall morphology of rabbit thigh fascia tissue was examined by Rhodin (1967,1968) who determined the following to be characteristics of pericytes:

1. Their perivascular location; most commonly adjacent to vessel walls of venous capillaries, postcapillary-sized venules and collecting venules up to 50 microns in diameter.

2. Pericytes, unlike veil cells, were surrounded by a thin basement membrane which is often absent on the side of the pericyte facing the endothelial cells.

3. They exhibit few intercellular contacts.

4. They had pinocytic vesicles located near or connected with the plasma membrane.

5. Pericytes had less granular endoplasmic reticulum than veil cells.

6. Pericytes had characteristically highly branched cytoplasm with extensions embracing the endothelial tube with frequent contacts without membrane fusion or tight junctions.

Seventy five percent of the pericytic volume of the eel rete mirabile was associated with arterially derived capillaries (Buchanan and Wagner, 1990). They suggested that pericytes were involved in the local control of capillary blood flow due to their alignment along capillaries and their similarities to smooth muscle cells. Histological studies have demonstrated the presence of tropomyosins, isomyosins, cyclic GMP-dependent protein kinase and muscle actins in pericytes (Joyce et al., 1985a,b; Herman and Jacobson, 1988; Buchanan and Wagner, 1990). These components were said to enable pericytes to act in a contractile-like manner and thereby influence local capillary blood flow.

Endothelial cell-pericyte interactions have been stated as playing a role in control of endothelial cell proliferation as well as controlling microvascular blood flow (Chakravarthy et al., 1992).

3.1.4 Pathways Across The Blood Vessel Wall

The microphysiology of transport pathways provided some insight into the permeability characteristics of the PDL vasculature (Castelli and Dempster, 1965; Cooper et al., 1990) in relation to its function in tooth support and its response to orthodontic forces (Clark, 1986; Lew, 1986; Crowe, 1989; Parlange, 1990).A number of pathways existed, such as across the cell or via a number of junctional specializations including close junctions, open junctions and fenestrae.

Several transport pathways across the capillary wall were proposed by Renkin (1977) and are shown in Figure 1. Pathways (1-6) were suggested to occur in all capillary endothelia, whereas pathways (7) and (8) were possible only in fenestrated endothelia.

Pathway (1) represented transendothelial transport and allowed the passage of water, lipid soluble, and some small unipolar solutes across endothelial cells. The processes of active transport and facilitated diffusion were important in this cell membrane dependent form of transport.

Micropinocytic vesicular transport was represented by pathway (2) where endocytosis occured at one cell membrane and exocytosis of the vesicle contents occured at the opposite cell membrane. The contents of the vesicles were considered to be extracellular as they did not mingle with the cell cytoplasmic contents. The rate of such microvesicular transport was dependent upon the turnover rate of the vesicles (Palade and Burns, 1968).

Passage through intercellular junctions was depicted by pathways (3-5). Pathway (3) showed lateral diffusion of water-insoluble lipids in the plasma membrane lining the junction. Pathway (4) represented the passive transport characteristics of the clefts of tight junctional complexes and these have been said to be the structural equivalent of the hypothetical "small pore" system proposed by Pappenheimer (1953), which allowed movement of water and small-to-moderate lipid insoluble molecules. Pathway (5) was thought to correspond to the "large pore" system proposed by Pappenheimer (1953), where fluid and large molecules such as plasma proteins crossed the capillary wall via wider intercellular junctions or transiently open junctions.

Pathway (6) represented transendothelial channels possibly created by fusion of a chain of micropinocytic vesicles. These channels were thought to be transitory (Wolff, 1967) and the areas of fusion between the vesicles were said to be narrow enough to encourage restricted permeability to small lipid-insoluble substances (Simionescu and Simionescu, 1975).

Open fenestrae which allowed large macromolecule transport was illustrated as pathway (7). Renkin (1977) postulated the presence of pathway (8) which was a closed fenestra with a diaphragm which was thought to be a specialized pathway for water-soluble substances, lipid-soluble substances and possibly even large lipid-insoluble molecules.

Fenestrated blood capillaries were suggested to be commonly found in specialized tissues where there was an interstitial accumulation of large molecules requiring removal (Casley-Smith, 1971), and the fraction of open and closed fenestrae varied with the capillary

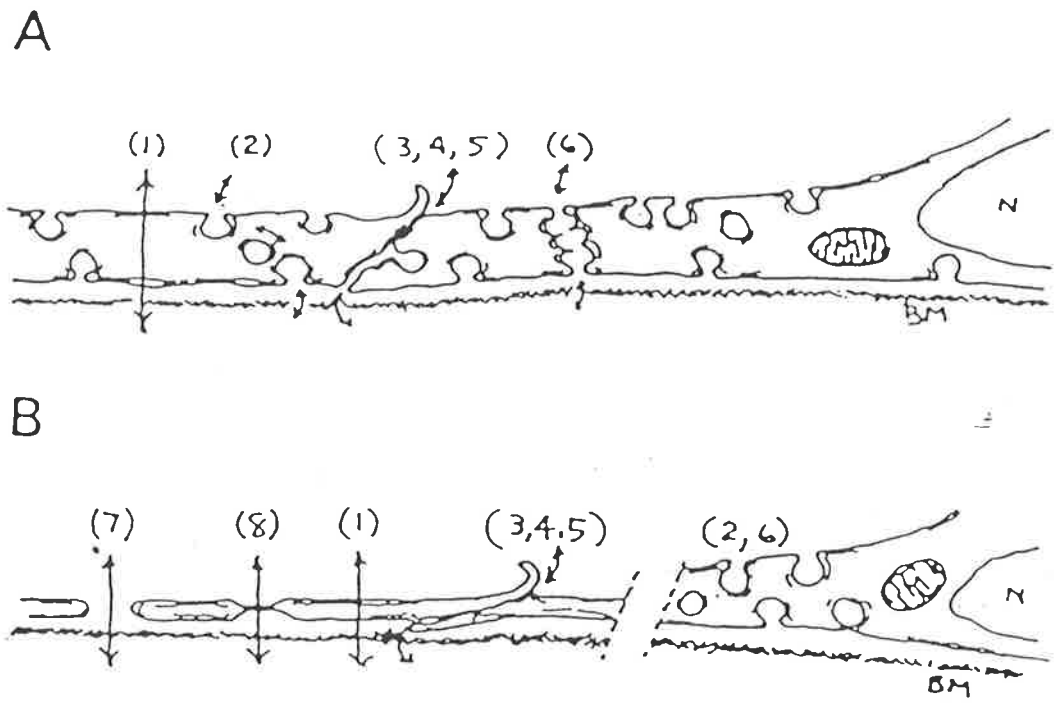


Figure 1. Representation of capillary endothelium transport pathways.
A, continuous capillaries; B, fenestrated capillaries; (1) direct cellular, (2) vesicular, (3) lateral diffusion, (4) narrow "small pore" junctions, (5) "large pore" junctions, (6) transitory open channels, (7) open fenestrae, (8) closed fenestrae. (Renkin, 1977)

bed. The techniques used for specimen preparation also influenced the fraction of open and closed fenestrae (Simionescu and Simionescu, 1984).

Corpron et al. (1976) described mouse PDL microvascular fenestrae as 30-50 micron openings in the endothelium. These authors suggested that a thin, continuous membrane attached to either side of the endothelial cells, was often found bridging the opening. They found the central capillaries in the mouse PDL to be devoid of fenestrae but demonstrated fenestrated capillaries close to the osteoblastic layer.

The vessels having the highest number of fenestrae in mouse PDL were the postcapillary-sized vessels (Sims, 1983). Pudney and Casley-Smith (1970) suggested that the venous ends of capillaries more commonly displayed fenestrae. They studied adrenal cortex during their study. Lew (1986) studied rat molar PDL and supported the claim of Pudney and Casley-Smith (1970) by rarely finding fenestrae in arterial capillaries.

The absolute nature of fenestrae function remains unclear. Casley-Smith et al. (1975) suggested that they were important in the establishment of a local extracellular capillary circulation of macromolecules. Corpron et al. (1976) postulated fenestrae as being involved in a rapid pathway of metabolite exchange across the endothelium to the periodontium where high metabolite requirements for growth or repair existed. If this was true then a knowledge of endothelial junctions may be an important part of understanding orthodontic tooth movement and tooth support. Lew (1986) hypothesised a role for fenestrated capillaries which was almost lymphatic in nature, by suggesting a rapid return of extracellular protein and metabolites to the vascular system. The presence of lymphatics in the PDL has been questionable (Edwall, 1982) although putative identification of lymphatic

vessels has been documented (Ruben et al., 1971; Gilchrist, 1978; Crowe, 1989; Foong, 1994). Casley-Smith (1977) suggested, that on morphological grounds alone, it was impossible to identify a particular vessel beyond doubt. He stated that the microprojection of tracers into the lymphatic system was the only certain way to positively identify lymphatics.

3.2 Endothelial Junctions

In describing the junctional complexes in a variety of epithelia, three components were distinguished by Farquar and Palade (1963):

1. Zonula occludens (tight junctions) were characterized by fusion of opposing cell membranes over a variable distance resulting in obliteration of the intercellular zone. These structures were reported close to the lumen.

2. Zonula adhaerens (intermediate junctions) were said to be involved in cellular adhesion. The cell membranes ran parallel for a length of 0.2 to 0.5 microns with an intercellular distance of approximately 20 nm. A dark strand of dense fibrillar material was noted in association with the cytoplasmic surfaces of each cell membrane.

3. Macula adhaerens (desmosomes) were characterized by dense plaques spaced at regular intervals in a circumferential row parallel to the zonula adhaerens. These maculae were also reported to be randomly distributed on the surfaces of adjoining cells.

Fawcett (1981) classified junctions into three types according to their function and permeability. Occluding junctions (zonula occludens or tight junctions) were implicated in the maintenance of the internal cell environment by preventing the passage of small molecules through the inter-epithelial cell space. Adhering junctions (zonula adhaerens and macula adhaerens or desmosome) were suggested as important sites for attachment of contractile and fibrillar cytoskeletal elements. As such they seemed to be important in maintaining cell cohesion. The gap junctions (communicating junctions) were described as allowing intercellular exchange of small molecules and ions, and hence played a role in coordinating the activities of various groups of cells.

3.2.1 Endothelial Junctions In The Microvasculature

Each element of the microvasculature was suggested as having characteristically organized endothelial junctions which reflected the degree of tightness and intercellular coupling. Simionescu and Simionescu (1984) described each component of the microvasculature and their junctions as follows:

Arterioles: were described as vessels of 30-100 microns in diameter with a continuous layer of smooth muscle cells in their tunica media. A combination of tight and gap junctions were said to be present between endothelial cells, enabling cell to cell communication via gap junctions and maintenance of the internal environment by tight junctions.

Capillaries: were said to be vessels of 5-10 microns diameter whose walls comprised a layer of endothelial cells and a variable number of pericytes. Gap junctions were

said to be absent. Those junctions present consisted of two to three successive rows, either branched and continuous, or staggered and discontinuous, of occluding junctions.

Postcapillary pericytic venules: were stated to be vessels of 10-50 microns in diameter whose walls consisted of a layer of endothelial cells plus an extensive, but not continuous, layer of pericytes. Again, gap junctions were said to be absent.

Muscular venules: vessels with diameters of 50-200 microns and a continuous layer of smooth muscles cells in their tunica media were designated as muscular venules. Junctional organization was said to resemble pericytic venules except that small, irregular gap junctions were described in association with (but not surrounded by) the tight junctions.

3.2.2 Tight Junctions

Tight junctions have been thought to be involved in formation and maintenance of structural, compositional and functional polarity of cells. Also, they were considered to act as a selective permeability barrier in epithelia and endothelia. Ultrastructurally, tight junctions have been said to consist of a series of punctate contacts and fusions of the outer sections of adjacent cell membranes (Staehelin, 1974). Freeze fracture studies by Simionescu et al. (1975a, 1975b, 1976) suggested a belt-like network, of varying complexity, with linearly arranged particles and/or strands.

The number of strands per tight junction was thought be an indicaton of its permeability, with an increased number of strands reducing the junctional permeability. Schneeberger and Lynch (1984) suggested renal proximal tubule (leaky) epithelia contained 2-4 strands per tight junction, whereas urinary bladder (tight) epithelia contained more than 8 strands per tight junction. Von Bulow et al. (1984), however, examined tight junctions in

frog choroid plexus and found no correlation between the number of strands per junction and the transepithelial resistance.

Epithelial and endothelial tight junctions were suggested to be structurally different (Simionescu et al., 1975a, 1975b, 1976). Epithelial tight junctions tended to show interconnected strands while endothelial tight junctions tended to remain particulate regardless of the length of fixation time used during preparation. Additionally, the structure of endothelial tight junctions varied with the segment of the microvasculature examined.

3.2.3 Close Junctions

Close junctions have been described as having a space of approximately 6nm between the adjacent cell membranes. The experimental technique for examination did not appear to alter the recorded intercellular distance as Casley-Smith et al. (1975) and Casley-Smith (1981) used both chemical fixation and freeze fracture techniques.

Close junctions were said to be more frequently found in postcapillary venules than in capillaries (Simionescu et al., 1978). This finding has been refuted (Bundit and Wissig, 1978; Casley-Smith et al. 1979) and the differences ascribed to examination technique. The use of a goniometer stage was said to be necessary for correct identification of close regions in the more convoluted junctions of capillaries.

Certain conditions such as inflammation or raised capillary pressure have been implicated in stretching or shortening close junctions, however, neither the width nor the depth of close and tight junctions were significantly altered by burning or histamine stimulae (Casley-Smith and Window, 1976).

3.2.4 Gap Junctions

"Communicating junction" was the term suggested by Simionescu et al. (1975a, 1978), to describe these junctions which were found in most animal tissue except skeletal muscle, many nerve cells and blood cells (Pitts, 1980). Other names used to describe these junctions include macula communicans, macula occludens or simply a nexus. Gap junctions were said to be important in the intercellular exchange of ions and small metabolites, and in the process of electron coupling.

Tissues which lacked morphologically distinct gap junctions, such as capillaries and pericytic venules, have been shown to still exhibit interendothelial transfer of dye particles. This anomaly was said to suggest that small gap junctions may have been missed during investigation due to technical proceedures, or that the transfer may be via tight junctions (Simionescu and Simionescu, 1984).

The precise function of gap junctions in endothelial cells remains unknown but their appearance has been described as a "spot weld" union of two cells. The inner region being hollowed out so that cytoplasmic processes of each cell could communicate via the gap junction.

Zampighi (1988) described the junctional membranes as containing a series of annular particles, called connexons, which were arranged in a regular hexagonal lattice. These connexons were said to traverse the entire membrane, protrude into the lumen and be coaxially aligned with those on the opposing side of the gap junction. Connexon configuration was variable and thought to be responsible for junctional permeability (Revel et al., 1980).

Intercellular channels have been found to open and close depending upon the prevailing physiological circumstances (Fawcett, 1981). Regenerating cells tended to show a reduction in size and number of gap junctions, while the number and dimensions of tight junctions remained relatively unchanged (Revel et al., 1980).

Oxygen deprivation and aldehyde fixation tended to change the resistance status of gap junctions from low to high. Hence most ultrastructural studies described gap junction appearance in their high resistance state (Casley-Smith, 1981).

3.2.5 Open Junctions

Open junctions were described by Casley-Smith and Window (1976) as having lumens greater than 30nm in diameter and they suggested that open junctions were found in lymphatics, normal sinusoidal endothelium and injured blood vessels. The reaction of junctions to inflammatory stimulae may be immediate (mediated) or delayed (non-mediated, possibly direct). Immediate response was thought to involve opening of endothelial intercellular junctions in capillaries, usually in the postcapillary venules, followed by closure approximately 30 minutes later. Delayed response involved opening of junctions in capillaries and venules. The opening extended for a variable period (Casley-Smith, 1983).

Open junctions allowed extravasation of blood borne molecules, and were considered as also allowing macromolecules to enter the venous circulation where hydrostatic pressure is low. Casley-Smith (1979, 1983) suggested that open venular junctions functioned as venous-limb fenestrae, and were actually involved in removal of a net amount of macromolecules from the tissues.

3.3 Junction Physiology And Ultrastructure

Morphological charcteristics of intercellular clefts or junctions have been described by various authors who have utilized electron microscopy (Casley-Smith et al., 1975; Simionescu and Simionescu, 1975; Bundgaard, 1984) and freeze fracture examination techniques (Casley-Smith, 1981; Okuda and Yamamoto, 1983). These authors described intercellular clefts which showed great variability in morphology. Some junctions were relatively straight in their course from lumen to ablumen, others were more convoluted. Tight, close and gap junctional zones were variable in terms of their presence or absence, and in terms of their position within the junctional complex.

Within intercellular junctions, close zones were considered to represent areas of reduced permeability whereas tight zones were considered to represent impermeable sites. Bundgaard (1984) was able to demonstrate discontinuities in the tight zone structure by serial section electron microscopy, thus demonstrating that junction morphology was more complex than initially suspected.

A series of studies by Curry, Huxley and Adamson (1983), Huxley, Curry and Adamson (1987), and Adamson, Huxley and Curry (1988) investigated the permeability of single capillary endothelial cell junctions to molecules of various sizes and with varying charges. These studies utilized frog mesenteric capillaries and a microscopic photometric technique to show that molecule charge had some influence on a specific molecule's permeability, but molecule size was an even greater determinant of a specific molecule's permeability.

