T.E.M. STEREOLOGY OF MICROVASCULAR AND NEURAL RECONSTITUTION IN MARMOSET INCISOR PERIODONTAL LIGAMENT FOLLOWING INCISOR DECORONATION, ENDODONTIC THERAPY, ORTHODONTIC EXTRUSION, AND LONG TERM RETENTION

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

by

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SUMMARY

Maxillary incisor fracture is a common dental injury. It is frequently treated by endodontic therapy, orthodontic extrusion, and a retention period. The principal aim of the present study was to mimic this regime and test the hypothesis that vascular and neural reconstitution of the periodontal ligament (PDL) was complete after a long term non-human primate retention of 30 weeks in the marmoset, which approximates 3 years in human terms.

This investigation was based on material prepared by Weir (1990), but not utilized in his study which was limited to the apical region. Weir used 7 male and 3 female marmosets (*Callithrix jacchus*) as the model to simulate this treatment regime. However, he finally selected 4 male marmosets to be a study group. His experimental procedure involved the upper left central incisor crown removal, root canal therapy, followed by magnetic extrusion of 1.2 mm. The fractured incisor was retained in the extruded position for 30 weeks. The upper right central incisors acted as the control.

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

The remaining non-apical segments of the blocks were sectioned at 150 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 e.g. orientation of the collagen fibres and cells perpendicular to the tooth surface. The most mesial PDL regions of the control and experimental central incisors were examined. At each level three micrographs were taken across the PDL width, at the bone, middle, and tooth circumferential thirds.

The micrographs were printed for morphometric and stereological analyses. Blood vessels were stereologically quantified, while nerves were

morphometrically quantified due to the limited sample of nerves. The effects of orthodontic extrusion on vascular and neural components of the PDL were assessed to determine if any changes occurred in the experimental PDL.

No significant differences were found in (1) total luminal and abluminal volume, (2) luminal and abluminal volume of each blood vessel type, (3) distribution of blood vessels across the PDL, (4) luminal diameter of each blood vessel type, and (5) nerve volume. These findings indicated that reconstitution of the PDL blood vessels and nerves was essentially complete after extrusion and a long term non-human primate retention period of 30 weeks. However, a significant reduction of the wall thickness of postcapillary-sized venules was present. This outcome suggested that while physiological re-establishment of the vascular system had occurred, the morphological reconstitution of postcapillary-sized venules was incomplete or they had undergone a longer term or permanent change.

The present study revealed that simulated incisor fracture followed by endodontic therapy, orthodontic extrusion, and a long term animal retention period resulted in repair of the PDL microvasculature and neural system. Therefore, it would seem that this procedure is a justifiable clinical treatment rationale.

The results from this study have clinical implications in that determination of the timing of the retention period is orthodontically important. As far as complete reconstitution of the PDL blood vessels and nerves is concerned, on the basis of this animal model, a retention period of between 1 and 3 years in human terms is suggested in order to maximise reconstitution of both systems.

SIGNED STATEMENT

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

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I give consent to this copy of my thesis, when deposited in the University Library, being available for photocopying and loan.

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ADDENDUM

p8	last para	add 'were used' after 'technique'
p8	2.4	magnetic force systems
p43	para 3	change 'incident to' to 'consequent upon'
p47	para 4	1st sentence, 'was' to 'is'
p60	Fig 3.6	2nd last line corner
p89	para 1	change 'replicating graticule' to 'grating replica'
p91	para 3, 1st line	'vessel' missing after 'blood'

CHAPTER 1
AIMS OF THE INVESTIGATION

Accidental fracture of incisor crowns frequently occurs in school children, athletes, and persons in vehicle collisions. In cases where the fracture extends subgingivally, exposure of the tooth structure becomes paramount to restore the tooth. Fugazzotto (1985) discussed the implications of subgingival crown margin placement which included an increase in the incidence of periodontal problems and the risk of recurrent decay. To avoid placing deep subgingival crown margins, Heithersay (1973) suggested the incisors should be treated by endodontic therapy, orthodontic extrusion and a retention period to allow for the construction of a post-core and crown.

The question is what happens to the ultrastructural arrangement of periodontal ligament (PDL) nerves and blood vessels after endodontics, tooth extrusion and a long term period of retention. Crowe (1989) mimicked this clinical regime in marmosets followed by a short term animal retention period. He demonstrated that reconstitution at the root apex was complete for nerves but not blood vessels. Parlange and Sims (1993) studied Crowe's PDL specimens between the cervical and apical region. Similarly, they found that re-establishment was complete for nerves but not all the vessels. Weir (1990) used the marmoset model to simulate long term animal retention for 30 weeks and demonstrated complete neural and blood vessel reconstitution in the PDL maxillary incisor apex. The PDL from the root end to the cervical region of the Weir marmosets has not been investigated after long term retention. This region has been studied in the present study. The aims of this investigation were to:

1. Section the remaining non-apical segments of the blocks prepared by Weir (1990) from four male 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 PDL at 150μm levels, between the alveolar crest and apical region.

2. Perform TEM examination of the marmoset incisor PDL.
3. Provide information regarding the vascular and neural distribution within the PDL with particular reference to the lateral and vertical thirds.
4. Use standard point counting methods to provide morphometric and stereological data.
5. Calculate the mean diameters ($\bar{d}$) and the stereological parameters (V_V, L_V, S_V, and N_A) for periodontal blood vessels and nerves.
6. Statistically analyse the microvasculature and nerves within the PDL following the treatment regime of endodontic therapy, orthodontic extrusion and a 30 week retention period.
7. Test the hypothesis that neural and vascular reconstitution of the PDL from the root end to the cervical region is complete after a relatively long term non-human primate retention period of 30 weeks.

CHAPTER 2
LITERATURE REVIEW

2.1 FRACTURE OF ANTERIOR TEETH

Fractured anterior teeth are a common sequelae of traumatic injuries to the facial area. The consequences to the appearance of the patient from such injuries cause considerable concern to both the patient and, in the case of a child, his or her parents.

Common aetiological factors include the battered child syndrome, school ground injuries, fight injuries, contact sport injuries, bicycle, and automobile injuries, etc. (Andreasen , 1981).

The incidence of these injuries was found to be higher in boys than in girls by 2:1 (Grundy, 1959; Zadik et al., 1980; Hargreaves, 1981; Burton et al., 1985). Edward and Nord (1968) found that 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. Burton et al. (1985) studied 12,287 students, mostly teenagers, and found that the total incidence of children with traumatised teeth was 6.06%.

In the case of subgingival fractures, adequate restoration of the teeth with a close fitting margin is difficult to achieve. This, in turn, may lead to the periodontal problems and an increased risk of recurrent caries (Fugazzotto, 1985). Exposure of subgingival margins by the periodontal surgery and localised osseous recontouring often created severe periodontal and aesthetic problems (Simon et al., 1980; Ingber, 1976; Potashnick and Rosenberg, 1982).

Heithersay (1973) suggested combined endodontic therapy and orthodontic extrusion to avoid placing deep subgingival crown margins. Subsequently, this procedure has been reported to avoid extraction of the root

and replacement, or periodontal surgery (Cooke and Scheer, 1980; Potashnick and Rosenberg, 1982; Garrett, 1985; Biggerstaff et al., 1986).

Ingber (1976) found that as a tooth is erupted, the gingiva and alveolar crest will follow and an increase in the attached gingiva zone causes a change in the position of the soft tissue. 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 practically unchanged, or possibly improve, in comparison to that of the treatment method of the osseous surgery.

