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A T.E.M. STEREOLOGICAL ANALYSIS OF BLOOD VESSELS AND NERVES IN MARMOSET PERIODONTAL LIGAMENT FOLLOWING ORTHODONTIC EXTRUSION

A research report submitted in partial fulfillment of requirements for the degree of Master of Dental Surgery

by

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1991

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SUMMARY

Incisor fracture is a common dental injury. It is treated by endodontic therapy, orthodontic extrusion and a retention period. No ultrastructural studies exist which have stereologically quantified the neural and vascular response of the periodontal ligament (PDL), above the subapical region, subsequent to this treatment regime.

The principal aim of this study was to test the hypothesis that neural and vascular reconstitution of the PDL is incomplete after a retention period of nine weeks.

This investigation was based on material prepared by Crowe (1989), but not utilized in his study which was limited to the apical region. Crowe (1989) used marmosets *(Callithrix jacchus)* as the model to mimic the common clinical treatment regime. His experimental procedure involved incisor crown removal, root canal therapy, followed by magnetic extrusion of 1.2mm. The fractured incisor was then retained in the extruded position for nine weeks.

Each animal was perfusion fixed with 5.6% glutaraldehyde and 0.9% osmium tetroxide mixture. The experimental and control incisor segments were isolated for TEM processing.

Four female marmoset animals were utilized in this present study. The blocks contained the mesial PDL from the cervical to apical region. The blocks were sectioned at 250 micron levels. Twelve slotted grids were taken at

each level and prepared for TEM examination. The zero level was taken as a "streaming" effect of the PDL i.e. orientation of the collagen fibres and cells perpendicular to the tooth surface. The most mesial PDL region of the central incisors was examined. Three micrographs were taken across the PDL width, at the bone, middle and tooth one-third, at each level.

The micrographs were printed for stereological evaluation using standard point counting techniques. The major effects on vascular and neural components of the ligament were assessed to determine if any stereological changes occurred in the experimental PDL.

Blood vessels and nerve axons were assessed for mean caliper diameter ($\bar{d}$), volume density (V_V), surface density (S_V), length density (L_V), and number per unit area (N_A).

The total luminal vascular volume density decreased from 11.26% to 7.66%. There was a statistically significant ($p<0.05$) increase in the mean number of venous capillaries per $560\mu m^2$ of PDL, from 0.07 to 0.17. The vessel wall and luminal volume densities (V_V) of postcapillary-sized venules decreased from 1.64% to 1.03% and 5.32% to 3.04%, at a statistically significant level ($p<0.05$). Mean luminal and abluminal diameters for postcapillary-sized venules were reduced ($p<0.05$). This study is the first to undertake the ultrastructural stereological quantification of both the vessel wall and luminal volume densities, of the marmoset PDL.

The neural volumetric density distribution in the middle, lateral third of the controls was found to be six times that of the bone third and the tooth third contained a negligible innervation. The neural parameters demonstrated no statistically significant change following the experimental regime, suggesting morphological reconstitution of the neural system.

The present study indicates that the PDL above the subapical region, after tooth extrusion with short-term retention (9 weeks), had overall maintained the same vascular volume density (V_V). However, a shift in volume density (V_V) and in number (N_A), for the different blood vessel categories was observed. A statistically significant shift occurred in the mesial venous bed, with evidence of venous capillary 'sprouting'. The significant decrease in postcapillary-sized venules, suggest revascularization was incomplete for this vessel-type.

This project does not support Crowe's essential hypothesis that reconstitution of the PDL is incomplete after a short-term retention of 9 weeks. On the contrary, the same material studied between the cervical and apical regions demonstrated almost complete morphological repair. There was, however, a slight shift remaining in the capillary bed.

This investigation reveals that simulated incisor fracture followed by endodontic and orthodontic treatment results in morphological repair of PDL microvasculature

and axon systems. Therefore, it would seem that this procedure is a justifiable clincial treatment rationale.

SIGNED STATEMENT

This report contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief, it contains no material previously published except where due reference in the text of the report.

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B.D.Sc. (Queensland)

ACKNOWLEDGEMENTS

I wish to thank the following people for their help and support over the past year:

Dr. M.R. Sims, Reader in Orthodontics at the University of Adelaide, for his supervision and invaluable advice;

Vicki Hargreaves, Margaret Leppard and Lorraine McMahon for their suggestions and technical assistance;

Mr. K. Crocker of the Electron Optical Centre for access and instruction in the use of the Transmission Electron Microscope;

Mr. K. Cellier of the Department of Statistics for conducting the statistical analyses of this study;

and special thanks are extended to my family for their continual support and encouragement.

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CHAPTER 1.

INTRODUCTION

Accidental fracture of incisor crowns frequently occurs in school children, athletes, and persons in vehicle collisions. In cases where the fracture extends subgingivally and pulpal exposure results, endodontic treatment is often followed by orthodontic extrusion to allow for the construction of a post-core and crown. Until Crowe (1989) simulated this clinical model in marmosets, there was no published information of the effects of these procedures on the ultrastructural morphology of the periodontal ligament.

Crowe demonstrated significant apical changes in the areal fractions and numbers per unit area of fibroblasts, epithelial cells, unmyelinated nerves and terminal arterioles. However, the Crowe study was confined to the maxillary incisor apex and comprised restricted sampling techniques.

The present study utilized Crowe's female marmoset blocks, except for one additional animal which was added to make a sample of four female marmosets. This study has differed significantly in aims to that of Crowe's study, by quantifying the experimental and control periodontal ligament between the alveolar crest and the apical region. The primary aim of this project was to test Crowe's principal hypothesis that reconstitution of the

periodontal ligament is incomplete after a retention period of nine weeks.

Silver sections of whole width periodontal ligament were cut with a diamond knife, at intervals of 250 microns. The sections were floated diagonally on R150A mesh uncoated multi-slotted copper grids. They were then stained with modified Reynolds' lead and uranyl acetate for examination and recording in a Jeol 100S transmission electron microscope. Magnifications were calibrated against a cross grating replica for measurement accuracy. Standard point counting methods (Williams, 1977; Weibel, 1979) were employed with the aim to provide morphometric and stereological data (Casley-Smith and Window, 1976; Cruz-Orive and Weibel, 1981; Atherton, Cabric and James, 1982; Baddeley et al., 1986; Gundersen et al., 1988a,b; Nyengaard et al.,1988; Gundersen, 1989) of the ligament components including blood vessels and unmyelinated and myelinated axons (Freezer and Sims, 1987) following short-term tooth retention.

CHAPTER 2.

AIMS OF THE INVESTIGATION

The purpose of this investigation was to section blocks prepared by Crowe (1989) from four female marmosets, in which the upper left central incisors had been endodontically treated and extruded. The right central incisors acted as the control. Sections were taken from the mesial aspect of the periodontal ligament at 250μm levels, between the alveolar crest and apical region.

The specific aims of this investigation were:

1. To prepare material for TEM examination of the marmoset incisor periodontal ligament

2. To provide information regarding the vascular and neural distribution within the periodontal ligament with particular reference to the lateral and vertical thirds.

3. To use standard point counting methods to provide morphometric and stereological data.

4. To calculate stereological parameters (V_V, L_V ,S_V and N_A) and mean diameters for periodontal blood vessels and nerve axons.

5. To statistically analyze the responses of the vasculature and neural axons within the periodontal ligament, following the treatment regime of endodontic therapy, orthodontic extrusion and a nine week retention period.

6. To test Crowe's principal hypothesis that reconstitution of the periodontal ligament is incomplete after a short-term retention period of nine weeks.

CHAPTER 3.

LITERATURE REVIEW

3.1 FRACTURES OF ANTERIOR TEETH

Traumatic injuries to the facial area often result in fractured anterior teeth. Common aetiological factors include: battered child syndrome, school ground injuries, bicycle injuries, automobile injuries, etc. (Andreasen, 1981).

The incidence of these injuries was found to be higher in boys than in girls (Burton et al., 1985; Zadik et al., 1980; Grundy, 1959; Hargreaves, 1981). The lack of muscle coordination and resultant inability to protect themselves by learned reflexes may possibly be the reason for the high incidence in young people (Edward and Nord, 1968).

If the fracture occurs at the gingival margin or below the alveolar crest, exposure of tooth structure becomes paramount to restore the tooth. Fugazzotto (1985) discussed the implications of subgingival crown margin placement, such as an increase in incidence of periodontal problems and the risk of recurrent decay.

To avoid placing deep subgingival crown margins, Heithersay (1973) suggested a combination of endodontic therapy and orthodontic extrusion. This procedure has been used clinically by others (Potashnick and Rosenberg,

1982; Garrett, 1985; Biggerstaff et al., 1986). Ingber (1976) reported that other methods to manage a fracture at the gingival margin are avoided i.e. extraction of the remaining root and replacement with a fixed prosthesis or periodontal surgery, which involve many compromises.

Ingber (1976) concluded from his experiment that as a tooth is erupted, the gingiva and alveolar crest will follow and the resultant change in position of soft tissue is due to an increase in the zone of attached gingiva. This finding supported his hypothesis that when a root segment is orthodontically erupted the supporting structures will be further coronal than the adjacent teeth and corroborated the earlier findings of Heithersay (1973).

Ingber (1976) also reported the crown to root ratio may remain virtually unchanged or possibly improve in comparison to that of the treatment method of osseous surgery.

3.2 THE EFFECT OF ENDODONTIC THERAPY ON THE PERIODONTIUM

Histological studies have been carried out to determine the effects of root canal therapy on the periapical tissues (Seltzer et al., 1969; Bhaskar and Rappaport, 1971; Malooley et al., 1979; Pascon and Spangberg, 1990).

Gutta-percha has been the most widely used solid-core root canal filling material (Ingle and Taintor, 1985). Recently, the inertness of gutta-percha has been challenged. Pascon and Spangberg (1990) evaluated a number of commercially available gutta-percha materials. It was demonstrated that pure raw gutta-percha was non-toxic but zinc-oxide, which is the major component of gutta-percha points, showed high toxicity.

Gutta-percha alone does not adhere to the dentine walls. The use of a sealer is recommended in order to achieve a satisfactory seal. Murzabal and Erausquin (1966) noted a mild periapical inflammatory reaction to AH26^{R} sealer cement.

A number of studies have demonstrated that in specimens in which the root canal fillings reached the constructed apical stops, less periapical inflammmatory reaction was observed. By contrast, there was more inflammation in those specimens which were overfilled (Bhaskar and Rappaport, 1971; Soares et al., 1990).

3.3 THE EFFECT OF EXTRUSION ON THE HISTOLOGY AND ULTRASTRUCTURE OF THE PERIODONTIUM

The microscopic effect of extrusion on the periodontal ligament has been examined by Huettner and Whitman (1958), Reitan (1967, 1974), Batenhorst et al. (1974), Rygh (1976), Sims (1978, 1980), Simon et al.

(1980), Van Venrooy and Yukna (1985), Lew (1986) and Melsen (1986).

Huettner and Whitman (1958) found that the direction of the periodontal fibres changed to follow the line of movement and new bone spicules formed in the apical region, during extrusion for 12 weeks of incisors in *Macaque rhesus* monkeys.

Reitan (1967) observed new bone formation in the apical region and later at the alveolar crest on extrusion of human teeth. The force recommended was not to exceed 0.24 to 0.30N. In 1974, Reitan studied the initial tissue reaction observed in the apical region of 30 human teeth after extrusion with various force levels. He observed a variation in the histology between cases, such as small resorptive lacunae, hyalinization of the ligament, resorption of the middle third of the root and some apical cemental deposition. The root resorption in areas of ligament tension was considered an exception rather than a rule.

The magnitude and duration of loads vary widely in the studies carried out on tooth extrusion (Parfitt, 1960; Moxham and Berkovitz, 1979; Cooke and Scheer, 1980), thus making comparisons difficult. Parfitt (1960) used a load of 0.15-0.3N on human incisors. Cooke and Scheer (1980) suggested that the ideal force is variable but usually within the range of 0.7-1.5N, which was based on clinical impressions.

Batenhorst et al. (1974) examined with light microscopy the effect of tipping forces, with which extrusion occurred inadvertently, on the histological structure of the periodontium. However, the sample size of two Rhesus monkeys was small. Changes which occurred labially, interdentally, lingually and apically were reported on in detail. Batenhorst et al. (1974) concluded that the gingiva and alveolar crest followed the extruding teeth as the result of an increase in the zone of attached gingiva.

Simon et al. (1980) examined the combined effect of root canal therapy and extrusion on the histological structure of the PDL in dogs. They noted that the alveolar housing reconstitutes occlusally with the tooth movement and is later followed by some bone deposition at the alveolar crest and throughout the interradicular portion. After seven weeks, the periodontal ligament appeared histologically normal and no large areas of resorption or nonattachment of PDL were observed.

Van Venrooy and Yukna (1985) studied the effects of extrusion on teeth with advanced periodontal disease in the beagle dog. The sample size was limited, consisting of three adult female dogs. The extruded teeth showed shallower pocket depths, less gingival inflammation and no bleeding on probing. After stabilization, new bone was seen coronal to the alveolar crest. This bone formation was verified radiographically and histologically. The

improved periodontal condition resulting from orthodontic extrusion may have been due to both physiologic and microbiologic changes in the local environment of the teeth.

The only ultrastructural studies of extrusion are those of Sims (1978, 1980), Lew (1986), Lew et al. (1989), and Crowe (1989).

The effects of extrusion on the apical vasculature of the rat molar PDL were studied by Lew (1986) and published by Lew et al. (1989). A large load of 1.0N was applied for 30 minutes. In particular, they found:

(1) The PDL width increased, resulting in the formation of a tension zone.

(2) There was an increase in the average vascular volume of each vessel type tested. The average endothelial surface area increased significantly. Lew suggested the increase in the functional microvascular bed may represent the increased metabolic demands in an injured tension zone. These vascular changes support the findings of Rygh et al. (1986) who implicated the vascular system as a "main mediator" in PDL remodelling following tooth movement.

Crowe (1989) studied the TEM effects of extrusion on the apical region of the marmoset periodontal ligament after a retention period of 9 weeks. A load of 0.2N was applied to extrude an upper central incisor. Crowe differed from the latter studies (Sims, 1978,1980; Lew, 1986; Lew et al., 1989), by also carrying out an ultrastructural examination of cellular and neural changes. He found:

(1) The mean areal fraction, number per unit area and profile boundary length per unit area of fibroblasts increased.

(2) There was a dramatic change in neural content. The ratio of unmyelinated nerves/myelinated nerve volume density changed from 1:4 to 3:2.

(3) The experimental teeth showed a change in make up of the vascular bed. The true capillary bed decreased while the areal fraction and boundary profile of the terminal arterioles increased. Thesleff et al. (1987) noted small arterioles had receptor sites for EGF. Crowe suggested from his study a possible link between arterial ingrowth and the increase in epithelial cell number.

(4) The vascular proportion of the marmoset control and experimental apical periodontal ligament was 11% and 10% of the total apical ligament respectively.

(5) The number of epithelial cells increased. This morphological change may be associated with the role of prevention of root resorption (Lindskog et al., 1983). The epithelial cells in other tissues have been shown to be involved in the formation of different chemotrophic substances, which may assist in the regeneration and repair of connective tissues. An example is neural growth factor which has a role in regeneration of neural tissue. It is interesting to note the increase Crowe found in proportions of epithelial cells and unmyelinated nerves.

Crowe's findings are only representative for the plane of section examined. No ultrastructural studies are present which demonstrate the response of the whole PDL to root canal therapy and orthodontic movement.

3.4 THE USE OF MAGNETS IN ORTHODONTICS

Blechman and Smiley (1978) first demonstrated magnetic force in an animal study as a viable alternative

to other force systems in orthodontics. Recent studies have demonstrated the use of magnets in orthodontic treatment cases (Blechman, 1985; Kaira et al., 1989).

Cerny (1982) claimed that a more physiological force is applied to the periodontal tissues using magnetic forces. Conventional orthodontic appliances place a larger initial force on the tooth and its periodontium which decreases with time. Magnetic attraction forces are the opposite. Using adult female dogs, Cerny (1980) concluded that cobalt-samarium magnets caused no histological dental tissue damage. The magnetic field flux ranged between 95 and 0.1mT.

3.5 RETENTION

Retention is that period of orthodontic treatment during which a passive appliance is used to maintain the postorthodontic correction. The retention requirements have not been examined histologically. Shapiro and Kokich (1981) discussed the aspect of retention based upon clinical observations due to the lack of research material. They came to no conclusive result and suggested the length of retention time should be determined by radiographic documentation of the width of the periodontal membrane space.

Moxham and Berkovitz (1979), after applying 0.05N extrusive load for a short period of time, noted at the

end of the extrusive recovery cycle the tooth did not return to its original position. The tooth showed a slightly extruded position. Fluid or vascular changes in the tissues were implicated as the possible mechanism by which this finding occurred.

Crowe (1989) examined the apical PDL in experimentally extruded marmoset teeth after a retention period of 9 weeks. He claimed the PDL was reconstituting by neural and vascular sprouting. Casley-Smith and Vincent (1980) studied the quantitative variations in tissue channels after subcutaneous tissue injury of mice. The tissue appeared relatively normal after 3 to 4 months, except for the tissue channels which returned to normal after 6 months. Revascularization appears to be slow in injured tissues. This may explain the findings reported by Crowe(1989), after a 9 week retention period, in the marmoset PDL.

3.6 THE MARMOSET AS AN EXPERIMENTAL ANIMAL

Marmosets are a group of New World monkeys (Figure 3.1, p.51). They have a similar dentition to man (Swindler, 1976), except that the marmoset has three premolars and two molars. They have been found to be suited for dental research as an analogue for man.

They appear to have a similar TMJ anatomy to man, and a comparable masticatory function (Wilson and Gardner,

1982). It would then seem that the periodontal ligament is put under similar functional load vectors to those of man.

Dreizen et al. (1967), Levy and Bernick (1968a,b), Bernick and Levy (1968a,b), Levy et al. (1970), Skougaard et al. (1970), Levy (1971), Levy et al. (1972), Cohn (1972), Grant et al. (1972), Page et al. (1974), Levy (1976), Bernick et al. (1977) and Douvartzidis (1984), found the periodontal ligament of the marmoset to be histologically similar to man.

3.7 THE MICROSCOPIC ANATOMY OF THE PERIODONTAL LIGAMENT

The periodontal ligament consists of cells, fibres, nerves, vascular components and ground substance. Berkovitz and Shore (1982), Lindhe (1983), Schroeder (1986) and Berkovitz (1990) recently reviewed these elements in detail.

This present study will examine the ultrastructural features of the PDL using the Transmission Electron Microscope (TEM). Hence, criteria used for profile identification and the distribution and volumetric proportion of structures that constitute the PDL will be dealt with in this review.

Freezer (1984) and Crowe (1989) examined the PDL of mouse mandibular molars and marmoset maxillary incisors, respectively. Freezer (1984) calculated stereologic

parameters, in contrast to Crowe's (1989) morphometric calculations. They provided the first general quantification of the PDL ultrastructure. Prior to their studies only limited quantification of individual components had been reported. (Beertsen and Everts, 1977; Gotze and Kindler, 1974; Gotze, 1976; Gould et al., 1977; Jonas and Riede, 1980; Deporter et al., 1982; Shore et al., 1982; McCulloch and Melcher, 1983; Shore et al., 1984).

3.7.1 CELLS OF THE PERIODONTAL LIGAMENT

Freezer (1984) categorized the cells into: fibroblasts, osteoblasts, cementoblasts, osteoclasts, progenitor cells, epithelial cells, vascular and perivascular cells, neural and perineural cells and macrophages. Since cementoblasts, fibroblasts, osteoblasts, and progenitor cells were similar morphologically, he grouped them together under the heading of synthetic cells.

The cells of the periodontal ligament are not a component of the present investigation, due to time constraints, although their relevance to the periodontal ligment can not be ignored. The principal studies of the various cells are as follows:

a. Fibroblasts

Fibroblasts are the most numerous cells of the periodontal ligament. In the mouse incisor, they occupy 50 - 60% of the volume of the densely collagenous portions (Beertsen and Everts, 1977). Freezer (1984) using a stereological technique found a lower proportion of fibroblasts in the mouse molar ligament. The bone side had the lowest value of 20.3%. However, Beertsen and Everts (1977) did not include neural and vascular components in their estimation which, therefore, is not the true proportion.

Crowe (1989) examined the apical PDL of the marmoset incisor and found that fibroblasts occupy 18.3% of PDL extra-vascular area.

Several reports have documented the morphologic and functional polarization of periodontal ligament fibroblasts. (Beertsen et al., 1979; Garant and Cho, 1979; Cho and Garant 1981a,b,c,d, 1984). The microtubular network supports the cytoplasmic polarization and the secretary pole was identified as the trailing end of the cell. Cytoplasmic polarization may be associated with cell migration and possibly tooth eruption.

The cytoplasmic processes of periodontal fibroblasts frequently contact each other and have been classified on the basis of morphological characteristics (Frank et al., 1976; Beertsen and Everts, 1980). Beertsen and Everts

(1980) and Shore et al. (1981) distinguished three intercellular contact types between fibroblasts in rat PDL, ie. simplified desmosomes, gap junctions and close contacts. The most frequent type seen was the simplified desmosome which was characterised by an increased density of the cell membranes, underlying cytoplasm and a narrow intercellular space.

The fibroblast is involved in collagen production (Beertsen et al., 1974a,b; Ten Cate and Deporter, 1974, 1975; Ten Cate et al., 1976; Frank et al., 1976; Beertsen and Everts, 1977; Shore and Berkovitz, 1979) and intracellular lysis (Ten Cate and Deporter, 1975; Garant, 1976; Deporter and Ten Cate, 1980; Berkovitz and Shore, 1982; Beertsen, 1987). Svoboda et al. (1979a,b) demonstrated phagocytosis of collagen in serial thin sections. Hirashita et al. (1985) reported ACP-ase and ATP-ase in the extracellular breakdown of the collagen profiles. Cho and Garant (1981a,b,c,d, 1986, 1987) demonstrated the role of the fibroblast in glycoprotein synthesis.

b. Osteoblasts

Osteoblasts of the periodontal ligament occur at and along the alveolar bone during active osteogenesis. They may form a cell chain covering osteoid in very active phases, while cells with little cytoplasm are present

during quiescent phases (Berkovitz and Shore, 1982; Schroeder, 1986).

Osteoblasts are similar in ultrastructure to fibroblasts. Recently, Berkovitz and Shore (1982) and Fawcett (1986) described their distinctive features including:

(1) a columnar or cuboidal cell shape with a basophilic cytoplasm;

(2) a nucleus polarized away from the bone surface;

(3) a more pronounced Golgi apparatus (situated between the nucleus and cell base) compared to fibroblasts;

(4) prominent microfilaments and microtubules near the bone surface; and

(5) small lipid droplets and membrane bound dense bodies within the cytoplasm.

Freezer (1984) found that osteoblasts occupy 0.3% of the overall periodontal ligament in mice, excluding the apical region. Crowe (1989) examined the apical PDL of marmosets and found osteoblasts to occupy 1.45% of PDL extra-vascular area.

c. Cementoblasts

Until recent work carried out by Yamasaki et al. (1986, 1987) and Rose et al. (1987), cementoblasts were very difficult to distinguish from fibroblasts. They described some differentiating characteristics:-

(1) cementoblasts consistently revealed a higher mean-volume density per tissue unit than the fibroblasts.