In an attempt to explain the variation in predicted and actual permeability of endothelial cell junctions to various molecules, a pore-matrix model (Weinbaum et al., 1992) and a fiber-matrix model (Schnitzer, 1992; Adamson, 1992) were proposed. These theories are based in complicated mathematics and authors such as Adamson and Michel (1993) have concluded that the ultrafiltration properties of the blood vessel wall could only be accounted for if a sieving matrix lies in series with the discontinuities in the tight zones. They proposed that plasma proteins and luminal endothelial glycocalyx may form the sieving matrix.

The surface of cells is composed of charge-bearing glycoproteins and glycosaminoglycans. It is well documented that plasma proteins modify microvascular permeability by reducing the hydraulic conductance of the vessel wall, and increasing its selectivity to other macromolecules (Adamson and Clough, 1992).

Despite the many different animal models utilized and differing methodology and examination techniques utilized, it may be concluded that intercellular junction structure and physiology is complex and still poorly understood.

3.4 Fluid And Macromolecule Movement During Tooth Movement

Force induced orthodontic tooth movement involves a chain of events which begins immediately upon application of a force. These events have been extremely well described by Davidovitch (1991) and Sandy (1992).

Davidovitch (1991) proposed a sequence of events beginning with fluid movement within the PDL which was suggested to initiate streaming potentials within the PDL. Streaming potentials affect the osmotic conditions around PDL and alveolar bone cells and lead to alteration in cellular shape, cytoskeletal configuration and ion channel permeability. Gradual distortion of PDL cells and matrix are also thought to lead to similar alterations in cell shape, cytoskeletal configuration and ion channel permeability. Orthodontic force application may also initiate bending of alveolar bone which produces piezoelectric effects which alter the electrical environment around PDL cells. Again, alterations in cellular shape, cytoskeletal configuration and ion channel permeability would follow.

Alternatively, distortion of PDL cells and matrix may lead to release of neuropeptides such as substance P and vasoactive intestinal peptide from PDL afferent nerve endings. These neuropeptides are known to stimulate capillary vasodilatation, migration of leukocytes into extravascular areas, as well as production and release of cytokines, growth factors, and prostaglandins. Such agents also alter the local osmotic conditions and some bind directly to cell membranes producing intracellular changes leading to further alteration of cellular shape, cytoskeletal configuration and ion channel permeability. Thus, on the biomechanical level, mechanical force application to a tooth can result in simultaneous exposure of PDL cell to signals from the nervous, immune, and endocrine systems, leading to intricate interactions, many of which involve the PDL blood vessels.

Although not directly mentioned by Davidovitch (1991), endothelial cell junction permeability must be altered by the chain of events described above. Any alteration in endothelial cell ionic conditions, osmotic conditions, or shape changes initiated by streaming

potentials or direct distortion of cells will alter junctional morphology. As the structure of tight junction zones is suggested to involve strands of cytoskeletal elements, any event which leads to an alteration in cytoskeletal configuration may alter tight junction zone and, therefore, total blood vessel permeability.

As indicated above, any change subsequent to orthodontic force application may alter endothelial cell junction morphology and permeability. Hence junction physiology and morphology is likely to be extremely important in homeostasis and orthodontic tooth movement.

3.5 Permeability Calculation Methods

Casley Smith et al. (1975) utilized Poiseuille's equation for flow between parallel plates to calculate capillary filtration coefficients for the dog skeletal muscle endothelial cell junctions used in their study. The equation utilized was:

$CFC = K/d/(w^3L)$, where CFC = capillary filtration coefficient

d = junction depth

w = junction width

L = junction length

K = constant

As stated above, Poiseuille's equation relates to flow between parallel plates. The assumption that flow within endothelial cell junctions was as between parallel plates was made by Casley-Smith et al. (1975). Their experimental design did not utilize specific tracer

molecules, so calculations were confined to values which would give an indication of junction permeability rather than directly calculating permeability for specific tracer molecules.

Utilizing bovine endothelial cell monolayers cultured in vivo, Oliver (1990) calculated junction permeability for a range of molecules of known size and charge. The tracer molecules were added to one side of the cell monolayers, and at various time intervals samples of fluid from the alternate side of the cell monolayer were analysed for the tracer molecule. The experimental design ensured that no hydrostatic pressure operated across the cell monolayer, and the author suggested that endothelial cell junction permeability alone was responsible for transport of the tracer molecule across the cell monolayer. This may not be true as transcellular transport, via vesicles or vacuoles, may be a major transport mechanism (Wagner and Chen, 1991; Predescu and Palade, 1993).

For the experimental procedure utilized by Oliver (1990), junction permeability was calculated using the following formula:

$P = J/\triangleright cpm$, where P = permeability

J = cpm/time

$\triangleright$cpm = difference in cpm across the cell monoloyer.

This equation was specific for each tracer molecule used rather than a theoretical calculation such as that used by Casley-Smith et al. (1975).

Schaeffer et al. (1993) also utilized cell monolayers to calculate endothelial solute permeability. The equation they used to calculate permeability was similar to that used by

Oliver (1990), but expressed in a slightly different manner. Schaeffer et al. (1993) utilized the equation:

$P = Js/S(C1-C2)$ where, P = solute permeability coefficient

Js = flux across the cell monolayer

S = a constant for each specific tracer

C1-C2 = solute concentration gradient

Again, these calculations were specific for each different tracer molecule being investigated.

Adamson and Michel (1993) were able to identify and directly cannulate mesenteric capillaries in frogs which had been pithed. This experimental procedure allowed the authors to directly measure hydraulic permeability within the cannulated capillaries. Unfortunately, this hydraulic permeability (Lp) was not a solute permeability coefficient (P), and so was only another indicator of permeability under the prevailing experimental conditions.

CHAPTER 4

MATERIALS AND METHODS

4.1 Summary Of Material Preparation

The material used in the present study was sourced from ten marmosets aged between sixteen and thirty-eight months originally prepared by Weir (1990). Seven of the experimental group were male and three were female. Under Saffan anaesthetic (Appendix 1), the upper left central incisor was decoronated, a vital pulpectomy performed and root canal filling placed using gutta percha and AH 26 cement.

Previously constructed cobalt-chromium (Rexillium, Generic Gold Co., Division of Generic Industries, Wallingford, Conneticutt, U.S.A.) splints were then cemented to the upper canines, lateral incisors and the upper right central incisor using Comspan (L.D. Caulk Division, Dentsply International, Milford, Delaware, U.S.A.). The decoronated upper left central incisor was then extruded in two stages utilizing Cobalt-Samarium magnets normally utilized as quartz digital watch rotors (Seiko Product No. 4146480) until 1.2 mm of extrusion had occurred. This amount of extrusion was considered to be proportionally equivalent to the amount of extrusion used in humans who require extrusion of a central incisor following trauma. Once the desired amount of extrusion had been achieved, the magnets were used to retain the extruded tooth in its new position for a period of thirty weeks.

The final group utilized for the Weir (1990), the subsequent study by Chintavalakorn (1994), and the present study consisted of four male marmosets. One sex of marmoset was used to reduce any possible hormonal influence. The other final selection criteria were uninterupted retention of the splint device, adequate perfusion at the time of sacrifice, a clinical assessment of healthy periodontal tissues prior to sacrifice, and adequacy of the root canal filling as assessed by radiographic examination.

Following intramuscular administration of Saffan anaesthesia (Appendix 1), Heparin (1000 units/ml., Commonwealth Serum Laboratories) was administered, using a 0.1 ml insulin syringe directly into the surgically exposed femoral vein, to prevent blood clotting during carotid perfusion with 5.6% glutaraldehyde and 0.9% osmium tetroxide in 0.06M cacodylate buffer (Appendix 2) via the carotid arteries.

Immediately following adequate perfusion the animals were decapitated, the maxillae mesial to the first premolars were dissected free, and the resulting tissue demineralized in 0.1M EDTA solution (Appendix 3). Adequate perfusion was obtained when the animal's jaws were locked together, the body was rigid, and the black colour of the osmium tetroxide was noted in the facial and mucosal arteries. Demineralization in EDTA was accompanied by constant agitation using a magnetic stirrer. Decalcification was deemed to be complete when probing in an unwanted area and radiographic investigation revealed no calcified tissue. Blocks of tissue containing the experimental upper left central incisor and the control upper right central incisor teeth were cut from the larger tissue sample and the apical regions, defined as 0.5mm coronal to the visible apex of each tooth, were removed from the remaining length of the specimens of tooth.

The non-apical regions produced by Weir (1990) were utilized by Chintavalakorn (1994) for stereological evaluation of the neural and vascular components of the non-apical regions. The sections of these non-apical regions produced by Chintavalakorn (1994) were re-examined for the present study. The sections were cut from the mesial aspect of the tooth root (Figure 2).

Ultrathin specimens of the marmoset PDL were prepared at each 150 micron level of PDL using a Reichart-Jung Om-U4 ultramicrotome and a Diatome diamond knife. The clearance angle of the knife was ten degrees and the cutting speed was 1mm per second. The ultrathin sections were floated onto a bath of millipored double distilled water, flattened with chlorofrom vapour and placed onto clean, uncoated R150A mesh multislotted copper grids. Grids thus produced were dried face upwards on Whatman's grade one filter paper in a covered Petri dish.

Zero level was determined as being that level when alveolar bone was first noted in the sections. Following the establishment of the zero level, sections were prepared at intervals of 150 microns until the most apical area was encountered (Figure 2). The number of levels achieved ranged from seven to twelve with the majority of specimens producing eight levels.

Dried grids with complete sections were selected and stained in preparation for viewing with the transmission electron microscope. Grids were placed tissue side down on droplets of 0.5% uranyl acetate (Appendix 4) for twelve minutes and were maintained at a temperature of thirty seven degrees Celsius. Grids were then rinsed for fifteen seconds in each of four beakers containing one hundred millilitres of double distilled water also at thirty

seven degrees Celcius. Four minutes of floating each grid, tissue side down, on a droplet of freshly prepared modified Reynolds' lead acetate (Appendix 4) completed the staining process. A container of sodium hydroxide was enclosed with the staining grids and Reynolds' lead in the covered Petri dish to absorb carbon dioxide and reduce the formation of lead carbonate precipitate on the tissue sections. The rinse cycle was again followed as above and the stained grids were left to dry, tissue face upwards, on fine grade filter paper in a covered Petri dish.

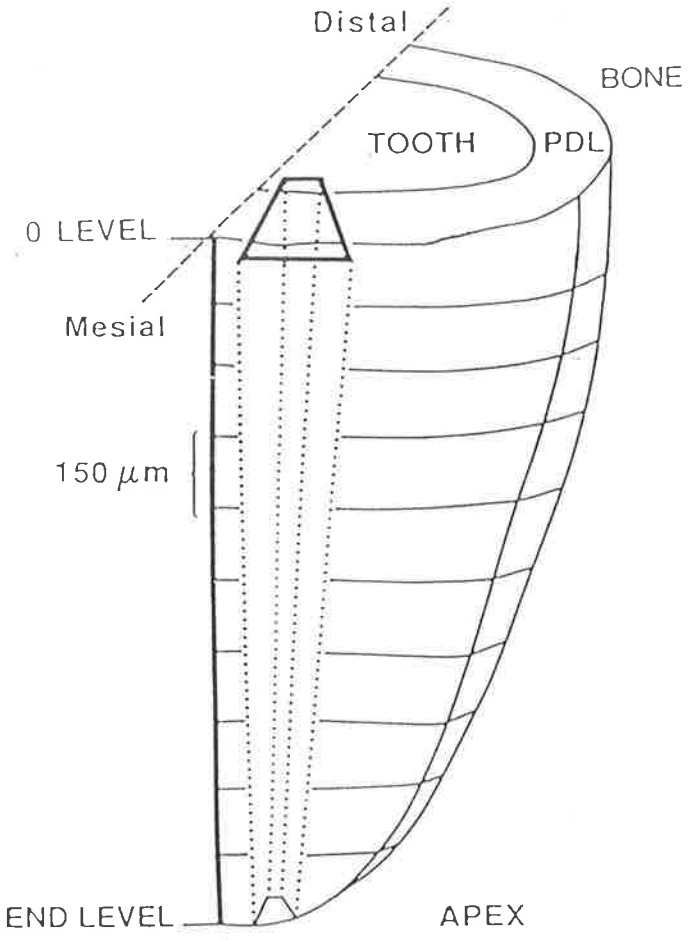


Figure 2. Diagram illustrating the sectioning orientation and region of the PDL examined. Ultrathin sections were collected at 150 micron intervals.

4.2 Transmission Electron Microscopy

Blood vessel classification was based on luminal diameter as well as the structure of the blood vessel walls according to the criteria suggested by Clark (1986) , namely:

1. arteriovenous anastomoses and terminal arterioles
2. arterial capillary
3. venous capillary - pericytic
4. venous capillary - non-pericytic
5. postcapillary-sized venule - pericytic
6. postcapillary-sized venule - non-pericytic
7. collecting venule

The narrowest diameters of the blood vessels were used for classification. Blood vessels were not classified as being situated in tooth, middle or bone thirds of the PDL as the sections were not clearly labeled in the storage facility following the Chintavalakorn (1994) project.

Each grid was examined in a Phillips CM 100 TEM using an accelerating voltage of 80 kV. No attempt was made to orient the grids prior to placement in the TEM specimen chamber. Beginning at the top left hand corner of each grid and examining across the grid from left to right and in the reverse direction for the adjacent row below, until blood vessels that were totally visible within the grid square and with complete junctions, were identified. A total of five postcapillary-sized venules with complete junctions were photographed per level for the control and experimental tissue. A complete junction was defined as one which

exhibited a distinct luminal and abluminal entrance/exit, and one which could be followed for its entire length. The number of junctions sampled per grid varied between one and five. If five different blood vessels each exhibiting one satisfactory junction could be photographed from the one grid, this was performed. Subsequent grids from the same PDL level were examined until the total number of junctions photographed was five. On several occasions it was necessary to examine up to four grids to achieve the desired sample of junctions per level.

Sampling of the blood vessels, and therefore the endothelial cell junctions, was random across the periodontal ligament as no attempt was made to select blood vessels other than as described above.

Electron micrographs of blood vessels were taken at a magnification of 900 x to permit classification of the blood vessel, measurement of its minimum luminal diameter, and to act as orientation when determining the luminal and abluminal extents of each junction. These low magnification photomicrographs also ensured sampling of different blood vessels at each level by enabling comparison of the features visible in the field of view. An image wobbler was used to assist in focussing at the lower magnification. Utilising the goniometer, complete junctions were viewed at a magnification of 73,000 x, at zero tilt and at various tilts (>15°) in either a clockwise or anti-clockwise direction around the axes of the specimen holder. This method was used to assess the presence or absence of space between the closely opposed external laminae of the plasma membranes and, therefore, classification of the junction as tight (no opening between external laminae) or close (opening between

external laminae). Further electron micrographs at 73,000 x, i.e., at zero and desired goniometer tilt angles were exposed.

Ilford Electron Microscope Film was used for micrographs, with an exposure time of 2.2 seconds and an automatic aperture opening. A micrograph of a replicating graticule (E.F. Fullam Inc., Schendectady, New York, 2160 lines/mm) was exposed at both the low and high magnifications at each viewing session to enable standardisation of magnification.

4.3 Developing And Printing

Electron micrographs were developed for four minutes at 20°C, using Kodak D19 developer, rinsed in deionised water for one minute and fixed for a minimum of five minutes in Ilford Hypam Rapid Fixer at 20°C. The micrographs were then rinsed for a further 15 minutes and air dried for two hours.

Using a Durst Laborator 54 enlarger, the negatives were printed on multigrade Ilfospeed photographic paper, using a grade 5 filter to obtain adequate contrast. The prints were developed for one minute using Ilfospeed paper developer and fixed for five minutes with Ilford Hypam Rapid Fixer, followed by rinsing and drying. Prints were enlarged to 146,000 x for measurement of junction dimensions.

4.4 Morphometry Of Junctional Dimensions

The junctional dimension assessments were completed on the enlarged printed electron micrographs, using the image analysis system, MOP-3 (Carl Zeiss Inc., Oberkorchen, West Germany) for the length measurements, and digital calipers for the

remaining measurements. Each measurement was repeated three times and the average used. The terminology for the dimensions of complete junctions used in the present study, were as follows (Casley-Smith, 1983):-

- Width : The distance between one endothelial cell and the next, that is, between the two external laminae of the two plasma membranes.
- Depth : The distance between the luminal and abluminal surfaces.
- Length : The length of the junction around the cell in the plane of the section.

Postcapillary-sized venules were recorded as either pericytic or apericytic depending upon the presence or absence of pericytes in the abluminal interstitium. Minimum intra-luminal diameter was measured for each blood vessel. This diameter was chosen to be consistant with stereological principles. Also, it could be more reliably determined and did not involve measurement of the variable postcapillary-sized venule wall thickness.

4.4.1 Length

The first length measurement, designated as L1, was recorded between the sites of measuring widths W1 and W6. The second length measurement, designated as L2, was recorded between the sites of measuring W2 and W5. Figure 3 illustrates these defined points.

4.4.2 Depth

The depth of the junctions was measured as the distance from the luminal cell membrane to the abluminal cell membrane across the junction. The depth was measured

perpendicular to the luminal cell membrane through a point mid-way along the length of the junction (Figure 3).