2.2 THE EFFECT OF ENDODONTIC THERAPY ON THE PDL

While no transmission electron microscope (TEM) studies have been carried out to investigate the effects of the root canal therapy on the periapical tissues, a number of histological studies have been performed (Muruzabal and Erausquin, 1966; Seltzer et al., 1969; Bhasker and Rappaport, 1971; Molooly et al., 1979; Pascon and Spanberg, 1990).

Gutta-percha has been the most widely used solid-core root canal filling material (Ingle and Taintor, 1976). Pascon and Spanberg (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 was 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 (Ingle and Taintor, 1976). Muruzabal 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

inflammation was observed. By contrast, there was more inflammation in those specimens which were overfilled (Bhaskar and Rappaport, 1971; Soares et al., 1990).

2.3 THE EFFECT OF EXTRUSION ON THE HISTOLOGY AND ULTRASTRUCTURE OF THE PDL

The microscopic effect of extrusion on the PDL has been investigated 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), and Lew (1986, 1989).

Huettner and Whitman (1958) extruded the central incisors in 10 *Macaque rhesus* monkeys for 12 weeks and found histologically that the direction of the PDL fibres changed their orientation to the line of movement and new bone spicules formed in the apical region.

Reitan (1967) reported that extrusion caused stretching of the principal PDL fibres, which resulted in the bone formation apically and at the alveolar crest. Similar features, in a histologic extrusion study using 14 teeth in 3 dogs, were described by Simon et al. (1980). In 1974, Reitan observed in the apical region of 30 human teeth after extrusion with various force levels. He found a variation in the histology between cases, such as small resorptive lacunae, hyalinisation of the ligament, resorption of the middle third of the root and some apical cemental deposition. The root resorption in the areas of ligament tension was considered an exception rather the rule.

Batenhorst et al. (1974) studied incisor extrusion in two *Macaca mulatta* monkeys and noted that at the apex of the extruded teeth, there was an increase in deposition of cellular cementum and trabeculation of the apical alveolar bone. However, the sample size of two rhesus monkeys was insufficient for a definitive finding e.g. to eliminate individual variation. Batenhorst et al.

(1974) concluded that the gingiva and alveolar crest followed the extruded teeth as the result of an increase in the zone of the attached gingiva.

The range of loads varied widely in the extrusion studies (Parfitt, 1960; Moxham and Berkovitz, 1979; Cooke and Scheer, 1980), thus making comparisons difficult. Parfitt (1960) applied a load of 0.15-0.3N on the human incisors. Cooke and Scheer (1980) found clinically that the ideal extrusive force laid within the range of 0.7-1.5N. There was no histological or ultramicroscopic study undertaken to confirm these clinical impressions.

The only ultrastructural studies of extrusion are those of Sims (1978, 1980), Lew et al. (1989), Crowe (1989), Cooper et al. (1990), Weir (1990), Tang and Sims (1992), Parlange and Sims (1993).

Lew et al. (1989) demonstrated statistically significant changes to the microvasculature of the rat molar PDL using relatively large extrusive loads of 1.0 N, applied for 30 minutes. These changes included an:

1) increase in the PDL width, resulting in the formation of a tension zone;
2) increase in the mean vascular volume as a percentage of the apical PDL volume;
3) increase in the mean endothelial cell surface area per cubic millimeter in all vessels; and
4) increase in fenestrae numbers per cubic millimeter of the PDL.

Lew et al. (1989) suggested the increase in the functional microvascular bed (MVB) may represent the increased metabolic demands in an injured tension zone. These vascular changes supported the findings of Rygh et al. (1986) who implicated the vascular system as a "main mediator" in the PDL remodelling following the tooth movement.

Crowe (1989) studied the TEM effects of extrusion on the apical region of the marmoset PDL after a retention period of 9 weeks. A load of 0.2 N was applied to extrude an upper central incisor. Crowe's investigation differed

from the studies of Sims (1978,1980) and 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 of the apical region of the marmoset incisor PDL. The ratio of unmyelinated nerve/myelinated nerve volume density changed from 1:4 to 3:2. Crowe (1989) hypothesised that there might be neural regeneration of unmyelinated nerves.

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

4) The vascular proportion of the marmoset control and experimental apical PDL was 11% and 10% of the total apical PDL, 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 the regeneration of neural tissue. It is interesting to note the increase Crowe found in the proportions of epithelial cells and unmyelinated nerves.

Weir (1990) studied the apical region of the marmoset PDL with the same method as Crowe's experiment except that Weir (1990) quantified the post-treatment effects on the fibrous stroma, neural and vascular elements after

a long term retention (30 weeks). He found that the reconstitution of the marmoset apical PDL was essentially complete following the experimental procedure and 30 week retention. Weir's findings are only representative for the plane of section examined. No ultrastructural studies are present which demonstrate the response of the whole PDL after the root canal therapy, orthodontic treatment and a long term retention.

2.4 THE USE OF MAGNETS IN ORTHODONTICS

Magnetic force system are mentioned in the dental literature. Initially they were used to aid retention of dental prostheses when used as jaw implants, as described by Behrman and Egan (1953), Behrman (1960, 1964) and Gilling (1981). The widespread use of magnets in prosthetic dentistry was prevented because the cost of early cobalt platinum magnets was prohibitive, and the magnetic properties were inadequate. Introduction of the small, powerful, and stable samarium-cobalt ($SmCO_3$) magnets, which are also reasonably priced, has led to magnets becoming more popular. Also, the magnet has a high corrosion resistance and is considered to be innocuous to tissues (Tsutsui et al., 1979), although some investigators express a cautionary viewpoint (Linder-Aronson and Lindskog, 1991).

The use of magnets for orthodontic tooth movement was first described by Blechman and Smiley (1978) who bonded aluminium-nickel-cobalt magnets to the teeth of adolescent cats to produce tooth movement. Their work suggested the biological safety of magnets and demonstrated an atraumatic, precise, and efficient method of moving teeth.

The first *in vivo* magnet studies using intermaxillary orthodontic forces were carried out by Blechman (1985), in which samarium-cobalt magnets with a sectional archwire technique. The conclusion from Blechman's study were that:

(1) the magnets provided the tooth moving force thereby removing the need for elastics;

(2) no discomfort during tooth movement was reported; possibly because of the force characteristics provided (a breakaway force of up to 250g was recorded during the study);

(3) unique vector control was possible.

Dellinger (1986) reported an appliance which he called the Active Vertical Corrector for use in open bite cases. It comprised upper and lower posterior bite planes with embedded samarium-cobalt magnets acting in repulsion. The advantages claimed over the traditional bite block, for the treatment of anterior open bites, were that instead of relying on occasional forces from the muscles of mastication, a constant force system was present which resulted in rapid tooth movement. The reduction of anterior facial height accompanying the posterior intrusion and mandibular autorotation, was another advantage of this system.

Kawata et al. (1987) described a new magnetised edgewise bracket which consisted of a samarium-cobalt magnet, plated with chromium to prevent corrosion and with nickel to allow soldering to the edgewise bracket. A mesh was attached to the underside of the bracket and this unit was then bonded directly to the teeth. Round archwire were used in the 0.018-inch slot and forces up to 250g were recorded. Unfortunately, when the distance between teeth was over 3mm an elastic chain had to be used as the magnetic force was insufficient to move the teeth. Despite this need for an elastic force, the authors claimed that the magnets allowed additional retraction forces and thereby reduced treatment times.