(2) relative volume of glycogen particles per cytoplasm was significantly higher in cementoblasts.

(3) microfilament (6nm diameter) bundles and mesh work densities of the contractile type tended to be seen more preferentially in cementoblasts (immature collagen-producing cells (CPC)).

(4) cementoblasts exhibiting cytological profiles of active CPC were not as common as the immature and resting groups.

Due to the specific techniques required to identify the glycogen, it is not a practical discriminating morphological feature. Yamasaki et al., (1986) also discussed possible roles associated with glycogen accumulations and microfilaments.

Freezer (1984) quantified the volumetric density of the cementoblasts in the mouse molar PDL to be 1.6% of the tooth third of the ligament and 0.5% of the total ligament. Crowe (1989) reported that cementoblasts occupy 3% of PDL extra-vascular area.

d. Osteoclasts

Osteoclasts are multinucleated giant cells whose primary function is the extracellular resorption of bone. The resorbing apparatus consists of a ruffled border and a surrounding 'clear zone' (Bonucci, 1981).

In the electron microscope, there are distinguishing features of the osteoclast which aid in identifying the

cells (Matthews et al., 1967; Gothlin and Ericsson, 1976; Berkovitz and Shore, 1982; Freezer, 1984; Fawcett, 1986):

(1) individual nuclei lack distinctive features and are located in the part of the cell which is remote from bone.

(2) cytoplasm contains a high concentration of lysosomes (0.5 to 3 microns in diameter).

(3) a circumnuclear configuration of the Golgi apparatus, placed at intervals around each nucleus.

(4) single membrane-bound "dense bodies" (0.2 to 0.5 microns in diameter).

(5) a ruffled border which constitutes a specialized area of the plasma membrane consisting of a complex system of cytoplasmic infoldings and microvillous projections. There is a distinctive coating of fine bundle-like structures.

(6) the cell surface next to bone is characterized by close adaptation of the plasma membrane and an adjacent organelle-free area, rich in actin filaments, the 'clear zone'.

Freezer (1984) carried out quantification of the mouse molar PDL osteoclast. He found the volume density to be 0.6% of the total ligament and 1.9% of the bone third of the ligament.

e. Progenitor Cells

Progenitor cells proliferate and produce differentiated cells which can synthesize bone, cementum and extracellular matrix within the mouse PDL (Melcher, 1980; McCulloch, 1985). The origin of these cells is unknown. McCulloch and Melcher (1983) reported that 40%

of the proliferative cells reside within 10 microns of periodontal blood vessels. Gould et al. (1977) reported some progenitor cells near the root surface.

Michaeli et al. (1979, 1987, 1988) noted that the apical PDL is the prime source of proliferating cells. Michaeli et al. (1987) also demonstrated a paracemental progenitor compartment, present along the length of the tooth which seemed to be supplied by progeny of the apical periodontal compartment.

f. Epithelial Cells

Epithelial cell rests of Malassez of the developing and established periodontal ligament are derived from the disintegrating sheath of Hertwig. Gurling (1982) reported in the mouse molar, that more coronally, they also arise from the reduced enamel epithelium.

Normally epithelial cells are inactive, but are capable of metabolizing and proliferating (Ten Cate, 1967). Wentz et al. (1950) distinguished three different morphologic types in the rat molar. Wentz et al. (1950) also noted that the incidence of epithelial remnants decreased with increasing age.

Reports have been made on the distribution of epithelial cells within the PDL and disparity in findings arise. This may possibly relate to differences in technique, age or species studied (Reeve and Wentz, 1962). Wentz et al. (1950) found the majority in the supra-

alveolar areas. Freezer (1984) found them to be most numerous around the apices of the teeth.

Lester (1969) examined the ultrastructure of the epithelial cells. Epithelial cells are initially surrounded by and separated from the connective tissue by a basal lamina, with hemidesmosomes and anchoring fibrils attached to it on either side. Two types of junctional complexes, desmosomes and gap junctions are seen between the epithelial cells. The cytoplasm contains tonofilament bundles, sparse endoplasmic reticulum, glycogen granules, abundant polyribosomes, numerous mitochondria and the Golgi complex. As the cells matured, the cytoplasmic volume decreased, tonofilament bundles became more prominent and rough endoplasmic reticulum assumed the form of small dilated sacs. Nuclear pores became more marked, membraneous vesicles near the Golgi apparatus dilated and lipid droplets became visible (Valderhaug and Nylen, 1966).

The function of the epithelial cells has not yet been resolved. Loe and Waerhaug (1961) and Lindskog et al. (1983) suggested a possible role in preventing root resorption. The epithelial cells may produce protease inhibitors which protect the root surface from osteoclastic resorption and contribute to the integrity of the PDL (Lindskog et al., 1983). Brice (1988) suggested a possible role in controlling the repair of resorbed surfaces.

Epithelial cells have been calculated to occupy 0.7% of the PDL volume (Freezer, 1984). Crowe (1989) found that following extrusion epithelial cells showed a marked increase in the number of epithelial cells per unit area.

g. Vascular and Perivascular Cells

i. Endothelial Cells

Blood vessel walls are comprised of an endothelial cell lining with or without surrounding perivascular cells. Avery et al. (1975) studied the ultrastructure of terminal blood vessels in the mouse molar periodontal ligament. Observations revealed endothelial cells with thin finger-like projections which extended into the lumen. The nucleus was also seen to protrude into the lumen. Distinctive features of the cytoplasm included numerous microvesicles, scattered ribosomes and small oval mitochondria. Tortuous intercellular junctions were seen between the endothelial cells. Endothelial and smooth muscle cells occasionally formed myoepithelial junctions. Corpron et al. (1976) reported occasional tight junctions which appeared between endothelial cells lined completely by a distinct basement lamina but incompletely encircled by pericytes.

ii. Pericytes

Rhodin (1967,1968) studied the vascular wall morphology in non-oral rabbit tissue. He described the

ultrastructure of the perivascular elements. Four perivascular cells were identified: veil cells (a form of fibroblast), pericytes, primitive smooth muscle cells and smooth muscle cells. Pericytes were considered to be precursor cells of smooth muscle cells, while the primitive smooth muscle cell represents an intermediate cell type between pericytes and "true" smooth muscle cells.

The ultrastructure of the pericyte is similar to the fibroblast. Rhodin (1968) described distinctive characteristics which included highly branched cytoplasm with extensions embracing the endothelial tubes, less granular endoplasmic reticulum, partially surrounded by a basement membrane, free ribosomes, few intercellular contacts and pinocytotic vesicles located preferentially on the side of the cell nearest the vessel lumen. These cells are most commonly seen adjacent to the vessel walls of venous capillaries, postcapillary-sized venules and collecting venules up to 50 microns in diameter.

Freezer (1984) described pericytes as enlongated cells with little cytoplasmic branching and a basement lamina that was continuous with that of the endothelium in the mouse molar PDL. These cells were more commonly around vessels with a luminal diameter of 4-8μm. In vessels where the luminal diameter was greater than 20 microns this cellular investment was usually absent or incomplete.

Smooth muscle cells are similar to pericytes but are completely surrounded by a basement membrane. They frequently contact other primitive smooth muscle cells, and contain an increased amount of intracellular filamentous material related to dense fusiform bodies. Other cytoplasmic components such as free ribosomes and rough endoplasmic reticulum are more scarce (Rhodin, 1968).

Buchanan and Wagner (1990) carried out a morphometric analysis of electron micrographs of the eel rete mirabile and found that 75% of the pericytic volume was associated with arterially derived capillaries.

The arrangement of pericytes along capillaries and similarities to smooth muscle cells have led to the proposal that they are involved in local control of capillary blood flow (Buchanan and Wagner, 1990). Histochemical studies have demonstrated the presence of tropomyosin, isomyosins, cyclic GMP-dependent protein kinase and muscle actins in pericytes (Joyce et al., 1985a,b; Herman and Jacobsen, 1988; Buchanan and Wagner, 1990).

iii. Pathways Across The Blood Vessel Wall

A number of pathways exist across the blood vascular endothelium such as across the cell, close junctions, vesicles and fenestrae and open junctions or leaks (Renkin, 1977; Casley-Smith, 1983). The microphysiology

of these transport mechanisms provides insight into the permeability characteristics of vessels in relation to function of tooth support and response to tooth-moving forces. A brief review is pertinent to this study.

Casley-Smith (1971) has defined criteria for the recognition and counting of fenestrae. Corpron et al. (1976) observed fenestrated capillaries peripherally and close to the osteoblastic layer, in the mouse PDL microvascular bed. The fenestrae appeared as 30-50nm openings and bridged by a thin membrane continuous with the adjacent endothelial cells. Fenestrated capillaries appeared peripherally and close to the osteoblastic layer. The highest incidence of fenestrae of all vessels was the postcapillary-sized vessels in the mouse PDL (Sims, 1983).

Moxham et al. (1985) carried out a quantitative ultrastructural study to assess the number and distribution of fenestrated capillaries in the PDL of the rat. The molar capillaries showed more fenestrations compared to those of the continuously erupting incisor. This variation may be related to a difference between the functional requirements for the vasculature of these teeth. It was also noted that fenestrae were not distributed uniformly along the incisor length.

Lew (1986) found fenestrae in arterial capillaries and postcapillary-sized venules. The fenestrae in the former were uncommon. Pudney and Casley-Smith (1970) noted a similar difference in fenestral populations

between the arterial and venous side in a number of organs.

Vesicles, endothelial channels and fenestrae possibly belong to the transendothelial transport system (Simionescu and Simionescu, 1983). Casley-Smith et al. (1975) postulated that fenestrae were necessary for increased vessel permeability in areas of large extravascular flow of macromolecules. While Corpron et al. (1976) suggested that fenestrations may represent pathways of rapid metabolites across endothelium to the periodontium where high metabolic requriements for growth or repair occur. Lew (1986) suggested that fenestrated capillaries may be responsible for the return of extravascular protein and metabolites to the vascular system, since the existence of lymphatics in the PDL remains unproven (Edwall, 1982).

Recently, Cooper (1988) and Cooper et al. (1990) investigated the transport of an ionic tracer molecule, via the 'tissue channel', across the PDL vascular endothelium. Cooper reported an increase of PDL tissue channels in the apical region of rat maxillary first molars incident to a 1.0N continuous extrusive load . Lew (1986) investigated the effects of a tension load on the fenestral number and dimensions of the small blood vessels. An increase occurred in both the number and diameter of fenestrae in arterial capillaries, venous capillaries and postcapillary-sized venules.

h. Neural and Perineural Cells and Nerve Endings

Ultrastructural features of human PDL Schwann cells have been described by Griffin and Harris (1968). Some distinguishing features included marginal condensations of chromatin and several nucleoli within cell nuclei, numerous mitochondria and a plasma membrane 7.5nm thick.

Another reported cell type is the K-cell (Beertsen et al., 1974b; Everts et al., 1977; Berkovitz and Shore, 1978; Maeda et al., 1989). K-cells were noted to occur adjacent to mitochondria rich unmyelinated nerves in rodent incisor PDL, while Freezer (1984) observed that they occurred predominantly near blood vessels. Freezer and Sims (1989) described distinguishing morphologic features such as rounded cross-section, kidney-shaped nucleus, prominent Golgi apparatus, abundant peripheral microvesicles and some extensions of rough endoplasmic reticulum with dilated cisternae.

Maeda et al. (1989), using immunohistochemistry and electrom microscopy, concluded that K-cells were terminal Schwann cells associated with Ruffini endings in the PDL of rat incisors. Byers (1985) failed to observe a similar finding in the periodontal ligament of rat molars.

Freezer and Sims (1988) reported a statistically significant association between K-cells and apericytic postcapillary-sized venules and between K-cells and

unmyelinated and myelinated nerves. It was postulated that these structural associations may comprise complex receptor or effector mechanisms.

There appear to be several types of nerve endings within the PDL that have been reported in various species (Bernick, 1952; Held and Baud 1955; Griffin and Harris, 1974a,b; Harris and Griffin 1974a,b). Everts et al. (1977) in a study of mouse periodontal ligament, classified them into three morphologically distinct groups:

(1) convoluted endings.

(2) endings of fibres terminating as lamellae, knob-, spindle- or leaf-like structures.

(3) fine delicate fibres which seem to terminate as free endings.

Griffin and Harris (1974a,b) using electron micrographs described encapsulated, convoluted nerve endings. These encapsulated nerve endings were designated mechanoreptors. There has been controversy over the type and structure of nerve endings. Byers (1985) interpreted the findings reported by Griffin and Harris (1974a,b) as preterminal axon bundles rather than encapsulated receptors. Corpron et al. (1980) reported corpuscles of the Golgi-Mazzoni type that may in fact correspond to large Ruffini endings (Byers 1985). Byers (1985) reported six receptor categories of Gasserian ganglion origin (Figure 3.2, p.52). No mention was made of lamellated receptors although they have been identified in other work

(Bonnaud et al. 1978 ; Berkovitz et al. 1983). Within the rat molar, unencapsulated Ruffini-like endings are the only type of mechanoreceptors (Byers 1985).

Cash and Linden (1981) reported that only one physiological receptor type exists within the ligament. The ultrastructural findings reported by Byers (1985) and Freezer and Sims (1989) contrast markedly with this suggestion.

Until work by Byers et al. 1986, little was known of the structure of mesencephalic mechanoreceptors. ^{3}H-proline was used to label the mesencephalic trigeminal nucleus (Mes-V) of the cat brain.

Mes-V receptors were confined to ipsilateral periodontal ligament, close to the root apex, with greater innervation on the posterior side. Some of the teeth had focal areas with dense innervation. Mes-V axons formed unencapsulated Ruffini-like mechanoreceptors in the PDL. The bundles of unmyelinated axons and the rare encapsulated endings were not labelled.

3.7.2 PERIODONTAL VASCULATURE

a. Anatomy and Distribution

Much of the current knowledge of the periodontal vasculature has been reviewed by Edwall (1982) and Schroeder (1986).

Arterial blood communicates with the periodontal ligament via intraosseal and periosteal channels. Arterioles approach the peridontal ligament through marrow spaces of the interdental and interradicular portions of the alveolar process and eventually enter at various levels (Kindlova and Matena, 1962; Kindlova, 1965; Castelli and Dempster, 1965; Carranza et al., 1966; Rohen et al., 1984).

The veins leave the periodontal ligament and join those of the alveolar septa, which in turn are linked with a venous network in the marrow spaces surrounding the apex of each tooth (Castelli and Dempster, 1965).

Birn (1966) investigated the vascular supply of the human periodontal ligament. The blood supply was found to increase towards the posterior teeth and in single-rooted teeth be greatest in the cervical third. Birn (1966) estimated the vascularity of the PDL on the unvalidated assumption that each alveolar wall perforation indicated the passage of a blood vessel (Freezer, 1984).

Sims (1987) and Freezer and Sims (1987) described the anisotropic distribution of microvascular volume in the PDL of the mouse mandibular molar. Vertically, the apical region was the most vascular area. The maximum vascular volume occured in the lingual quadrants. The vascular volumes in the mesial and the distal quadrants were slightly greater than those in the buccal quadrants.

The main vessels of the periodontal ligament were arranged in palisades and ran parallel to the long axis of the root. The vessels branched to form capillaries arranged in a flat network, ie. a venous plexus. These vessels were located nearer to the alveolar wall than to the root surface. In the cervical region, this network gave off single capillaries which form coiled structures resembling glomeruli (Lenz, 1968; Freezer, 1984).

Using the SEM vascular casting technique, Weekes and Sims (1986) described the rat molar PDL as a predominantly venous microcirculatory bed. A TEM investigation of the PDL of the mouse mandibular molar by Sims (1983) demonstrated postcapillary-sized venules as the predominant vessels in the venous bed.

Wong and Sims (1983,1987) examined the microvascular bed of the mouse PDL. Scanning electron microscopy of methyl methacrylate vascular perfusion casts with stereopair imaging was used. Characteristic microvascular patterns occurred in the cervical, middle and apical thirds. Cervical third venous vessels were orientated occluso-apically, either as single vessels or grouped to form tracts. Polygonal anastomoses, from which vascular branches arose to link with medullary vessels, were accompanied by an intertwining capillary network. The middle third microvascular bed was characterized by vertically - orientated capillary loops which connected

the arterial and venous vessels. The apical third had a distinct, irregular, anastomosing venous pattern.

Using corrosion castings, Lee (1989) examined the periodontal microvasculature of marmoset molars and premolars. The cervical and middle thirds comprised mainly of postcapillary-sized venules running occluso-apically. Arterioles were less commonly observed and branched less frequently compared to venules.

b. Classification

Bevelander and Nakahara (1968) and Frank et al. (1976) studied the ultrastructure of the periodontal ligament. They observed that the vessels differed in calibre and were all thin-walled, but no method of categorization was attempted.

Bennet et al. (1959) and Rhodin (1967, 1968) have classified blood vessels in other tissues. Bennet et al. (1959) based their classification on the presence or absence of a continuous basement membrane, on the number and position of fenestrae and on the presence or absence of pericytes. Rhodin's classification was based on the lumen calibre, the endothelial cell lining, the patterns of vessel branching and anastamosis and the constituents of the blood vessel wall (Table 3.1, p.48 and Table 3.2, p.49).

Wiedeman (1984) pointed out the problems associated with classifications based on diameter alone. In the

living animal, vessel diameter is not constant due to autoregulatory mechanisms. According to Wiedeman (1984), position and function of vessels should be considered in classfying vessels.

Due to the shortcomings of the Bennet et al. (1959) classification i.e. capillaries defined as any small blood vessel, Majno (1965) classified capillaries into three groups based on the type of endothelial wall:-

Type I (continuous),

Type II (fenestrated) and

Type III (discontinuous).

No attempt was made to classify the capillaries as venous or arterial.

Avery et al. (1975) carried out a TEM examination of mouse PDL and demonstrated capillaries and precapillary vessels. The capillaries had occasional pericytes and endothelial, finger-like projections extending into the lumen. Precapillary vessels displayed thicker endothelial walls and an incomplete muscular coating. A basement lamina separated the endothelium and the surrounding connective tissue from the muscular layer.

Simionescu and Simionescu (1984) defined capillaries as as the smallest ramification of the microvascular system, with a luminal diameter of 5-10μm.

Clark (1986) and Lew (1986) examined the apical microvascular bed of the mouse molar as part of experiments investigating the respective effects of

intrusion and extrusion. The terminal arterioles had 1-2 layers of smooth muscle cells, each cell being 0.5-1.5μm thick. Clark (1986) found that the luminal diameter ranged between 10-30 μm, while Lew (1986) found a range between 18-24μm. The arterial capillaries had a luminal diameter of 5-10μm with 1 to 2 endothelial cells encompassing the lumen. Lew (1986) described the endothelial cells to be 0.09-0.25μm thick and to have numerous knob-like microvilli projecting into the lumen.

Fenestrated venous capillaries had a luminal diameter of 8-10 μm surrounded by 1-3 endothelial cells of 0.08-0.18μm thickness. Pericytes were occasionally noticed. Both Clark (1986) and Lew (1986) reported the nuclei of cells bulged abluminally. Clark (1986) noted venous capillaries with up to 5 fenestrae in the area, while Lew (1986) suggested that the fenestrae occur singly or as pairs.

Postcapillary-sized venules had a luminal diameter of 10-30μm, while collecting venules reached 50μm. The endothelial thickness ranged between 0.15-0.30μm in nonfenestrated and 0.05-0.25μm in fenestrated vessels. The pericyte investment was incomplete and up to 8 endothelial cells surround the lumen. Myelinated and unmyelinated nerves were found in the vessel walls (Lew, 1986).

Lew (1986) reported arteriovenous anastamoses. They were thick-walled and had a luminal diameter of 12-15μm.

The endothelium rested on a sphincter-like media. Myelinated and unmyelinated nerves were observed in the adventitia.

In the mouse PDL, Freezer (1984) devised a classification of microvessels, based on the vessel diameter and the appearance of pericytes. Sims (1983) suggested the term 'apericytic' venules for the venules that did not have complete layers of pericytes and veil cells. Freezer and Sims (1987) reported that 70% of postcapillary-sized venules were devoid of a pericytic cellular investment.

c. Quantification

Freezer and Sims (1987) classified vessels, within the moused PDL, as capillary-sized and postcapillary-sized venules and calculated stereological parameters (Table 3.3, p.50). In comparing their study with previous investigations, Freezer and Sims (1987) confirmed the finding by Gould et al. (1977) that 73.4 percent of the vessel volume is in the bone half of the ligament and by McCulloch and Melcher (1983) that the bone-half of the ligament is four times more vascular than the tooth-half. However, the sampling techniques of McCulloch and Melcher (1983) were unacceptable since they examined only selected regions. Hence, their results are not directly comparable to those of Freezer and Sims (1987). Sims (1980) reported regional human premolar volumetric proportions as high as

20 percent which contrast with the low vascular proportions reported by Wills et al. (1976) and Gotze (1976).

Crowe (1989) found the vascular proportion of the control marmoset apical PDL to be 11% of the total apical ligament. The proportion of the experimental PDL was 10%. The marmoset incisor PDL had a predominately venous vascular bed with venous vessels occupying 95% of the vascular area and 76% of the vessel length.

Rygh et al. (1986) noted that in tipped rat molars, the vascular proportion increased in the tension zone. His assessment was unreliable since it was only a visual assessment and could have been due to observer error or animal variability. Lew (1986) showed vascular dilatation occurred after the short term extrusion of a rat molar.

Crowe (1989) noted some interesting changes in experimentally extruded teeth. He reported a dramatic increase in the areal fraction and boundary profile length of the terminal arteriole group. A fall in caliper diameter was noted across most vessel groups. Crowe (1989) concluded that revascularization of the apical PDL was still incomplete in marmosets, nine weeks after cessation of extrusion. Revascularization appeared to occur by an increase in length of terminal arterioles, supplying blood to a sparser capillary network, and through a reasonably intact postcapillary-sized venule and collecting venule network.

Lew (1986) found continuous capillaries to comprise 91% of all capillaries. Lew et al. (1989) reported 95% of total vascular volume was provided by capillaries and postcapillary-sized venules. Comparison of the control and experimental PDL, following short-term extrusion, showed a significant increase in mean vascular volumes for postcapillary-sized venules, venous capillaries, arterial capillaries and terminal arterioles.

Douvartzidis (1984) carried out a light microscopic morphometric investigation of the periodontal ligament vascular distribution of the marmoset monkey mandibular second molar. Serial horizontal sections were taken at 8μm from the the cervical to apical region. Lateral vascular distribution reported was tooth third (2.6%), middle third (13.1%) and alveolar third (8.9%). The overall vascular volume was 8.3%. In general, the vascular volume increased from the cervical to apical region.

Crowe (1989) reported the vascular proportion of the marmoset control periodontal ligament was 11% of the total ligament. The microvascular bed was predominantly venous, with the venous vessels occupying 95.36% of the control vascular area.

d. Function

The functional role of the periodontal ligament vasculature has not yet been fully resolved.

Investigations have implicated it in the mechanisms of tooth eruption and tooth support (reviewed by Moxham and Berkovitz, 1982).

Due to anatomical considerations and reliable techniques for use, experiments determining properties such as rates of blood flow are difficult (Berkovitz, 1990). Limited information can be deduced from structural observation on fixed material.