4.4.3 Width

The width measurements for each junction were divided into six components as shown in Figure 3:

1. W1: the width of the junction measured perpendicular to the line of the junction from the point of maximum convexity of the smallest junction flap at the luminal end.
2. W2: the width of the junction measured at the site where the plasma membranes at the luminal end first become parallel.
3. W3: the width of the junction measured at a point one third along the junction length as measured between W2 and W5.
4. W4: the width of the junction measured at a point two thirds along the junction length.
5. W5: the width of the junction measured at the last site where the plasma membranes at the abluminal end are parallel.
6. W6: the width of the junction measured perpendicular to the line of the junction from the point of maximum convexity of the most luminal reflection of the plasma membranes.

4.4.4 Meander

To ascertain whether there was a relationship between postcapillary-sized venule depth and junction length, the variable termed meander was calculated for each junction. Meander was defined as the ratio of junction length to junction depth. Two values for meander, designated as M1 and M2 were determined per junction due to the measurement of two junction lengths (L1 and L2) per junction. M1 was defined as L1/D and M2 was defined as L2/D.

4.4.5 Junction Types

Junctions were classified as close if one or more close zones only were evident in any part of that junction. A close zone was defined as a segment of a junction where the adjacent cell plasma membranes were parallel, but at an intercellular distance which was reduced when compared with the majority of the junction. Tight junctions exhibited at least one tight zone, and any number of close zones, in any part of that junction. A tight zone was defined as an area where the adjacent cell membranes appeared to fuse. Junctions with no close or tight zones were defined as no tight/close junctions.

Tight and close zones along the junction length were classified as occurring in one of three regions:

1. The luminal third being between the sites of measuring W2 and W3.
2. The middle third being between the sites of measuring W3 and W4.
3. The abluminal third being between the sites of measuring W4 and W5.

The number of tight and/or close zones, and the total length of tight and/or close zones was determined for each of the three junction regions. Width measurements of the close zones were also performed.

Junctions were also divided into those which were straight along their course, or convoluted along their course. Convoluted junctions were described as those whose direction changed by one hundred and eighty degrees, or more, at least once along their length.

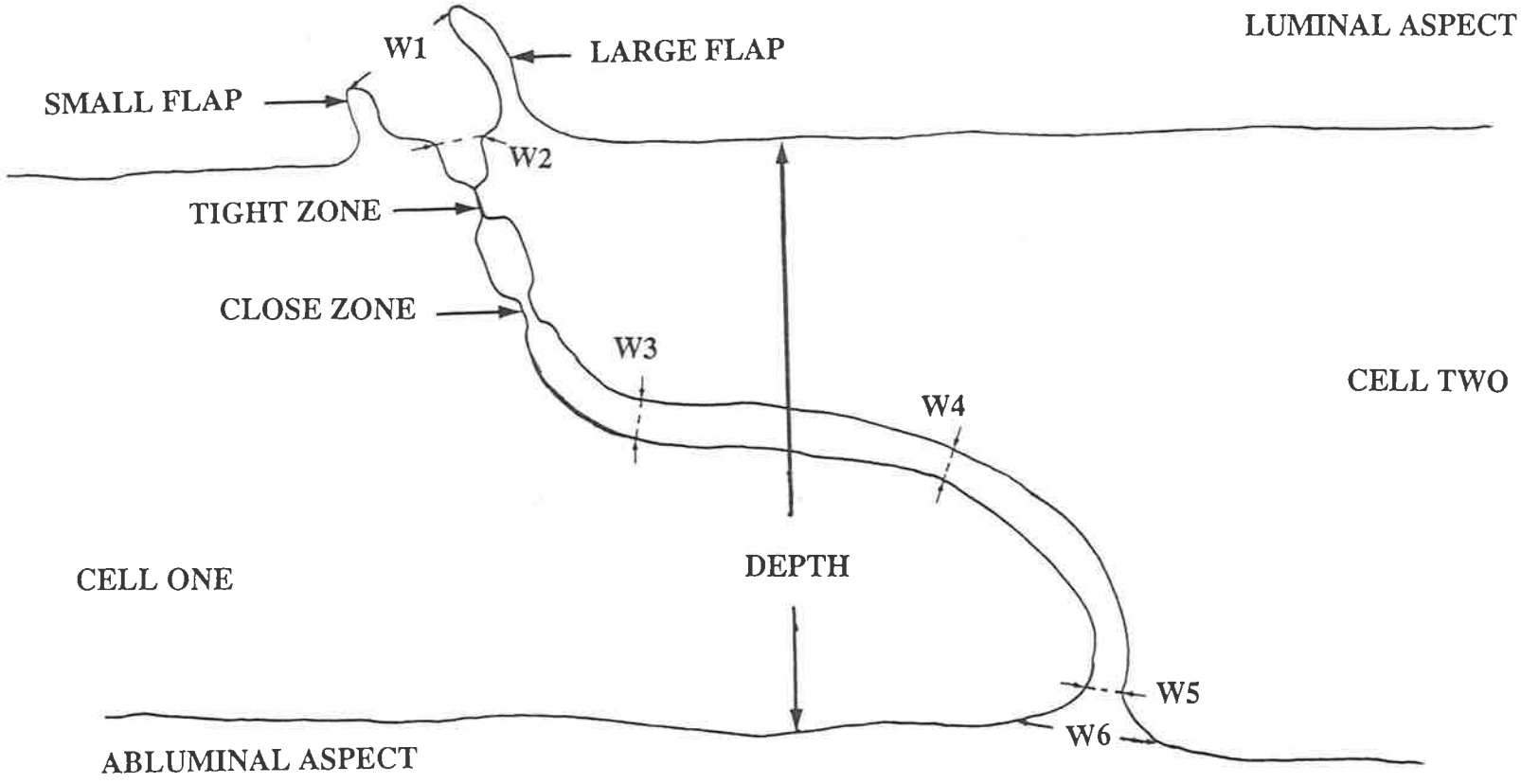
4.4.6 Junction Zone Size

Junction zone size was defined as the length, in nm, of any tight or close zones noted in each endothelial cell junction. For tight zones the junction zone size was measured as the

length over which the adjacent cell membranes appeared to be fused. For close zones the junction zone size was measured as the length over which the adjacent cell membranes displayed parallelism at a reduced intercellular distance (Figure 3).

4.4.7 Junction Flaps

An assessment of the size, number and frequency of junction flaps was also performed. The luminal end of each junction was described as having either zero, one or two flaps and the flaps were either large or small. A large junction flap was deemed to project at least 50 microns into the vessel lumen.



LEGEND

- L1 measured from W1 - W6
- Region 1 = luminal 1/3 = W2 - W3
- Region 3 = abluminal 1/3 = W4 - W5
- L2 measured from W2 - W5
- Region 2 = middle 1/3 = W3 - W4
- Tight and close zones are depicted
- Depth (D) measured perpendicular to luminal surface through point midway between W3 and W4

Figure 3. Representation of definitions for measuring junction lengths, depth, and widths.

An approximation had to be used for the segments between W1-W2 and W5-W6 due to the lack of parallelism of the plasmalemma membranes of these segments. The width used in these segments was the average of W1 and W2 or W5 and W6 respectively.

The present study was not designed to calculate stereologically the length density (L_v), or the length in the plane of the endothelium, as required by the above equation. Chintavalakorn (1994) utilized the same material and calculated the necessary length, so that value was used. Chintavalakorn (1994) calculated L_v for postcapillary-sized venules as 30.97×10^3 cm/cm^3.

For each junction, the total CFC was calculated by adding the CFC's for each of its segments. These total CFC values were used to calculate mean CFC per level.

4.6 Statistical Analysis

For each variable, the arithmetic mean and standard deviations for each animal was obtained to assess inter-animal variability.

For the variables, L1, L2, W1-W6, BVD or blood vessel diameter, D or junction depth, M1, M2, junction size (total length) and CFC it was determined whether their means were significantly different down the PDL levels and between control and experimental groups.

Initially data was tabulated to give arithmetic means with their respective standard deviations and classification by treatment and PDL level. Where junction morphology was

found to be ambiguous, in that the abluminal end could not be clearly determined, the junctions were eliminated from the study. The arithmetic means did not account for the variation in the number of measurements used to calculate the means at each PDL level. Consequently, the means for each PDL level and group were estimated using an analysis of variance, based on the means of the measurements taken at each PDL level.

Associations between the continuous variables were investigated using correlation analyses and these associations were tested to see if they were significantly different from zero. The effects of animal, PDL level and treatment were not considered in the correlation analysis because they were not continuous variables.

Mean blood vessel diameters for apericytic and pericytic blood vessel types were tested for significant differences using a t-test. Mean CFC for close, no tight/close and tight junctions were also tested for differences using a t-test.

Chi-square analyses were used to determine whether the proportion of junction types sampled were the same for the presence and absence of flaps, whether the proportion of junction types sampled were the same for large and small flaps, and whether the proportion of junction types sampled were the same for apericytic and pericytic blood vessel types.

For the junction types close, no tight/close and tight, it was determined by analysis of variance whether the mean numbers in each category for the control and experimental treatments were significantly different down the PDL levels.

For the junction types close and tight it was also determined, by analysis of variance, whether the mean numbers and the mean junction zone size in each category for the control and experimental groups differed significantly between the junction regional locations of

luminal, middle, and abluminal thirds. That is, did the control and experimental groups differ significantly for mean number and mean junction zone size when the junctions were located in the luminal, middle, and abluminal thirds.

Statistical analysis was completed using Genstat™ 5 Release 3 (AFRC Institute of Arable Crops Research, Harpenden, UK) and was performed at the Biometrics Unit of the CSIRO, Waite Campus, The University of Adelaide.

4.7 Error Study

To determine the error of the method, negatives of thirty randomly selected junctions were reprinted and all of the measurements and classification of junction types, eg. close or tight, were repeated. Once the repeat measurements and classification of junction type were completed, the first and second measurements of length, width and depth were compared for any statistically significant differences. If the data were normally distributed, a paired t-test was used for comparison. Where data was not normally distributed, a Wilcoxon signed rank nonparametric test was used.

For the parameters of length, width and depth, no statistically significant differences were found between the first and second measurements. The coefficient of variability for all of the parameters was less than five percent.

To compare the first and second assessments of junction classification, a kappa coefficient (Cohen, 1968) was calculated to determine the degree of agreement between the first and second assessments. The kappa coefficient was found to be 0.8 indicating:

1. good correlation between the first and second assessments of junction classification
2. no statistically significant differences between the two classifications.

CHAPTER 5

RESULTS

5.1 Blood Vessel Type

Postcapillary-sized venules of two types were identified and examined in this study; pericytic and apericytic (Figures 4 and 5).

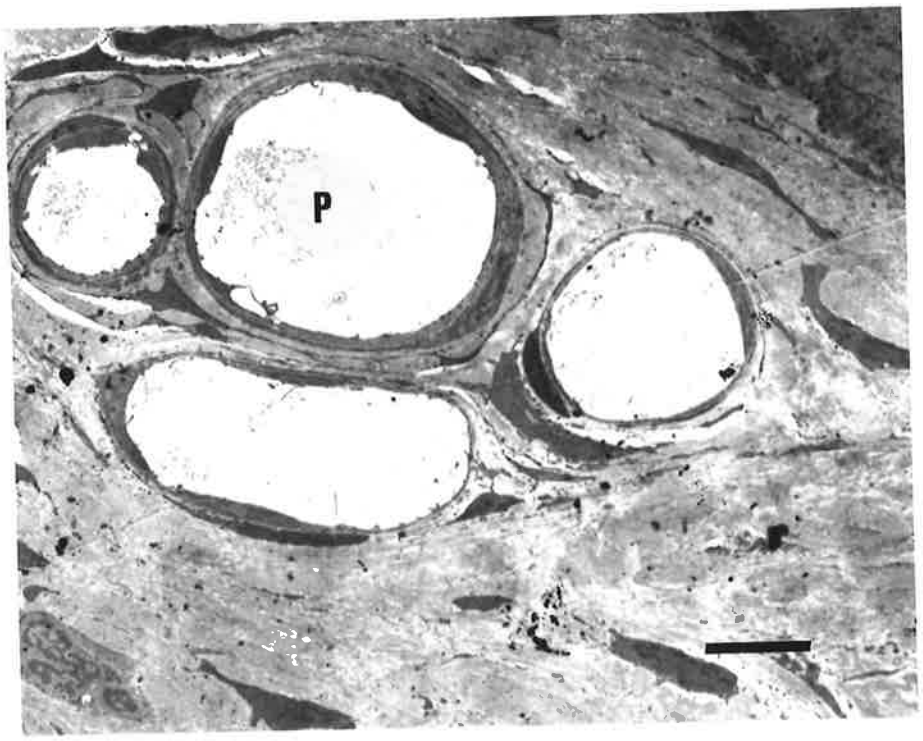


Figure 4. Pericytic postcapillary-sized venule (P) with pericyte shown by arrow.
Uranyl acetate and Reynolds' lead stain.
Original magnification = x 900
Bar = 10μm

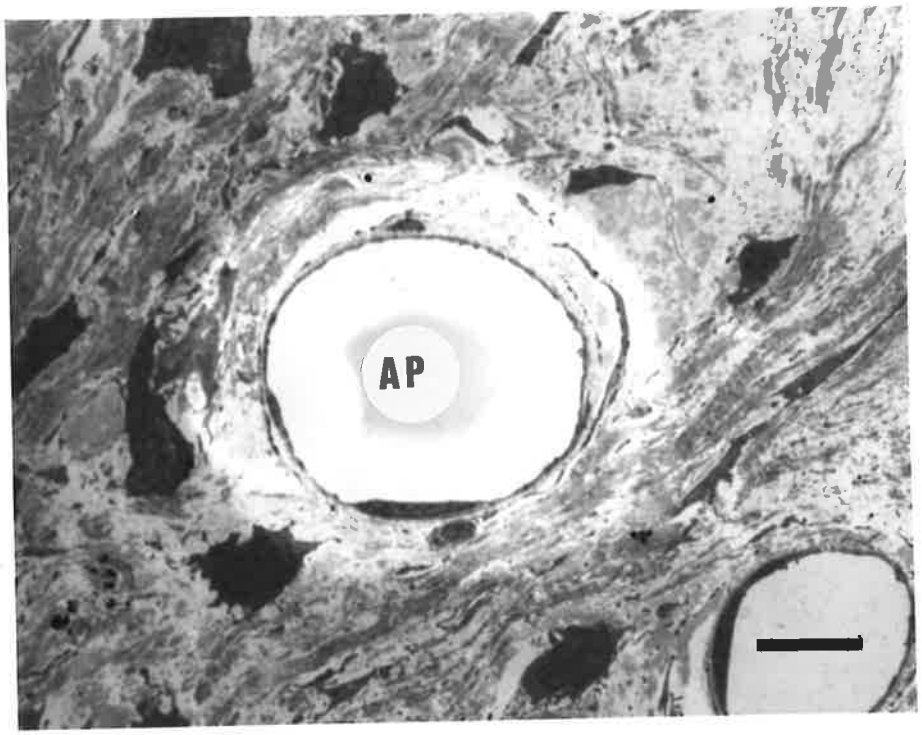


Figure 5. Apericytic postcapillary-sized venule (AP).
Uranyl acetate and Reynolds' lead stain.
Original magnification = x 900
Bar = 10 μm

The analysis of variance showed that there were no significant differences between the mean number of apericytic blood vessels for the control and experimental groups ($p=0.135$), or down the PDL levels ($p=0.260$). Thus, the mean number of apericytic blood vessel types did not vary with the treatment or PDL level.

Differences in the mean number of pericytic blood vessels among the PDL levels indicated that they were dependent on the treatment ($p=0.015$). That is, there was a significant treatment by PDL level interaction. This was attributed to the control group, where the mean number of pericytic blood vessels was significantly higher in the > 900μm PDL level than in the other PDL levels. The control group had a greater mean number of pericytic blood vessels than the experimental group at the >900 μm level ($p=0.05$). The interaction effect and the relationships highlighted above are illustrated in Figure 6.

In total, apericytic blood vessels were more common than pericytic blood vessels in both control and experimental treatments at all PDL levels ($p=0.04$) as shown by Table 2.

Table 2. Total number of apericytic and pericytic PCV at each PDL level for control and experimental groups.