Using four juvenile female *Macaca fascicularis* monkeys, Vardimon et al. (1987) investigated the effects of force magnitude (high versus low) and point of force application (teeth versus direct palatal endoosseous pins) on palatal expansion treatment. Three subjects received one of the following

appliances: (1) a conventional type jackscrew maxillary plate with a high force magnitude of 2033 g bonded to the posterior teeth, (2) a similar tooth-borne appliance having a low force of 258 g, or (3) a specially designed palatal acrylic appliance pinned directly to the palatal shelves also utilising rare earth repulsive magnets with a low force of 258 g. A fourth animal, the control, received a passive sham appliance bonded to the abutment teeth. The authors claimed that the palatally pinned magnetic appliance induced bodily tooth movement, the greatest increase in the intermolar distance, and a superior repositioning of the maxillopalatine region. The latter two effects were caused by selective excitation of the transverse suture over the premaxillary suture. There was an eightfold reduction over conventional force with the rare earth magnets.

Because of ongoing interest in the use of magnetically induced tooth movement (Blechman and Smiley, 1978; Blechman, 1985; Dellinger, 1986; Vardimon et al., 1987; Kawata et al., 1987), and the claim that magnetic forces are more physiological than conventional orthodontic appliances (Cerny, 1980, 1982), the present study was conducted using samarium-cobalt magnetic extrusion.

Biological effects of the static magnetic fields

The reaction of the dental tissues of dogs to a magnetic field was examined by Cerny (1980). He concluded that under the influence of a field strength up to 95 milliteslas (mT) over a six month period, no clinical or histological evidence of tissue damage could be noted. He extended this study to examine the effects of similar field strengths on blood cells and tissues in rats (Cerny, 1983a, b) and again noted no discernible difference between the experimental and control animals.

Blechman (1985) addressed the biological safety of the magnets. The magnets were embedded in biocompatible epoxy resin to prevent corrosion

products leaking into the tissues. Frequent physical examination by a paediatric consultant was performed and urinary cobalt studies were carried out every 6 months during treatment. No abnormal results were reported and it was concluded that no biological hazard existed.

Linder-Aronson et al. (1992), using two male monkeys, examined soft and hard oral tissues in contact with or close to samarium-cobalt magnets following an extended exposure time. The monkeys were injected with tetracycline at the start of the experiment and after 4 weeks. Linder-Aronson et al. (1992) found that there was a thinner epithelium compared to the controls and a patchy tetracycline-incorporation in the bone adjacent to orthodontic magnets in contrast to a homogenous tetracycline fluorescent pattern in the controls. They concluded that the orthodontic magnets produced biological responses, although it could not be concluded whether this was due to the magnetic field, corrosion of the magnets, or even a combination thereof.

2.5 RETENTION

Retention is the period of the orthodontic treatment during which a passive appliance is used to maintain the post-orthodontic correction. The retention requirements have not been examined histologically. Shapiro and Kokich (1981) discussed the aspect of the retention based upon the clinical observations due to the lack of research material. They came to no conclusive result and suggested the length of the retention period should be determined by radiographic documentation of the width of the periodontal space, which is not true. The retention period should be based on biological data. It should be determined by reconstitution of the ultrastructure of the PDL including cells and collagen turnover (Reitan, 1967), tissue channels (Tang and Sims, 1992), and blood vessels and nerves (Crowe, 1989; Weir, 1990; and Parlange and Sims, 1993). The present study was specifically focussed on blood vessels and nerves.

Moxham and Berkovitz (1979), after applying a 0.05N extrusive load for a short period of time, noted that 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 the experimentally extruded marmoset incisor tooth after a retention period of 9 weeks. He claimed that the PDL was reconstituting by neural and vascular sprouting. Casley-Smith and Vincent (1980) studied the quantitative variations in tissue channels after the subcutaneous tissue injury of the mice. The tissue appeared relatively normal after 3 to 4 months, except for the tissue channels which returned to normal after 6 months. Revascularisation appears to be slow in injured tissues. This may explain the findings reported by Crowe (1989), after a short term retention period of 9 weeks, in the marmoset PDL.

Using TEM stereology, Weir (1990) investigated the apical PDL in the experimentally extruded marmoset incisor tooth after a long term animal retention period of 30 weeks, approximating 3 years in the human. He concluded that following 30 week retention in the marmoset, there were no significant differences between the apical PDL of experimental and control teeth, except for the collecting-sized venule parameter. Weir (1990) concluded that the apical PDL is capable of full reconstitution following crown fracture, endodontic therapy, orthodontic extrusion, and a long term animal retention period. However, Weir (1990) examined only the apical PDL. Reconstitution of the whole PDL after a long term animal retention period will be investigated in the present study.

Parlange and Sims (1993) studied a TEM stereological analysis of blood vessels and nerves in the marmoset maxillary incisor PDL following endodontics, magnetic extrusion, and a 9-week retention period, approximating 1 year in the human. They found that, for rapid tooth movement with fixed

short-term retention, PDL reconstitution was incomplete for the microvascular bed (MVB), but essentially complete for the nerve population. However, animal tissues generally heal very rapidly. Therefore, they concluded that, for human patients, retention periods in excess of 1 year are indicated for complete microvascular and neural reconstitution. The interesting question is how long complete re-establishment of blood vessels and nerves takes. The present study will investigate these events after an animal long term retention of 30 weeks, which approximates 3 years in human terms.

2.6 THE MARMOSET AS AN EXPERIMENTAL ANIMAL

Marmosets (Figure 2.1, p.14) are a group of New World monkeys which have been found to be suitable for dental research as an analogue of man in the following areas:

(1) They have a similar dentition to man (Swindler, 1976), except that the marmoset has three premolars and two molars.

(2) According to Wilson and Gardner (1982), they appear to have a similar TMJ anatomy to man, and a comparable masticatory function. It would seem that the marmoset PDL is subjected to similar functional load demands to those of man.

(3) The marmoset PDL has been examined in some detail by Dreizen et al. (1967); Levy and Bernick (1968a, b); Bernick and Levy (1968a, b); Levy et al. (1970); Skougaard et al. (1972); Page et al. (1974); Levy (1976); Bernick et al. (1977); Douvartzidis (1984), Crowe (1989), Weir (1990), and Parlange and Sims (1993), and has been found to provide an acceptable analogue for the study of human PDL, owing to its histological similarity.

Figure 2.1 The physiognomy of the marmoset (Crowe, 1989)

2.7 THE MICROSCOPIC ANATOMY OF THE PDL

The PDL comprises cells, fibres, nerves, vascular components and ground substance. Recent reviews of these elements have been provided by Berkovitz and Shore (1982), Lindhe (1983), Schroeder (1986) and Berkovitz (1990).

This present study will examine the ultrastructural features of the PDL using the TEM. Thus, the aim of this review is to indicate the criteria used for the identification of the PDL components and to describe present concepts relating to the distribution and volumetric proportion of all these components.

The quantification of the individual components of the PDL has been provided by Beertsen and Everts (1977), Gotze and Kindler (1974), Gotze (1976, 1980), Gould et al. (1977), Jonas and Reide (1980), Deporter et al. (1982), Shore et al (1982, 1984), and McCulloch and Melcher (1983).

The first general quantification of the PDL ultrastructure was provided by Freezer (1984) and Crowe (1989). They investigated the PDL of the mouse mandibular molars and marmoset maxillary incisors, respectively. Freezer (1984) quantified stereologic parameters, in contrast to Crowe's (1989) morphometric quantification.