Kvinnsland et al. (1989) measured blood flow rate in rat molars, subjected to 0.3-0.5N orthodontic forces. Blood flow rate was measured by injecting the animals with fluorescent microspheres of appropriate size to become lodged in the precapillary arterioles. This allowed measurement of blood flow at the time of injection. An increase in blood flow was reported in all experimental teeth. The response of the vasculature was after short-term application of orthodontic forces for 5 days.

Investigation of the structure and microphysiology of the junctions, vesicles, fenestrae and tissue channels would allow understanding of their role in vascular function, in relation to the passage of substances across the endothelial barrier (Casley-Smith and Vincent, 1980). Casley-Smith (1983) speculated that venous capillary and apericytic postcapillary wall structure facilitates the rapid passage of fluid between vessels and extravascular compartment.

3.7.3 LYMPHATICS

Casley-Smith (1977) described distinctive features of lymphatic vessels:

(1) usually larger than blood capillaries

(2) appear partially or totally collapsed

(3) have an irregular outline

(4) open juctions present between endothelial cells; lack of fenestrae

(5) have abluminal projections where collagenous filaments attach to endothelial cells.

Bloom and Fawcett (1975) noted that pericytes are not found near lymphatic vessels but are frequently observed near blood capillaries.

The presence of lymphatic vessels in the periodontal ligament has been controversial. Levy and Bernick (1968b) reported lymphatic vessels in marmoset PDL, using 20-30μm thick histological sections. Lymphatic vessels originated as blind endings and terminal lymphatic capillaries drained into thin walled collecting vessels which contained valves.

Casley-Smith (1977) stated that lymphatic vessels are difficult to differentiate from blood vessels without prior injection of tracers.

Ruben et al. (1971) using retrograde perfusion of large regional lymphatic collecting vessels with carbon solution, claimed to demonstrate periodontal lymphatic channels in dogs. The light microscope was used to

examine the sections. The lymphatic vessels were described as coursing apically on the alveolar side of the ligament with some passing into the periosteum and the perivascular intraosseus channels. No detailed criteria for identifying the vessels was discussed.

Gilchrist (1978) reported vessels resembling lymphatic vessels in human PDL. Lymphatic vessels in human alveolar bone have also been reported (Barker, 1982). Ultrastructural studies on mice have not confirmed the presence of lymphatic vessels in the PDL (Freezer, 1984).

Crowe (1989) noted one putative lymphatic vessel adjacent to a collecting venule in the apical region of the marmoset PDL. The vessel had valves like a lymphatic capillary wall structure.

3.7.4 PERIODONTAL NEURAL SUPPLY

a. Anatomy and Distribution

Recent reviews on the innervation of the periodontal ligament have been discussed by Hannam (1982) and Schroeder (1986) which follow the earlier synopses by Fearnhead (1967), Anderson et al. (1970) and Van Steenberghe (1979).

The origin of innervation is the trigeminal ganglion which was demonstrated through tritiated-L-proline

injections into the Gasserian ganglion. Autoradiographic labelling was then used to look at the distribution of the nerve endings in the rat molar (Byers and Holland, 1977; Byers, 1985). The periodontal ligament was not heavily labelled, hence supporting the view that some nerves are derived from cell bodies in the mesencephalic nucleus .

The entry of the nerve branches occurs from two locations. Robinson (1979) demonstrated that one approach is via peripheral branches which arise from the dental nerve prior to entering the apical foramen and take a course towards the gingiva. The other approach is via the alveolar bone and take a coronal or an apical course. The above fibre groups join together to form a nerve plexus comprised of coarse fibre bundles running parallel to the long axis of the root and thin fibre bundles giving off terminal branches and individual fibres.

Both myelinated and unmyelinated fibres have been described within the periodontal ligament (Bernick, 1952; Simpson, 1966; Griffin and Harris, 1968; Freezer and Sims, 1987). Van Steenberghe (1979) and Hannam (1982) found that the unmyelinated afferent sensory fibres of the periodontal ligament varied between 0.5 and 1μm in diameter while the myelinated fibres were between 1 and 16μm . Van Steenberghe (1979) demonstrated variations in the neural ultrastructure between species.

Berkovitz and Shore (1978) examined the rat molar PDL and reported that unmyelinated nerve fibres varied from

0.5-4.0μm in diameter. The larger fibres were found to be situated close to the main neurovascular bundles.

Small unmyelinated fibres in mouse molar PDL, were reported to exhibit either a Schwann cell coating or a covering of thin cellular investment rich in microvesicles. Other fibres had a thin basement lamina between themselves and the surrounding collaqen. The terminal ends are bulbous and contain an accumulation of mitochondria, microvesicles and dark granules (Corpron et al., 1974). In the rat molar periodontal ligament it was not clarified whether unmyelinated nerves rich in mitochondria arise from larger unmyelinated nerve fibres or are the distal ends of myelinated fibres (Berkovitz and Shore, 1978). In the human, unmyelinated nerve fibres were reported to arise from myelinated ones (Griffin and Harris, 1968).

The distribution of the terminal branches and nerve fibres (and their endings) forming the periodontal ligament plexus, are often not evenly distributed throughout the whole ligament space (Byers and Holland, 1977; Everts et al., 1977; Kubota and Osanai, 1977). Byers and Holland (1977) and Byers (1985) found that the incidence of nerve endings was greater in the apical third of the ligament than in the coronal portion. Nerve endings were also reported in the avascular, tooth related region. Morphometric analysis of light microscopic and electron microscopic autoradiograms were used.

Kubota and Osanai (1977) histologically observed the nerve distribution density to be greater in the apical region than in the intermediate zone region of the Japanese shrew mole. A greater density was also reported for maxillary, in contrast to mandibular apical regions. Everts et al. (1977) using the mouse incisor found the neural elements to be exclusively in the alveolar compartment.

Freezer (1984) examined the lateral periodontal ligament thirds in the mouse mandibular first molar. A greater mean number of nerve axons per unit area was found in the bone third and decreased towards the avascular tooth region. These findings differ from those of Byers (1985), where most neural components were found in the tooth region of the apical third of the rat molar PDL.

b. Quantification

There is limited information on the quantification of periodontal ligament axons. Recently, there have been transmission electron microscopic studies (Freezer and Sims, 1987,1988,1989; Crowe, 1989), while previous information was derived from light microscopy studies (Kubota and Osanai, 1977; Byers, 1985).

Freezer and Sims (1987) looked at the neural distribution in the mouse molar PDL using stereological parameters. Myelinated nerve fibres constituted 5% of the length of periodontal nerve axons and comprised 26% of the

neural volume. The majority were found close to bone and within the apical one half. Unmyelinated fibres were found in the middle and bone third of the PDL. They constituted 95% of the nerve fibre length. No statistically significant variation in the neural elements was detected along the tooth root.

Freezer and Sims (1989) stereologically quantified the neural structures in the mouse molar PDL. Myelinated and unmyelinated axons were observed throughout the ligament, with 45% of all axons located in the parenchyma of the ligament and 55% occurring in the blood vessel wall. The alveolar annular third of the ligament had twice the innervation of the middle annular third and the tooth third contained only 3% of the periodontal innnervation. The number of periodontal nerves was found to be statistically significant across the width of the ligament ($p<0.01$).

Crowe (1989) looked at the apical PDL of the marmoset monkey. The mean caliper diameter of myelinated and unmyelinated nerves was 4.59μm and 1.32μm, respectively. In the control teeth, the ratio of unmyelinated to myelinated nerves was approximately 1:4. The myelinated nerves comprised 81% of the total neural area. The myelinated nerves were found near the apical foramen. In contrast, the unmyelinated nerve axons were found in all areas of the ligament. The experimentally extruded teeth demonstrated an alteration in myelinated and unmyelinated

nerve ratio of approximately 3:2. Nerve regeneration occurred by neuronal sprouting.

3.8 SUMMARY

Descriptions of the ultrastructural anatomy of the periodontal ligament are numerous. However, until recent investigations (Freezer, 1984; Crowe, 1989), only limited quantifications of the individual components have been provided. Some studies estimated the relative proportion of the cellular components of the ligament, but excluded the vascular and neural components (Beertsen and Everts, 1977 and Deporter et al., 1982).

Until Crowe's (1989) study, no TEM effects of extrusion and root canal therapy on the ultrastructural composition of the PDL had been performed. Crowe reported dramatic changes in the vascular and neural proportions, after short-term retention of 9 weeks. It appeared that the apical PDL was reconstituted by vessel and nerve sprouting, although a statistical analysis was not performed. A thorough stereological analysis needs to be carried out to determine if Crowe's morphometric findings are representative for the whole PDL.

Freezer (1984) and Freezer and Sims (1987) carried out the only studies to stereologically investigate the PDL vascular volume proportions. These studies fulfilled the sampling requirements for a stereological study of

blood vessels and nerves (Nyengaard et al., 1988; Gundersen, 1989). It is difficult to make comparisons between studies, since previous techniques are inadequate compared to the new sampling and mathematical methods discussed by Gundersen et al., 1988a,b). For example, Gould et al. (1977) and McCulloch and Melcher (1983) used planimetric methods of restricted sample section numbers and PDL regions, to compare the percentage of blood vessel area to total ligament area. It is necessary to be aware that differences in reports may be due to animal variability, tooth difference and site specificity, as well as quantification procedures.

Kubota and Osanai (1977), Freezer and Sims (1987) and Crowe (1989) published data on the proportions of nerves in the PDL. Neural growth seems to be assisted by substances such as NGF and cyclic AMP (Thoenen et al., 1988; Lumsden, 1988; Davies, 1988).

A quantitative investigation of the whole PDL using vertical sectioning (Baddeley et al., 1986) may assist in providing insight in explaining the fact that the PDL and its constituent structures can adapt to orthodontic tooth movement over large distances. This adaptation particularly relates to the maintenance of their neural and vascular supply and in allowing the PDL to regenerate.

Blood Vessel Type	Lumen Calibre	Wall Thickness	Endothelial Cell Morphology	Peri-endothelial Cells	Other
Arteriole	100-50μm	Greater than 6μm	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 50μm	Less than 6μm	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 elastic interna, nerves closer to vessel wall with more frequent contacts with the smooth muscle layer, some myoendothelial junctions.
Precapillary	7-15μm	Less than 5μm	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 myo-endothelial junctions.

Table 3.1 Ultrastructural features of arterial blood vessels (Rhodin, 1967).

Blood Vessel Type	Lumen Calibre	Wall Thickness	Endothelial Cell Morphology	Peri-endothelial Cells	Other
Venous Capillary	4-7μm	0.3-1.3μm	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	8-30μm	1-5μm	Cell rarely less than 0.2μm thick and generally larger than that of venous capillary. Slight over-lapping 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 Venule	30-50μm	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	50-100μm	2.0μm	As above.	1-2 layers of smooth muscle cells.	Veil cells form a complete layer arourd vessel wall. Myoendothelial junctions present.
Small collecting vein	100-300μm	2-3μm	As above but with specific endothelial granules.	2 or more layers of smooth muscle cells.	Unmyelinated nerves situated 5-10 microns from muscular layer.

Table 3.2 Ultrastructural features of venous blood vessels (Rhodin, 1968).

Vessel Type	Mean Diameter	% of PDL Blood volume	% of PDL vessel length	% of vessels in Tooth third of PDL	% of vessel mid third of PDL	% of vessels in bone third of PDL
Type A (Capillary-sized)	6.4 µm	12%	60%	100%	67%	57%
Type B (Post-capillary venule sized)	20.9 µm	88%	40% (70% Apericytic) (30% Pericytic)	0%	33%	43%

Table 3.3 Classification of vessel types (Freezer, 1984).

Figure 3.1
The physiognomy of the marmoset (Crowe, 1989).

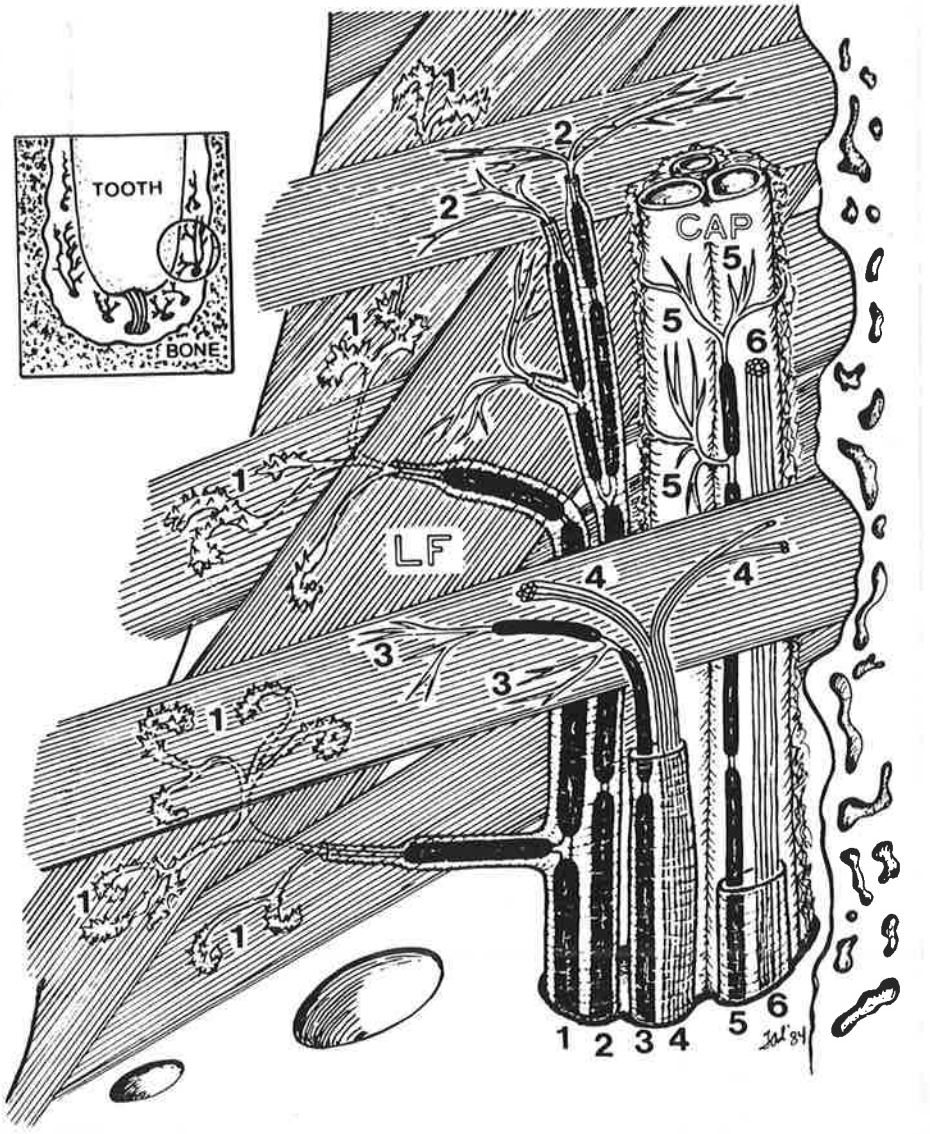

Figure 3.2

This drawing shows representative types of preterminal axons and sensory endings in the periodontal ligament of a rat molar.
Receptors among ligament fibres (LF):
(1) complex Ruffini-like endings, ensheathed preterminal axon; (2) simple Ruffini-like endings, ensheathed preterminal axons - preterminal axons can form paired branches;
(3) simple Ruffini-like endings, branching from free, small, myelinated axons; (4) bundles of free unmyelinated axons. Receptors in loose connective tissue around capillaries (CAP);
(5) simple Ruffini-like endings, branching from free, small, myelinated axons; (6) bundles of free, unmyelinated axons.

(Byers, 1985)

CHAPTER 4

MATERIALS AND METHODS

4.1 REVIEW OF CROWE'S EXPERIMENTAL PROCEDURES

Crowe (1989) used marmosets (*Callithrix jacchus*), as a model to simulate a routine clinical form of treatment. The material for this study came from Crowe's investigation (1989 - "A T.E.M. investigation of extrusion and R.C.T. on the marmoset periodontal ligament"). In total there was tissue used from four female marmosets. Three specimens had already been embedded in resin block form (animal numbers 237, 227, and 236). Specimen number 229 reached the stage of demineralization in preparation of the tissue for sectioning.

Crowe used cotton eared marmosets (*Callithrix jacchus*) from a colony maintained by the CSIRO, Division of Human Nutrition, Glenthorne Laboratories, South Australia (Table 4.1).

Table 4.1 ANIMAL BIRTHDATES

Animal number	Birthdate
237	16/12/1985
227	02/12/1985
236	16/12/1985
229	02/12/1985

During the first five weeks all of the animals had their teeth cleaned every two days with a soft toothbrush

and swabbed with 0.02% chlorhexidine digluconate. This procedure was discontinued when no marked improvement was noted of the gingival condition and the excessive brushing technique harmed delicate gingival tissue. A weekly prophylaxis was substituted.

Magnets were used as the extrusive mechanism. The magnets were of the Samarium-Cobalt type, normally used as quartz digital watch rotors (Seiko product no. 4146235). The magnets were calibrated by the Department of Mechanical Engineering, University of Adelaide, South Australia. A magnet separation of 0.6mm corresponded to an attraction force of 2 grams (~0.02 N).

Under Saffan anaesthesia, the left central incisor of each animal was sectioned at the gingival margin. A vital pulpectomy was then performed and the root canal was filled with gutta percha points and AH 26^{R} (De Trey, U.S.A.). The average working length was 4mm.

One magnet was embedded in the sectioned face of the endodontic treated root and the other was placed in a housing built into the chrome cobalt splint. The splint magnet was fixed 0.6mm coronal to the magnet embedded in the root. A prepared stainless steel spacer was used for this purpose. The splint was electrolytically etched and cemented against the canines and the incisors adjacent to the experimental tooth. As soon as the magnets approximated each other (indicating tooth extrusion), the

magnets in the splints were repositioned further occlusally by 0.6mm.

A fractured human incisor, approximately 22mm long, is usually extruded an average of 4-6mm after a traumatic injury. A corresponding proportional movement in a marmoset incisor, approximately 6mm long, is 1.2mm. The total amount of extrusion was 1.2mm and took an average of two weeks. Crowe had originally aimed for 12 weeks of retention but was limited due to time constraints. The retention period was 9 weeks prior to sacrifice, using the opposed magnets as a retentive device.

The animals were anaesthetized with an I.M. dosage of Saffan (18mg per kg). Heparin (0.04ml) was injected following the onset of anaesthesia to prevent blood clotting. The surgical preparation was carried out followed by perfusion.

The maxillae were then dissected free, and a transverse cut was made mesial to the third premolars. These premaxillary blocks were then demineralized in EDTA solution (Appendix 8.1).

Three specimens were already embedded in resin blocks when received from Crowe. Specimen 229 required the following procedures:

4.2 TRIMMING

A razor blade was used to make bilateral diagonal cuts in the block extending through the middle of the primate space, and running medially to intersect in the midline of the palate. This separated the pre-maxilla from the maxilla proper. During this procedure the tissue was kept moist in 0.06M cacodylate buffer. A mid-sagittal cut was then made to separate the experimental from the control blocks. They were then embedded in alignate and a para-sagittal cut was made through the distal third of the central incisors. The cuts were made with new razor blades (Blu-Strike, U.S.A.). Trimming was facilitated by the use of a dissecting stereo-microscope. The blocks were numbered, and diagrams were drawn to facilitate orientation during embedding.

4.3 TISSUE PROCESSING SEQUENCE

Tissue segments were then transferred to separate, metal-capped, 2 ml soda glass vials which were half-filled with 0.06 M cacodylate buffer. During processing, tissue portions were continuously rotated, and solutions were changed at room temperature with soda glass Pasteur pipettes. The following tissue processing regime was adhered to:

1. Wash

   The trimmed tissues were washed overnight in 0.06M cacodylate buffer (Appendix 8.2).

2. Post-fixation

   4% OsO_4 in double distilled (d.d.) water for 1 hour (Appendix 8.3).

3. Wash

   0.06M cacodylate buffer for 20 minutes.

4. Wash

   70% ethanol for 30 minutes.

5. Block stain

   1% uranyl nitrate in 70% ethanol for 1 hour (Appendix 8.4).

6. Wash

   70% alcohol for 30 minutes.

7. Dehydration

   2 x 30 minutes in 70% ethanol.

   2 x 30 minutes in 80% ethanol.

   2 x 30 minutes in 90% ethanol.

   2 x 30 minutes in 100% anhydrous ethanol.

   2 x 30 minutes in propylene oxide.

   2 x 30 minutes in propylene oxide.

   All diluted alcohols were prepared with d.d. water.

8. Infiltration

   14 hours 1:1 (propylene oxide : Agar 100)

4 hours 1:3 (propylene oxide : Agar 100)

4 hours in Agar 100 embedding resin (Appendix 8.7)

20 hours in Agar 100 embedding resin (Appendix 8.7)

4.4 EMBEDDING

Fresh Agar 100 (Appendix 8.7), prepared for final embedding, was allowed to stand overnight. Using a stereomicroscope, the tissue samples were removed from their vials and then placed with known orientation into silicone rubber moulds, each of which had previously had 1ml. of Agar resin added. The resin filled moulds were incubated at 37°C for 48 hours, and then at 60°C for a further 48 hours, before being coded and stored at room temperature.

4.5 SAMPLE SELECTION

The paired blocks containing the mesial periodontal ligament from both the control and the experimental sides were used.

4.6 SECTIONING

Each block was reduced in size, secured in a Reichert specimen holder for flat trimming, and mounted on a Reichert-Jung OM-U4 ultramicrotome. A number 11 scalpel blade was used to trim the most coronal aspect of the block face to a divergent sided trapezoidal mesa and to complete other initial trimming. The block and chuck were then transferred to the specimen arm of the ultramicrotome and a glass knife aligned in the knife holder. Glass knives were prepared using a LKB type 780 1B knife maker.

A one micron thick orientation section was transferred with a wire loop to a pool of millipored double distilled water on a clean glass microscope slide. This section was flattened onto the slide atop a 70°C hotplate and stained for 2 minutes at 70°C with millipored solutions of 0.5% toluidine blue and 1% borax (Appendix 8.5). Sections were rinsed with millipored d.d. water and dried on the hotplate. The tissue was examined microscopically and appropriate orientation photographs were taken with a Polariod Type 107 black and white land film. The zero level was defined when a "streaming" effect of the periodontal ligament was detected across the mesial aspect of the tooth i.e. collagen fibres and cells orientated perpendicular to the tooth surface (Figure 4.1, p.67).

The final trapezoidal mesa was outlined to cover the PDL at the most mesial part of the incisor. Bone and

tooth substance was included at the base and top of the trapezium, respectively. This gave better support for the section and allowed for orientation during TEM viewing. From each block, thin sections (silver/gold 60-80nm) were cut with a Diatome diamond knife. At each level 36 sections were taken to get 3 sections on each grid. On the basis of previous investigations (Freezer, 1984), the levels were at 250 micron intervals (Figure 4.2, p.68).

The clearance angle of the knife was 10° and the cutting speed 1mm/second. These sections were floated on to a bath of millipored, d.d water, flattened with chloroform vapour and placed on to a clean, uncoated, R150A mesh multi-slotted copper grid. Three sections were collected at a time, by holding the copper grid with its dull surface towards the water bath, slowly lowering the grid to contact the sections and then lifting the grid and attached sections perpendicularly from the water surface. Grids were dried face upwards on Whatman's grade 1 filter paper in a covered petri dish and stored in a LKB specimen grid holder.