	Apericytic				Pericytic			
PDL Level	Control	Range	Exp.	Range	Control	Range	Exp.	Range
0	12	2-4	11	2-3	6	1-2	7	1-2
150	11	2-4	13	3-4	9	1-3	7	1-2
300	10	2-3	10	2-3	8	2-2	10	2-3
450	13	2-4	8	1-4	7	1-3	4	0-2
600	13	2-4	8	0-3	6	1-3	6	0-3
750	10	2-3	15	3-4	9	1-3	4	1-1
900	12	2-4	8	0-4	8	1-3	11	1-5
> 900	25	1-10	12	0-9	22	2-8	7	0-5
Total	106		85		75		56	

- range from the smallest to largest number of junctions found in all PCV measured at each PDL level

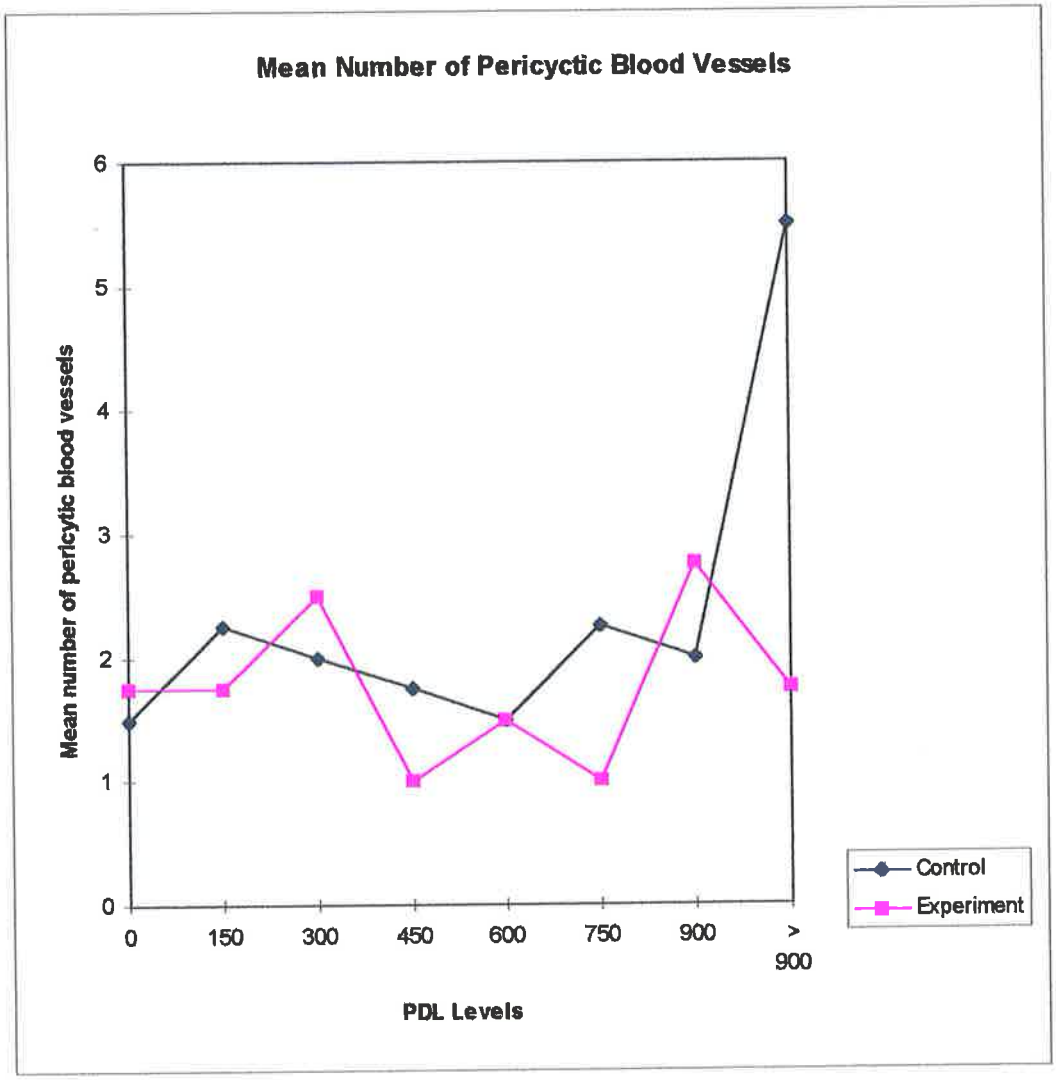


Figure 6. Plot of mean number of pericytic blood vessels per PDL level for the control and experimental groups.

A chi-square analysis indicated that the proportion of junction types (close, no tight/close, tight) sampled were the same for apericytic and pericytic blood vessel types (Chi-square=2.09, p=0.15). The number of junction types for apericytic and pericytic blood vessel types is given in Table 3.

Table 3. The number of junction types for apericytic and pericytic blood vessel types.

	Close	No Close/Tight	Tight	TOTAL
Apericytic	58	40	93	191
Pericytic	47	20	64	131
TOTAL	105	60	157	322

5.2 Blood Vessel Diameter

The mean blood vessel diameter for apericytic blood vessel profiles (Table 4) was significantly smaller than the mean blood vessel diameter for pericytic blood vessel types ($p<0.05$).

Table 4. Mean blood vessel diameter for apericytic and pericytic blood vessel types.

Blood Vessel Type	Mean μm	Standard Error of Differences
Apericytic	15.88	0.44
Pericytic	16.93	0.44

Treatment had a significant effect on PCV blood vessel diameter (BVD) ($p<0.001$), with the control group having a larger mean BVD than the experimental group at each PDL level. There were no significant differences down the PDL levels ($p=0.364$). Therefore, mean BVD changed with treatment, but not down PDL levels (Table 5).

The arithmetic and predicted means for BVD at each PDL level for each group are shown in Table 5. Only the predicted means differed significantly. The predicted means are shown graphically in Figure 7.

Table 5. Mean blood vessel diameter (BVD in μm) of all apericytic and all pericytic PCV for control and experimental animals at each PDL level.

PDL Level	Control		Experiment	
0	15.1† (4.3)	15.1‡	14.0† (2.9)	13.9‡
150	17.8 (3.6)	17.8	14.1 (4.6)	14.1
300	17.4 (5.0)	17.4	14.2 (2.3)	14.2
450	19.7 (3.2)	19.7	13.2 (3.0)	14.2
600	16.4 (3.8)	16.6	15.3 (4.3)	15.5
750	18.0 (2.4)	18.0	15.8 (3.5)	15.9
900	16.8 (4.0)	16.8	17.0 (4.0)	16.8
> 900	17.1 (3.3)	17.6	16.3 (3.1)	16.9
Mean	17.3	17.4***	15.1	15.2***

*** Indicates significant differences between the control and experimental groups for mean blood vessel diameter ($p<0.001$).
† Arithmetic means with standard error values in parentheses.
‡ Mean values calculated using analysis of variance. They were adjusted because of different numbers of values at each PDL level.

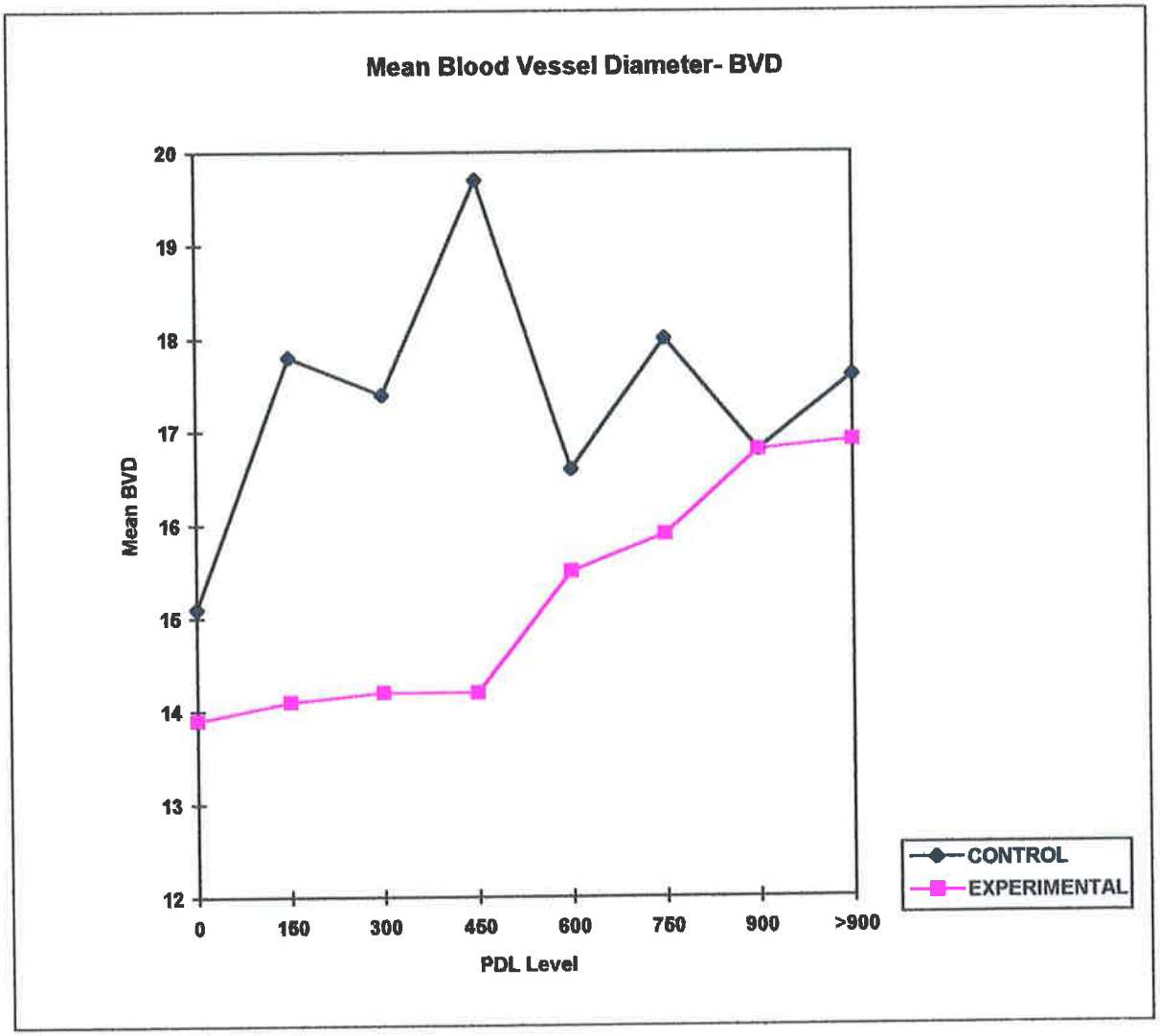


Figure 7. Plot of mean blood vessel diameter of all pericytic and all apericytic PCV for control and experimental groups at each PDL level.

5.3 Junction Type

Three types of endothelial cell junctions were found. Tight junctions which contained at least one tight zone and any number of close zones, close junctions which contained close zones only, and junctions containing no close or tight zones. No gap or open junctions were located.

Junctions classified as tight, close, and one with no tight or close zones are illustrated in Figures 8, 9 and 10.

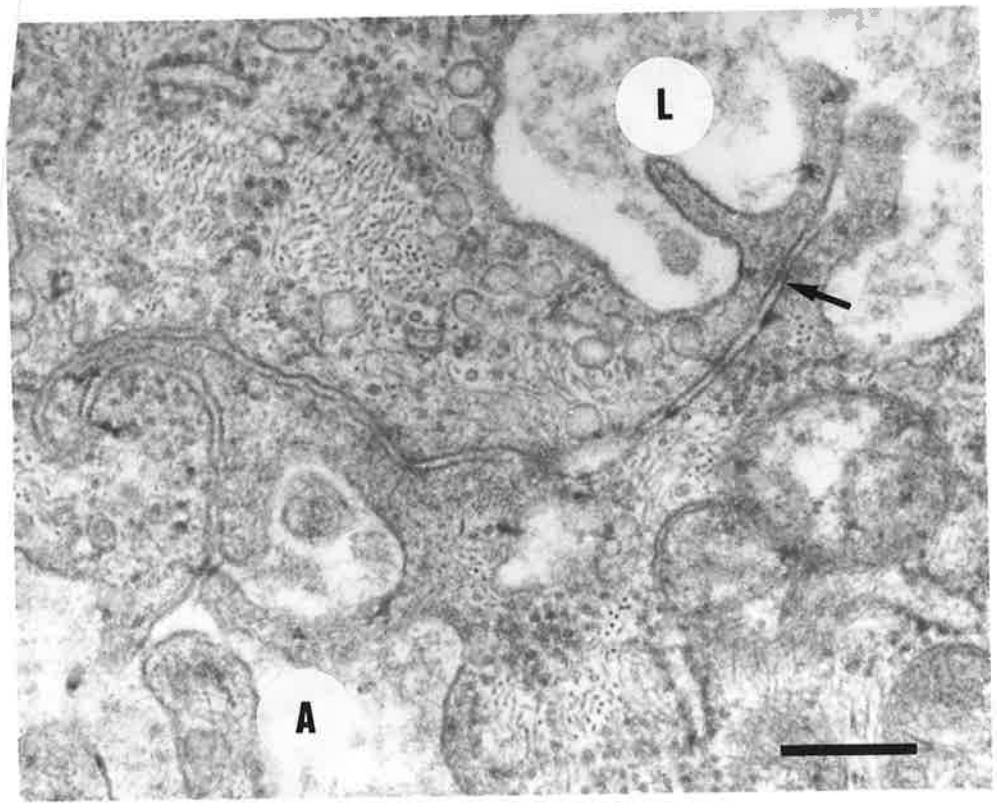


Figure 8. Tight junction with tight zone arrowed. L = luminal side. A = Abluminal side.
Uranyl acetate and Reynolds' lead stain.
Original magnification = x 73,000
Bar = 300 nm

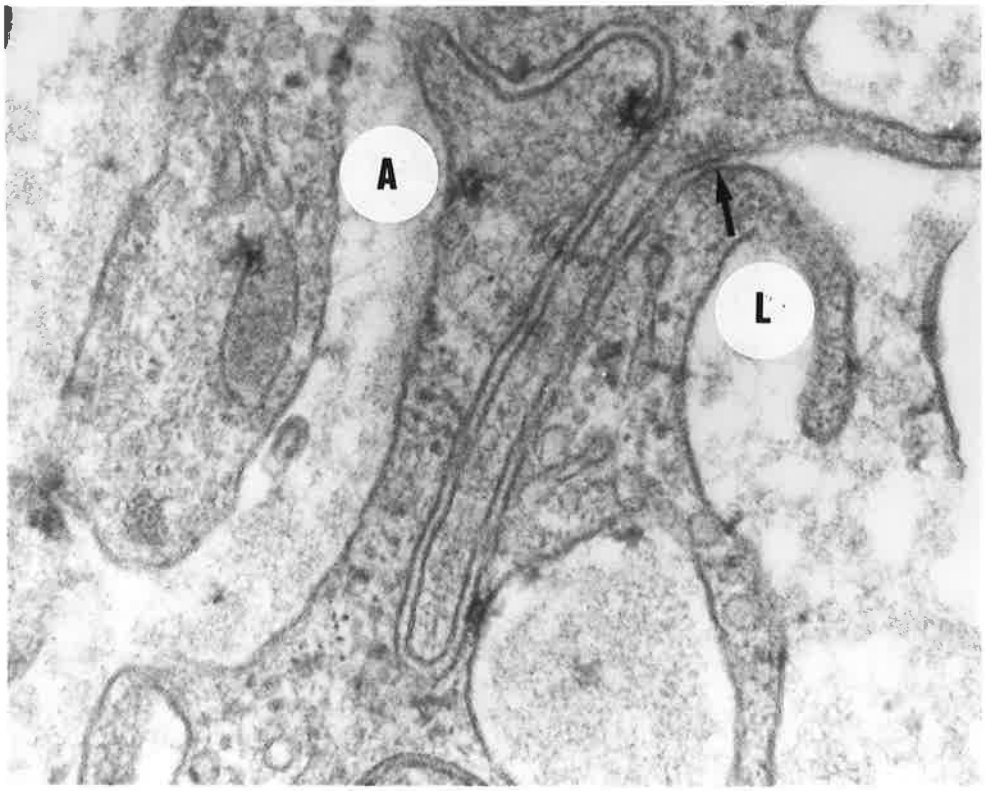


Figure 9. Close junction with close zone indicated by arrow.
L = luminal side. A = abluminal side.
Uranyl acetate and Reynolds' lead stain.
Original magnification = x 73,000
Bar = 300 nm.

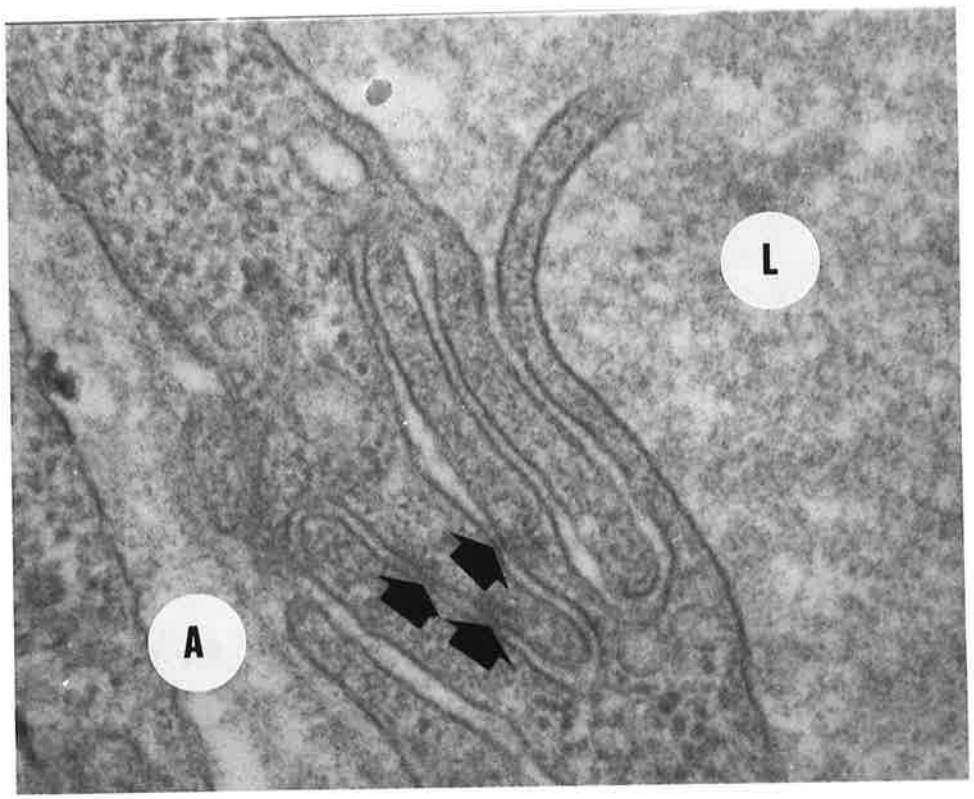


Figure 10. Junction with no tight or close zones. The arrows indicate what appear to be close zones. These were found not to be close when tilted with the goniometer and patency of the second convolution from the lumen was confirmed. L = luminal side. A = abluminal side.
Uranyl acetate and Reynolds' lead stain.
Original magnification = x 73,000
Bar = 300 nm.