2.7.1 CELLS OF THE PDL

Freezer (1984) categorised the PDL 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, these cells were grouped together under the heading of synthetic cells.

The cells of the PDL are not a component of the present investigation, although their relevance to the PDL can not be ignored. The principal studies of the various cells are as follows:

a. FIBROBLASTS

Fibroblasts are the most numerous cell of the PDL. In the rodent incisor PDL, they occupy about 50-60% of the volume of densely collagenous portions (Beertsen and Everts, 1977). However, this figure is not the true proportion because these researchers assume that all cells present in the PDL were fibroblasts and did not separate the neural and vascular components. Freezer (1984) using a stereological technique determined the volume density of fibroblasts in the rat molar PDL to be as low as 20.3%. Crowe (1989) investigated the apical PDL of the marmoset incisor and found that fibroblasts occupied 18.3% of the PDL extravascular area.

Shape of fibroblasts

Numerous authors have offered descriptions of fibroblast shape (Beertsen et al., 1974a; Brunette et al., 1976; Beertsen et al., 1979; Roberts and Chamberlain, 1978; and Freezer and Sims, 1987).

Roberts and Chamberlain (1978) identified four general fibroblast cell types in a scanning microscope (SEM) examination of the rat molar PDL:

(1) Irregular, oblong cells 16-22μm in length, with roughened surfaces and an absence of cell processes, oriented along principal PDL fibres.

(2) Stellate-shaped cells, 8-13μm in length, with multiple cellular processes found in lacunar spaces among principal PDL fibres.

(3) Nodular, spheroid cells 7-12μm in diameter located perivascularly.

(4) Elongated, stellate cells up to 60μm long, with numerous pseudopodic-like cellular processes, orientated along principal PDL fibres, possibly migrating.

However Berkovitz and Shore (1982) warned that artifacts resulting from specimen preparation could have been produced, and this classification must be accepted with care. Further, it is possible that, lacking preferred orientation, cells of similar shape may appear plemomorphic (Berkovitz, 1990). Also, the difference may be due to tooth type.

Ultrastructure of fibroblasts

Ultrastructurally, fibroblasts can be seen to contain all the intracellular organelles necessary for the synthesis of the extracellular proteins of connective tissues. In the PDL where there is a rapid turnover of extracellular protein, these organelles are particularly abundant (Rippen, 1976).

Ultrastructural features include:

(1) A prominent nucleus which may occupy 25% of the cell volume (Yamasaki et al., 1987a). In the rat PDL, the nucleus is a flattened disc conforming to the general cell shape (Shore and Berkovitz, 1979).

(2) Abundant rough endoplasmic reticulum, vesicles, a well developed Golgi complex and mitochondria (Berkovitz and Shore, 1982).

(3) Lysosomes of varying sizes and densities (Beertsen et al., 1974a)

(4) Microfilaments 4-8nm in diameter, probably composed of actin, form a meshwork and peripheral bundles (Schroeder, 1986). Beertsen et al. (1974a) postulated that such filaments were included in cell locomotion. Abercrombie et al. (1971) considered that the plasma membrane associated with these cell processes, termed the undulating membrane, was the main locomotory organelle of single migratory cells. Fawcett (1981) suggested that the undulating membrane was associated with pinocytosis and hence microfilaments might be included in this cell function. Farsi and Aubin (1983) indicated that, in the porcine PDL fibroblast, microfilaments were responsible for generating the force required to align and compact collagen.

(5) A well defined system of microtubules, with a diameter of 20-24μm, which may be part of a mechanism for collagen granule translocation from Golgi complex to the cell periphery (Cho and Garant, 1981 a,b,c,d). Berkovitz and Shore, 1982; Beertsen et al., 1974a suggested that these microtubules were probably responsible for the formation and maintenance of cell shape.

(6) The microtubules are associated with centrioles and cilia. Beertsen et al. (1975) reported that, in the mouse incisor PDL, at least 70% of the fibroblasts were ciliated and suggested that, since this frequency did not alter between motile and non-motile cells, the presence of this organelle was not directly associated with locomotion.

(7) The numerous fibroblast cytoplasmic processes frequently contact each other. Berkovitz and Shore (1982) found three types of cell contacts between the PDL fibroblasts: desmosomes (macula adherens), gap junctions (nexus), and tight junctions (zonula occludens). Little information exists concerning the distribution and functions of each cell

contact type, although desmosomes were the most frequent type of contact between fibroblasts in rat molar and incisor PDL (Shore et al., 1981).

(8) Mitochondria randomly distributed throughout the PDL fibroblasts have been described by Beertsen et al. (1974a), Melcher (1980), and Berkovitz and Shore (1982). These structures have been variously described as elongated to round, possibly due to an artifact of the plane of section.

Cells morphologically indistinct from fibroblasts appear in conjunction with blood vessels (Rhodin, 1968) and nerves (Bonnaud et al., 1978). The former were termed veil cells and the latter formed the sheath of Henle.

Function

Fibroblasts appear to have a dual role in the PDL - as a collagen synthesising cell and as a phagocytosing cell (Schroeder, 1986; Garant, 1976; Ten Cate et al., 1976). The extracellular formation of collagen associated with fibroblasts has been described by numerous authors (Ten Cate, 1972; Listgarten, 1973; Beertsen et al., 1974a; Ten Cate and Deporter, 1974; Ten Cate and Deporter, 1975; Ten Cate et al., 1976; Frank et al., 1976; Beertsen and Everts, 1977; and Shore and Berkovitz, 1979).

Ten Cate (1972) was the first to publish and clearly demonstrate the extensive distribution of collagen within fibroblasts of oral connective tissues. He suggested that fibroblasts had both degradative and synthetic roles in collagen metabolism. This hypothesis has subsequently been well supported in the literature (Ten Cate and Deporter, 1975; Eley and Harrison, 1975; Garant, 1976; Ten Cate et al., 1976; Deporter and Ten Cate, 1980; and Berkovitz and Shore, 1982).

Using serial thin-sectioning, Svoboda et al. (1979a, b) demonstrated that fibroblasts did phagocytose collagen fibrils and that intracellular collagen was not endogenous. These studies substantiated the suggestion that the fibroblasts had a degradative role in collagen metabolism.

The position and orientation of these cells has been reviewed by Berkovitz and Shore (1982). The cells appear to have a polarity which may be associated with cell migration and possibly tooth eruption (Beertsen et al., 1979; Garant and Cho, 1979; Cho and Garant, 1984), although this polarisation varies with age (Cho and Garant, 1984).

Cho and Garant (1981a,b,c,d, 1986, 1987) demonstrated that glycoprotein synthesis was another role of the fibroblast.

b. OSTEOBLASTS

The PDL osteoblasts are located adjacent to 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). The ultrastructural features of osteoblasts closely resemble those of fibroblasts and have been reviewed by Fawcett (1986) and Berkovitz and Shore (1982).

The volume density of the osteoblasts in the mouse PDL, excluding the apical region, has been reported as 0.3% (Freezer and Sims, 1987). Crowe (1989) investigated the apical PDL of marmosets and found osteoblasts to occupy 1.45% of the PDL extravascular area.

c. CEMENTOBLASTS

Despite studies by Yamasaki et al. (1986, 1987a, b) and Rose et al. (1987), these cells remain difficult to distinguish from fibroblasts. The proximity to the tooth remains their main distinguishing feature (Berkovitz and Shore, 1982; Schroeder, 1986).