4.7 GRID STAINING

Dried grids with complete sections were selected and stained. The tissue side was placed down, for 12 minutes on a droplet of 0.5% uranyl acetate (Appendix 8.6) maintained at 37° C. This was done by placing grids on

microfiltered droplets of freshly prepared stain on Parafilm 'M' laboratory film in a covered petri dish that had been been preheated on a thermostatically controlled hotplate. Grids were rinsed by agitating them for 15 seconds in each of four beakers containing 100 ml of millipored, d.d water at 37°C.

The grids were stained with lead citrate for 4 minutes by floating them in microfiltered droplets of freshly prepared modified Reynolds' lead (Appendix 8.6) on a square of Parafilm 'M' laboratory film in a covered petri dish. Also enclosed in this petri dish were sodium hydroxide pellets to absorb contained carbon dioxide and minimize the formation of lead carbonate precipate on tissue sections.

The tissue was rinsed again in fresh double-distilled, millipored water and dried, tissue surface uppermost, on fine grade filter paper. Grids were then stored in an LKB specimen grid holder until required for transmission electron microscopy.

4.8 END LEVEL

The tissue blocks were sectioned down apically and were collected at 250μm intervals for approximately 11 to 12 levels (2,750μm - 3,250μm). The end level was determined by the appearance of a small area of cellular

cementum, which indicated the termination of the root length.

4.9 TRANSMISSION ELECTRON MICROSCOPY

A Jeol 100S transmission electron microscope (Appendix 8.9) was used to examine the tissue sections. Grids were placed tissue side downwards in the vacuum column of the microscope and conditioned in the electron beam before being examined.

4.10 MICROGRAPHY

The most mesial, rectangular grid slot was selected, which was parallel to the tooth and without technical imperfections, at each sampling level. Consecutive micrographs were taken across the PDL to bone, at a magnification of 3,000x (Figure 4.3, p.69). An orientation micrograph of the whole area was taken at a magnification of 500x (Figure 4.4, p.70), which was used as an aid in the identification of the different type of blood vessels. A replicating graticule at a magnification of 3,000x and 500x was also exposed at this stage.

Ultramicrographs were obtained with Kodak Electron Microscope Film using the inbuilt photographic equipment of the Joel 100S. This equipment has an automatic aperture setting. An exposure time of 3 seconds and 1

second were used for the magnifications of 3,000x and 500x, respectively. Focusing was assisted by use of an image wobbler which was switched off immediately prior to micrograph exposure.

4.11 DEVELOPING AND PRINTING

Ultramicrographs were agitated in Kodak D19 Developer for 4 minutes at 20°C, rinsed in running water and fixed in Hypam Rapid Fixer at 20°C for 7 minutes. Following fixation, negatives were again washed under running water for 15 minutes and rinsed in a mixture of wetting agent and deionized water. After drying, negatives were identified and stored in cellophane envelopes.

All micrographs used for morphometric and stereological analysis were printed at a final magnification of 8,500x using a Simmon Omega D-3 enlarger. Replicating graticules were used to ensure that variation in magnification between micrographs was recorded. Micrographs were printed on Ilford Multigrade glossy medium weight photographic paper, processed with Ilford multigrade paper developer and fixed in Hypam rapid fixer.

4.12 POINT COUNTING PROCEDURES

Each photomicrograph print was masked to leave an area of 170 x 238 mm uncovered as this corresponded to an

area of 20 x 28 μm of periodontal ligament. A 140 point square lattice grid, with adjacent points spaced 17mm apart, were placed over the micrograph (Figure 4.5, p.71). Profiles underlying each point were scored in accordance with the categories listed in Appendix 8.11. In those instances where a point of the test system lay on a boundary between two profiles, the profile above and to the right of test point was scored. The number of profiles of blood vessels and nerves on each photomicrograph were counted using the convention of forbidden lines (Figure 4.6, p.72), and the data was tabulated for stereological analyses. The analyses were made using the equations presented in Appendix 8.10.

4.13 MORPHOMETRIC CRITERIA USED IN TEM INVESTIGATION

a. Blood Vessel Identification

The classification used by the author was based broadly upon the classification of Rhodin (1967,1968). Important contributions were included from Freezer (1984), Cooper (1989) and Crowe (1989). The luminal diameter and the structure of the blood vessel walls formed the main criteria for blood vessel identification.

Blood vessel luminal diameters were measured across the narrowest part of the vessel where the vessel was cut in an oblique or longitudinal direction.

Cells were included in the outer vessel wall if at least 3/4 of the cell enclosed the outer endothelial layer and the 'gap' between the cell membrane and outer endothelial layer was no greater than 1μm.

b. Nerve Axon Identification

The classification used for unmyelinated and myelinated axons was based upon the location in the periodontal ligament and morphological features described by Freezer (1984), Freezer and Sims (1989), and Crowe (1989). Axons were located in the parenchyma of the ligament or in close apposition to the endothelium of the blood vessel walls.

A 10x eyepiece from an Olympus binocular microscope was used for identification of unmyelinated axons. Schwann cell cytoplasm was not included in the criteria for identifying myelinated and unmyelinated axons.

4.14 STATISTICAL ANALYSIS

The data were entered into the Vax computer at the University of Adelaide. The stereologic parameters (V_V, L_V, S_V and N_A) and mean diameter of the control and treated animals were compared by means of a two-way analysis of covariance with unequal numbers of observations in the tissue, using the residual maximum likelihood option (Program 3V of BMDP-81, UCLA).

The mean vessel diameters were compared after adjusting for the depth down the tooth by means of an analysis of covariance which is incorporated in the 3V program above.

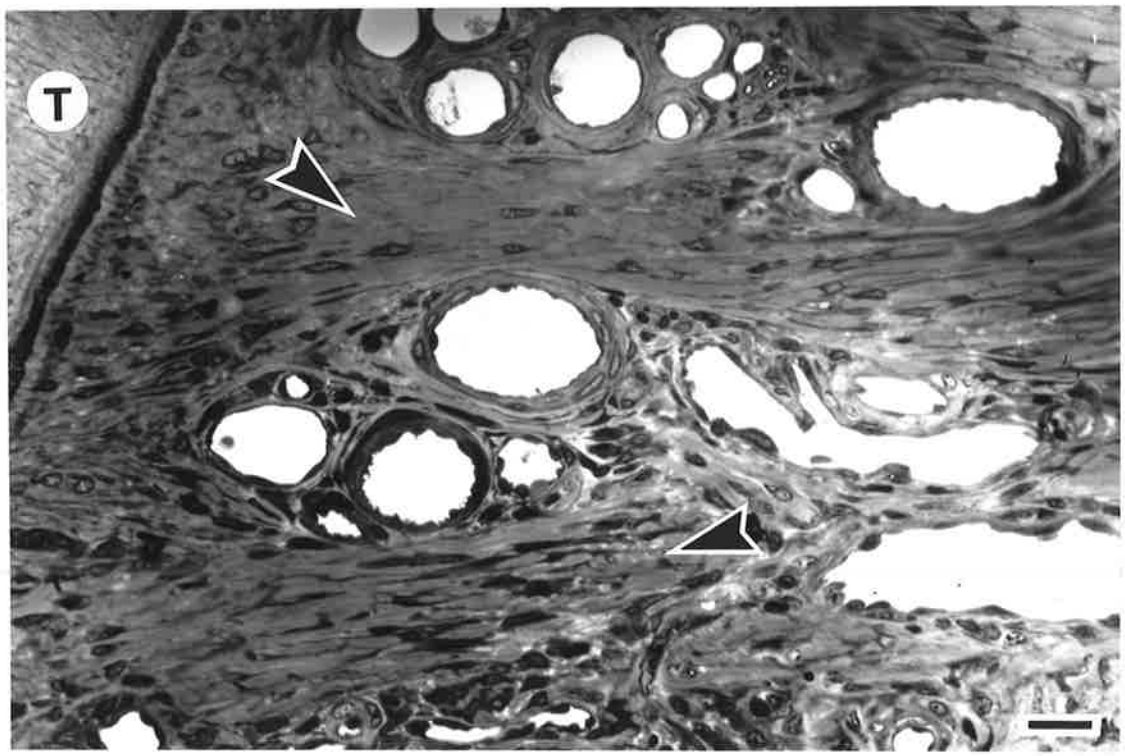

Figure 4.1
"Streaming" effect perpendicular to the tooth surface, at zero level, of the periodontal ligament (arrowed), showing collagen fibres and cells orientated perpendicular to the tooth surface. (T) Tooth. Toluidine blue stain.

Print Magnification: x390.
Bar = 20μm.

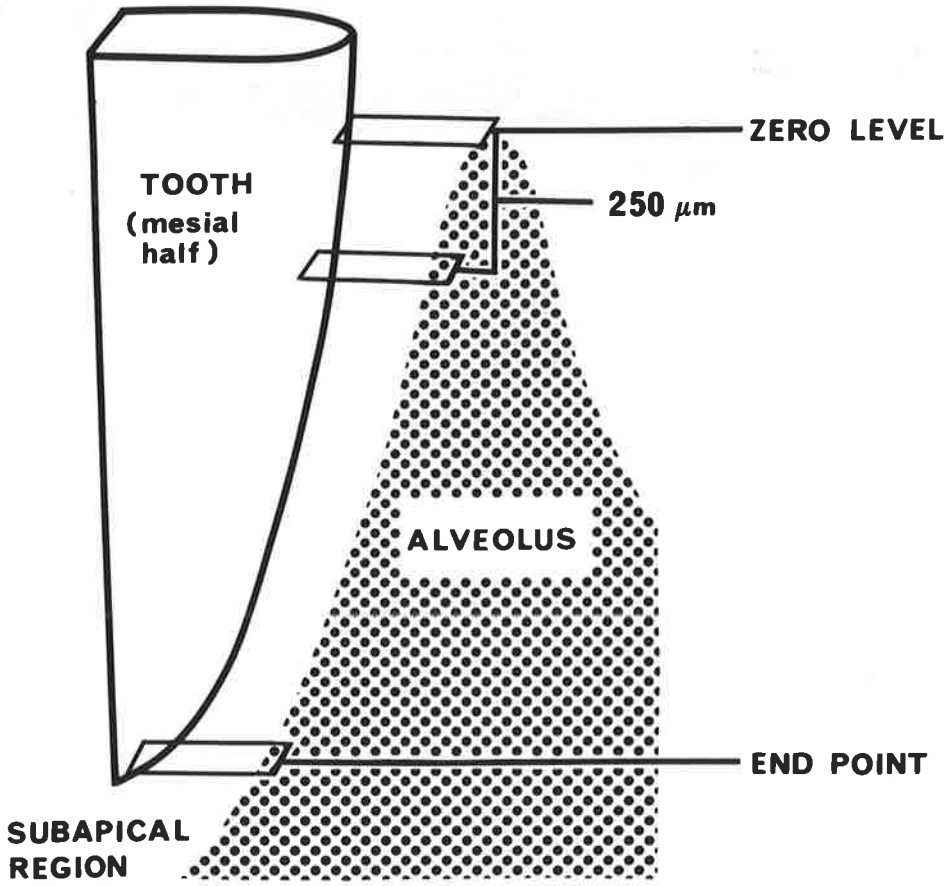

Figure 4.2
Diagram illustrating the region of the periodontal ligament examined. Ultra-thin sections were collected at 250μm intervals.

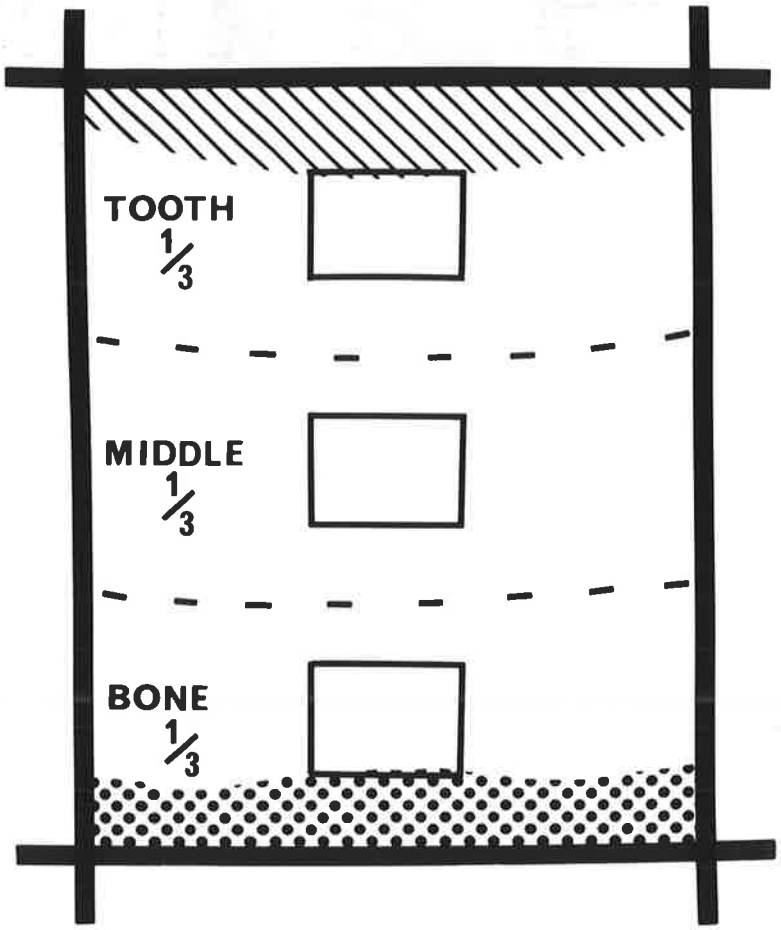

Figure 4.3
Diagram illustrating the site selection of quadrats in the tooth, middle and bone thirds of the ligament using a 150-slotted grid.

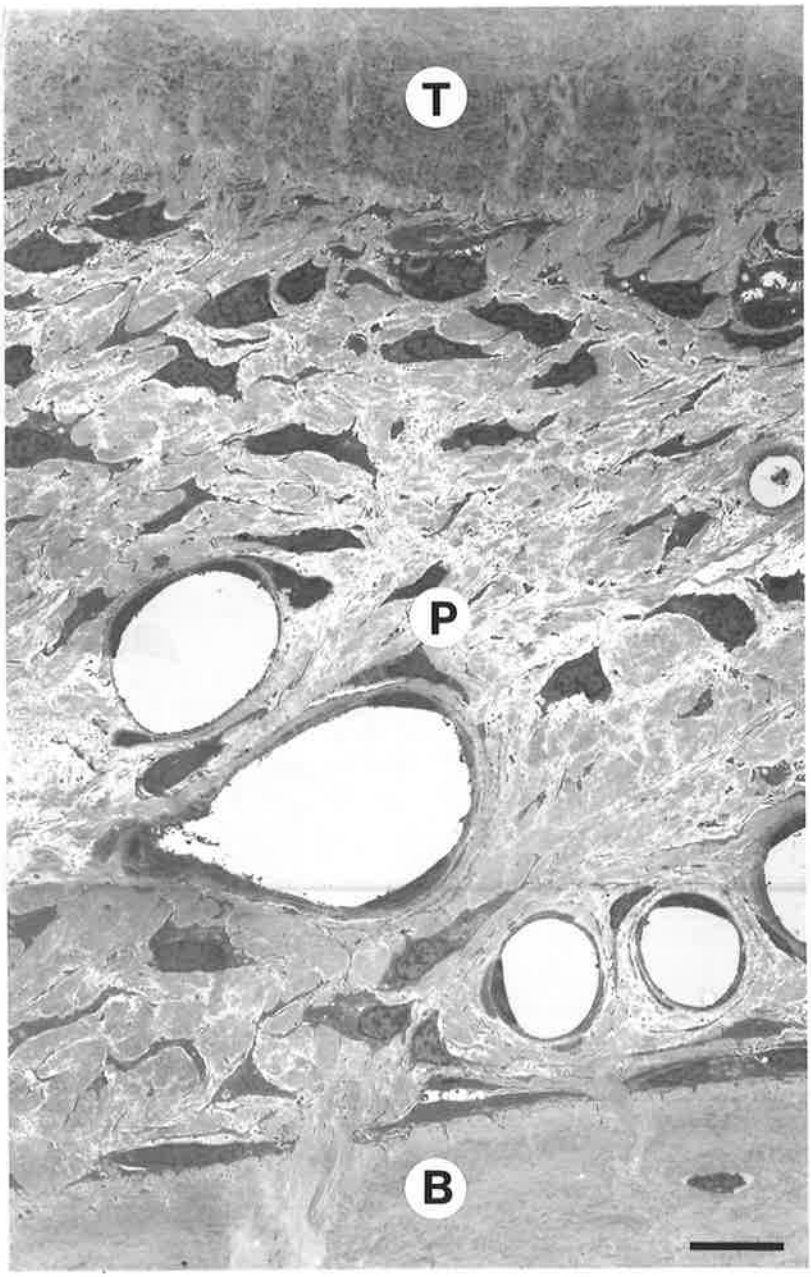

Figure 4.4
Orientation micrograph of the control block across the width of the periodontal ligament. (T) Tooth; (P) Periodontal ligament; (B) Bone.

Depth: 1500μm.
Print Magnification: x1,240.
Bar = 10μm.

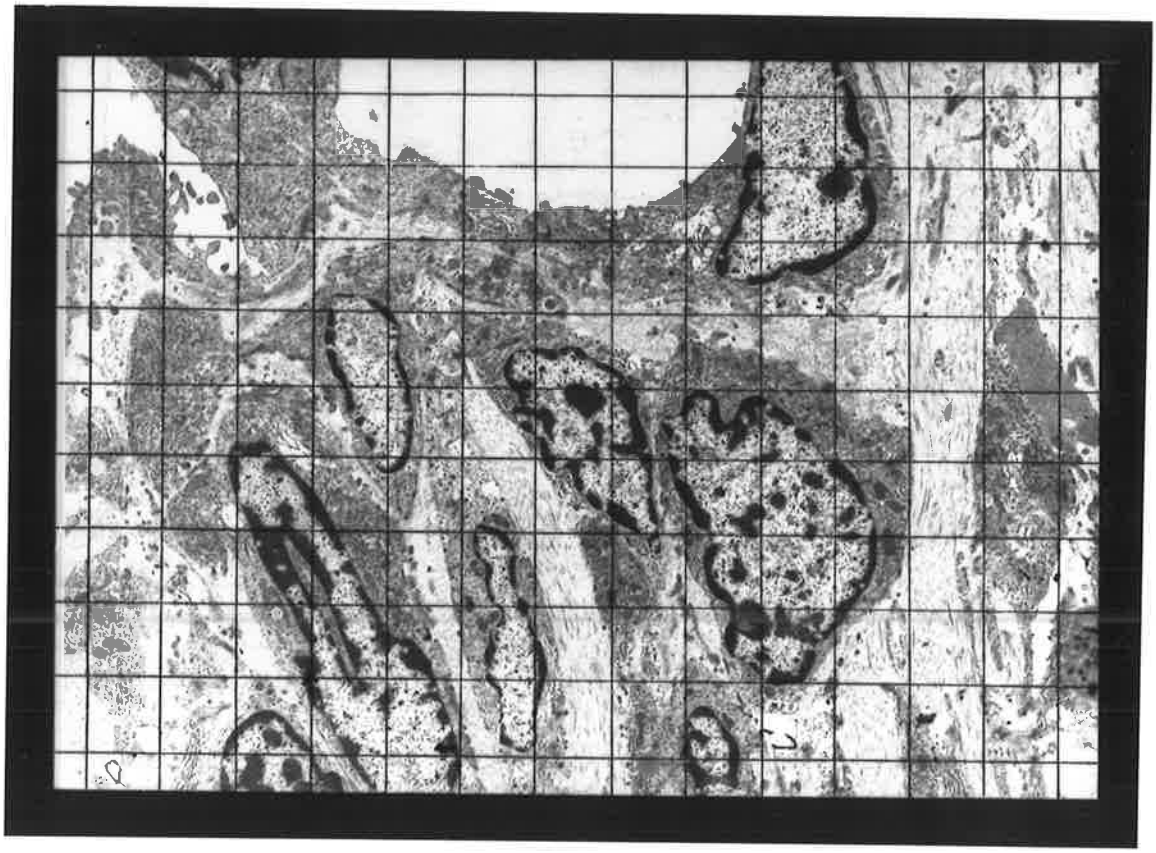

Figure 4.5
A 140 point square grid superimposed on a photomicrograph at zero level. Original print magnification: x8,500.

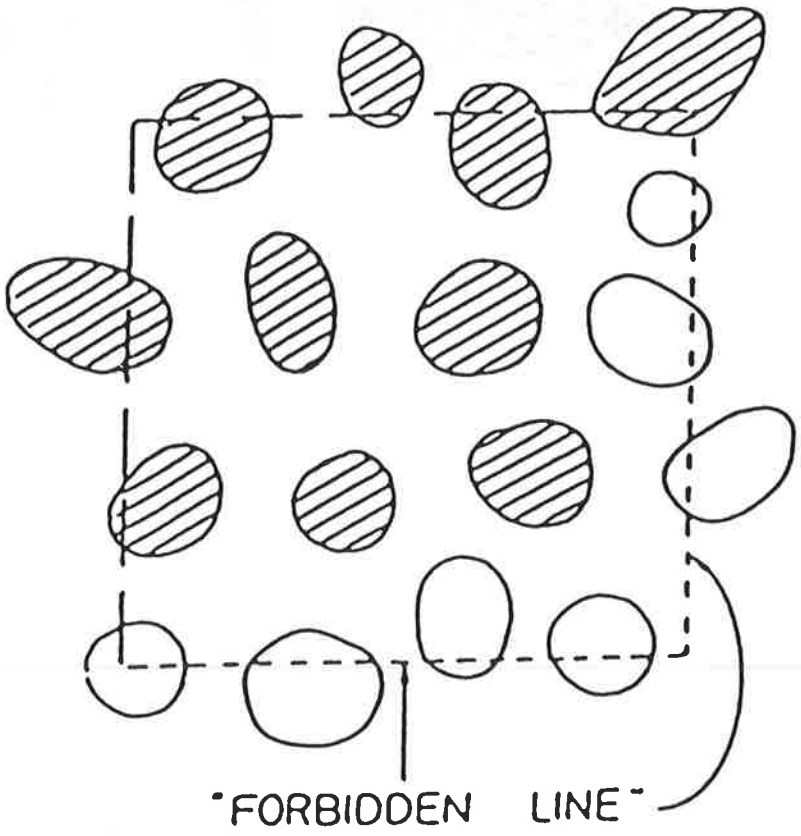

Figure 4.6
The convention of forbidden lines (Weibel 1979). Diagramatic representation of the convention of forbidden lines (Weibel, 1979) with a modification suggested by Casley-Smith (1984). Profiles that intersect both the "accept" and "forbidden" lines are accepted only when the intersection is greater along the "accept" than the "forbidden" line. If the intersection is greater along the "forbidden" line, then the profile is not counted. Consequently, the shaded profile in the upper right corner is counted but the one in the lower left corner is not.

CHAPTER 5

RESULTS OF INVESTIGATION

5.1 EVALUATION OF TISSUE PREPARATION

Adequate penetration of the embedding material into the animal tissue was of concern due to the size of the final block of animal number 229. This potential problem was overcome by a similar procedure carried out by Crowe (1989) i.e. increasing the time spent in each of the processing solutions from the standard 15 minutes used in this laboratory to 30 minutes per alcohol solution. Softening of the blocks was also minimized by keeping them in the desiccator.