A chi-square analysis indicated that the proportion of junction types (close, no tight/close, tight) were the same for the control and experimental groups (Chi-square=0.53, p=0.46) . The mean percentage of tight zones for control and experimental groups was 33.7% and 31.2% respectively (Table 6). The mean percentage of close zones for the control and experimental groups was 47.0% and 51.1% respectively, while the mean percentage for no close/tight junctions was 19.3% and 17.7% respectively (Table 6).

Table 6. The mean percentage of tight and close zones in relation to the total number of tight and close junctional zones observed for control and experimental groups.

	Tight	No Close/Tight	Close
Control	33.7	19.3	47.0
Experiment	31.2	17.7	51.1
Difference	2.1	1.6	4.1

There were significantly different mean numbers of close regions at each PDL level (p=0.027) but no overall significant differences between the control and experimental groups (p=0.090). The control group at the 900 and >900 μm, and the experimental group at the 750 and 900μm levels did show significant differences (Table 7).

No significant differences existed in the mean number of no tight/close region junctions at each PDL level (p=0.750), or between the two groups (p=0.094).

There were no overall significant differences in mean number of tight regions for each PDL level (p=0.070). At the >900μm PDL level only, there were significantly higher mean numbers of tight junctions than all the other PDL levels ($p<0.05$). There were no significant differences between the control and experimental groups (p=0.332) except at the > 900μm level as described above and expressed in Table 7.

Table 7. Effect of PDL level on the total number of close and tight regions for control and experimental groups.

PDL Level	Junction	Control	n	Range	Experimental	n	Range	All Animals
0	c§	6	4	1-3	5	3	0-2	11
	oψ	2	2	0-1	5	4	0-2	7
	t¶	10	4	1-4	8	4	1-4	18
150	c	7	4	1-3	6	4	1-2	13
	o	4	4	1-1	4	3	1-2	8
	t	9	4	1-3	10	4	1-4	19
300	c	5	3	0-2	5	3	0-2	10
	o	5	3	0-3	5	4	0-2	10
	t	8	3	0-3	10	4	2-3	18
450	c	7	4	1-3	6	3	0-2	13
	o	5	4	1-2	1	1	0-2	6
	t	8	4	1-3	5	2	0-3	13
600	c	7	4	1-3	5	3	0-3	12
	o	4	4	1-1	3	2	0-3	7
	t	8	4	1-3	6	3	0-3	14
750	c	7	4	1-3	10^{b*}	4	1-4	17
	o	3	2	0-2	2	2	1-4	5
	t	9	3	0-4	7	4	1-3	16
900	c	4^{a*}	2	0-2	1^{b*}	1	0-1	5
	o	5	4	1-2	3	2	0-1	8
	t	11	4	2-4	15	4	2-5	26
> 900	c	$18^{a* c*}$	4	2-6	6^{c*}	2	0-5	24
	o	7	3	0-3	2	1	0-5	9
	t	22	4	1-8	11^{d*}	2	0-7	33
Count	c	61		4-18	44		1-10	105
	o	35		2-4	25		1-5	60
	t	85		8-22	72		5-15	157
Total		181			141			322

[a*] Indicates a significant difference between the mean number of close junction types in the control group for PDL level 900 and > 900μm ($p<0.05$).

[b*] Indicates a significant difference between the mean number of close junction types in the experimental group for PDL level 750 and 900μm ($p<0.05$).

[c*] Indicates a significant difference between the mean number of close junctions in the PDL level >900μm for the control and experimental group ($p<0.05$).

[d*] Indicates a significantly higher number of tight junctions in the experimental group for PDL level >900μm than the other PDL levels ($p<0.05$).

§ close zones

ψ no tight/close zones

¶ tight zones

n is number of animals contributing to the number of junctions at each PDL level

range from the smallest to largest number of junctions found in all animals at each PDL level

A summary (from Table 7) of the percentages of close, tight, and no close/tight junctions is given in Table 8.

Table 8. Summary of the percentages of close, no tight/close and tight junctions in relation to the total number of junctions observed for control and experimental groups against PDL level (summarised from Table 7).

PDL	Close		No Tight/Close		Tight	
Level	control	experimental	control	experimental	control	experimental
0	9.8	11.4	5.7	20.0	11.8	11.1
150	11.5	13.6	11.4	16.0	10.6	13.9
300	8.2	11.4	14.3	20.0	9.4	13.9
450	11.5	13.6	14.3	4.0	9.4	6.9
600	11.5	11.4	11.4	12.0	9.4	8.3
750	11.5	22.7	8.6	8.0	10.6	9.7
900	6.6	2.3	14.3	12.0	12.9	20.8
> 900	29.5	13.6	20.0	8.0	25.9	15.3

Tight regions (85.3% in the control and 79.0% in the experimental) and close regions (76.6 % in the control and 60.9% in the experimental) were largely confined to the luminal third of the PCV ($p<.001$) as shown in Table 9.

Table 9. Relationship between junction type and location of the junction zone (tight or close) within the luminal, middle and abluminal thirds of the junction.

		Region Location		
Treatment	Junction Type	L1/3	M1/3	Ab1/3
Control	Close	141	28	15***
	Tight	139	20	4***
Experimental	Close	70	34	11
	Tight	128	28	6

*** Indicates a significant effect between each region ($p<0.001$)

No significant differences existed between control and experimental groups ($p=0.21$).

Table 9 represents the total number of junctional zones present, hence the totals added across Table 9 differ from the Table 7 totals where the classification was only open, close, no tight/close, regardless of the number of junction zones per junction. That is, some junctions exhibited more than one tight or close zone so the totals for Table 9 are greater than for Table 7.

5.4 Junction Dimensions

5.4.1 Junction Length

Two junction lengths were measured L1 and L2. The results for each measured length L1 and L2 are presented separately.

Treatment had a significant effect on the mean length of the junctions for L1 ($p<0.001$). There were no significant effects of PDL level on the mean length of the junctions (Table 10).

Treatment had a significant effect on the mean length of the junctions for L2 ($p<0.001$). There was no significant effect of PDL level on the mean length of the junctions for L2 ($p=0.119$). Therefore, mean junction length for L2 did change with treatment, but not between PDL levels (Table 11).

Tables 10 and 11 and Figure 11 confirm that L1 and L2 show the same trends down the PDL levels. The only differences being the greater absolute values for L1.

Table 10. Mean junction length L1 in nm for control and experimental groups down PDL levels.

PDL Level	Control		Experiment	
0	1415†	1413‡	1396†	1391‡
	(266)		(323)	
150	1598	1598	1237	1237
	(283)		(234)	
300	1486	1499	1260	1260
	(335)		(233)	
450	1548	1548	1189	1313
	(355)		(240)	
600	1618	1631	1299	1293
	(502)		(235)	
750	1706	1706	1309	1303
	(547)		(289)	
900	1458	1458	1296	1300
	(406)		(343)	
> 900	1702	1663	1462	1570
	(383)		(477)	
Mean	1588	1565***	1311	1333***

*** Indicates significant differences between the control and experimental groups for mean junction length L1 ($p<0.001$).

† Arithmetic means with standard error values in parentheses.

‡ Mean values calculated using analysis of variance. They were adjusted because of different numbers of values at each PDL level.

Table 11. Mean junction length L2 in nm for control and experimental groups down PDL levels.

PDL Level	Control		Experiment	
0	1250†	1253‡	1269†	1265‡
	(303)		(328)	
150	1431	1431	1127	1127
	(296)		(247)	
300	1326	1338	1178	1178
	(323)		(230)	
450	1421	1421	1049	1151
	(319)		(214)	
600	1462	1474	1185	1169
	(489)		(252)	
750	1573	1571	1180	1177
	(555)		(304)	
900	1319	1319	1160	1167
	(381)		(358)	
> 900	1540	1480	1296	1451
	(363)		(489)	
Mean	1436	1411***	1186	1211***

*** Indicates significant differences between the control and experimental groups for mean junction length L2 ($p<0.001$).

† Arithmetic means with standard error values in parentheses.

‡ Mean values calculated using analysis of variance. They were adjusted because of different numbers of values at each PDL level.

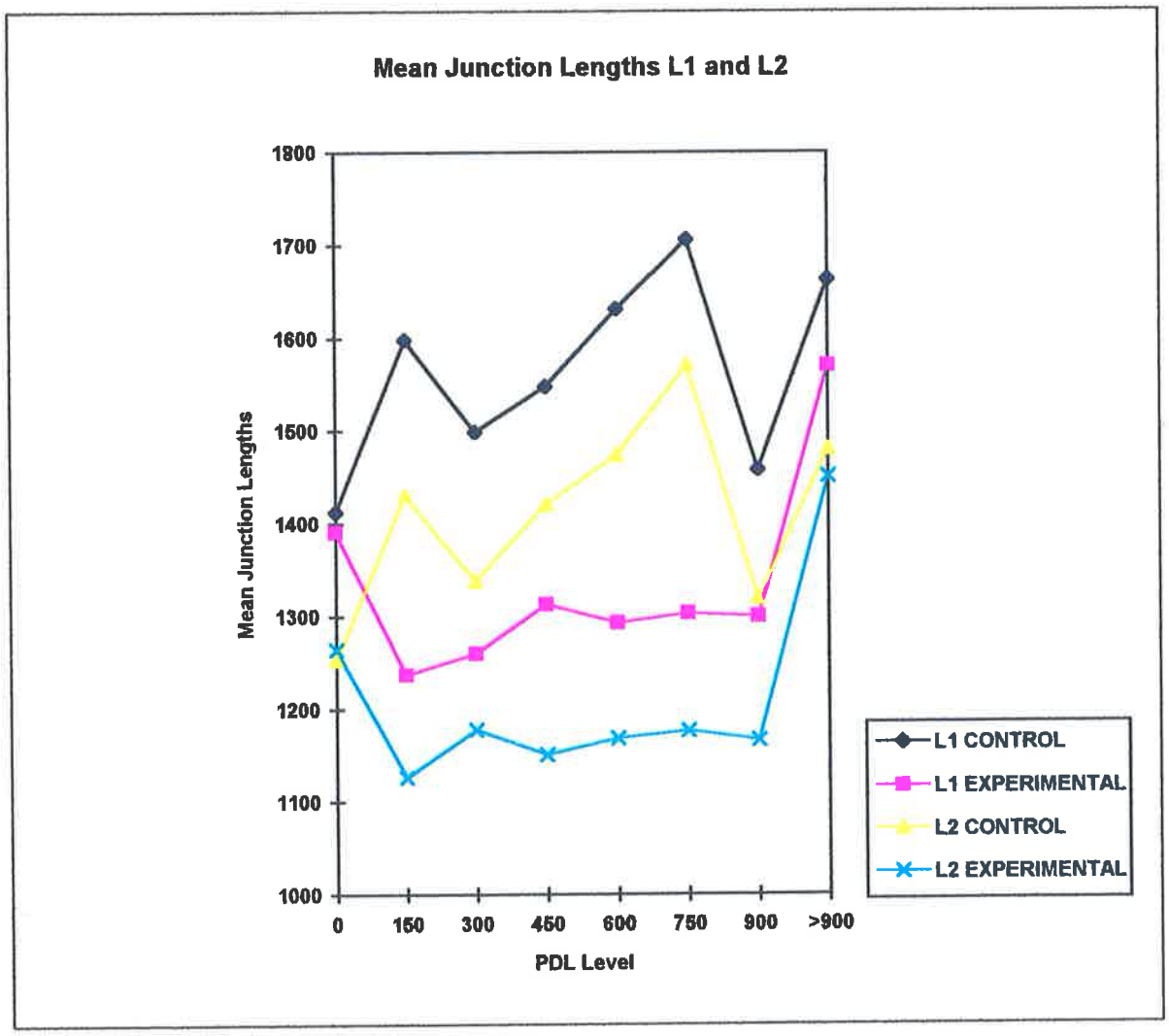


Figure 11. Plot of mean junction lengths L1 and L2 for control and experimental groups at each PDL level. Summarized from Tables 10 and 11.

5.4.2 Junction Depth (D)

Treatment had no significant effect on the mean depth of the junctions ($p=0.089$). Mean junction depth did not change with treatment, or down PDL levels (Appendix 6).

5.4.3 Junction Widths

For junction width measurements W1, W2, W3, W4 and W6 mean junction width did not change with treatment, or down the PDL levels (Appendices 7, and 8). Table 12 and Figure 12 show the significant difference with respect to width measurement W5.

For mean junction width 5 there was a significant effect of PDL level between the two groups ie a significant interaction effect ($p=0.029$). This was attributed to the experimental group, where mean W5 was significantly larger in the >900μm PDL level than the mean junction widths (W5's) at the other PDL levels ($p<0.05$). Overall, treatments did not have a significant effect on mean W5 ($p=0.429$), and PDL level did not have a significant effect on mean W5 ($p=0.392$).

Table 12. Mean junction widths W5 and W6 in nm for control and experimental groups at each PDL level.

	W5				W6			
PDL Level	Control		Experiment		Control		Experiment	
0	11.6†	11.7‡	11.3†	11.3‡	29.9†	29.3‡	31.6†	30.9‡
	(1.5)		(1.4)		(10.0)		(17.2)	
150	11.1	11.1	10.6	10.6	27.8	27.8	23.4	23.4
	(0.9)		(0.4)		(6.6)		(9.8)	
300	11.3	11.2	11.1	11.1	28.1	28.0	28.4	28.4
	(0.6)		(0.7)		(7.7)		(9.0)	
450	11.0	11.0	11.3	11.6	28.6	28.6	32.8	41.0
	(0.6)		(1.2)		(12.5)		(24.1)	
600	11.1	11.1	11.2	11.2	28.2	28.4	23.0	23.6
	(0.6)		(1.0)		(7.4)		(8.5)	
750	10.8	10.9	11.0	11.0	28.8	28.6	27.7	27.4
	(0.9)		(1.5)		(9.8)		(16.9)	
900	11.0	11.0	10.8	10.7	26.7	26.7	28.2	27.8
	(0.7)		(0.7)		(7.9)		(10.0)	
> 900	10.6	10.5	12.2	12.2*	26.9	27.6	34.5	30.5
	(1.0)		(1.0)		(8.5)		(7.2)	
Mean	11.0	11.1	11.2	11.2	27.9	28.1	28.6	29.1

* Indicates significant difference in mean junction width W5 for >900μm PDL level and the other PDL levels for the experimental treatments ($p<0.05$).

† Arithmetic means with standard error values in parentheses.

‡ Mean values calculated using analysis of variance. They were adjusted because of different numbers of values at each PDL level.

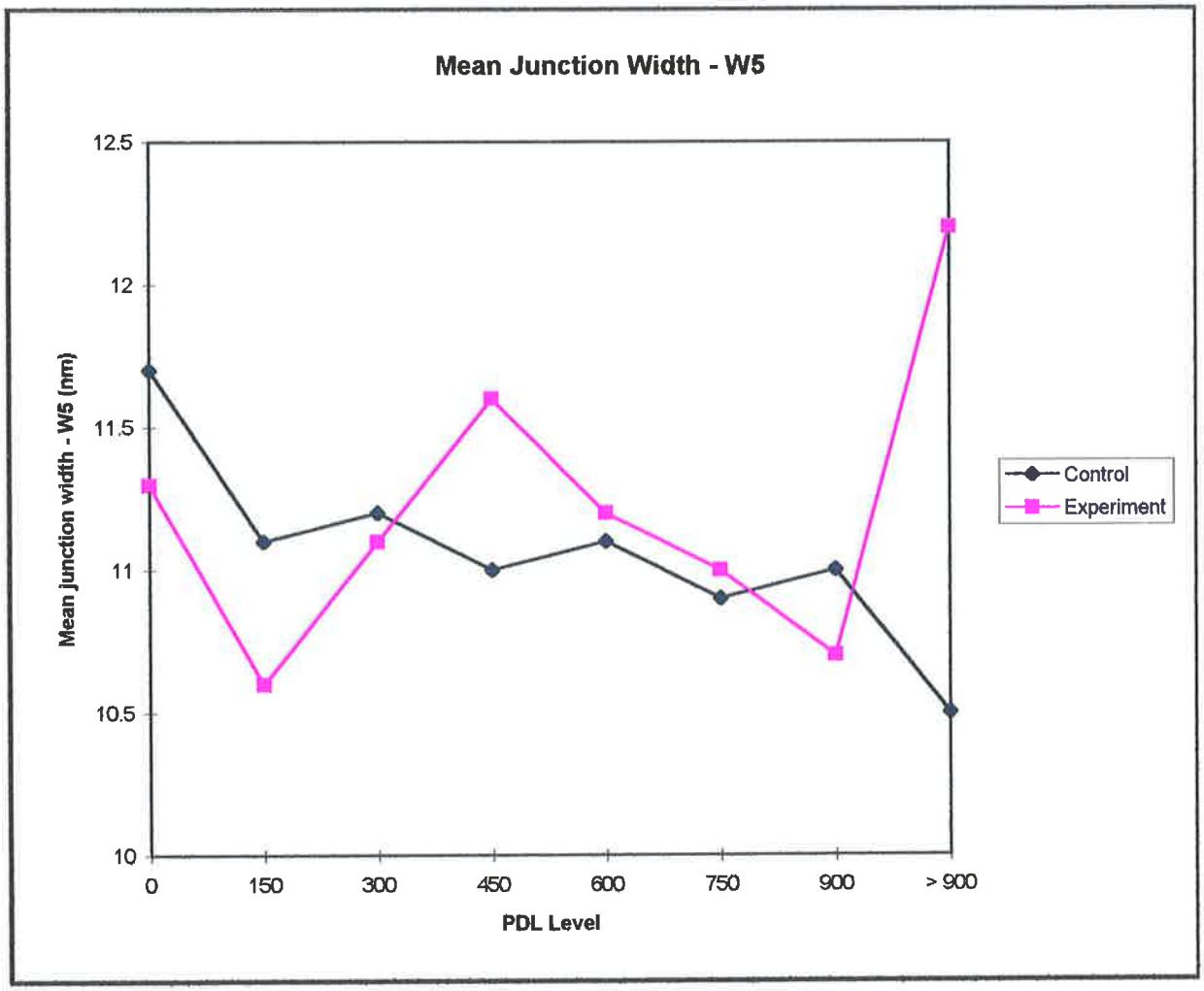


Figure 12. Plot of mean junction width W5 for the control and experimental groups down PDL levels.