Freezer and Sims (1987) quantified the volumetric density of the cementoblasts in the mouse molar PDL to be 1.6% of the tooth third of the PDL but only 0.5% of the total PDL. Crowe (1989) reported that cementoblasts occupy 3% of the PDL apical extravascular area.

d. OSTEOCLASTS

Osteoclasts are multinucleated giant cells, 20-100μm in diameter. Their primary function is the extracellular resorption of the bone. The ultrastructural features of the osteoclasts have been reported by Gothlin and Ericsson (1976), Berkovitz and Shore (1982), Freezer and Sims (1987) and Fawcett (1986).

Freezer and Sims (1987) stereologically quantified the mouse molar PDL osteoclast. They found the volume density to be 0.6% of the total PDL and 1.9% of the bone third of the PDL.

e. PROGENITOR CELLS

The PDL, as with all connective tissue, contains progenitor cells which have the capacity to undergo mitosis. They proliferate and produce differentiated cells which can synthesise bone, cementum and extracellular matrix within the mouse PDL (Melcher, 1980 ; McCulloch, 1985). The origin of these cells is unknown. Gould et al. (1977) found some progenitor cells near the root surface. McCulloch and Melcher (1983) reported that 40% of the proliferative cells resided within 10μm of the PDL blood vessels.

f. EPITHELIAL CELLS

Epithelial cells occur in the PDL as epithelial cell rests of Malassez derived from the disintegrating sheath of Hertwig. In the mouse molar, the epithelial rests of Malassez also arise from the reduced enamel epithelium (Gurling, 1982).

Normally epithelial cells are inactive, but are capable of metabolizing and perforating (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 the epithelial remnants decreased with increasing age.

Controversy exists concerning the distribution of the epithelial cells within the PDL. 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 area, whilst Freezer (1984) found them to be most numerous around the apices of the teeth.

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

The role of the epithelial cells within the PDL is largely undetermined (Melcher, 1980). Loe and Waerhaug (1961) and Lindskog et al. (1983) suggested a possible role in preventing root resorption by producing protease inhibitors which protected the root surface from the osteoclastic resorption and contributed to the integrity of the PDL (Lindskog et al., 1983). Brice (1988) suggested a possible role in controlling the repair of the resorbed surfaces.

g. VASCULAR AND PERIVASCULAR CELLS

i. Endothelial cells

Blood vessel walls comprise an endothelial cell lining with or without surrounding perivascular cells. Avery et al. (1975) investigated the ultrastructure of the terminal blood vessels in the mouse molar PDL. They found endothelial cells with thin finger-like projections which extended into the vessel lumen. The nuclei also extended into the lumen. The characteristics of the cytoplasm included numerous microvesicles, scattered ribosomes and small oval mitochondria. Tortuous intercellular junctions with occasional tight junction were seen between the endothelial cells (Corpron et al., 1976). Endothelial and smooth muscle cells occasionally formed myoepithelial junctions.

ii. Pericytes

While pericytes and fibroblasts share many common features, Rhodin (1967, 1968) studied the vascular wall morphology in the non-oral rabbit tissue and described the following characteristics identifying the pericytes :

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

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

(3) They exhibited few intercellular contacts.

(4) They had pinocytotic vesicles located near or connected with the plasma membrane.

(5) Pericytes had less granular endoplasmic reticulum.

(6) Pericytes had characteristic highly branched cytoplasm with extrusions embracing the endothelial tube at frequent membranous contacts without fusion or tight junctions.

Freezer and Sims (1987) described pericytes as elongated cells with little cytoplasmic branching and a basement lamina which was continuous with that of the endothelium in the mouse molar PDL. These cells were commonly located around vessels with a luminal diameter of 4μm, while in vessels with a luminal diameter exceeding 20μm, the pericytic cellular investment was usually incomplete or absent.

Buchanan and Wagner (1990) carried out a morphometric analysis of the electron micrograph 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 the local control of the capillary blood

flow (Buchanan and Wagner, 1990). Histochemical studies have demonstrated the presence of tropomyosins, isomyosins, cyclic GMP-dependent proteinkinase and muscle actins in pericytes (Joyce et al., 1985a, b ; Herman and Jacobson, 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, fenestrae and open junction or leaks (Renkin, 1977 ; Casley-Smith, 1983). The microphysiology of these transport mechanisms provides an insight into the permeability characteristics of vessels in relation to their function in the tooth support and their response to tooth-moving forces.

Renkin (1977) proposed possible transport pathways across the capillary wall (Figure 2.2). Pathways (1-6) were supposed to be present in all capillary endothelia although the properties of the specific pathways might differ in different MVB. Pathways (7) and (8) were found only in fenestrated endothelia.

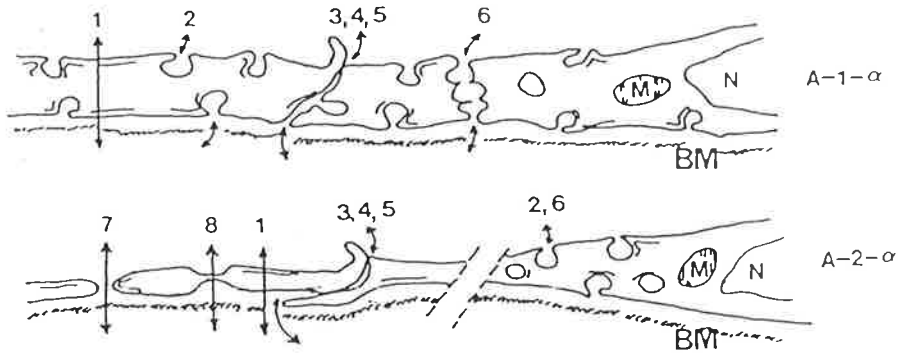


Fig. 2.2 Capillary transport pathways, partly or wholly conjectural, based on electron microscopic observations (Renkin, 1977). A-1-alpha, non-fenestrated capillaries. A-2-alpha, fenestrated capillaries. BM, basement membrane. N, nucleus. M, mitochondria. See text for explanation of numbers. Classification is according to Bennett et al. (1959).

Pathway (1) represented the transendothelial pathway. This pathway allowed the passage of water, lipid-soluble and possibly small unipolar solutes. Facilitated diffusion or active transport of specific substances represented a special characteristic of the cell membrane pathway.

Pathway (2) represented the micropinocytic vesicles which transited across the endothelial cells. Topologically, this was an "extracellular" route since the material transported were lined with cell membranes and, therefore, their contents were, strictly speaking, "outside" the cell cytoplasm and did not mingle with the endothelial cell cytoplasm. Theoretically, these vesicles might include anything within the plasma or interstitial fluid which could fit into a vesicle. The rate of transport was determined by the turnover rate of the vesicles (Palade and Bruns, 1968).

Pathways (3-5) represented different modes by which molecules could get through the intercellular junctions. Pathway (3), proposed by Scow et al. (1976), transported water-insoluble lipids by lateral diffusion in the plasma membrane lining the junction. Pathway (4) represented the passive transport characteristics of the clefts of tight junctional complexes between endothelial cells. This might be the structural equivalent of the hypothetical "small pore" system (Pappenheimer, 1953) which allowed movement of water and small-to-moderate lipid-insoluble molecules. Pathway (5) proposed that fluid and macromolecular solutes, such as plasma protein, crossed the capillary wall through wider intercellular junctions or transiently open junctions, corresponding to the hypothetical "large pore" system (Pappenheimer, 1953).