There was good fixation of the vessel walls, using the criteria of Hayat (1970). The definition of cell and nuclear membranes was quite distinct. Ultrastructurally, the integrity of mitochondrial cristae was sometimes not intact in the unmyelinated axons. However, overall the mitochondrial cristae remained adequately intact in all regions of the ligament examined under the TEM.

5.2 SECTIONING, GRID SELECTION AND TISSUE STAININING

After the completion of sectioning of animals 236 and 227, continual use of the same diamond knife caused

"chatter" on commencement of animal 237. Switching to a resharpened diamond knife, eliminated the "chatter".

The bars of the slotted grids interfered less with the viewing of a combined area of tooth, periodontal ligament and bone, in contrast to square mesh grids. However, the 150-slotted grids provided less tissue support. Initially, some of the sections were damaged during the rinsing process. This became an infrequent occurrence by using a less vigorous agitation in the rinsing technique, although the chances of tissue contamination from the combined uranyl acetate and lead citrate stain increased. The possible use of coated grids to help support the tissue sections should be assessed to overcome this problem.

The combined uranyl acetate and lead citrate staining method provided excellant contrast of the different cell components. Endothelial plasma membranes were well defined and junctions were readily identified.

5.3 MORPHOLOGICAL FINDINGS

5.3.1 BLOOD VESSEL TYPES AND MORPHOLOGY

Six morphologically distinct types of vessels present within the ligament were :

(i). arterial capillaries;

(ii). venous capillaries with or without a partial pericytic cellular investment;

(iii). postcapillary-sized venules with or without a partial pericytic cellular investment;

(iv). collecting venules;

(v). arteriovenous anastomoses;

(vi). terminal arterioles.

The periodontal blood vessels were most frequently orientated perpendicularly to the plane of section and located within the middle third of the ligament.

No distinctive differences in blood vessel morphology were observed between the control and experimental samples.

(i). **Arterial capillaries**

The vessel wall varied in thickness from 3-5μm. The endothelial cell nucleus bulged into the lumen and was thicker and more lobulated than its venous counterpart. Microvillous projections were often noted along the luminal surface in the region of the endothelial cell junctions (Figures 5.1, p.79 and 5.2, p.80).

(ii). **Venous capillaries**

The vessel wall ranged in thickness from 1-4μm, consisting of a single layer of endothelium. Very few venous capillaries had a complete pericytic layer. The

basement lumina of the pericytes formed a close continuous contact with the neighbouring endothelium. The pericytes were elongated cells with minimal cytoplasmic branching. Their cytoplasmic content was sparse in contrast to endothelial cells. Little endoplasmic reticulin was present, mitochondria were infrequent.

(iii). **Postcapillary-sized venules**

The vessels had a range of 10-26μm in internal luminal diameter. The cell wall was relatively thin averageing 2μm. The pericytic layer if present was usually incomplete with similar ultrastructural characteristics as those associated with venous capillaries. The organelles present with the pericytes were evenly distributed throughout the cytoplasm (Figure 5.3, p.81).

(iv). **Collecting venules**

Collecting venules had the largest luminal diameter ranging from 32-80μm. The vessel wall consisted of a single endothelial layer. Luminal microvillus extensions were rarely present (Figure 5.4, p.82).

(v). **Arteriovenous anastomoses**

Very few arteriovenous anastomoses were observed. The internal luminal diameter ranged from 9-12μm (Figure 5.5, p.83).

(vi). **Terminal arterioles**

Terminal arterioles had the largest cell wall, ranging between 3-10μm. The vessel walls were characterized by a single or double layer of smooth muscle cells which encircled the luminal endothelium (Figure 5.3, p.81). Endothelial processes occasionally were observed to come into contact with the investing smooth muscle cells forming myoendothelial junctions. The endothelial cells overlapped one another at intercellular junctions and their nuclei tended to bulge into the vessel lumen.

5.3.2 NERVE AXON TYPES AND MORPHOLOGY

Myelinated and unmyelinated axons were categorised according to location within the periodontal ligament :

a). within the parenchyma of the ligament;

b). subjacent to the endothelial lining of blood vessel walls.

(i). **Myelinated axons**

Most myelinated axons were located in the parenchyma of the ligament and were frequently surrounded by a thin capsular wall.

Ultrastructurally, myelinated axons contained neurofilaments and varying accumulations of mitochondria. The apical third of the periodontal ligament demonstrated

neural morphology which differed from the middle and cervical regions. The apical-third tissues were distinguished by the presence of numerous myelinated axons arranged in encapsulated bundles, surrounded by a thin, multi-layered capsule (Figure 5.6, p.84).

Accumulations of mitochondria were present within many of the myelinated nerves and the cytoplasm of their Schwann cells. The Schwann cells were surrounded by a basement membrane.

(ii). **Unmyelinated axons**

Unmyelinated nerve axons were present as groups of axons or single axons, which were frequently related to extensions of the Schwann cell cytoplasm (Figure 5.6, p.84). The unmyelinated axons varied in diameter from 1-9μm.

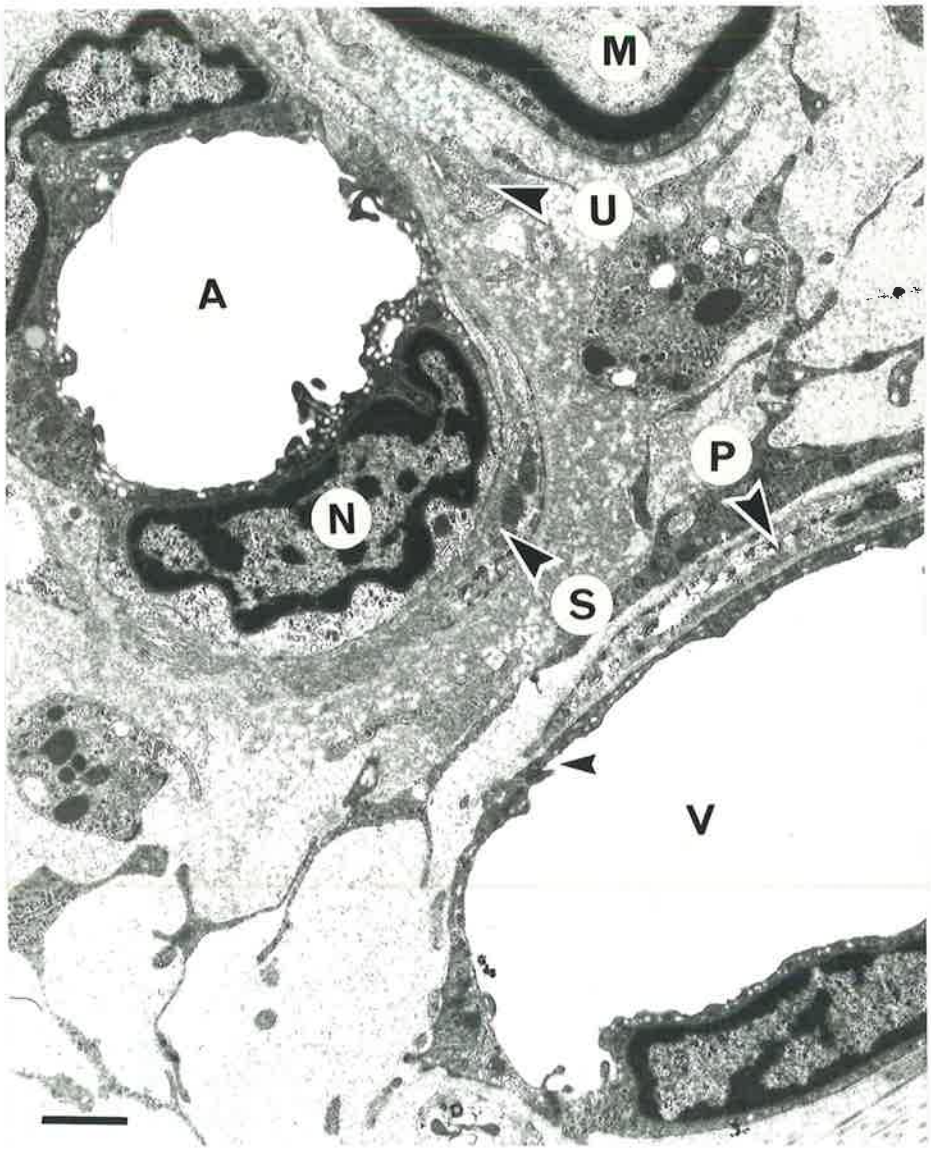

Figure 5.1

An arterial capillary vessel (A) with lobulated endothelial nuclei (N). Smooth muscle cell investment (S) adapted to the endothelial layer. Numerous luminal microvillus extensions present in comparison to the venous capillary (V). A venous capillary (V) with a single endothelial layer, partially surrounded by a pericytic cell (P). Junctions noted in the region of the luminal microvillus extensions are arrowed. Small encapsulated unmyelinated (U) and myelinated (M) axons are present.

Print Magnification: x10,960.
Bar = 1μm.

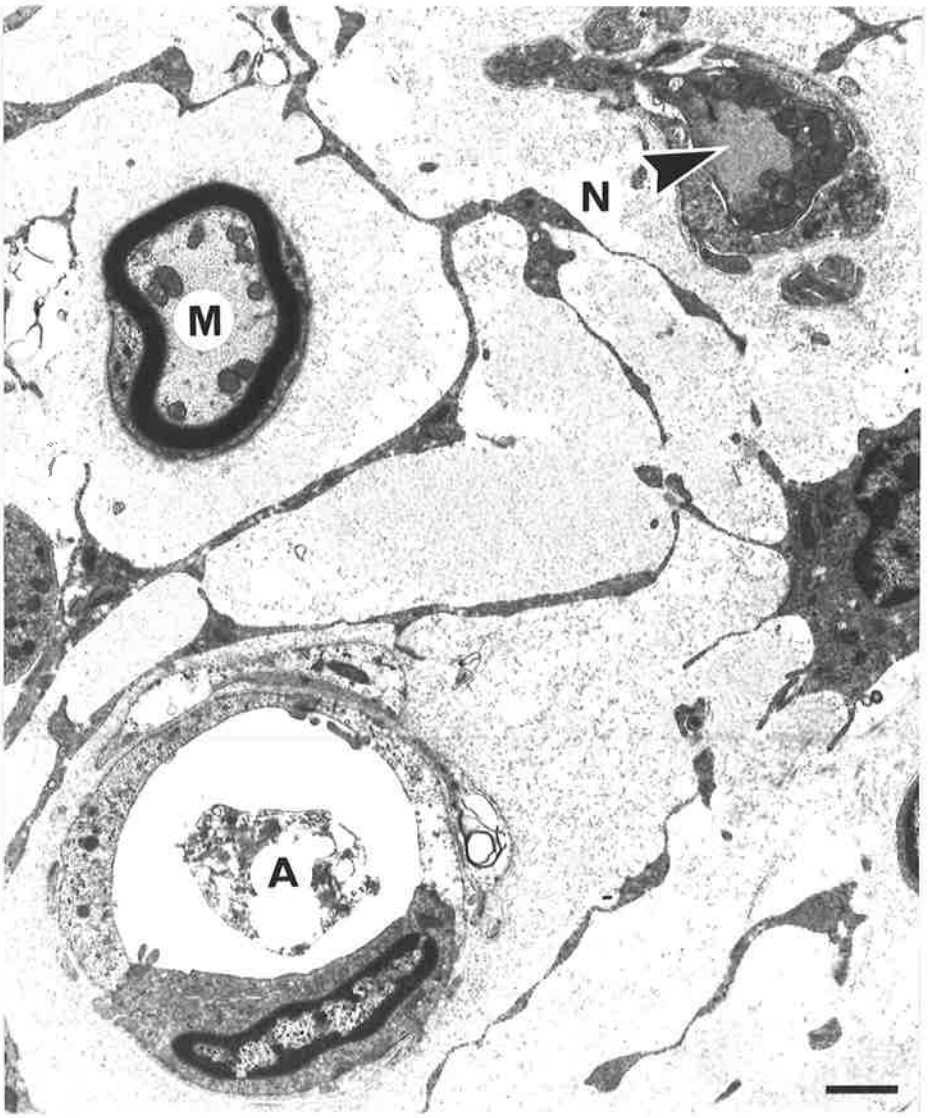

Figure 5.2

An arterial capillary (A) with a luminal diameter of 4μm. A mitochondria-rich nerve ending (N) within the surrounding parenchyma. Myelinated axon (M).

Print Magnification: x8,970.
Bar = 1μm.

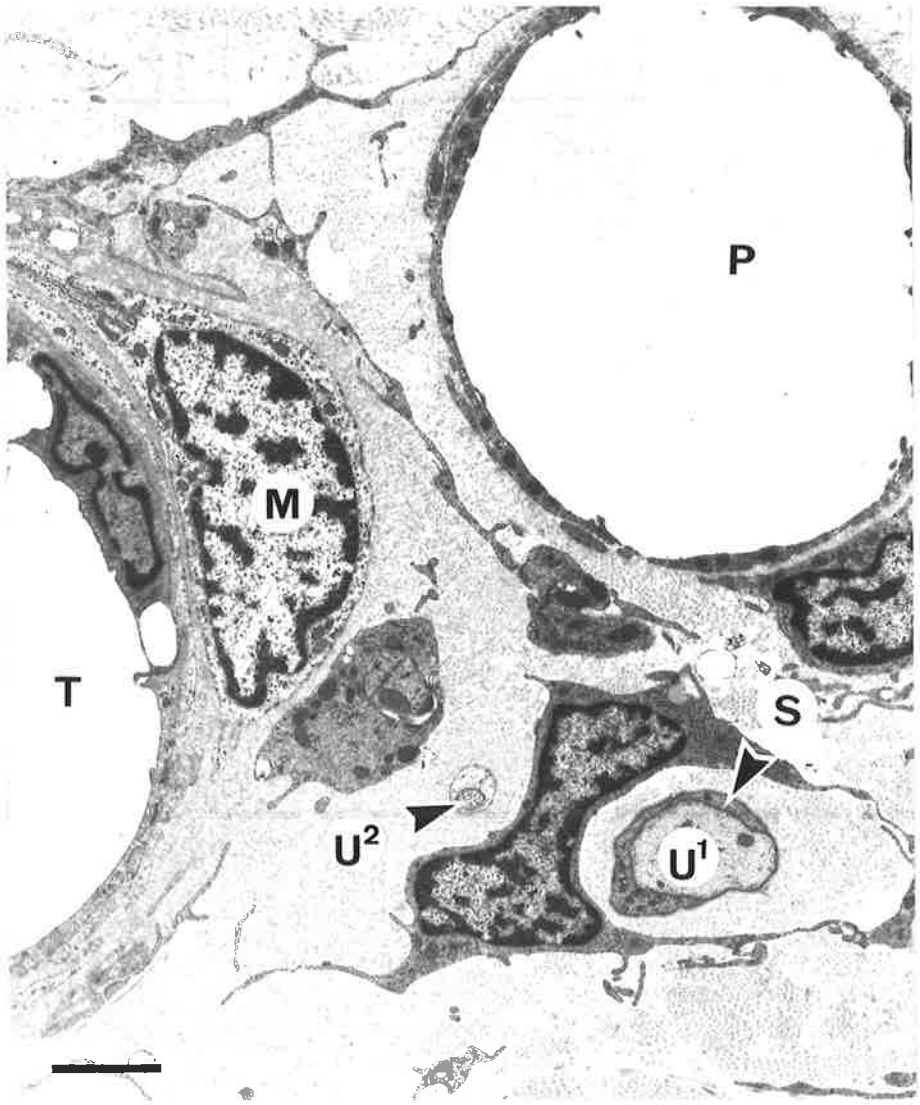

Figure 5.3

An apericytic postcapillary-sized venule (P). The terminal arteriole (T) has a multi-layered cell wall with a smooth muscle cell (M) in close juxtaposition. An encapsulated unmyelinated axon (U^1) associated with Schwann cell cytoplasm (S), which contains dense staining, small mitochondria. Another unmyelinated axon (U^2) is partially enclosed by a single enfolding of investing Schwann cell cytoplasm.

Print Magnification: x7,150.
Bar = 2μm.

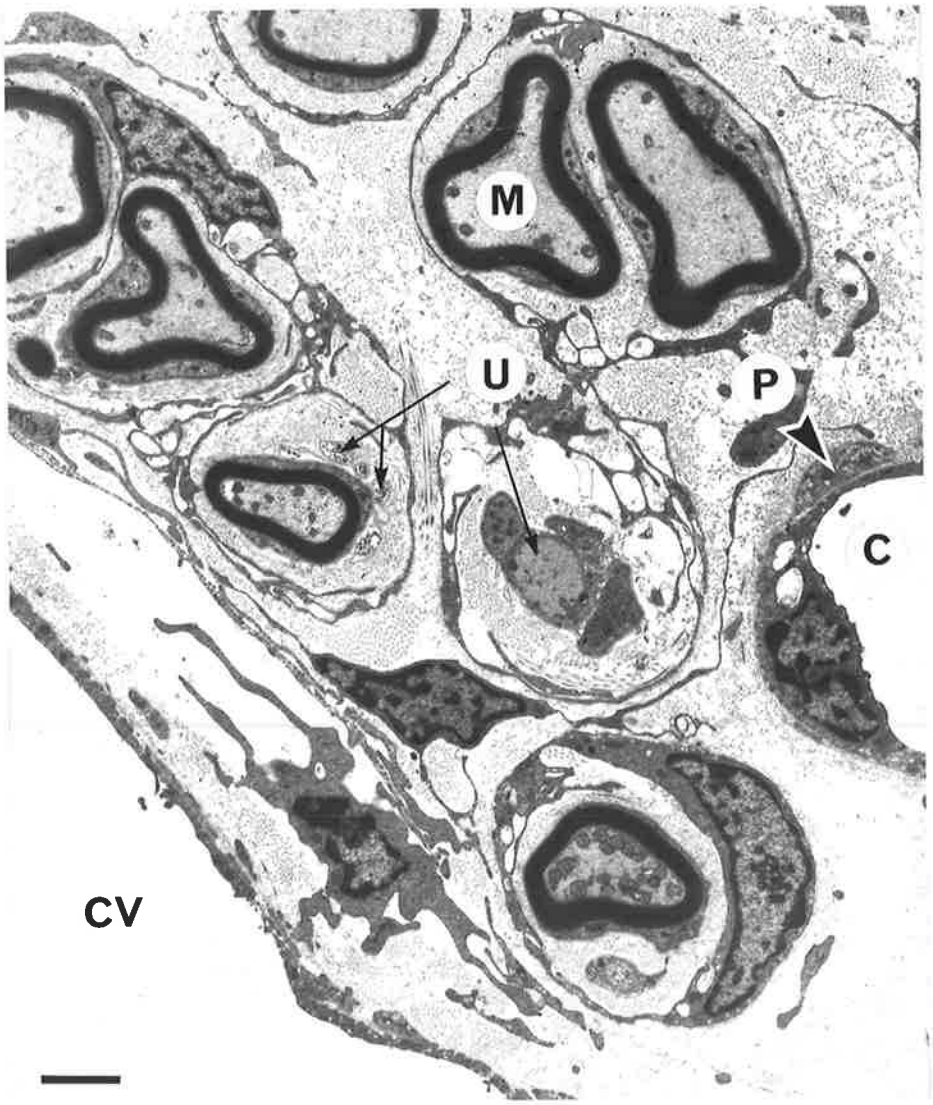

Figure 5.4

A collecting venule (CV), with a thin endothelial layer. Numerous encapsulated myelinated axons (M) in close proximity. Some unmyelinated axons (U) are also present within the encapsulations. A capillary-sized vessel (C) with a presumptive smooth muscle cell or pericyte (P) situated in the vessel wall.

Print Magnification: x5,140.
Bar = 2μm.

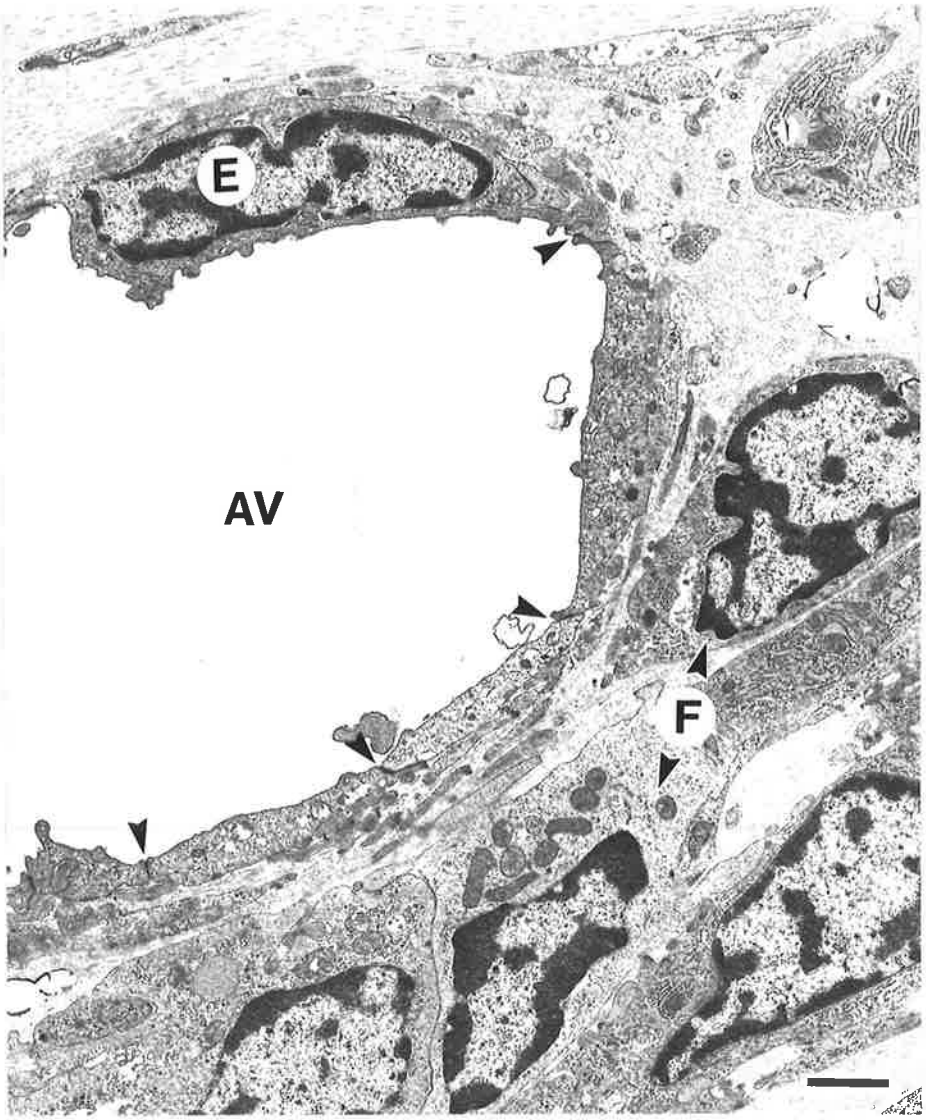

Figure 5.5

Venous end of an arteriovenous anastomosis (AV), with a diameter of 11μm. Junctions (arrowheads) are evident, passing through the endothelial wall.
E = Endothelial cell F = Fibroblasts

Print Magnification: x5,230.
Bar = 2μm.