5.4.4 Meander

The term meander was used to describe the ratio of junction length to junction thickness. As two measures of length were recorded, two meander values were also calculated and designated M1 and M2.

Treatment did have a significant effect on mean M1 and M2 ($p=0.019$ and $p=0.042$, respectively), with the control group having a larger mean meander than the experimental group. This is not unexpected given the reported differences for L1 and L2 between control and experimental groups (Tables 10 and 11) and the finding of no significant difference between control and experimental groups for junction depth. There were no significant differences down the PDL levels ($p=0.140$ and $p=0.216$ for Meander 1 and Meander 2 respectively). Therefore, mean M1 and M2 did change with treatment, but not down PDL levels (Table 13 and Figure 13).

5.4.5 Capillary Filtration Coefficient

Treatment did have a significant effect on mean CFC ($p=0.002$), with the control group having a smaller mean capillary filtration coefficient than the experimental group at each PDL level. There were no significant differences across the PDL levels ($p=0.672$). Therefore, mean CFC did change with treatment, but not down PDL levels (Table 14 and Figure 14).

Table 13. Mean meander M1 and M2 for control and experimental groups at each PDL level.

	Meander 1				Meander 2			
PDL Level	Control		Experiment		Control		Experiment	
0	0.7167†	0.7178‡	0.7314†	0.7323‡	0.6575†	0.6610‡	0.6873†	0.6885
	(0.0931)		(0.1843)		(0.1338)		(0.1939)	
150	0.8135	0.8135	0.7658	0.7658	0.7632	0.7632	0.7225	0.7225
	(0.1308)		(0.1122)		(0.1329)		(0.1222)	
300	0.7718	0.7735	0.7297	0.7297	0.7210	0.7244	0.6994	0.6994
	(0.1450)		(0.1546)		(0.1388)		(0.1646)	
450	0.7998	0.7998	0.7536	0.7897	0.7630	0.7630	0.6986	0.7318
	(0.1113)		(0.1467)		(0.1099)		(0.1600)	
600	0.8364	0.8424	0.7377	0.7416	0.7895	0.7951	0.6951	0.6960
	(0.1624)		(0.1463)		(0.1744)		(0.1604)	
750	0.8228	0.8258	0.7286	0.7294	0.7836	0.7861	0.6756	0.6778
	(0.1650)		(0.1305)		(0.1745)		(0.1790)	
900	0.7658	0.7658	0.6957	0.6944	0.7206	0.7206	0.6414	0.6415
	(0.1475)		(0.1294)		(0.1506)		(0.1396)	
> 900	0.8143	0.8024	0.7389	0.8195	0.7697	0.7496	0.6809	0.7811
	(0.1577)		(0.2119)		(0.1581)		(0.2242)	
Mean	0.7965	0.7928*	0.7344	0.7503*	0.7504	0.7454*	0.6871	0.7048

* Indicates significant differences between the control and experimental groups for mean M1 and M2 ($p<0.05$).

† Arithmetic means with standard error values in parentheses.

‡ Mean values calculated using analysis of variance. They were adjusted because of different numbers of values at each PDL level.

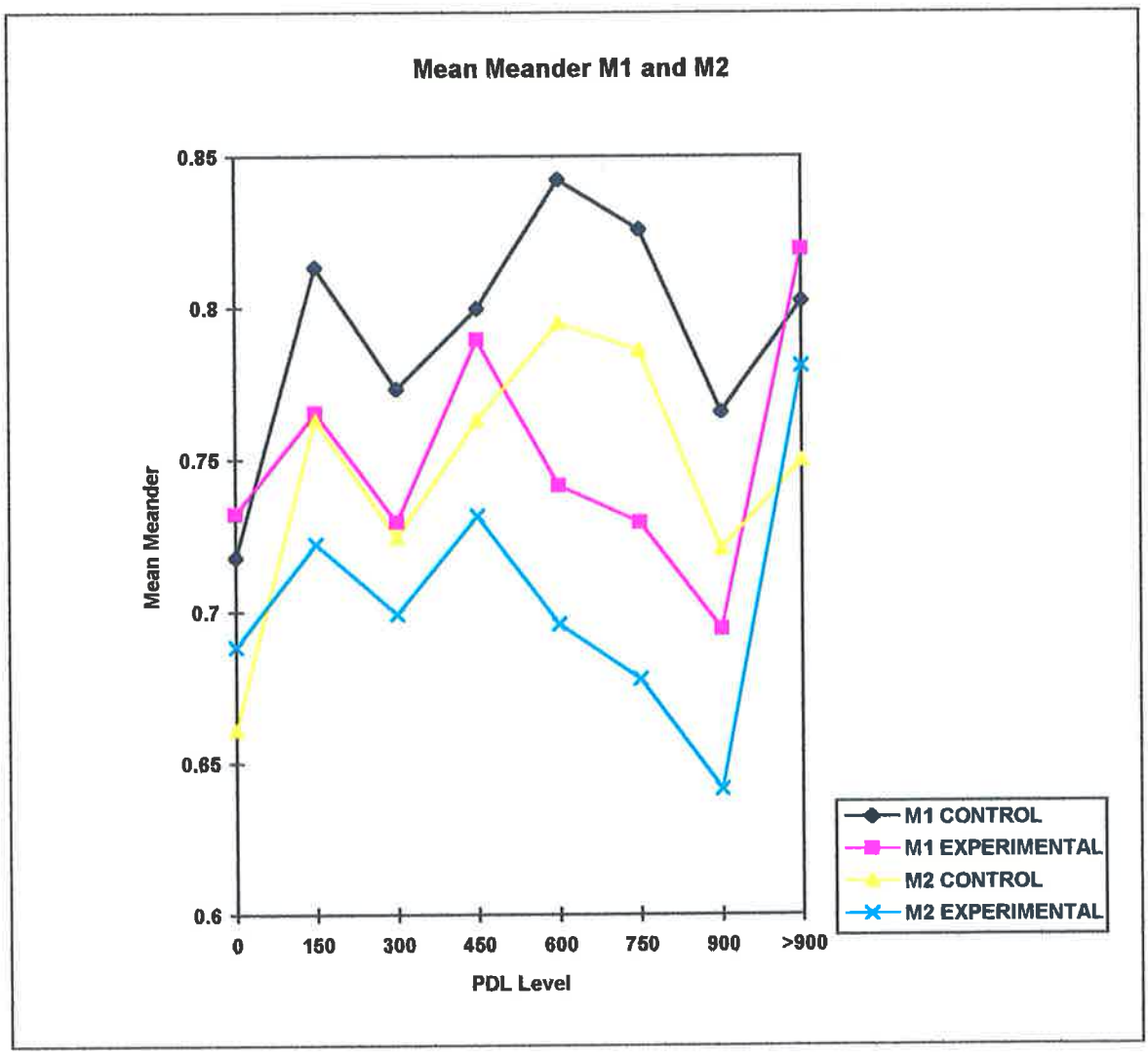


Figure 13. Plot of mean meander M1 and M2 for control and experimental groups at each PDL level. Summarized from Table 13.

Table 14. Mean capillary filtration coefficient for control and experimental groups at each PDL level.

PDL Level	Control		Experiment	
0	0.0502† (0.0256)	0.0498‡	0.0421† (0.0135)	0.0416‡
150	0.0351 (0.0123)	0.0351	0.0451 (0.0151)	0.0451
300	0.0364 (0.0157)	0.0354	0.0474 (0.0232)	0.0474
450	0.0323 (0.0106)	0.0323	0.0448 (0.0141)	0.0409
600	0.0334 (0.0133)	0.0336	0.0474 (0.0221)	0.0452
750	0.0331 (0.0149)	0.0330	0.0601 (0.0973)	0.0600
900	0.0403 (0.0159)	0.0403	0.0578 (0.0455)	0.0572
> 900	0.0320 (0.0127)	0.0315	0.0499 (0.0178)	0.0484
Mean	0.0358	0.0364**	0.0496	0.0482**

** Indicates significant differences between the control and experimental groups for the mean capillary filtration coefficient ($p<0.01$).
† Arithmetic means with standard error values in parentheses.
‡ Mean values calculated using analysis of variance. They were adjusted because of different numbers of values at each PDL level.

Significant differences in the mean CFC were found for the three junction types ($p<0.05$), with the mean of each junction type being significantly different from all of the others. This pattern is shown in Table 15.

Table 15. Table of mean capillary filtration coefficient for the three different junction types.

Junction Type	Mean	Standard Error of Mean
Close	2.91×10^{-2}	8.54×10^{-5}
No Close/Tight	5.01×10^{-2}	1.13×10^{-4}
Tight	4.73×10^{-2}	6.98×10^{-5}

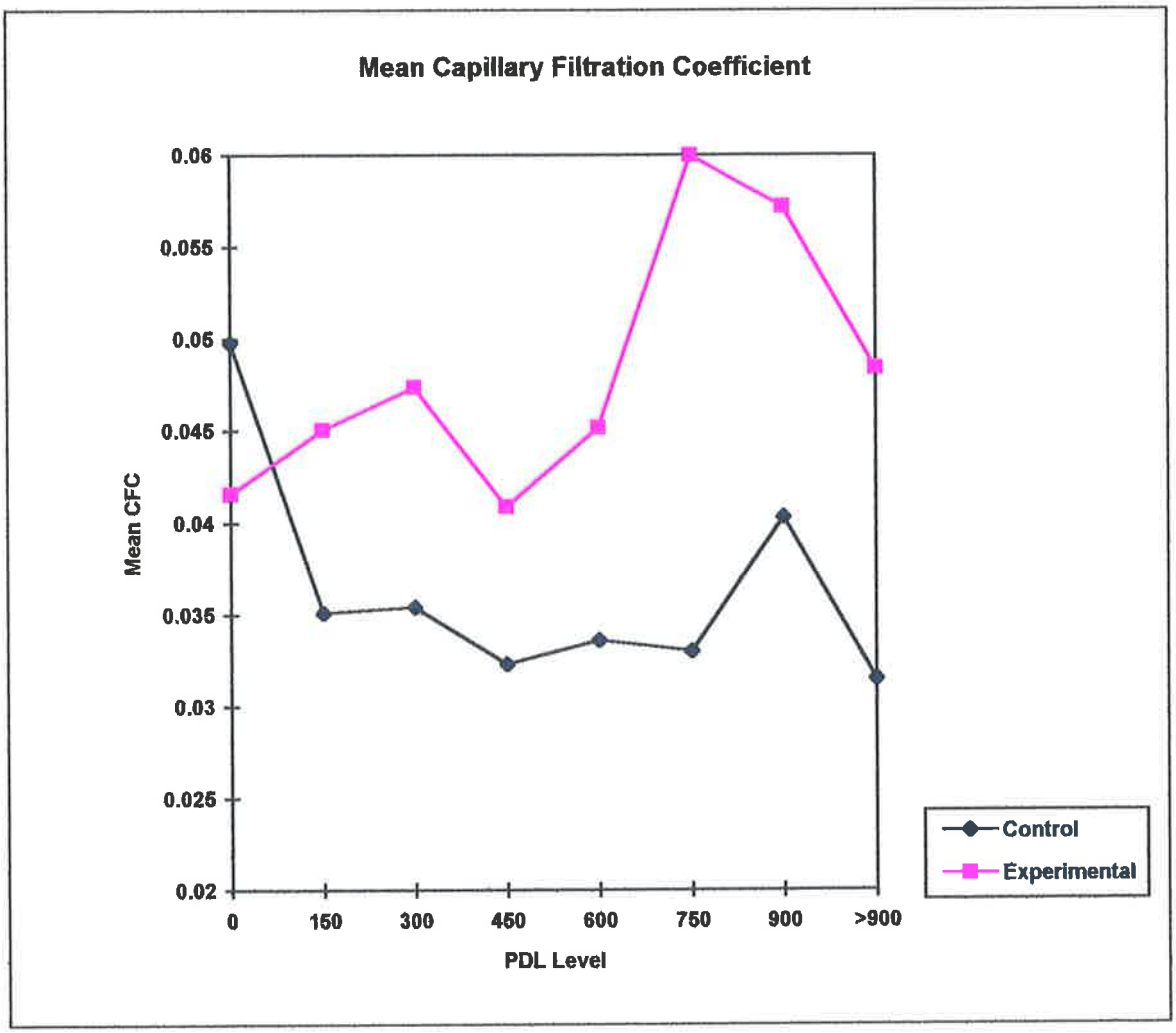


Figure 14. Plot of mean capillary filtration coefficient for control and experimental groups at each PDL level. Summarized from Table 14.

5.4.6 Junction Zone Size

There was a significant interaction between the effects of PDL level and treatment for mean junction zone size ($p<0.05$), i.e. differences in mean junction zone size between the groups are not independent of PDL level. This finding implies that there was a different trend down the PDL levels for the control and experimental groups.

Standard errors were used to locate differences among the PDL levels for the two groups. For the control treatments, PDL level 0μm was significantly different from PDL levels 150, 300, 450, 600, 750, and > 900μm; PDL levels 150, 600 and 750μm were significantly different from the PDL level 900μm; PDL level 300μm was significantly different from 600 and 750μm; and PDL level 900 was significantly different from PDL > 900μm ($p <0.05$). Refer to Table 16.

For the experimental treatment, PDL levels 0, 150, 300, 450, 600, 750 and 900μm were significantly different from PDL level > 900μm; PDL level 0μm was also significantly different from PDL levels 750 and 900μm; and PDL level 150μm was also significantly different from PDL levels 300,450, 600, 750 and 900μm ($p<0.05$). An indication of a linear trend for increasing mean junction zone size also existed down the PDL levels for the experimental group ($p<0.05$) and is shown by Table 16.

The interaction effect and the relationships noted above are illustrated in Figure 15.

Table 16. Mean junction zone size in nm for control and experimental groups at each PDL level.

PDL Level	Control		Experiment	
0	52.2† (29.3)	48.6‡	50.8† (23.3)	48.2‡
150	75.0 (41.3)	71.4	44.0 (16.3)	40.8
300	69.2 (35.2)	62.9	54.9 (9.0)	55.4
450	73.9 (37.4)	72.0	47.4 (25.3)	55.7
600	68.9 (33.9)	73.0	53.4 (36.0)	53.7
750	83.1 (43.5)	81.4	63.6 (37.3)	61.2
900	58.7 (29.2)	55.3	63.2 (32.4)	60.0
> 900	70.3 (38.2)	69.0	81.6 (52.6)	78.7
Mean	69.0	66.7	58.8	56.7

† Arithmetic means with standard error values in parentheses.
‡ Mean values calculated using analysis of variance. They were adjusted because of different numbers of values at each PDL level.