Pathway (6) represented transendothelial channels which were possibly made up of chains of fused micropinocytic vesicles forming continuous channels from the luminal to the abluminal surface. According to the "vesiculation" theory proposed by Wolff (1967), these channels were transitory. Simionescu et al. (1975) also suggested that the areas of fusion

between vesicles were narrow enough to provide a basis for restrictive permeability to small lipid-insoluble substances.

Pathway (7) represented an open fenestra which allowed macromolecules to penetrate through it. Renkin (1977) postulated that pathway (8): a closed fenestra with a diaphragm might be a specialized pathway for water-soluble substances, lipid-soluble substances and/or even large, lipid-insoluble molecules. The fraction of open and closed fenestrae varied amongst different capillary beds and also, to some extent, between preparatory techniques (Simionescu and Simionescu, 1984).

Fenestrated blood capillaries are commonly seen in specialized tissues where there is an interstitial accumulation of large molecules needing removal (Casley-Smith, 1971). Corpron et al. (1976) studied the fenestrae in the mouse PDL microvascular bed and described them as 30-50 nm openings in the endothelium, bridged by a thin membrane continuous with either side of the endothelial cell. While the central capillaries in the PDL appeared devoid of the fenestrations, the fenestrated capillaries appeared peripherally close to the osteoblastic layer. Sims (1983) reported that the highest incidence of the fenestrae in all vessels was found in the postcapillary-sized vessels in the mouse PDL.

Moxham et al. (1985) quantified the number and distribution of the fenestrated capillaries in the rat PDL. The molar capillaries showed more fenestrations compared with those of the continuously erupting incisor. This variation may indicate the differing functional requirements for the vasculature of these teeth.

Pudney and Casley-Smith (1970) found that the fenestrae were more common at the venous ends of capillaries in the adrenal cortex than at the arterial ends. Similarly, Lew (1986), in a study on the rat molar PDL, found the fenestrae to be uncommon in the arterial capillaries.

Casley-Smith et al. (1975) suggested that the fenestrae may be of greater importance in the establishment of a local extracellular capillary circulation of macromolecules. Corpron et al. (1976) postulated that the fenestrations may represent rapid pathways of metabolites across the endothelium to the periodontium where high metabolite requirements for growth or repair occur. Lew (1986) suggested that the fenestrated capillaries may be responsible for the return of extracellular protein and metabolites to the vascular system , since the existence of a lymphatic system in the PDL is uncertain (Edwall, 1982).

h. NEURAL AND PERINEURAL CELLS AND NERVE ENDINGS.

Two types of cells which invest neurites have been described in the PDL:- the Schwann cell and the kidney-shaped cell (or K-cell)

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

The K-cell has been reported by Beertsen et al. (1974 b), Everts et al. (1977), Berkovitz and Shore (1978), and Maeda et al. (1989). Ultrastructural features of K-cell in the mouse PDL were described by Everts et al. (1977), Freezer and Sims (1989) as:

1) Rounded cell bodies with cytoplasmic extensions, each forming part of a sheath around the terminal region of a small nerve fibre.
2) Kidney-shaped nuclei, positioned eccentrically in the cytoplasm.
3) Prominent, centrally positioned Golgi apparatuses.

The functional significance of the K-cell has not been determined (Hannam, 1982 ; Maeda et al., 1989). Freezer and Sims (1988) reported a statistically significant association between the K-cells and the apericytic postcapillary-sized venules and between the K-cells and both unmyelinated and myelinated nerves in the PDL parenchyma. It was postulated that these

structural associations may comprise complex receptor or effector mechanisms. Maeda et al. (1989), using immunohistochemistry and electronmicroscopy, determined that, in the continuously erupting rat incisor, K-cells were associated with Ruffini endings and showed evidence of active protein synthesis. These researchers concluded that K-cells represented terminal Schwann cells associated with Ruffini endings, presumably corresponding to the lamellar cells in the inner bulb of sensory corpuscles. However, the existence of cells resembling PDL terminal Schwann cell has not been reported in the Ruffini endings of other tissues. Byers (1985) did not observe terminal Schwann cells associated with the Ruffini-like endings in the rat premolar PDL. Maeda et al. (1989) concluded that functions of terminal Schwann cells were possibly related to some characteristic conditions of the rodent incisor.

Nerve endings

A detailed description of nerve endings is difficult to provide, due to species variation, and the source of the human material e.g. whether tissue was obtained post mortem or from extracted teeth (Schroeder, 1986). In addition, the difficulty in determining the level of sectioning (whether terminal or subterminal), has affected the accuracy of description (Bonnaud et al., 1978). Descriptions of nerve terminals in various species have been reported by Bernick (1952), Held and Baud (1955), Simpson (1966), Bernick and Levy (1968 b), Harris and Griffin (1974a, b), Everts et al. (1977), Kubota and Osanai (1977), Berkovitz and Shore (1978), Bonnaud et al. (1978), Corpron et al. (1980), Berkovitz et al. (1983), Byers (1985), and van Steenberghe et al. (1992).

Everts et al. (1977), in a study on the mouse PDL, classified nerve endings into three morphologically distinct groups :

1) Convoluted endings ("end ring") - ovoid, encapsulated structures.

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

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

While Cash and Linden (1981) reported that only one type of physiological receptor exists in the PDL, the ultrastructural findings of Byers (1985), and Freezer and Sims (1989), provide evidence to the contrary.

Byers (1985) used light and electron microscopy and autoradiography to establish a detailed ultrastructural description of PDL neural receptors in the rat molar. Byers (1985) reported six receptor categories of Gasserian ganglion origin (Figure 2.3).

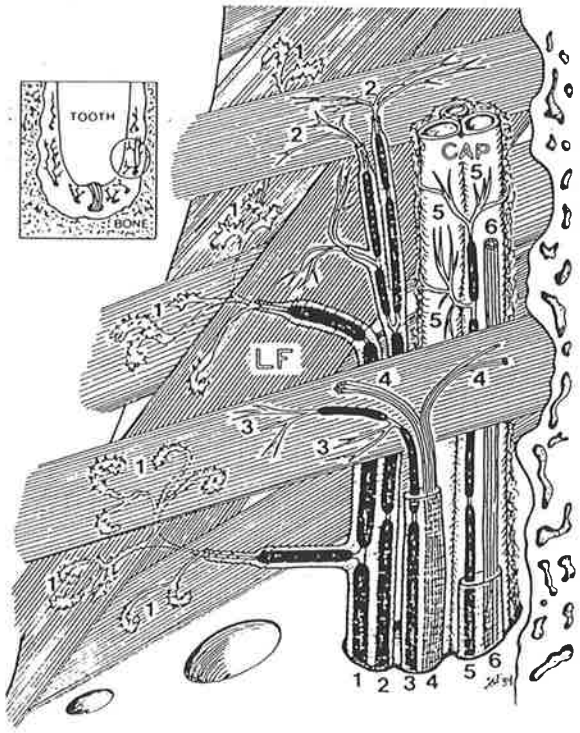


Figure 2.3 This drawing shows representative types of preterminal axons and sensory endings in the PDL of a rat molar.
Receptors among ligament fibres (LF) :
(1) complex Ruffini-like endings, ensheathed preterminal axon; (2) simple Ruffini-like endings, ensheathed preterminal axons - preterminal axons can form paired branches; (3) simple Ruffini-like endings, branching from free, small, myelinated axons; (4) bundles of free myelinated 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)

Using electron microscopy, Lambricht et al. (1992) studied the ultrastructure of sensory nerve endings in the human PDL and found three types of them: free nerve endings, Ruffini-like endings and lamellated corpuscles. Free nerve endings stemed from unmyelinated or from myelinated nerve fibres. Ruffini-like receptors were mostly found in the apical part of PDL. The relationship between morphology and function of neural receptors remains mostly speculative. Free nerve endings were regarded as receptors for pain and heat. The lamellated endings might be mechanoreceptors of the rapidly-adapting type (McCloskey, 1978; Tracey, 1978). Lambricht et al. (1992) found the direct contact between the receptor and the oriented collagen fibres, suggesting the tactile function of the PDL.