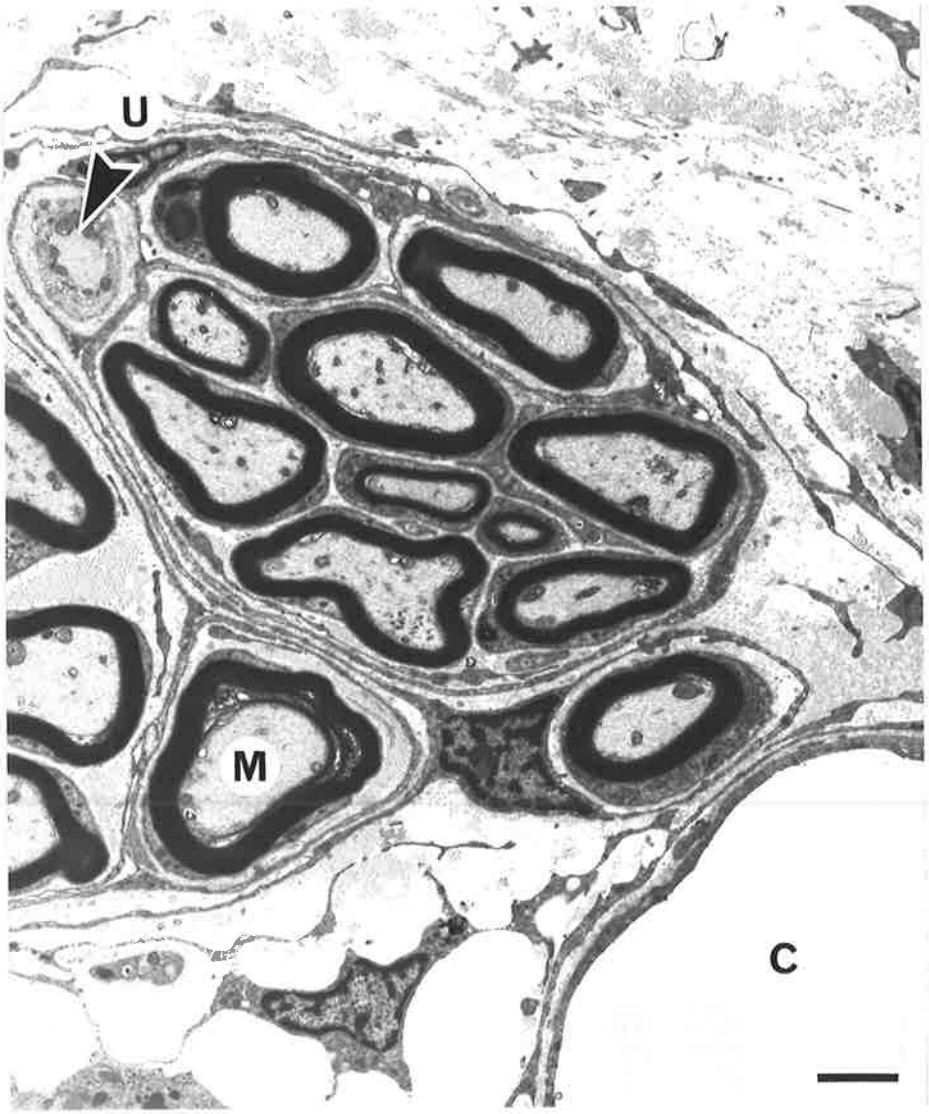

Figure 5.6

A bundle of myelinated axons (M) and one unmyelinated axon (U) enclosed by a thin, multi-layered capsule, alongside a collecting venule (C).

Print Magnification: x5,170.
Bar = 2μm.

5.4 STATISTICAL ANALYSIS

The data, which were recorded in the categories on the data collection sheet (Appendix 8.11), were collated in the following groups to make the data more manageable, because some of the observations were rather small in number, e.g. arteriovenous anastomoses.

A distinction between pericytic and non-pericytic vessels could not be conducted accurately. This was due to the low magnification used in identification of blood vessels and the difficulties in differentiating between pericyte and fibroblast cytoplasmic processes.

GROUPS:

A. (i). Venous Capillaries
   (ii). Postcapillary-Sized Venules
   (iii). Arterial Capillaries
   (iv). Terminal Arterioles (including Arteriovenous Anastomoses)
   (v). Collecting Venules

B. (i). Unmyelinated Axons
   (ii). Myelinated Axons

Assessment of the experimental data involved assigning values for specific stereologic parameters to the major vascular and neural components examined. Blood vessels were assessed for volume density (V_V), surface density (S_V), length density (L_V), number per unit area (N_A) and mean caliper diameter ($\bar{d}$).

Statistical significance is indicated by the use of the asterisk (*) convention, where a difference is significant at $P<0.05$ is indicated by *, $P<0.01$ by **, and $P<0.001$ ***.

The stereologic parameters were calculated for individual blood vessels and nerve axons and for the total vascular and neural bed. The analysis takes into account the different number of observations from each animal. Therefore, the weighting given to the observations for individual blood vessel and nerve axons, was slightly different from the totals. This explains the slight difference noted, when addition of stereologic parameters for the individual blood vessels and nerve axons, is compared to the total readings.

5.4.1 BLOOD VESSELS

Mean values and standard errors for stereologic parameters and mean caliper diameters for the various blood vessel types investigated in the control and experimental periodontal ligament tissue, are presented in Tables 5.1 to 5.7 (pp.93 - 96).

The regressions of luminal and abluminal diameters on depth down the length of the tooth were found by using the analysis of covariance referred to in section 4.13 (Tables 5.9 and 5.10, p.98). There was a negative correlation in position down the tooth for terminal arteriole mean

luminal diameter at $p<0.01$, and for postcapillary-sized venule abluminal diameter at $p<0.05$. This result implies there was an effect of depth associated with an increase in diameter progressively down the length of the tooth.

a. Control Side

The total mean luminal blood vessel volume within the mesial region studied was 11.26%. When also taking into account the vessel wall, the total blood vessel volume increased to 14.85%.

The total mean blood vessel length within the periodontal ligament was 46.0 x cm/cm^3. Of this length, the capillary-sized vessels contributed 33% of the total vascular length, with the arterial capillaries supplying 16% and the venous capillaries the remaining 17%. The postcapillary-sized venules and collecting venules contributed a further 50% of the total blood vessel length. The terminal arterioles contributed 17%.

The vascular volume varied in a similar way to the vessel length with the capillary bed constituting 3.55% the total periodontal vasculature studied. Of this capillary bed, the venous capillaries provide 2.40% and the arterial capillaries provided the remaining 1.15% of the capillary vessel volume (Table 5.8, p.97).

The larger venous vessels constitute 88.19% of the vascular bed volume. The postcapillary-sized venules and

the collecting venules constituted 47.25% and 40.94%, respectively. The postcapillary-sized venules were also the major contributor to the total periodontal vessel luminal surface area with 44.2%. Terminal arterioles constituted 13.19%. The total vascular surface density was 231.19 cm^2/cm^3, and varied with the different types of blood vessels in a similar way to the length and volume parameters.

Table 5.6 (p.95) provides the mean number of vessels types per 560 μm^2 (micrograph) of PDL tissue. Postcapillary-sized venules occurred with the most frequency at 0.48 / 560 μm^2.

The method of sampling gave a mean caliper diameter for both luminal and abluminal (Tables 5.1 and 5.2, p.93).

No vessels were observed in the tooth third. A difference in the luminal blood vessel volume occurred within the lateral zones of the ligament. The vascular volume comprised 24.89% of the total PDL in the middle third and 8.8% in the bone third (Table 5.11, p.99).

Investigation revealed little variation in luminal blood vessel volume down the length of the tooth. The vascular volume comprised 7.67% of the PDL in the cervical third, 4.58% in the middle third and 4.61% in the apical third. However, there was marked animal variation in the vascular volume for the vertical thirds and no specific trend was observed.

b. Experimental Side

The total luminal vascular volume of the PDL decreased from 11.26% to 7.66% and the vessel wall volume decreased from 3.59% to 2.82%.

The mean number of blood vessels per 560 μm^2 reduced from 1.10 to 0.98.

Changes in the vessel volume density of individual blood vessels as a proportion of the total PDL examined, due to the experimental regime, were minor. The only statistically significant change occurring in both vessel wall and luminal volume density (V_V) was for postcapillary-sized venules ($p<0.05$). The reduction for vessel wall volume density was from 1.64% to 1.03% and the reduction for luminal volume density was from 5.32% to 3.04% (Table 5.3, p.94 and Table 5.7, p.96).

The overall luminal venous volume reduced as a percentage of the total vascular volume from 90.59% to 75.18%. However, there was a slight increase in the venous capillary proportion from 2.4% to 6.8%.

The mean number (N_A) of terminal arterioles and venous capillaries per 560 μm^2 increased, while all other blood vessel-types reduced. The change observed for venous capillaries as a result of experimental regime was significant at $p<0.05$.

Mean luminal caliper diameter of the different types of vessels also changed (Table 5.1, p.93) with the

postcapillary-sized venules decreasing from 18.75μm to 15.98 μm ($p<0.05$). The changes observed for the other vessel types were minor. There was a marked difference in range of diameters for terminal arterioles from 8-26μm control to 9-60μm experimental.

Mean abluminal caliper diameter decreased for all blood vessels (Table 5.2, p.93) except for terminal arterioles where there was an increase from 24.99μm to 29.0μm ($p<0.01$). The decrease in abluminal diameter for postcapillary-sized venules was from 10.41μm to 7.85μm and was statistically significant at $p<0.05$.

No statistically significant changes occurred due to treatment regime for the parameters of length (L_V) and surface (S_V) density. The total vascular length density of the PDL reduced from 46.0 to 37.69 x 10^3 cm/cm^3.

5.4.2 NERVE AXONS

The mean values and standard errors for the stereologic parameters and mean caliper diameters, for umyelinated and myelinated axons are presented in Tables 5.13 to 5.17 (pp.101 - 102).

Axons within the blood vessel walls comprised a negligible percentage of the periodontal ligament volume and no myelinated axons were found in the blood vessel walls of the control animals. Therefore, in applying stereological parameters (V_V,L_V,S_V,N_A) and mean caliper

diameter ($\bar{d}$), nerve axons were classified as myelinated or unmyelinated and were not separated further into subgroups based on location.

a. Control and Experimental Sides

Myelinated axons constituted the largest percentage (72.10% control and 76.79% experimental) of the total periodontal neural volume.

Stereological parameters for nerve axons varied across the width of the ligament and down the length of the tooth. Comparison of unmyelinated and myelinated volume density in the different lateral thirds of the ligament (Tables 5.18 and 5.19, p.103), demonstrated no myelinated axons in the tooth third. Both myelinated and unmyelinated axons were found in the highest proportion in the middle third, followed by the bone third.

The volume proportion for both myelinated and unmyelinated axons increased from the cervical third to the apical third in both control and experimental sides (Tables 5.20 and 5.21, p.104). No myelinated axons were observed in the cervical third on the control side.

The neural stereological parameters (V_V,S_V,L_V,N_A) and mean caliper diameter for the PDL region examined, demonstrated change following the experimental regime. However, the differences observed were not of statistical significance. The periodontal neural volume comprised

0.43% (control) and 0.56% (experimental) of the total periodontal ligament examined (Table 5.14, p.101). The parameters of S_V, L_V and N_A demonstrated a slight reduction (Tables 5.15 to 5.17, pp.101 - 102).

Unmyelinated axons had a mean caliper diameter of 0.67μm (control) and 0.80μm (experimental) and constituted 0.12% (control) and 0.13% (experimental) of the periodontal volume.

Myelinated axons had a mean caliper diameter of 2.94μm and 3.68μm for the control and experimental sides, respectively. Numerically the myelinated axons constituted 11% control and 14% experimental of the periodontal nerve axons. However, due to their size they comprised the largest proportion (72% control and 77% experimental) of the total periodontal neural volume.

Overall, there was an increase in neural volume in the experimental teeth of both myelinated and unmyelinated axon volume density. The change observed was slight from 0.12% to 0.13% and 0.31% to 0.43% for unmyelinated and myelinated axons, respectively.

TABLE 5.1 MEAN CALIPER LUMINAL DIAMETER OF PDL BLOOD VESSEL TYPES

$\bar{d}$ Blood Vessels (μm) ± S.E.

	CONTROL	EXPERIMENTAL	
VENOUS CAPILLARIES	7.59 ± 0.90	6.66 ± 0.67	NS
POSTCAPILLARY -SIZED VENULES	18.75 ± 0.87	15.98 ± 0.89	*
ARTERIAL CAPILLARIES	5.24 ± 0.54	6.01 ± 0.62	NS
TERMINAL ARTERIOLES	20.23 ± 4.72	23.46 ± 3.82	NS
COLLECTING VENULES	40.90 ± 2.47	40.31 ± 3.39	NS

TABLE 5.2 MEAN CALIPER ABLUMINAL DIAMETER OF PDL BLOOD VESSEL TYPES

$\bar{d}$ Blood Vessels (μm) ± S.E.

	CONTROL	EXPERIMENTAL	
VENOUS CAPILLARIES	10.41 ± 1.26	7.85 ± 0.98	NS
POSTCAPILLARY -SIZED VENULES	20.94 ± 0.86	17.98 ± 0.89	*
ARTERIAL CAPILLARIES	9.98 ± 0.90	10.18 ± 0.85	NS
TERMINAL ARTERIOLES	24.99 ± 5.29	29.0 ± 4.27	**
COLLECTING VENULES	43.40 ± 2.51	42.84 ± 3.42	NS

TABLE 5.3 LUMINAL VOLUME DENSITY OF PDL BLOOD VESSEL TYPES AS A PERCENTAGE OF TOTAL PDL VOLUME

V_V (%) ± S.E

	CONTROL	EXPERIMENTAL	
VENOUS CAPILLARIES	0.27 ± 0.11	0.52 ± 0.11	NS
POSTCAPILLARY-SIZED VENULES	5.32 ± 0.61	3.04 ± 0.61	*
ARTERIAL CAPILLARIES	0.13 ± 0.08	0.16 ± 0.08	NS
TERMINAL ATERIOLES	0.93 ± 0.46	1.64 ± 0.46	NS
COLLECTING VENULES	4.61 ± 1.90	2.29 ± 1.90	NS
TOTAL	11.26 ± 2.72	7.66 ± 2.72	NS

TABLE 5.4 LUMINAL LENGTH DENSITY OF PDL BLOOD VESSEL TYPES

L_V (x 10^3 cm/cm^3)

	CONTROL	EXPERIMENTAL	
VENOUS CAPILLARIES	7.30 ± 1.72	9.59 ± 1.49	NS
POSTCAPILLARY-SIZED VENULES	18.75 ± 3.59	14.98 ± 3.59	NS
ARTERIAL CAPILLARIES	7.0 ± 1.94	5.58 ± 2.86	NS
TERMINAL ARTERIOLES	7.60 ± 1.94	3.03 ± 1.69	NS
COLLECTING VENULES	3.44 ± 1.40	2.63 ± 1.58	NS
TOTAL	46.0 ± 8.40	37.69 ± 7.95	NS

TABLE 5.5 LUMINAL SURFACE DENSITY OF PDL BLOOD VESSEL TYPES

S_V (x cm^2/cm^3) ± S.E.

	CONTROL	EXPERIMENTAL	
VENOUS CAPILLARIES	17.94 ± 4.69	24.85 ± 4.06	NS
POSTCAPILLARY-SIZED VENULES	111.86 ± 16.41	75.49 ± 16.41	NS
ARTERIAL CAPILLARIES	15.39 ± 5.22	11.43 ± 4.27	NS
TERMINAL ARTERIOLES	33.32 ± 8.95	22.93 ± 7.75	NS
COLLECTING VENULES	44.55 ± 18.57	30.65 ± 21.15	NS
TOTAL	253.19 ± 47.48	173.64 ± 44.75	NS

TABLE 5.6 NUMBER OF PDL BLOOD VESSEL TYPES / 560 μm^2 OF PDL TISSUE.

N_A MEAN (/ 560 μm^2) ± S.E.

	CONTROL	EXPERIMENTAL	
VENOUS CAPILLARIES	0.07 ± 0.03	0.17 ± 0.03	*
POSTCAPILLARY-SIZED VENULES	0.48 ± 0.09	0.41 ± 0.09	NS
ARTERIAL CAPILLARIES	0.13 ± 0.07	0.07 ± 0.07	NS
TERMINAL ARTERIOLES	0.16 ± 0.05	0.15 ± 0.05	NS
COLLECTING VENULES	0.26 ± 0.10	0.17 ± 0.10	NS
TOTAL	1.10 ± 0.21	0.98 ± 0.21	NS

TABLE 5.7 VESSEL WALL VOLUME DENSITY OF PDL BLOOD VESSEL TYPES AS A PERCENTAGE OF TOTAL PDL VOLUME.

V_V (%) ± S.E

	CONTROL	EXPERIMENTAL	
VENOUS CAPILLARIES	0.19 ± 0.09	0.24 ± 0.09	NS
POSTCAPILLARY-SIZED VENULES	1.64 ± 0.16	1.03 ± 0.16	*
ARTERIAL CAPILLARIES	0.26 ± 0.17	0.21 ± 0.17	NS
TERMINAL ATERIOLES	0.70 ± 0.26	1.07 ± 0.26	NS
COLLECTING VENULES	0.80 ± 0.20	0.27 ± 0.20	NS
TOTAL	3.59 ± 0.64	2.82 ± 0.64	NS

TABLE 5.8 PERCENTAGE OF THE TOTAL PERIODONTAL VASCULAR (LUMINAL) VOLUME OCCUPIED BY DIFFERENT BLOOD VESSEL TYPES.

	CONTROL	EXPERIMENTAL
ARTERIAL CAPILLARIES	1.15%	2.10%
VENOUS CAPILLARIES :APERICYTIC	2.40%	5.64%
VENOUS CAPILLARIES :PERICYTIC	0%	1.16%
POSTCAPILLARY-SIZED VENULES :APERICYTIC	42.51%	34.19%
POSTCAPILLARY-SIZED VENULES :PERICYTIC	4.74%	5.55%
COLLECTING VENULES	40.94%	28.64%
TERMINAL ARTERIOLES	7.36%	21.44%
ARTERIOVENOUS ANASTOMOSES	0.90%	1.29%

TABLE 5.9 MEAN CALIPER LUMINAL DIAMETER ($\bar{d}$) OF PDL BLOOD VESSEL TYPES

	SIGNIFICANCE OF THE REGRESSION OF DIAMETER ON DEPTH
VENOUS CAPILLARIES	NS
POSTCAPILLARY -SIZED VENULES	NS
ARTERIAL CAPILLARIES	NS
TERMINAL ARTERIOLES	** (-ve)
COLLECTING VENULES	NS

TABLE 5.10 MEAN CALIPER ABLUMINAL DIAMETER ($\bar{d}$) OF PDL BLOOD VESSEL TYPES

	SIGNIFICANCE OF THE REGRESSION OF DIAMETER ON DEPTH
VENOUS CAPILLARIES	NS
POSTCAPILLARY -SIZED VENULES	* (-ve)
ARTERIAL CAPILLARIES	NS
TERMINAL ARTERIOLES	NS
COLLECTING VENULES	NS

TABLE 5.11

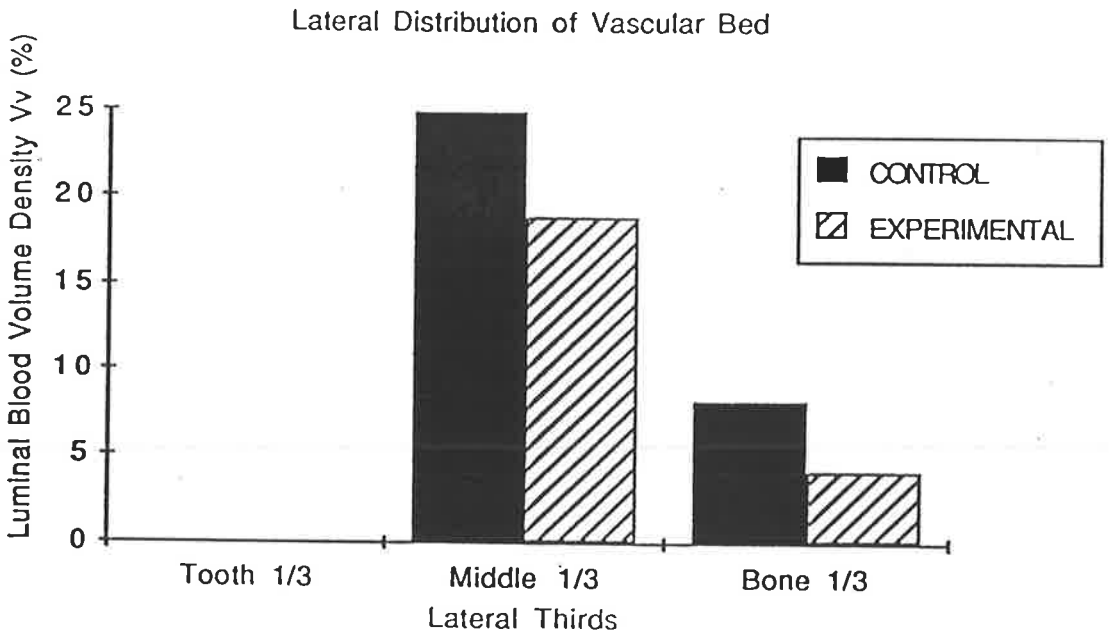

Table 5.11

Histogram illustrating the distribution of vascular (luminal) volume density (V_V) as a percentage of the total PDL volume in the lateral thirds.

TABLE 5.12

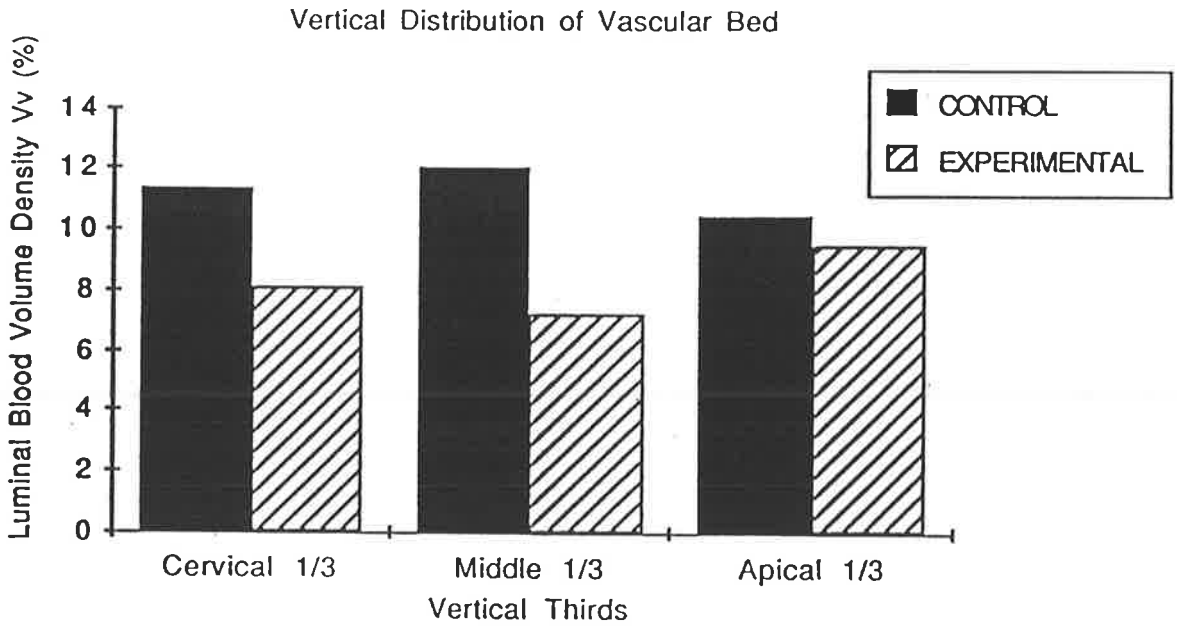

Table 5.12
Histogram illustrating the distribution of vascular (luminal) volume density (V_V) as a percentage of the total PDL volume in the vertical thirds.