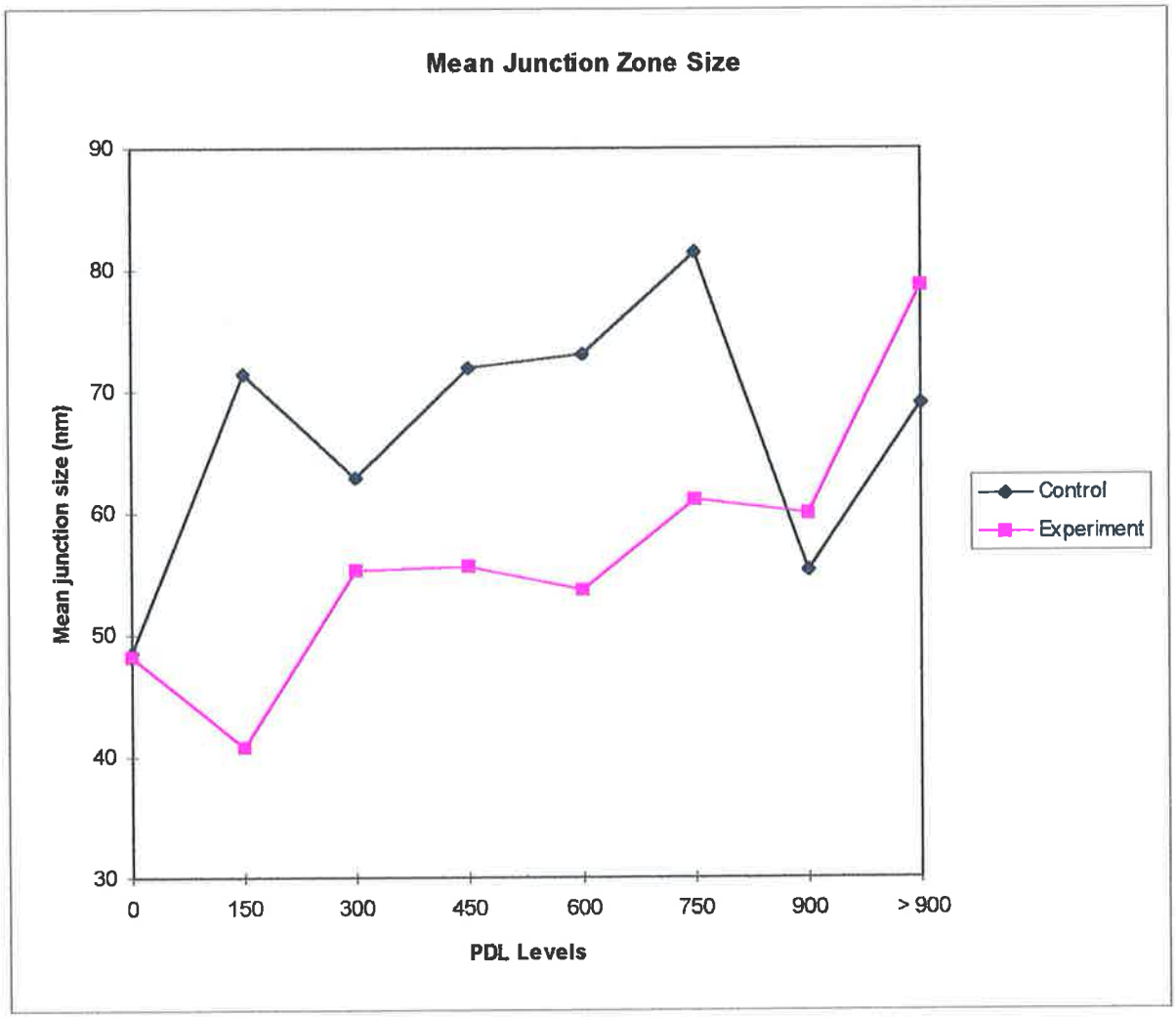


Figure 15. Plot of mean junction zone size for the control and experimental treatments across PDL levels.

There was a significant difference in mean junction size between close and tight junctions ($p<0.001$), with the close junctions having a larger mean junction size than the tight junctions. The values being 62.6 nm and 50.5 nm, respectively. There was also a

significant difference in mean junction zone size for the three different junction regions ($p<0.001$), with the mean junction zone size decreasing from the luminal 67.1 nm, to the middle 54.9 nm and abluminal thirds 47.7 nm. There were significant treatment effects ($p<0.001$), with the control group having significantly larger mean junction zone size than the experimental group (61.4 nm and 51.8 nm respectively). All interactions between the factors of junction type, region or treatment were not significant ($p>0.45$). Refer to Table 17.

Table 17. Mean size in nm of tight and close zones located in control and experimental sections in the luminal, middle, or abluminal thirds along the length of the junction.

Treatment[a***]	Junction Type[b***]	Region Location[c***]					
		L1/3		M1/3		Ab1/3	
Control	Close	81.2† (43.0)	80.0‡	63.4† (22.9)	63.3‡	60.4† (36.5)	61.9‡
	Tight	62.9 (33.3)	63.0	49.8 (14.6)	56.0	62.6 (5.8)	44.0
Experiment	Close	67.8 (33.9)	67.8	53.1 (24.2)	56.1	49.0 (25.7)	46.9
	Tight	61.8 (43.2)	57.7	46.5 (25.5)	44.3	37.8 (10.4)	37.9

[a***] Indicates significant differences between control and experimental groups for mean junction zone size ($p<0.001$).
[b***] Indicates significant differences between the junction types for mean junction zone size ($p<0.001$).
[c***] Indicates significant differences between the region locations for mean junction zone size ($p<0.001$).
† Arithmetic means with standard error values in parentheses.
‡ Mean values calculated using analysis of variance. They were adjusted because of different numbers of values at each PDL level.

5.4.7 Luminal Flaps

A chi-square analysis indicated that the proportion of junction types (close, no tight/close, tight) sampled were not the same for the presence and absence of flaps (Chi-square=4.51, p=0.03). The number of junction types for the presence and absence of flaps is given in Table 18 and the percentages in Table 19. From Table 19 it seems that for the presence or absence of flaps the main differences were in the no tight/close and tight junction types.

Table 18. The number of junction types for the presence and absence of flaps.

FLAPS	Close	No Close/Tight	Tight	TOTAL
present	72	33	110	215
absent	33	27	47	107
TOTAL	105	60	157	322

Table 19. The percentage of junction types for the presence and absence of flaps.

FLAPS	Close	No Close/Tight	Tight
present	33.5	15.3	51.2
absent	30.8	25.2	43.9

A chi-square analysis indicated that the proportion of junction classification types (close, no tight/close, tight) sampled were the same for the large and small flaps (Chi-square=1.56, p=0.21). The number of junction types for large and small flaps is given in Table 20.

Table 20. The number of junction types for large and small flaps.

FLAPS	Close	No Close/Tight	Tight	TOTAL
large	33	13	40	86
small	46	22	81	149
TOTAL	79	35	121	235

The totals noted in Tables 18 and 20 differ as Table 18 related only to the presence or absence of luminal flaps, whereas Table 19 related to the number of large and small flaps noted. That is, some junctions exhibited two small, two large, or one large and one small luminal flaps.

5.4.8 Correlations Between Continuous Variables

The continuous variables measured or calculated included length (L1, L2), width (W1, W2, W3, W4, W5, W6), junction depth (D), blood vessel diameter (BVD), and capillary filtration coefficient (CFC).

Table 21 depicts the correlation values and shows that junction length 1 and junction length 2 had a positive correlation which was significantly ($p<0.05$) different from zero. Junction widths W2, W3, W4, W5 and W6 all had positive correlations which were significantly different from zero ($p<0.01$). Junction width 1 (W1) was not highly correlated with the other junction widths, but it did have a coefficient significantly different from zero ($p<0.01$). CFC had negative correlations significantly different from zero with L1 and L2 ($p<0.01$), and positive correlations significantly different from zero with W2, W3, W4, W5 and W6 ($p<0.01$). In Table 21 the figures in bold type showed the largest positive or negative correlations. All figures indicated with an asterix were significant at $p<0.01$. This result reflected the fact that the calculation of CFC was based on junction length and junction width.

	L1	L2	W1	W2	W3	W4	W5	W6	D	BVD	CFC
L1	1.000	0.968**	-0.059	0.007	0.006	0.083	0.031	0.039	0.294**	0.132	-0.413**
L2		1.000	-0.178**	-0.055	-0.029	0.046	-0.013	-0.019	0.256**	0.113	-0.449**
W1			1.000	0.202**	0.073	0.074	0.023	0.151**	-0.049	0.078	0.044
W2				1.000	0.663**	0.600**	0.538**	0.336**	0.163**	0.223**	0.360**
W3					1.000	0.703**	0.626**	0.332**	0.117	0.178**	0.434**
W4						1.000	0.648**	0.362**	0.153**	0.169**	0.377**
W5							1.000	0.417**	0.080	0.089	0.358**
W6								1.000	0.154**	0.129	0.196**
D									1.000	0.082	-0.079
BVD										1.000	-0.010
CFC											1.000

** Indicates a correlation coefficient significantly different from zero ($p<0.01$).

Table 21. Correlation matrix for the continuous variables.

CHAPTER 6

DISCUSSION

6.1 Assumptions In The Present Study

1. It has been assumed that the static, two dimensional photographs of endothelial cell junctions provides relevant data on the morphometry and permeability of those junctions as indicated by the capillary filtration coefficient calculations. This assumption may be questioned as endothelial cells are able to change the architecture of their junctions rapidly to allow the passage of plasma proteins and cells such as leukocytes (Lampugnani et al., 1993). Therefore the data provided by this study is relevant only for the time at which the animal was sacrificed.

Tight zones were found to be only tight within the plane of the section examined (Bundgaard, 1984). He showed, with serial section electron microscopy, that there were discontinuities within tight regions at different vertical levels. As such the tight junctions are not strictly impermeable as has been assumed in this study.

More recently, pore-matrix model (Weinbaum et al., 1992) and fiber-matrix model (Schnitzer, 1992; Adamson, 1992) theories have been developed to explain the differences in permeability of the microvasculature to molecules of different sizes. These models agree with the postulates of Renkin (1977) as they assume that the major path of transport from blood vessel lumen to interstitial tissues is via the endothelial cell junction. This assumption has been questioned by studies from Wagner and Chen (1991) and Predescu and Palade

(1993) who have suggested that the major path for transportation, especially of macromolecules, is transcellular via microvesicles.

2. The presence of tight and close regions is assumed to be actual rather than a deviation of the junction in the vertical plane. Also, a tight region may, in fact, represent a close junction with a vertical plane deviation at that point of the junction. This is a possibility as the tissue sections used were in the silver to gold interference range of 60-80 nanometres thickness. With the width of the junctions in areas not defined as tight or close zones in the order of 11 nanometres, a deviation of the junction in the vertical plane could bias the number of tight and close junction zones noted. This possibility was limited by the study design where only junctions which could be followed for their entire course were utilized. Smith and Shine (1992) reported on a technique where tight junctions of the blood-brain barrier were labelled with an antibody that recognized the high molecular weight protein, ZO-1 which is associated with tight junctions. Some definitive staining of tight and close junctions would eliminate the uncertainty referred to above.

3. In utilizing only junctions which could be followed for their entire course, the present study may bias against longer endothelial cell junctions. Hence, the length measurements cited may under estimate actual junction length.

4. The presence of junction lumen flaps was noted in approximately 70% of the junctions studied. Whether these flaps project into the lumen, as depicted by the TEM photographs, when there is blood flowing through the vessels is a matter for speculation. Exactly what functional significance may be attributed to these flaps is also debatable. Three-dimensional computer reconstruction of multiple thin sections, in the same manner in

which three-dimensional reconstructions are used with magnetic resonance imaging, would be of value for increasing the understanding of the functional significance of the luminal flaps. There would still be the problem of visualizing the flaps during true physiological function.

6.2 Methodological Problems

1. The grids used for TEM viewing had several sections from the same level on each grid. When following the grid bars systematically to identify postcapillary-sized venules, it was possible to sample more than one endothelial cell junction from the same blood vessel. The project aimed to sample junctions from five different postcapillary-sized venules. Sampling multiple junctions from one blood vessel was minimized by viewing the whole blood vessel and its surrounding tissue prior to junction photography. Some sampling of multiple junctions from the same blood vessel was inevitable. However, as not all of the sections were placed on the grids with the same orientation this would enable the same blood vessel to appear different due to orientation differences.

2. Inter-animal variability was assessed by calclating means and standard deviations for each of the measured and calculated variables. The variables W1, W6 and CFC had large standard deviations indicating a large amount of variability for these values. W1 and W6 were likely to have high standard deviations due to the variability of the number and size of luminal flaps (W1) and the inconsistent abluminal junctional morphology. For CFC, the stated statistically significant difference between the control and experimental treatments

may, in fact, represent a high degree of inter-animal variability. A larger sample size and a uniform age of animal would be necessary to further test the association between treatments and CFC.

3. Capillary filtration coefficient calculations required utilization of L_v (luminal length density) as the measure of the length of endothelial cell junctions in the vertical plane. This value was calculated by Chintavalakorn (1994) using the same tissue sections as were used in this study. Chintavalakorn (1994) calculated L_v as 30.97 X 10^3 cm/cm^3 for postcapillary-sized venules. This figure is approximately twice the value for L_v as calculated by Parlange (1990) for her group of four female marmosets. The differences could represent sex differences. However, the calculated CFC values cited in this study may be over estimated by a factor of two.

The CFC values of this study were approximately half the value of those calculated by Casley-Smith (1975). He examined skeletal muscle vasculature of canine hind legs where postcapillary-sized venules were rarely found. It is possible that different tissues could contain different blood vessel types and proportions and represent different functional requirements.

As the material used in the present study was sourced from the tissue of animals sacrificed by Weir (1990), the author was not able to alter the experimental procedure or utilize specific molecules to assess endothelial cell junction permeability in a more specific fashion. The calculation of capillary filtration coefficients as an indicator of junction permeability was chosen as it would enable comparison with the work of Casley-Smith et al.

(1975) even though the methodology was outdated. It would be interesting if future studies could analyse permeability of specific tracer molecules.

4. The tissue used as controls in the present experiment were contra-lateral to the experimental teeth (i.e. from the same animal) and were also covered by the splint designed to retain the experimental tooth in its extruded position. Not having performed the experimentation, the present author has no knowledge of the presence/absence/amount of occlusal interference caused by the splint. It is conceivable that the control teeth were subjected to unusual occlusal forces which may invalidate them as accurate controls. Alternatively, being incorporated into the splint may have reduced the amount of function on the control teeth and therefore altered the surrounding PDL. With the small sample size available, the control tissue could not come from separate animals even though this would be desirable.

6.3 Blood Vessel Diameter

The present study found the mean diameter for pericytic PCV to be 16.93 (0.44) microns and apericytic PCV to be 15.88 (0.44) microns. The figure in parentheses represents the standard error. The predicted mean diameter for pericytic and apericytic PCV for the control group was 17.4 microns and for experimental group was 15.2 microns. Chintavalakorn (1994) examined the same material using stereology and point counting techniques and found the mean diameter for control and experimental PCV to be 17.02 (0.77) microns and 16.23 (0.89) microns respectively. The difference in the means found in

this study were slightly greater than that found by Chintavalakorn (1994), however the trend of control PCV being slightly larger than experimental PCV was similar. It is possible that measuring different profiles of the same vessels as Chintavalakorn (1994) may account for the differences noted in blood vessel diameter.

Normal and tensioned rat maxillary molar PDL was examined by Chintakanon (1990) who found mean PCV diameter for control and experimental groups to be 15.26 (0.67) and 14.87 (1.03). The same trend as noted in the current study, of the control group having slightly larger PCV than the experimental group was noted. The differences were not statistically significant due to the large standard error for the experimental group (Chintakanon, 1990).

Examining young and old mice, Cameron (1995) found the mean PCV diameter for young mice to be 14.6 (1.2) and for old mice to be 13.3 (1.1). If the young mice were considered to be controls and the old mice experimental, the same trend as noted above was found.

6.4 Junction Type

The present study found a significant difference between the mean number of close junctions in the control group for PDL levels 900 and >900 microns. The experimental group showed a significant difference between the mean number of close junctions for PDL levels 750 and 900 microns. Overall, however, the mean number of tight and close junctions did not differ significantly between the control and experimental groups. The proportions of

the different types of junctions (close, tight, not close/tight) were found to be the same for control and experimental groups. Cameron (1995) found old mice to have more tight and less close junctions, whereas young mice had more close and less tight junctions. Cameron (1995) also found more tight junctions at the alveolar crest and apex. The present study did not find similar results for tight junctions. However close junctions did show an increase towards the more apical PDL levels for the control and experimental groups. The experimental group only showed an increase in the number of tight junctions at the >900 micron PDL level. This could be due to the pooling of a variable number of levels to create the >900 micron data. The differences in the material (young and old mice versus control and experimental marmoset) and the fact that Cameron (1995) examined the apex, whereas the present study did not, could account for the differences in the trends noted.

As for the percentages of tight and close junctions found, the present study found 33.7% tight and 47.0% close for the control group and 31.2% tight and 51.1% close for the experimental group. Chintakanon (1990) found, in normal and tensioned rat maxillary molar PDL, 15.6% tight and 84.4% close for the control group and 21.3% tight and 78.4% close for the experimental group. Cameron (1995) found for young mice 11.2% tight and 88.8% close and for old mice 25.3% tight and 74.7% close junctions. If the young mice used by Cameron (1995) were considered as controls and the old mice as experimentals, the results of Cameron (1995) and Chintakanon (1990) are remarkably similar. The results found for the experimental group in the present study were not greatly dissimilar to those of the other two studies, but the control group of the present study showed almost twice the number of tight junctions, and half the number of close junctions as the other studies cited. This could

imply that the experimental proceedure of using the contra-lateral central incisor, which was included in the splint used to retain the experimental tooth, is suspect. Alternatively, the present classification of no tight/close junctions may underestimate the number of close junctions, or the studies of Chintakanon (1990) and Cameron (1995) may have overstated the number of close junctions. Species differences may be another explanation of the differences noted above.

The luminal third of endothelial cell junctions was found to be the most likely location for tight and/or close zones to be located. The present study found 85.3% of tight and 76.6% of close zones were located in the luminal third of junctions for the control group. For the experimental group, 79% of tight and 60.9% of close zones were located in the luminal third of the junctions studied. Chintakanon (1990) located 87% of control tight junctions, and 88% of experimental tight zones in the luminal third of the junctions examined. No percentages were cited for close junctions. Cameron (1995) located 86.1% of tight and 76.6% of close zones in the luminal third of young mouse junctions. Old mouse junctions showed 90% of tight and 65.5% of close zones in the luminal third of the junctions examined.