2.7.2 PDL VASCULATURE

a. ANATOMY AND DISTRIBUTION

The vasculature of the PDL has been reviewed by Edwall (1982) and Schroeder (1986).

The PDL receives its arterial blood supply from intraosseous and periosteal channels. Arterioles approach the PDL through the marrow spaces of the interdental and interradicular portions of the alveolar bone, and eventually enter at various levels (Kindlova and Matena, 1962; Kindlova, 1965; Castelli and Dempster, 1965; Carranza et al., 1966; Lenz, 1968; Rohen et al., 1984).

The venous drainage from the PDL has been described by Castelli and Dempster (1965). In monkey and man, veins in the alveolar bone and the PDL join to drain into larger veins in the interalveolar and interradicular septi which in turn are connected to a rich venous network surrounding the apex of each alveolus.

Birn (1966) estimated the vascularity of the human PDL by examining 84 tooth sockets. The blood supply was found to increase towards the

posterior teeth and in single-rooted teeth be greatest in the cervical third, less in the apical third, and least in the middle third. However, Birn (1966) estimated the vascularity of the PDL on the unvalidated assumption that each alveolar wall perforation indicated the passage of a blood vessel (Kishi Y. and Takahashi K., 1977; Freezer and Sims, 1987)

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

In man and non-human primates, the main vessels of the PDL were arranged in palisades that ran parallel to the long axis of the root. These vessels branched to form capillaries arranged in a flat network, e.g. a venous plexus that surrounded the root surface. This network was located nearer to the alveolar wall than to the root surface. In the cervical region, the network became condensed into a narrow band, with single capillaries being given off to form coiled structures resembling glomeruli. The PDL vessels anastomosed with those of the gingiva (Lenz, 1968 ; Freezer, 1984).

Weekes and Sims (1986), using the SEM vascular corrosion casting technique, described the rat molar PDL as a predominantly venous microvascular bed (MVB). 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 MVB.

Using SEM of methacrylate vascular perfusion casts with stereopair imaging, Wong and Sims (1987) examined the MVB of the mouse PDL. Characteristic MVB 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 characterised by vertically-orientated capillary loops which connected the arterial and venous vessels. The apical third had a distinct, irregular, anastomosing venous pattern.

Lee (1989), using corrosion castings, studied the periodontal and palatal gingival microvasculature of marmoset molars and premolars in both maxilla and mandible. He reported that the cervical and middle thirds comprised mainly postcapillary-sized venules running occluso-apically. Frequent anastomoses were noted between these vessels as they coursed 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 PDL. They observed that the vessels differed in calibre and were all thin-walled, but no method of categorisation was attempted.

Blood vessels of the microcirculation in other tissues were classified by Bennett et al. (1959) and Rhodin (1967 , 1968). Bennett et al. based their classification on:

1) Basement membrane

   Type A - complete, continuous basement membrane.

   Type B - without a complete investment of basement membrane.

2) Fenestrations or pores

   Type 1 - capillaries without fenestrae or perforations.

   Type 2 - capillaries with intracellular fenestrations.

   Type 3 - capillaries with intercellular perforations.

3) Pericapillary cellular investment

Type alpha - capillaries without a complete pericapillary cellular investment.

Type beta - capillaries with a complete pericapillary cellular investment.

Due to the shortcomings of the Bennett 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.

Rhodin's classification was based on the lumen diameter, the endothelial cell lining, the patterns of vessel branching and anastomosis, and the constituents of the blood vessel wall (Table 2.1, P. 33).

A TEM study by Avery et al. (1975) on the mouse molar PDL showed that capillaries which exhibit finger-like endothelial projections into the lumen were present in the PDL. Occasionally, pericytes were found in association with these vessels. Precapillary vessels with a thicker endothelial lining and incomplete muscular coating were distributed throughout the PDL. The muscular layer was separated from the endothelium and surrounding connective tissue by a distinct basal lamina which was breached at myoendothelial junctions.

Simionescu and Simionescu (1984), in a review of the ultrastructure of the microvascular wall, defined capillaries as the smallest ramification of the MVB, with a thin wall composed of endothelial lining, a basal lamina and a few pericytes surrounding a lumen with a diameter of 5-10 μm.

In a stereological TEM study of the rat molar PDL, Freezer (1984) described four vessel types :

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 30μm	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 3μm	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.
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	1-3μm	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 around 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 3-10 microns from muscular layer.

Table 2.1 Ultrastructural features of blood vessels (Rhodin, 1967, 1968)

1) Capillary-sized vessels (internal diameter of 4-7μm) with a partial or complete pericytic cellular investment.
2) Capillary-sized vessels without a pericytic cellular investment.
3) Postcapillary-sized vessels (internal diameter of 8-30μm) with associated pericytes being few in number or absent.
4) Postcapillary-sized vessels with a complete pericytic investment.

The normal apical MVB of the rat molar PDL was studied by both Clark (1986) and Lew (1986) as part of experiments investigating the respective

effects of intrusion and extrusion on the rat molars. They described the following features of the microvasculature :

1. Terminal arterioles - these had luminal diameters of 10-30 μm (Clark , 1986), or 18-24 μm (Lew, 1986), surrounded by one to two layers of smooth muscles cells.

2. Arterial capillaries - these had luminal diameters of 5-10μm with one to two endothelial cells encompassing the lumen and sending numerous microvilli projecting into the lumen. Occasional fenestrae occurred , always singly.

3. Continuous capillaries - these comprised 91% of all capillaries in Lew's sample. They were morphologically similar to fenestrated capillaries although they naturally lacked fenestrae.

4. Fenestrated venous capillaries - these had lumina 8-10 μm in diameter, surrounded by a layer of one to three flattened endothelial cells and occasional pericytes. Endothelial cell nuclei bulged abluminally.

5. Postcapillary-sized venules - these had lumina 10-30μm in diameter, surrounded by a layer of one to eight endothelial cells, and incompletely surrounded by 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.

6. Collecting venules - these had luminal diameters up to 50μm, surrounded by a layer of endothelial cells.

7. Arteriovenous anastomoses - these were seen as relatively thick-walled vessels with a luminal diameter of 12-15μm and an endothelium which was surrounded by smooth muscle cells. Both myelinated and unmyelinated nerves were present in the adventitia.

However, Freezer (1984) reported that most of the vessels found in the mouse PDL as either type A (capillary-sized) or type B (postcapillary-sized). Lew (1986) postulated that the arterial elements of the type A vessels may be responsible for pumping up the PDL venous reservoir because Freezer (1984) found that the postcapillary-sized venules handled about 3 times more blood volume per unit surface area of the endothelium than type A vessels.

c. QUANTIFICATION

Freezer and Sims (1987) calculated stereological parameters for vessels within the mouse PDL. Their findings are summarised in Table 2.2.