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TABLE 5.13 MEAN DIAMETER OF PDL NERVE AXONS

$\bar{d}$ Nerve Axons (μm) ± S.E.

	CONTROL	EXPERIMENTAL	
UNMELINATED AXONS	0.67 ± 0.10	0.80 ± 0.10	NS
MYELINATED AXONS	2.94 ± 0.25	3.68 ± 0.25	NS

TABLE 5.14 VOLUME DENSITY OF PDL NERVE AXONS AS A PERCENTAGE OF TOTAL PDL VOLUME

V_V (%) ± S.E.

	CONTROL	EXPERIMENTAL	
UNMYELINATED AXONS	0.12 ± 0.05	0.13 ± 0.05	NS
MYELINATED AXONS	0.31 ± 0.10	0.43 ± 0.10	NS
TOTAL	0.43 ± 0.35	0.56 ± 0.35	NS

TABLE 5.15 LENGTH DENSITY OF PDL NERVE AXONS

L_V (x 10^3 cm/cm^3) ± S.E.

	CONTROL	EXPERIMENTAL	
UNMYELINATED AXONS	347.47 ± 114.80	254.91 ± 114.80	NS
MYELINATED AXONS	43.40 ± 13.04	42.64 ± 13.04	NS
TOTAL	390.86 ± 364.55	297.54 ± 145.82	NS

TABLE 5.16 SURFACE DENSITY OF PDL NERVE AXONS

S_V (cm^2/cm^3) ± S.E.

	CONTROL	EXPERIMENTAL	
UNMYELINATED AXONS	71.48 ± 26.28	62.47 ± 26.30	NS
MYELINATED AXONS	41.14 ± 12.31	47.53 ± 12.30	NS
TOTAL	112.54 ± 28.33	110.0 ± 28.33	NS

TABLE 5.17 NUMBER OF PDL AXONS / 560 μm^2 OF PDL

N_A (/ 560 μm^2) ± S.E.

	CONTROL	EXPERIMENTAL	
UNMYELINATED AXONS	1.94 ± 0.64	1.43 ± 0.64	NS
MYELINATED AXONS	0.24 ± 0.64	0.24 ± 0.64	NS
TOTAL	2.19 ± 0.82	1.67 ± 0.82	NS

TABLE 5.18

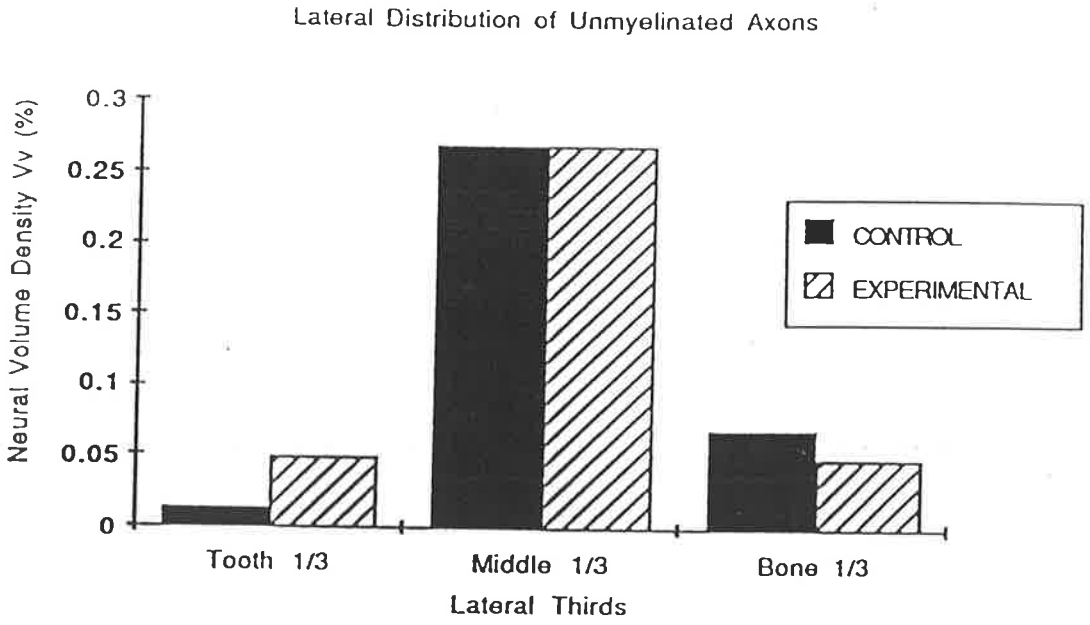
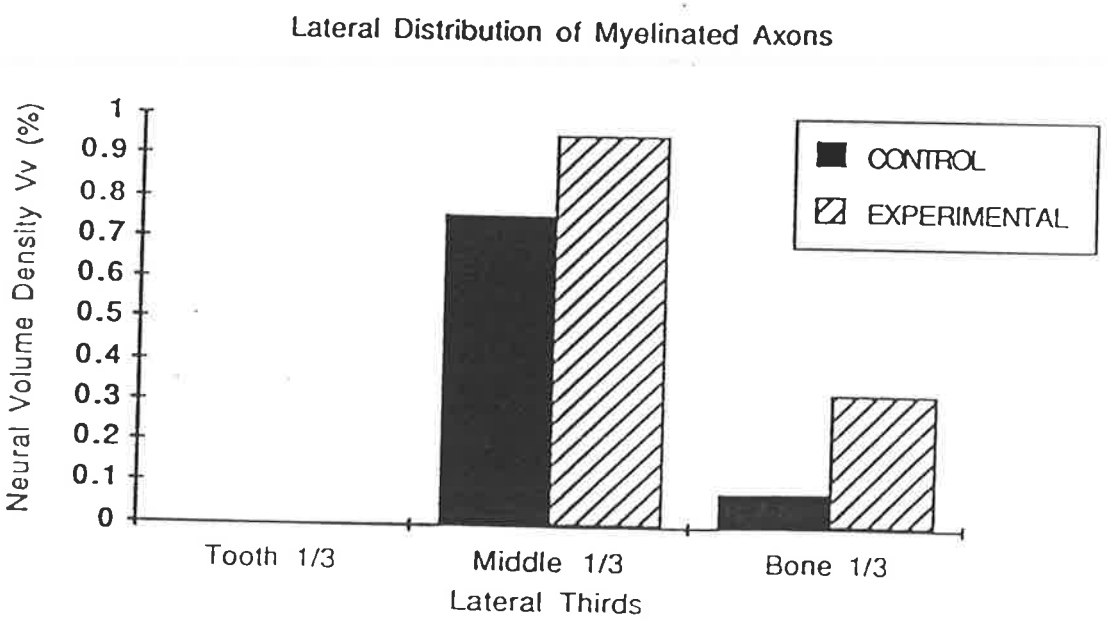


TABLE 5.19



Tables 5.18 and 5.19

Histograms illustrating the distribution of unmyelinated and myelinated axon volume density (V_V) as a percentage of the total PDL volume in the lateral thirds.

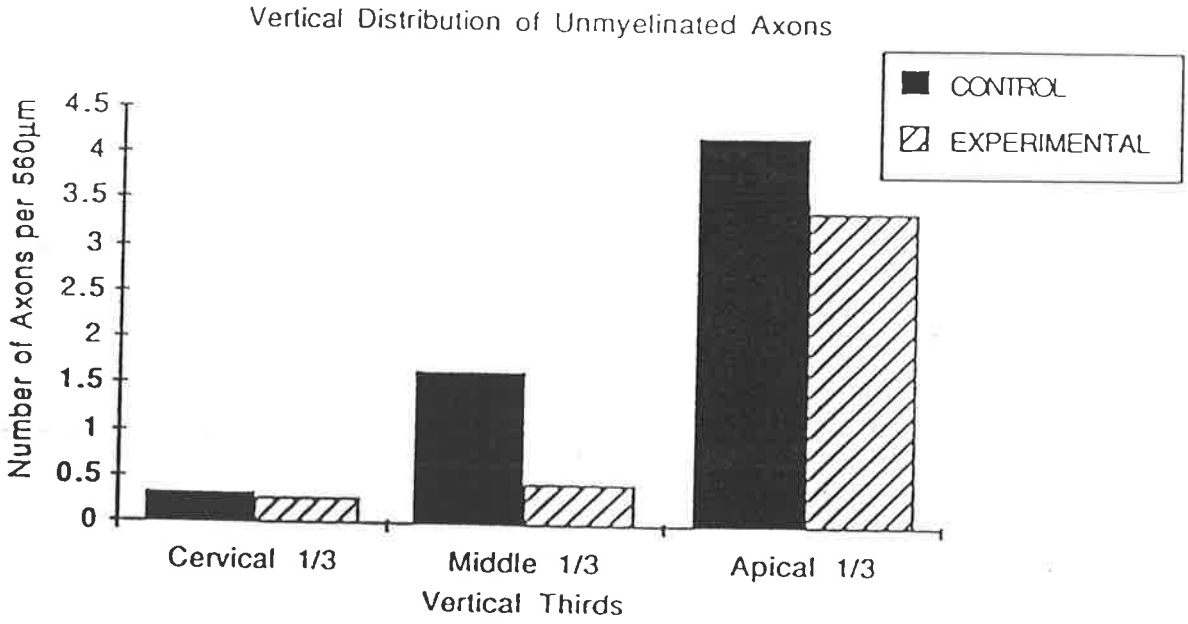
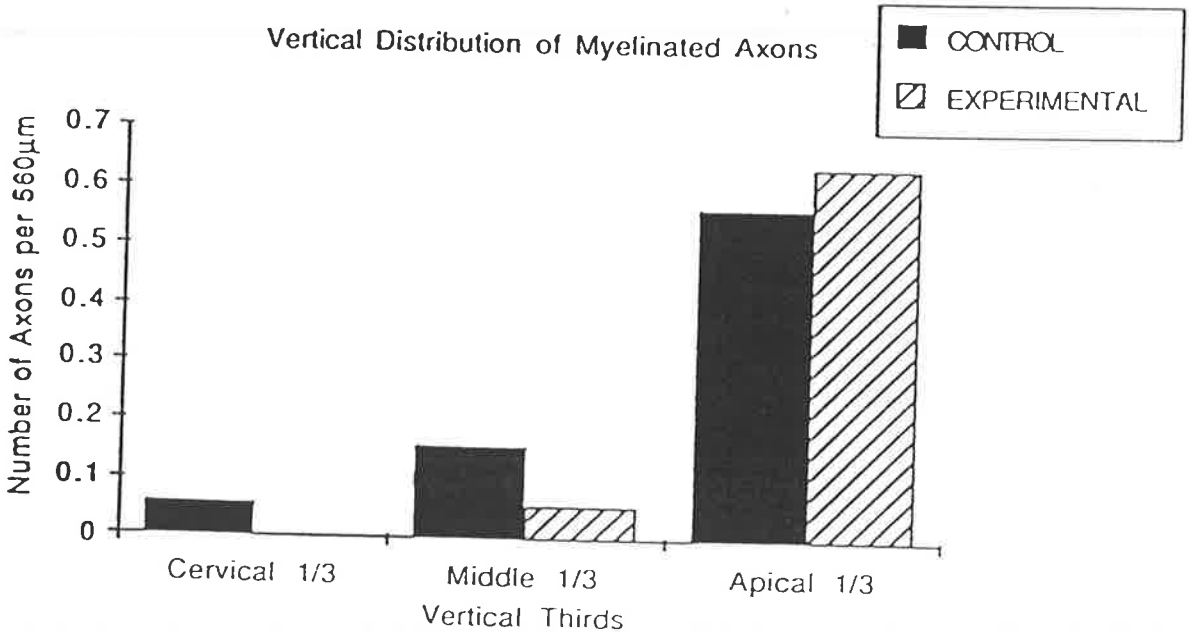


TABLE 5.21



Tables 5.20 and 5.21

Histograms illustrating the distribution in the mean number of unmyelinated and myelinated axons per unit area down the length of the tooth in the vertical thirds.

CHAPTER 6.

DISCUSSION

6.1 TECHNICAL CONSIDERATIONS

a. Anaesthetic and Anticoagulant

Saffan was used by Crowe (1989) as the anaesthetic and was administered via the intramuscular route. The Therapeutic Index of Saffan is high (Glaxo Laboratories Ltd, Greenford, Middlesex, England, 1974). Crowe found it provided good relaxation of the abdominal muscles, and respiration was well maintained.

Crowe used heparinization following anaesthesia to circumvent the coagulation of blood elements which might prevent adequate vascular washout with fixative solution.

b. Fixation Procedures for the TEM

Trump and Bulger (1966) demonstrated that administration of osmium tetroxide and glutaraldehyde simultaneously, minimized most of the deleterious effects of using these fixatives sequentially. Franke et al. (1969) reported that the advantages of this technique were the preservation of cytoplasmic microtubules and ribosomes and the increased contrast distinctiveness of the membranes. Precipitation contaminants frequently caused by fixation were also noted to be eliminated.

c. Perfusion Fixation

Uniform fixation of tissue for electron microscopy can be achieved through perfusion fixation (Veerman et al., 1974). Preservation of the periodontal ligament using this technique was first used by Bernick in 1962.

d. Dimensional Changes During Tissue Processing

EDTA demineralization at 4°C was used in this study in preparation of animal number 229. This was the same technique used by Crowe (1989) in preparation of the other animals used. The original decision to use this procedure was based on findings reported by Fejerskov (1971). EDTA allowed the best preservation of cytological detail and demineralization at 4°C reduced the amount of cellular dissociation in contrast to the effects of other agents studied.

Tissue dehydration has been reported to result in cell shrinkage (Bahr et al., 1957; Kushida, 1962). Weibel and Knight (1964) demonstrated that dimensional changes were minimized when dhydration in alcohol schedules were started in 70% ethanol in comparison to using 50% ethanol.

Kushida (1962) found that cellular shrinkage of 0.6% occurred during the polymerization of Epon 812. Weibel and Knight (1964) also considered that errors arising from block shrinkage when using embedding material, were of

little importance. Agar 100 (Agar Aids, Essex, England) was the embedding material used for all the animals. This material is in the same family as Epon 812 and, therefore, similar effects can be expected with it.

Consideration of the work by Kushida (1962) and Weibel and Knight (1964) has led to the consclusion that dimensional changes in this study were likely to be less than 1% linearly and less than 2% volumetrically. As dimensional changes during tissue processing were considered minimal and do not effect estimation of volume proportion (Weibel, 1979), they were ignored.

Williams (1977) advocated the use of heavy metal stains to achieve maximum contrast. Poor section contrast can result in under estimation of tissue components. Therefore, sections were stained in this present study using modified Reynolds' lead and uranyl accetate.

Dimensional changes in periodontal ligament using similar fixation and tissue processing techniques to this project are not available, as reported by Crowe (1989). Dimensional changes during fixation and tissue preparation are tissue and methodology dependent (Bahr et al., 1957; Kushida, 1962), therefore, results from other studies cannot be extrapolated directly.

6.2 TEM MORPHOMETRIC AND STEREOLOGIC TECHNIQUES

a. Tissue Sampling

Gundersen et al. (1988a,b) discussed techniques necessary to ensure unbiased tissue specimens for statistical information to be "true". In standard methods of stereology, the planes of section must be isotropic, uniform random (IUR), or the structure itself must be statistically isotropic.

Biological structures such as the PDL are always anisotropic and also it may be impossible to make IUR tissue sections. A solution to the problem of anisotropy is based on the principle of vertical sections. This technique ensures that completely unbiased tissue specimens are obtained for stereological analysis. Baddeley et al. (1986) discussed the four requirements which must be fulfilled in handling of tissue for use in vertical section stereology.

Morphometry provides results which are true for the particular plane of section studied and not to the tissue as a whole. However, Crowe (1989) due to time constraints was only able to quantify one section per side in the apical region. In the present study sections were taken down the whole length of the PDL.

Recently, Gundersen (1989) has discussed the lack of need of vertical sectioning for stereological analysis of tubular structures i.e. blood vessels and nerve axons. This analysis was based on the branching and irregularity

in distribution of blood vessels and axons, which provides adequate randomness to ensure a completely unbiased specimen to gather stereological information.

Stereological calculations are derived from the mathematical probability with which section profiles coincide with an appropriate bias free test system, and that the measurements of stereological parameters assume a random orientation of profiles measured against the plane of section (Weibel, 1979). Micrographs should be well dispersed throughout the tissue to ensure a rigorous random sampling. Ebbeson and Tang (1967) demonstrated that when an inherent periodicity is not present, stratified or systematic sampling gives rise to the smallest standard errors.

Weibel (1979) reported on the importance of section thickness. If the section thickness approaches 1/12 of the profile diameter, then correction for the Holmes effect should be considered. Ultrathin sectioning presents the problems of compression and distortion. Compression of the section is inversely proportional to section thickness. The compression and distortion was kept to a minimum by cutting sections using a new diamond knife in the silver interference range. Additionally, sections were expanded with chloroform vapour before collection.

b. Point Counting

A TEM micrograph magnification of 3,000x is adequate for the recognition of cell and blood vessel types. All photomicrographs were printed at a final magnification of 8,500x. The magnification was standardized using a replicating graticule.

Weibel (1979) concluded that systematic point counting was most accurate in comparison to a number of other methods available for measuring volumetric proportion. This method is also good for estimating areal fraction (Gundersen et al. 1988a,b).

Square lattice test grids are preferred to triangular grids because direct analysis of surface density need not be undertaken. Weibel (1979) also concluded that test points should be spaced such that only one point lay on the same profile. Mathieu et al. (1980) stated that to attain the accuracy of a manual optical picture analyser, 64 points should be used. Freezer (1984) tested a 35 point grid against a 140 point grid and found no significant difference. According to Weibel (1979) a 35 point grid does not adequately recall rare or small profiles, but can be overcome by using a large enough number of micrographs. In this investigation, a 140 point grid was used so as to fulfill the requirements for point counting axons.

The convention of forbidden lines (Figure 4.6, p.72) was used in this study to determine the number of profiles

per unit area. The size of periodontal blood vessels makes the convention of forbidden lines inaccurate for determining the number of vessels per unit area (Casley-Smith, 1984). A more appropriate system of ascribing the value of 1/4 for each side of the micrograph print that intersected with a blood vessel was used.

6.3 BLOOD VESSELS

a. Control Side

Douvartzidis (1984) found the vascular volume of the marmoset molar to be 8.3%. A volume of 11.26% was found in the present study of the marmoset maxillary incisor. This difference may reflect site specificity or vascular anatomy arising from a different root number and arrangement, or differences in TEM and light microscopy accuracy.

Crowe (1989) investigated the subapical vasculature of the material used in this present study and found the vascular areal fraction of the control to be 11%. This is similar to the volume density found in the present study, despite the differences in regions investigated.

Freezer (1984) and Freezer and Sims (1987) carried out the only studies, other than the present study, to stereologically investigate PDL vascular volume proportions. The periodontal ligament in the mouse molar between the alveolar crest and apex had a vascular volume

of 7.5%. Freezer and Sims (1987) did not include the region of the PDL below the root apex (subapical area) in their examination. This present study looked at a comparable region in the marmoset PDL and provides the first stereologically estimation of normal primate incisor vasculature for this region. As methodological, site and species differences apply, the results are not immediately comparable.

Gould et al. (1977) investigated the periodontal ligament mesial to the mouse mandibular first molar and found that 73.4% of vessel volume was in the bone half. McCulloch and Melcher (1983) found that the bone half is 4 times more vascular than the tooth half. Gould et al. 1977 based their estimates of mouse molar vascularity on tissues from a single animal. McCulloch and Melcher (1983) provided only limited data restricted to coronal samples from an isolated mesial site. Both studies reflect limited sampling techniques.

Freezer(1984) and Freezer and Sims (1987) found that in the tooth, middle, and bone thirds, blood vessels occupied 0.2%, 6.2% and 15.8% of the mouse PDL volume, respectively. Their findings were not supported by the present study, but are consistent with that of Douvartzidis (1984), that the vascular volume of the marmoset is greatest in the middle, lateral thirds of the ligament. In this present study no blood vessels were found in the tooth third of the control animals.

Interstudy comparison is difficult due to different experimental techniques or methods of sample selection, animal variability, tooth differences and site specificity, as well as quantification.

Little consideration has been given to depth effects in previous morphometric studies, although vascular pattern changes are said to occur down the root length in the rat molar (Garfunkel and Sciaky, 1971). Gotze (1976) reported a progressive increase in the vascular volume in man from the cervical to apical thirds. Sims(1987) found the effect of depth to be quadratic in nature. The larger vascular volumes at the cervical and apical regions and lower vascular volumes opposite the middle third of the root. Douvartzidis (1984) demonstrated similar findings to Gotze (1976) in the marmoset. The present study found the mean vascular volumes of the coronal, middle and apical thirds to be 11.20%, 12.09% and 10.33% of the PDL, respectively. This data corresponds to that reported by Sims (1987). However, this was not a trend seen when looking at the control animals individually i.e. there was marked animal variation.

It was observed in this study that the luminal and abluminal diameter of postcapillary-sized venules and abluminal diameter of terminal arterioles, increased going down the root length towards the apex.

Previous studies Freezer and Sims (1987), Sims (1987), Crowe (1989) have looked at the luminal vascular

volume only. No attempts have been made to quantify the vessel wall vasculature volume. This may possibly be related to difficulties in assessing accurately the total blood vessel wall perimeter. The vessel wall volume was found to be 3.57% of the total PDL giving a total vascular volume of 14.83%.

The present study demonstrates that the marmoset incisor periodontal ligament has a predominantly venous luminal vascular bed comprising 90.59% of the total vascular pool. A similar finding was reported by Crowe 1989 in the subapical region. Wong and Sims (1987) also observed that a venous pool characterizes the mouse PDL.

Blood vessel stereological parameters indicated that the mean blood vessel length per unit volume within the ligament was 46.0 x 10^3 cm/cm^3 and the mean surface-area per unit volume was 253.19 cm^2/cm^3. Freezer and Sims 1987 reported a similar length density (L_V) in the rat molar ligament of 46.5 x $10^3 cm/cm^3$. A slightly lower value of 208.8 cm^2/cm^3 was reported for surface density which may reflect a smaller overall mean diameter and/or may be related to the method of calculation.

Unlike Rhodin (1968) and Freezer (1984), the present author found few vessels in sections with complete pericytic coverings. No pericytic venous capillaries were identified in the control. Pericytic postcapillary-sized venules comprised 4.74% of the total vascular pool (V_V) as compared to 42.51% for apericytic postcapillary-sized

venules. It is difficult to accurately identify pericytic processes and easy to confuse them with abluminal endothelial processes. The pericytic layer was usually incomplete and was in juxtaposition with the abluminal surface of the endothelial cells, separated usually by only the intervening basement membrane.