6.5 Junction Dimensions

6.5.1 Junction Length

Junction length was measured two ways in the present study. Both measures showed the same trends for PDL levels and between the control and experimental groups. This

would suggest that either method is suitable for endothelial junction length measurement. Any calculations based on length measurement will be numerically influenced by which length measure is used, but the trends displayed will not vary because of the length measurement used.

Mean junction length (L1) for the present study was 1588 nanometers for the control and 1311 nanometers for the experimental group. Mean junction length (L2) was 1436 nanometers for the control and 1186 nanometers for the experimental group. Cameron (1995) found mean junction length to be in the 800 - 1200 nanometer range. Chintakanon (1990) did not cite figures for mean junction length. Casley-Smith et al. (1975) found an average junction length (equivalent to the present studies L1) of 624 nanometers in dog hind leg skeletal muscle. Casley-Smith et al. (1975) stated that PCV were rarely found in the skeletal muscle sample, so this could explain the difference in mean junction length found in their study.

The difference between control and experimental groups for junction length were highly significant ($p<0.001$). This finding is consistent with the results relating to mean PCV wall thickness as measured by Chintavalakorn (1990). Postcapillary-sized venules were the only blood vessel type to show a statistically significant difference between control and experimental groups, with the PCV wall thickness reduced following orthodontic extrusion (Chintavalakorn, 1994). The amount of convolution of endothelial junctions may mask some of the reduction in PCV wall thickness. However, the calculated values for meander confirm the conclusion of reduced PCV wall thickness and junction length. Mean junction depth, although not a direct measure of PCV wall thickness, showed a similar trend with the

experimental group showing a reduced junction thickness. The differences were not statistically significant.

6.5.2 Junction Width

Junction width W1 and W6 showed greater variability due to the presence or absence of large or small luminal flaps, and the morphology of the abluminal end of the junctions. Junction widths W1, W2, W3, W4 and W6 did not alter down the length of the PDL and were not significantly different between control and experimental groups.

Only at the > 900 micron PDL level, for the experimental group, did junction width W5 show a significant increase with respect to the other PDL levels. It is possible that this finding is due to the pooling of results at the > 900 micron PDL levels, and was therefore not an accurate reflection of the real W5 junction width. Junction width W5 was significantly correlated to junction widths W2, W3, W4 and W6 as shown by Table 21. The correlation matrices varied from 0.417 to 0.648 which indicated a moderate level of correlation. It is possible that some of the methodological problems associated with the present study could account for the lack of significant differences noted between the control and experimental groups with respect to junction widths other than junction width W5. A study with similar methodology to the present project, examining the apical region, would be able to test whether the noted difference in the experimental junction width W5 was real or artifactual due to the pooling of data at the > 900 micron PDL level.

Excluding the more variable junction widths W1 and W6, the mean junction width for the current sample was in the 11-12 nm range. This value is lower than that cited by Casley-Smith et al. (1975) and Chintakanon (1990) who respectively found mean junction

width to be between 19 nm and 23 nm. Casley-Smith et al. (1975) examined dog hind leg skeletal muscle, and Chintakanon examined normal and tensioned rat maxillary molar PDL. The differences in animal models, different teeth studied, and the fact that the present study examined only postcapillary-sized blood vessels where the cited studies examined all blood vessel types, could explain the differences noted.

6.5.3 Capillary Filtration Coefficient

Calculations of CFC revealed that the most important region in determining the total CFC per junction is that part of the junction where the cell external lamina were parallel. That is, the length from the site of measuring W2 to the site of measuring W5 (i.e. the portion defined as L2), excluding the total length of any tight or close zones present within that section of the junction. This was true until the number of close junction zones exceeded four. Then the CFC for the total length of close zones became the dominant factor in determining the total CFC.

Given that the total CFC was most dependent on the junction length and width, and that the length varied significantly between the control and experimental groups, but not down the PDL, and that junction width varied significantly only for the > 900 micron experimental group PDL level, it is not surprising that total CFC showed a similar trend to that of junction length. The correlation matrix shows the interdependence of junction length, junction width and CFC.

The values for mean CFC found in this study are of the same order of magnitude as those found by Casley-Smith et al. (1975). This suggests that the permeability of the different microvascular beds of the present study and that of Casley-Smith et al. (1975), are

similar despite their different functional reqiurements. Possibly, permeability has more to do with junction length than any other factor so far measured, and may reflect the fact that cells have a finite upper limit to their size.

6.5.4 Junction Zone Size

Mean junction zone size was found to be greater in the control group. At every PDL level up to 750 micron, the mean junction zone size was greater for the control group. At the 900 micron and > 900 micron levels the experimental group mean junction zone size was greater than the control group. It is possible that the differences in junction zone size noted reflect a permanent change resulting from the orthodontic extrusion, or incomplete reconstitution at the endothelial cell junction level. Alternatively, it could also reflect the presence of more close junctions in the control group as shown in Table 9.

Mean junction zone size was also noted to vary depending upon where the zone was located in the junction. Those junction zones located in the luminal third were larger than those located in the middle third, which were in turn larger than those in the abluminal third. Such a change in zone size, and the finding that the vast majority of tight and close junction zones are located in the luminal third, may illustrate the occurrence of greater control over blood vessel permeability close to the lumen.

6.6 Relevance Of This Study To Orthodontics

Periodontal ligament blood vessels play an extremely important role in periodontal homeostasis and are intimately involved in the chain of events associated with orthodontic

tooth movement (Davidovitch, 1991; Sandy, 1992). The PDL provides cushioning for the teeth and the blood vessels act as a reservoir for fluid involved in the cushioning process (Berkovitz, 1990). The PDL blood vessels also provide nutrients for such processes as bone and PDL remodeling involved in orthodontic tooth movement, as well as removing waste products from areas undergoing degradation.

To enable blood vessels to act efficiently in these roles, the permeability of the vessel wall must be under strict control and also be able to alter rapidly. It is still uncertain exactly how control of endothelial cell junction permeability is modulated, but the endothelial cells, and therefore the junctional complexes, are influenced by signals from the nervous, immune, and endocrine systems (Davidovitch, 1991). From an orthodontic perspective, application of a mechanical force produces fluid movement in the PDL which can alter ionic and osmotic conditions, produce direct distortion of cells, and induce chemical changes in cell metabolism which initiate the bone and PDL remodeling associated with tooth movement.

The present study was not designed to analyze changes in endothelial cell junction permeability over a time frame following application of an orthodontic force. This would be an interesting project for the future. The present study did provide insight into the normal morphological features of endothelial cell junctions of marmoset PDL and demonstrated that junction morphology was altered following the experimental procedure and retention period. This suggests that either the PDL blood vessels are altered irrevocably by orthodontic tooth movement, or that the time necessary for complete reconstitution is greater than that utilized in the present study.

The problems with the present discussed earlier in this chapter may enable criticism of the present study, but hopefully will lead to further refinement for study in this area. Isolating of PDL blood vessels as per the study of Adamson and Michel (1993) may prove to be impossible. Collection of PDL endothelial cell to create cell monolayers as per the studies of Oliver (1990) and Schaeffer et al. (1993) may also prove to be difficult. Perhaps in the future PDL blood vessels may be reconstructed three dimensionally using computer aided reconstruction of photomicrographs of serial sections so that a clearer picture of junction morphology could be obtained.

CHAPTER 7

CONCLUSIONS

1. Apericytic postcapillary-sized venules were more numerous than pericytic postcapillary-sized venules at all PDL levels. The number of pericytic PCV were significantly greater in the control group at the > 900 micron PDL level only.
2. Apericytic PCV (15.88 μm) exhibited smaller mean intraluminal diameter than pericytic PCV (16.93 μm). For the combined apericytic and pericytic PCV, the control group showed a greater mean diameter than the experimental group.
3. The proportions of tight, close, and no tight/close junctions were not different between the control and experimental groups. There were significantly greater numbers of close junctions for the control group at the 900 and >900 micron PDL levels.
4. Close and tight junction zones were largely confined to the luminal third of the junctions studied; 85.3% of control and 79.0% of experimental tight junctions, and 76.6% of control and 60.9% of experimental close zones were located in the luminal third. The middle third showed the next highest number of junction zones with the abluminal third exhibiting the least number of tight or close zones.
5. The mean junction zone size was greater for the control than experimental groups. Close zones (62.6 nm) had a greater mean size than tight zones (50.5 nm). Tight or close zones located in the luminal third were larger than those found in the middle third which were larger than those found in the abluminal third.

6. Mean junction length was measured in two ways. Both junction length measurements L1 and L2 showed the same differences between control and experimental groups so either definition of junction length is adequate for future studies. The control group (1565 nm) showed greater junction length than the experimental group (1333 nm). This finding is in agreement with the finding of reduced PCV wall thickness (Chintavalakorn, 1994) following orthodontic extrusion.
7. Junction widths W1, W2, W3, W4, and W6 showed no significant differences between control and experimental groups or down the PDL levels. Junction width W5 showed a significantly greater mean at the > 900 micron PDL level only. This difference could be due to pooling of data to create the > 900 micron level. Furthes studies examining the apical region could clarify this finding. Mean junction width W1 and W6 showed greater variability than the other widths due to the variable luminal and abluminal extents of the junctions. For junction widths W2-W5 the mean width was approximately 11 nm.
8. Meander showed no significant effects down the PDL levels. The control mean meander was significantly greater than the experimental meander indicating that the experimental junctions were less convoluted. This may be an indication that endothelial cell junctions reconstituted following orthodontic tooth extrusion are of a simpler morphology.
9. Capillary filtration coefficient calculations showed the control group to have significantly smaller mean values than the experimental group suggesting that the experimental PCV were more permeable. This finding is not surprising given the findings of greater junction length and increased numbers of close zones in the control group.

10. Following decoronation, vital pulpectomy, endodontic therapy, orthodontic extrusion with magnets and retention for thirty weeks, there were differences noted between the control and experimental marmoset incisor PDL postcapillary-sized venule endothelial cell junctions. The null hypothesis that there would be no significant differences between the control and experimental groups was rejected.

CHAPTER 8

APPENDICES

1. ANAESTHETIC SOLUTION

Solution : Saffan (Glaxovet, a division of Glaxo Australia P/L., Victoria, Australia) The active constituents are Alphaxalone (3α - hydroxy - 5α - pregnane -11,20 - dione) and alphadolone acetate (21 -acetoxy -3α - hydroxy -5α pregnane -11,20 - dione), solubilized in saline by a 20% w/v polyoxyethylated castor oil.

Presentation : 10ml vials.

Dosage : Sedation: 10-12 mg per kg.

Anaesthesia: 18mg per kg.

Shelf Life : Indicated on package if stored at room temperature. Refrigeration is contra-indicated as it may precipitate the solid constituents.

2. FIXATIVE FOR PERFUSION

Solution : 5.6% glutaraldehyde, 0.9% osmium tetroxide and 1% sodium nitrate in 0.06% M sodium cacodylate buffer (final pH 7.4).

Preparation : 105 ml 0.6 M cacodylate buffer, pH 7.4, 42ml 25% glutaraldehyde (25% aqueous solution; TAAB Laboratory Equipment, England)

42ml 4% osmium tetroxide

7.56 g Dextran 70

1.9 g Sodium Nitrate

Dissolve Dextran 70 and sodium nitrate in the 0.06 M cacodylate buffer solution and add glutaraldehyde.

Adjust to pH 7.4 using 1N HC1, 2-3 hours before perfusing.

Immediately before perfusing add the 4% osmium tetroxide solution.

Shelf Life : 10-15 minutes at room temperature.

Route : Via the common carotid arteries.

3. DECALCIFYING SOLUTION

Solution : 0.1 M EDTA in 2.5% glutaraldehyde.

Preparation : 74.48 gm EDTA (Ethylenediaminetetra-acetic acid as di-sodium salt ; Ajax Chemicals, Sydney, Aust.);1,800 ml 0.06 M cacodylate buffer, pH 7.4; 200ml 25% glutaraldehyde. Dissolve EDTA in 0.06M cacodylate buffer by gentle heating. Cool to 4°C, add glutaraldehyde (pH 6.0 at 4°C using 1 N HC1).

Shelf Life : 7 days at 4°C.

4. GRID STAINS

A. Uranyl Acetate

Solution : 0.5% uranyl acetate in 70% alcohol.

Preparation : 0.125g uranyl acetate ; 7.5ml alcohol made up to 25ml with millipored water. Shake to dissolve. Exclude light by wrapping in Alfoil.

Shelf Life : 3 months at room temperature.

B. Reynolds' Lead (REYNOLDS, 1963)

Solution : Modified Reynolds lead.

Preparation : (i) 1.33 gms lead nitrate ; 1.76 gms sodium citrate ; 30 ml dd water.

(ii) 8 ml 1 N sodium hydroxide.

Vigorously shake (i) and allow to stand for 30 minutes; add (ii) then dilute to 50 mls with dd water, mixing by inversion.

Shelf Life : 30 days at 4°C. Discard if pH drops below 11.

5. TRANSMISSION ELECTRON MICROSCOPE

Phillips CM 100

Accelerating voltage of 80 kV

Resolution to 1 micron

Tilt and rotation goniometer utilized.

Low magnification photography taken at 900 x magnification.

High magnification photography taken at 73,000 x magnification.

Replicating graticule 2160 lines/mm

6. MEAN JUNCTION THICKNESS IN NM FOR EACH TREATMENT DOWN PDL LEVELS.

PDL Level	Control		Experiment	
0	272.9† (56.5)	272.5‡	257.8† (58.3)	256.5‡
150	249.0 (61.2)	249.0	213.9 (47.0)	213.9
300	257.2 (78.8)	257.9	243.2 (82.3)	243.2
450	245.7 (59.7)	245.7	212.1 (56.0)	214.3
600	232.9 (60.1)	231.2	240.3 (57.4)	237.0
750	252.6 (61.9)	251.2	244.8 (54.7)	243.2
900	250.9 (75.3)	250.9	262.6 (78.3)	264.2
> 900	268.4 (89.1)	268.9	265.2 (64.3)	238.2
Mean	255.8	253.4	243.8	238.8

† Arithmetic means with standard error values in parentheses.
‡ Mean values calculated using analysis of variance. They were adjusted because of different numbers of values at each PDL level.

7. MEAN JUNCTION WIDTHS W1 AND W 2 IN NM FOR CONTROL AND EXPERIMENTAL ANIMALS AT EACH PDL LEVEL.

	W1				W2			
PDL Level	Control		Experiment		Control		Experiment	
0	66.0†	62.6‡	64.4†	63.2‡	11.5†	11.5‡	11.7†	11.6‡
	(58.9)		(29.9)		(1.4)		(1.8)	
150	70.1	70.1	74.6	74.5	11.3	11.3	10.7	10.7
	(36.5)		(95.1)		(1.1)		(0.7)	
300	76.0	76.8	66.0	66.0	11.5	11.5	12.0	12.0
	(41.2)		(55.5)		(0.9)		(2.2)	
450	60.4	60.4	54.4	52.5	11.6	11.6	11.3	11.3
	(44.6)		(28.2)		(0.9)		(0.9)	
600	69.1	68.9	68.8	70.8	11.1	11.1	11.5	11.5
	(39.9)		(59.1)		(0.7)		(1.4)	
750	57.9	58.2	67.4	66.6	11.4	11.4	11.7	11.6
	(42.9)		(46.6)		(0.9)		(2.4)	
900	43.9	43.9	72.1	70.0	11.2	11.2	11.6	11.5
	(26.6)		(52.9)		(0.9)		(1.2)	
> 900	79.0	93.6	69.1	47.9	11.3	11.2	12.6	12.4
	(66.7)		(33.5)		(1.3)		(1.3)	
Mean	67.2	66.8	67.7	63.9	11.3	11.4	11.6	11.6

† Arithmetic means with standard error values in parentheses.
‡ Mean values calculated using analysis of variance. They were adjusted because of different numbers of values at each PDL level.

8. MEAN JUNCTION WIDTHS W3 AND W4 IN NM FOR CONTROL AND EXPERIMENTAL ANIMALS AT EACH PDL LEVEL.

	W3				W4			
PDL Level	Control		Experiment		Control		Experiment	
0	11.7†	11.7‡	11.6†	11.6‡	11.8†	11.9‡	11.3†	11.3‡
	(1.5)		(1.2)		(1.7)		(1.5)	
150	11.2	11.2	10.8	10.8	11.3	11.3	10.8	10.8
	(0.8)		(0.4)		(0.9)		(0.5)	
300	11.4	11.4	11.5	11.5	11.2	11.2	11.0	11.0
	(0.8)		(1.6)		(0.7)		(0.6)	
450	11.2	11.2	11.5	11.6	11.1	11.1	11.2	11.3
	(0.8)		(1.2)		(0.8)		(0.8)	
600	11.1	11.1	11.5	11.5	11.3	11.3	11.2	11.2
	(0.5)		(1.5)		(0.7)		(1.2)	
750	10.9	10.9	11.4	11.4	11.0	11.0	11.2	11.2
	(0.9)		(1.8)		(0.5)		(1.8)	
900	11.1	11.1	11.0	11.0	11.1	11.1	11.3	11.2
	(0.8)		(1.0)		(0.7)		(0.8)	
> 900	11.1	11.0	12.6	12.5	11.1	11.0	12.5	12.5
	(1.0)		(1.4)		(0.9)		(1.0)	
Mean	11.2	11.2	11.5	11.5	11.2	11.2	11.3	11.3

† Arithmetic means with standard error values in parentheses.
‡ Mean values calculated using analysis of variance. They were adjusted because of different numbers of values at each PDL level.

CHAPTER 9

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