Investigations by Parfitt (1960), Wills et al. (1976), Gotze (1980) and others, have emphasized the role of the vasculature in the tooth support mechanism. The various postulations proposed have been based on subjective interpretation and little quantitative, experimental substantiation.

Parfitt (1960) and Bien (1966) proposed that the pulsatile nature of blood flow within the PDL is responsible for supporting the tooth when subjected to axial loading. Wills et al. (1976) and Walker (1980) employed models to explain the relative contribution of the PDL vasculature to tooth support. Walker (1980) proposed that collagen is loaded in parallel with the periodontal vasculature. The hypothesized model of vascular tooth support also presupposes that vascular elements in compression participate in tooth support and not in tension states (Walker, 1980). However, the present study supports previous studies (Lew 1986; Crowe 1989), that there is a change in vascular proportions when tensional loads are applied to teeth.

b. Experimental Side

There have been previous studies which have investigated vascular changes subsequent to orthodontic tooth movement. Rygh et al. (1986) demonstrated increased vascularity in the tension zones of tipped rat molars. No morphometric or stereological assessments were carried out. Assessment of dilation according to vessel diameter, perimeter and area may be a reflection of the plane of section. The actual increased vascular activity could be representative of a number of factors, including an increase in vascular volume or length, or flow rate, animal variability, or a combination of these factors, but they are difficult to define from Rygh's study.

Macapanpan et al. (1954) and Khouw and Goldhaber (1970) reported dilation of vessels in tension zones after the application of continuous forces of various durations. Rygh (1976) investigated the ultrastructural changes in the tension zones of the rat molar PDL. Rygh reported dilation and distension of blood vessels in the direction of strain. Lew (1986) reported vascular dilatation following short-term extrusion of a rat molar.

Crowe (1989) was the first to quantify the long-term effects on the subapical vasculature following Marmoset incisor extrusion. The postcapillary-sized vessels and collecting venules, as a group, reduced as a proportion of the vascular area by 9%. A dramatic increase in the areal fraction (0.24% to 1.73%) and profile boundary length

(1.01% to 2.45%) was reported in the terminal arteriole group. Comparable findings were reported in the present study, although no statistical analysis following treatment effects were reported by Crowe (1989).

There was a fall in mean luminal caliper diameter across most vessel groups except for terminal arterioles, which showed an increase. The decrease in luminal diameter of postcapillary-sized venules was significant at $p<0.05$. There also was observed a significant decrease abluminal in diameter at $p<0.05$. The mean caliper abluminal diameter of terminal arterioles increased from 24.99μm to 29.0μm ($p<0.01$).

Unlike Crowe's (1989) study, an increase in volume of the capillary bed was noted. The only statistically significant change in volume density (V_V) was a decrease in postcapillary-sized venules.

The number of venous capillaries per 560μm of PDL tissue increased from 0.07 to 0.17, which was significant ($p<0.01$). All other vessel types were not statistically significant.

These changes suggest that revascularization is still incomplete for the venous pool. The implication is that revascularization is characterized by venous capillary sprouting. The significant decrease in postcapillary-sized venules, suggest that revascularization is incomplete. It can not be predicted what would happen in the long-term. The postcapillary-sized venules appear to

be the more sensitive component and the effect may be permanent or transitory i.e. no impairment to morphology of the ligament. Due to the lack of statistical significance in vascular changes, conclusions must be considered quite tentative. Weir (1990) found no change in postcapillary-sized venules in the apical PDL region after a 30 week retention period. This suggests changes in postcapillary-sized venules to be transitory.

Overall, the results from the present study indicate that revascularization was essentially complete by the end of the retention period. The only differences still evident, being in the proportions of the various blood vessel types occupying the PDL examined.

A nine week retention period in the marmoset has a lifespan which approximates an eighth of a human lifespan. Therefore, extrapolation of lifespans between the human and marmoset indicate a retention period of nine weeks in the marmoset would correspond to approximately one year in the human.

Recommended orthodontic retention periods in the literature are rarely based on biological data. The data from the present study implies that, for rapid tooth movement with fixed short-term retention, PDL reconstitution is essentially complete. However animal tissues generally heal very rapidly. Therefore for some human patients retention periods in excess of one year are indicated for microvascular reconstitution.

6.4. NERVE AXONS

a. Control Side

The ultrastructure of the myelinated and unmyelinated nerves found in the present study was similar to that reported by Griffin and Harris (1968),Corpron et al. (1974) and Freezer and Sims (1989).

There are fewer studies quantifying periodontal axons than for other periodontal structures. Kubota and Osanai (1977) quantified the periodontal free nerve endings and the nerve fibres, of the Japanese shrew mole using light microscopy. Comparison was made between the innervation at different depths, using 30μm thick celloidin sections. The periodontium was found to be more densely supplied by the nerve endings at the apical region than at the intermediate zone by a ratio of 8:1 in the maxillary dentition.

Freezer (1984) and Freezer and Sims (1987,1989) published TEM investigations of the periodontium of the mouse molar. Morphometric data indicated that unmyelinated axons constituted approximately 95% of all periodontal axons (N_A). Similarily, in the present study, unmyelinated nerves comprised 88.58% of all periodontal axons (N_A).

The mean caliper diameters of unmyelinated and myelinated axons , 0.67μm and 2.94μm respectively, are

smaller than the diameters (1.32μm and 4.59μm) reported by Crowe (1989) in the subapical region, where the principal nerves enter the PDL. The range in diameters for both unmyelinated and myelinated axons in the present investigation was 0.12μm - 1.18μm and 1.53μm - 5.76μm, respectively. This may be indicative of site specificity. The variations in diameter of unmyelinated nerve axons, correlated with the size range reported by Berkovitz and Shore (1978) and Freezer and Sims (1989). The observed sizes of myelinated axons also correlated to the range reported by Freezer and Sims (1989) in the mouse molar. These diameters were far less than for other species (Hannan, 1982), where periodontal myelinated nerves up to 14 μm in diameter were reported.

In the present study the prime location of unmyelinated axons was within the parenchyma of the ligament. This is in contrast to Freezer and Sims (1989) who reported that the majority of unmyelinated axons, in the mouse molar PDL, occurred in close apposition to the abluminal endothelium of blood vessel.

The present investigation demonstrated unmyelinated axons forming a close relationship to postcapillary-sized venules, terminal arterioles and collecting venules. The juxtapostion of free nerve endings to blood vessel abluminal endothelium is at variance with the classic texts on the ultrastructure of blood vessel walls in other connective tissues (Rhodin 1967,1968). But unlike the

report of Freezer and Sims (1989), both the present study and that of Crowe (1989) found this type of vessel to nerve relationship very uncommon, although it was not quantified by Crowe (1989). The significance of this difference is uncertain, but may be related to a species difference or regional anatomical differences.

Freezer and Sims (1989) reported a total volumetric density for all nerve axons of 0.5% and excluded the subapical PDL area, making their results comparable to the present study. The total volumetric neural density in the present study was found to be 0.43% of the Marmoset periodontal ligament.

However, unlike Freezer and Sims (1989) the distribution in the lateral thirds was dissimilar. Freezer and Sims (1989) found that the alveolar annular third of the ligament had twice the innervation of the middle third, while the tooth third contained minimal amount of periodontal innervation. The present author found the middle third to have a neural volumetric density approximately 6 times that of the bone third, with negligible innervation in the tooth third. This difference, expressed as a ratio of 6:1, may be indicative of species specificity.

This study supports the finding of Byers and Holland (1977) and Byers (1985) that innervation of the cementum via the PDL, does not seem to occur. The tooth third in the present study, lacked nerve axons and was found to

constitute only 1.16% neural volumetric density of the periodontal ligament. These findings question the validity of cementum innervation as reported in the histological investigations by Bernick (1956,1957) and Bernick and Levy (1968b).

The investigation by Freezer and Sims (1989) is comparable to the present study regarding changes in neural volumetric density at different levels i.e. both studies indicated greater innervation (V_V) in the apical region. Crowe (1989) reported an areal fraction of 1.6% in the subapical area of the marmoset incisor. This is not unusual, since the subapical area is known to be the main portal of entry of neural fibres from the bone into the periodontal ligament. Byers et al. (1986) injected tritiated proline into the mesencephalic nucleus of the cat and reported labelled structures in the area close to the root apex. However, while Linden and Scott (1988) reported that receptors in the cat PDL, which are represented in the mesencephalic nucleus, are unequally distributed around the tooth root. The difference in findings may possibly be related to technique variations.

b.Experimental Side

There are very limited studies pertaining to neural changes incident to orthodontic tooth movements, making comparisons between studies difficult. Specific data incident to orthodontic extrusion is lacking.

Crowe (1989) is the only study which quantified neural changes incident to orthodontic extrusion, although direct comparison is difficult as no statistical analysis was carried out by Crowe. Crowe (1989) reported on changes in the subapical region of the marmoset incisor. The present study is, therefore, the first to quantify the effects on the neural component of the PDL (excluding the subapical region) of the extrusive process.

In the present study the neural volume of the ligament increased from 0.43% to 0.56%. Crowe (1989) also reported an increase in neural area. A more outstanding feature reported by Crowe (1989) was the alteration in the proportion of unmyelinated axons to myelinated axons, from a ratio of approximately 1:4 to one of 3:2. In the present study the neural volume density of the PDL changed from 0.12% to 0.13% and from 0.31% to 0.43% for unmyelinated and myelinated axons, i.e. 1:2.5 to 1:3.3 respectively. However, although the neural volume density increased, the proportion of unmyelinated to myelinated axons also changed. The mean number of unmyelinated axons of the total number of axons changed from 88.58% to 85.62%. The lack of statistical significance for the neural parameters makes any hypothesis tentative. Within the limits of the the experimental procedures, it would appear that reconstitution of the neural system was complete.

Like Crowe (1989) who found an increase in mean caliper diameter for myelinated and unmyelinated axons, the present study showed a subtle increase from 0.67μm to 0.80μm and 2.94μm to 3.68μm for unmyelinated and myelinated axons, respectively.

Ultrastructural studies of neural regeneration, generally concentrate on reponses to axotomy. Whether this is analogous to the present experimental situation is uncertain, as the amount of tooth movement was probably not traumatic enough to cause nerve axon rupture. Murphy et al.(1990) investigated the effects of CNS and PNS axotomy of the IVth nerve in the cat, on cell death, soma size, axon size and axon number. Counts and measurements of axons revealed that mean axon diameter of regenerated axons was much smaller than normal, 3 months after axotomy. There was an increase in diameter during the 3rd to 6th postoperative months, but then showed no subsequent increase in and remained below normal. Measurements were made for myelinated axons only.

The findings of the present study suggest that nerve regeneration is complete as indicated by the return of values within that of the control. Recent investigation by Weir (1990), on the long-term response of the neural component of the PDL to orthodontic tooth movement, reported neural regeneration as complete after 6-7 months in the marmoset.

Schubert (1984) reported that neural growth seems to be assisted by substances such as nerve growth factor (NGF), cyclic AMP and prostaglandins E_1 and E_2. Davidovitch and Shandfield (1975) have shown that in areas of alveolar bone tension, there is an initial fall in cyclic AMP level which may be related to an increase in cellular division. The levels of cyclic AMP later increased and were significantly higher than in the controls two weeks after treatment.

Cells which produce NGF include epithelial cells, smooth muscle cells, fibroblasts and Schwann cells (Thoenen et al., 1988; Lumsden, 1988). Bunge et al. 1987 found neural growth enhanced substrates on Schwann cell extracellular matrix and also on Schwann cells minus their extracellular matrix. Davies et al. (1987) have shown that NGF for mice trigeminal cells to be primarily produced in epithelial tissue and the levels of NGF in these cells is ten times that found in underlying mesenchymal cells. It has been shown that NGF synthesis in a target field commences with the arrival of the earliest nerve fibres.

Lumsden and Davies (1983,1986,1987) in "in vitro" embryonic tissue studies have further supported the role of epithelium in the target fields as being responsible for neural growth. It is suggested that NGF is responsible for nerve growth, but not necessarily

directional growth. Early directional growth is possibly due to unknown chemotrophic influences (Davies, 1988).

It is of interest that Crowe (1989) found that the areal fraction and number of Schwann cells per unit area increased in a similar manner to the change in unmyelinated nerve number, in the subapical periodontal ligament. This finding supports the hypothesis, that epithelium and epithelial cells seem to be the source of many of the growth factors responsible for neural cell formation and regeneration. The present study did not quantify cellular profiles in the remaining non-apical periodontal ligament. Further investigation of the cellular profiles in the present material may confirm the findings reported by Crowe (1989) and may correlate to the dramatic increases in the stereological parameters for myelinated nerves.

CHAPTER 7

CONCLUSIONS

1. The marmoset proved to be an adequate experimental model for the analysis of the periodontal ligament above the subapical region.

2. The periodontal tissue was well preserved using osmium tetroxide and glutaraldehyde as the fixative.

3. The size of the tissue blocks limited the amount of resin penetration. This affect resulted in some variation in hardness through the blocks. This variable difference affected the ease of tissue sectioning to some degree.

4. This investigation is the first to stereologically quantify the neural and vascular components of the marmoset PDL above the subapical region, following endodontic therapy, orthodontic extrusion and a nine week retention period.

5. The experimental parameters of V_V, L_V, S_V, N_A and $\bar{d}$, calculated for myelinated and unmyelinated axons demonstrated no statistical differences from the control, thus suggesting complete neural reconstitution.

6. The vessel wall and luminal volumetric densities (V_V) of postcapillary-sized venules as percentages of the total PDL volume, decreased from 1.64 ± 0.16% (control) to 1.03 ± 0.16% (experimental) and 5.32 ± 0.61% (control) to 3.04 ± 0.61% (experimental), respectively. Both findings were statistically significant at $p<0.05$. These changes may imply that regeneration of this vessel-type was incomplete.

7. Compared to the "control" PDL, the "experimental" PDL demonstrated a statistically significant ($p<0.05$) increase in the mean number of venous capillaries per 560μm^2 of PDL tissue. The venous capillaries increased in number by 2.4 times in the "experimental" PDL (from 0.07 ± 0.03 / 560μm^2 to 0.17 ± 0.03 / 560μm^2). This change suggests possible reconstitution of the PDL tissue through venous capillary "sprouting". Therefore, venous capillaries are very active in the repair process.

8. Overall, despite the limited statistically significant changes reported in individual vessel-types, the statistical analysis of the stereological data from the control and experimental PDL tissue above the subapical region, indicates reconstitution is essentially complete following the treatment carried out.

9. A statistically significant trend exists in the mean luminal diameter of terminal arterioles for depth (negative correlation) i.e. the diameter increases towards the apex. A similar finding occurred for the mean abluminal diameter of the postcapillary-sized venules.

10. The total mesial luminal vascular volume in the marmoset incisor was 11.26%. In contrast, Douvartzidis (1984), on histological evidence, reported the marmoset molar to be 8.3%.

11. Although not statistically tested, the lateral vascular volume distribution appears region dependent. The greatest vascular volume density was observed in the middle, lateral third. This distribution variation was also noted by Douvartzidis (1984), possibly reflecting species specificity.

12. Very few pericytic vessels were noted, which is in contrast to the report by Freezer (1984) for the mouse molar. Future investigation on the possible significance of this finding may shed light on the function of the pericytic cells.

13. The neural volumetric density distribution in the middle, lateral third of the controls was found to be six times that of the bone third and the tooth third contained

a negligible innervation. In contrast, Freezer and Sims (1989), in the mouse molar PDL, found the bone third innervation to be three times that of the middle third.

14. SUGGESTED AREAS OF FUTURE RESEARCH

a. A true stereological quantification and statistical analysis of the subapical PDL from the same animals, would allow a more accurate and direct comparison to be made with the present study. It would also provide an assessment of regional differences.

b. Comparison of a similar region of the PDL tissue, in teeth which have been moved laterally, where definite bone resorption and apposition have occurred. This may possibly demonstrate a totally different vascular/neural regeneration.

c. As this study used female marmosets, an evaluation and comparison with male marmosets may indicate the influence of sex variations.

d. This study was evaluated in only one time-frame (nine weeks retention). Carrying out time-course experiments would allow comparisons to be made for the vascular and neural parameters examined. Time-course studies would also allow determination if the

shift in the vascular components, observed in the present study, resolved.

e. The true experimental effects of this study could be determined by having a control group which had endodontic therapy only and another control group which had orthodontic extrusion only. These procedures would allow true determination that the changes reported in this study were as a result of orthodontic extrusion only.

f. Using a larger sample, for example more tissue from the same animals or 8-12 animals in each group, would likely reduce the standard errors of the parameters under study and improve the accuracy of the results obtained.

g. The use of cell markers to identify cell types, especially pericytes, which proved to be difficult to distinguish form fibroblasts.

h. Investigation of the microphysiology of the vasculature transport mechanisms, using tracer molecules to observe the pathways across the endothelium. This would provide insight into any changes in the permeability characteristics of

vessels, in reponse to orthodontic extrusion and short-term retention.

CHAPTER 8

APPENDICES

8.1 DECALCIFYING SOLUTION

Solution:	0.1M EDTA in 2.5% glutaraldehyde
Preparation:	74.45g EDTA (Ethylenediaminetetra-acetic acid as disodium; Ajax Chemicals, Sydney, Australia). 1,800ml 0.06M cacodylate buffer by gentle heating. Cool to 4°C, add glutaraldehyde, ph to 6.0 at 4°C using 1N HCL.
Shelf life:	7 days at 4°C.

8.2 CACODYLATE BUFFER (sodium cacodylate; BDH chemicals Ltd., Poole, U.K.)

Solution:	0.06M sodium cacodylate.
Preparation:	25.68 gms sodium cacodylate in 2,000 mls d.d. water. Adjust to ph 7.4 using 1N HCL at 20°C.
Shelf life:	7 days at 4°C.

8.3 4% OSMIUM TETROXIDE (OsO_4) SOLUTION

Solution:	4% Osmium Tetroxide

Preparation: Place 2g of osmium tetroxide in 50 ml of double distilled (d.d.) water. Place the ampoule in hot water to melt the osmium tetroxide crystals. Remove from Water and rotate the ampoule to allow the melted osmium tetroxide to form an even film over the inside. When the osmium tetroxide has again solidified, remove the label and clean the outside the ampoules thoroughly with ethyl alcohol. Then drop the ampoule into a thick-walled bottle containing d.d. water and shake to break the ampoule. Wrap in foil to exclude light and leave in the fume cupboard.

Shelf life: 7 - 10 days. This solution can only be used when clear. If it becomes straw-coloured or darker, then its fixative properties are greatly reduced. Refrigeration is not advised as it increased the rate of oxidation and because osmium tetroxide is so highly toxic.

8.4 BLOCK STAIN

Solution:	1% uranyl nitrate in 70% alcohol.
Preparation:	1 gm uranyl nitrate.
	70 ml ethyl alcohol.
	30 ml d.d. water.
Shelf Life:	7 days at room temperature.

8.5 LIGHT MICROSCOPE STAINS

a) 0.05% TOLUIDINE BLUE

Solution:	0.05% Toluidine Blue in d.d. water.
Preparation:	0.05g Toluidine Blue.
	100 ml d.d. water.
	Dissolve by stirring.
Shelf life:	6 months at room temperature.
Precautions:	Avoid skin contact.

b) 1% BORAX

Solution:	1% Borax in d.d. water.
Preparation:	1 g of sodium Thiosulphate (Borax).
	100 ml of d.d. water.
	Dissolve by stirring.
Shelf life:	6 months at room temperature.
Precautions:	Avoid skin contact.

8.6 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

Solution: Modified Reynolds' lead.

Preparation: (i) 1.33 gms lead nitrate
1.76 gms sodium citrate
30 ml d.d. 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 d.d. water, mixing by inversion.

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

8.7 **AGAR EMBEDDING MEDIUM** (Ladd Research Industries Inc.)

The ratio of anhydride to epoxy resin can be varied to obtain blocks of different hardness but, for the purpose of this study, the hardest resin was used. The ratio of anhydride to epoxy equivalent is, therefore, 0.7 : 1 (Luft, 1961).

The two anhydrides used are NMA (MW = 178) and DDSA (MW = 266). The total quantity of resin required from each anhydride is varied in the ratio of 1 DDSA : 2 NMA.

a) Embedding Resin

Mixture A: Agar = 5.00 ml
DDSA = 6.42 ml
11.42 ml

Mixture B: Agar = 10.00 ml
NMA = 8.59 ml
18.59 ml

Total A + B = 30.01 ml

Therefore: BDMA = 0.14 ml/10 ml = 0.42 ml

Thorough mixing is essential, otherwise blocks with poor sectioning properties will be obtained.

b) Infiltration Resin: Ratio 1:2 (15-30)

Mixture A: Agar = 15.00 ml
DDSA = 19.26 ml
34.26 ml

Mixture B: Agar = 30.00 ml
MNA = 25.78 ml
55.78 ml

Total A + B = 90.04 ml

Therefore: BDMA = 0.14 ml/10 ml = 1.26 ml

Precautions: Agar 100 is a suspected carcinogen and should thererfore be handled with great care. Agar, NMA, DDSA and BDMA are all toxic if swallowed, and can cause skin irritation. Work with the components must be conducted in a fume cupboard, avoiding exposure to vapours, and avoiding contact with skin, eyes and clothing. Gloves and gown should be worn.

8.8 RADIOGRAPHIC EQUIPMENT

Kodak periapical ultraspeed film: 22 x 35 mm.,

Siemens Heliodent machine.

Voltage:	50kV
Current:	7mA
Exposure time:	0.1sec

8.9 TRANSMISSION ELECTRON MICROSCOPE

JOEL 100S (JEOL LTD., TOKYO, JAPAN)

1) Accelerating voltage of 60kV.
2) Beam current of 50 micro amps.
3) Gun bias setting of 5.
4) Objective lens aperature of 1.
5) Field limiting aperture setting of 2.

8.10 STEREOLOGICAL EQUATIONS

$$N_A = N / 560$$

Adapted from Atherton, Cabric and James, 1982

$$V_V = \frac{V(i)}{V(ref)}$$

$$L_V = \frac{V_V}{\bar{d}^2 \ \pi/4}$$

$$S_V = \bar{d} \ \pi \ L_V \qquad \text{Gundersen, 1989}$$

Symbols used in stereological equations

Vv	Number of test points falling on profiles of a component divided by the total number of test points.
N	Number of profiles per micrograph (using the convention of forbidden lines).
$\bar{d}$	Mean caliper diameter of a component.
N_A	Number of profiles per unit area.
V_V	Volume density.
S_V	Surface density.
L_V	Length density. -Compartment of interest (i) -Reference compartment (ref)

8.11 RECORDING SHEET

Micrograph Number	
Animal Number	
Depth (microns)	
Region (tooth=1, middle=2, bone=3)	

PROFILE		FINE GRID	NUMBER (N)
VESSEL LUMEN			
(1).Arterial Capillaries			
(2).Venous Capillaries (a). pericytic			
(b). non pericytic			
(3).Postcapillary-Sized Venules (a). pericytic			
(b). non pericytic			
(4).Collecting Venules			
(5).A-V Anastomoses			
(6).Terminal Arterioles			
Unmyelinated Nerves (a). in Collagen			
(b). in BV walls			
Myelinated Nerves (a). in Collagen			
(b). in BV walls			

CHAPTER 9.

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