	Type A	Type B
Mean diameter	6.4μm (capillary-sized)	20.9μm (postcapillary-sized)
Pericytes	Frequently contain a partial or complete pericytic investment	Few pericytic cells surrounding blood vessel (70% of vessels devoid of pericytic cellular investment).
Total periodontal blood volume	12%	88%
Total blood vessel surface area	31%	69%
Total blood vessel length	60%	40%

Table 2.2 Vessel types found in the PDL of mouse mandibular first molar (Freezer, 1984)

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 PDL and the McCulloch and Melcher (1983) estimate that the bone-half of the PDL is four times more vascular than

the tooth-half. However, the sampling techniques of McCulloch and Melcher (1983) were unacceptable because they examined only selected regions. Thus, their results are not directly comparable to those of Freezer and Sims (1987). Freezer and Sims (1987) reported a volumetric density of 7.5% for the mouse molar PDL, excluding the apical region. Sims (1980) reported regional human premolar volumetric proportions as high as 20 percent which contrast with the vascular proportion of 1-2% reported for Macaque monkey PDL (Wills, Picton and Davies, 1976) and with the study of Gotze (1976), according to whom blood vessels occupied only 1.63-3.50% of the human PDL.

Using a TEM investigation of marmoset PDL following magnetic incisor extrusion and a short term retention period, Crowe (1989) found that the vascular proportion of the control marmoset apical PDL was 11% of the total apical PDL. Parlange & Sims (1993), using the non-apical regions of Crowe's specimens, reported a similar vascular volume of 11.26% for the total PDL. The marmoset incisor PDL had a predominately venous MVB with venous vessels occupying 95% of the vascular area and 76% of the vessel length. Crowe (1989) reported a dramatic change in the areal fraction and boundary profile length of the terminal arteriole group. A fall in mean caliper diameter was noted across most vessel groups. He concluded that revascularisation of the apical PDL was still incomplete in marmosets after a nine week retention period. Revascularisation 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) reported that continuous capillaries comprised 91% of all capillaries. Lew et al. (1989) found that 95% of total vascular volume was provided by capillaries and postcapillary-sized venules. Comparison of the control and experimental PDL, following short term extrusion, demonstrated

a significant increase in mean vascular volumes for postcapillary-sized venules, venous capillaries, arterial capillaries and terminal arterioles.

Using a TEM investigation of extrusion and a long term retention period in the marmoset PDL, Weir (1990) reported a total control vascular volume of 10.76% for the apical PDL tissue, which closely matched that of 11% reported by Crowe (1989). Weir (1990) found that the apical volume of the PDL occupied by larger venous vessels (postcapillary-sized and collecting venules) was 89.81%, almost exactly matching the apical volume of 88% determined by Crowe (1989) for vessels in these categories. Weir's (1990) study also confirmed the finding of Crowe (1989) that the marmoset incisor apical PDL had a predominantly venous MVB. The volumetric density of venous vessels in the control PDL in Weir's study was 91.24%, compared with the 95% found by Crowe.

A synopsis of the various quantitative studies on the PDL vascular proportion is outlined in Table 2.3.

Study	Region of PDL	Vascular Proportion (%)
Humans		
- Gotze (1976)	Anterior and premolar teeth	1.63-3.50%
- Sims (1980)	Mandibular premolars	11% (Regional areas: up to 20%)
Monkeys		
- Wills et al. (1976)	Mandibular incisors	1-2%
- Douvartzidis (1984)	Molars	8.3%
- Crowe (1989)	Control maxillary incisors (Apical region)	11%
- Weir (1990)	Control maxillary incisors (Apical region)	10.76%
- Parlange & Sims (1993)	Control maxillary incisors	11.26%
Mouse		
- Gould et al. (1977)	Mesial to mandibular first molars	7.7 ± 0.6%
- Sims (1980)	Mandibular first and second molars	17%

Study	Region of PDL	Vascular Proportion (%)
Mouse		
- McCulloch & Melcher (1983)	Mesial to mandibular first molars	7.25 ± 0.75%
- Freezer and Sims (1987)	Mesial to mandibular first molars (excluding the apical region)	7.5%
- Sims et al. (1994)	Mesial to mandibular first molars	8.5%
Rat		
- Moxham et al. (1985)	Molars	22.1 ±5.6%
	Incisors: 3mm from alveolar crest	6.7 ± 1.6%
	Incisors: 12mm from alveolar crest	52 ± 6.7%
- Clark (1986)	Maxillary first molars - apical	23.1%
- Lew (1986)	Maxillary first molars - apical	19.9%
- Blaushild et al. (1992)	Incisors - apical half	47 ± 2%
	- incisal half	4 ± 2%

Table 2.3 Summary of studies on the vascular proportion of the PDL.

d. Function

The functional role of the PDL vasculature has not 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 the lack of reliable techniques for use, experiments determining properties such as rates of blood flow are difficult to undertake (Berkovitz, 1990). Even with fixed tissues only, limited information can be deduced from structural observations.

Investigation of the structure and microphysiology of the junctions, vesicles, fenestrae and tissue channels would allow a better 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) hypothesised that venous capillary and apericytic post-capillary wall structure facilitated the rapid passage of fluid between vessels and extravascular compartment.

Kvinnsland et al. (1989) measured the blood flow rate in the rat molars subjected to 0.3-0.5N orthodontic forces for five days. Blood rate was measured by injecting the animals with fluorescent microspheres of appropriate size to become lodged in the precapillary arterioles, thereby gaining a measure of blood flow at the time of injection. A substantial and relatively uniform increase in blood flow was found in all areas of the PDL of all experimental teeth over controls by Kvinnsland et al. (1989). This study, however, only reports on the early response of the PDL vasculature to orthodontic forces, and longer term studies are required to improve understanding of the response of this tissue.

2.7.3 LYMPHATICS

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

(1) usually larger than blood capillaries
(2) appeared partially or totally collapsed
(3) had an irregular outline
(4) open junctions were present between endothelial cells; fenestrae were lacking
(5) abluminal projections existed where collagenous filaments attached to endothelial cells

The existence of lymphatic vessels in the PDL has been a matter of debate. A study by Levy and Bernick (1968b), of 20-30μm thick histological sections of marmoset PDL, described lymphatic vessels originating as blind endings within the body of the PDL. Casley-Smith (1977) stated that on morphological grounds alone, it is impossible to identify a particular vessel beyond any doubt, and advised that microprojection of tracers into the lymphatic system is the only certain way.

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 PDL with some passing into the periosteum and the perivascular intraosseous channels. Criteria used to identify vessels as lymphatic vessels were not given, and the number of experimental animals used was not reported.

Gilchrist (1978) reported vessels resembling lymphatic vessels in human PDL, while Barker (1982), in a ultramicroscopic study, claimed that lymphatic vessels were present in human alveolar bone. Ultrastructural studies on mice have not confirmed the presence of lymphatic vessels in the PDL. (Freezer, 1984).

Crowe (1989) noted a 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. Similarly, Foong (1994), in the human premolar PDL, found a presumptive lymphatic vessel in the apical region.

2.7.4 PDL NEURAL SUPPLY

a. Anatomy and distribution

The innervation of PDL has been reviewed by Fearnhead (1967), Anderson et al. (1970), van Steenberghe (1979), Hannam (1982), Schroeder (1986).

Generally, the PDL is supplied by sensory and autonomic nerves. In the maxilla, the sensory nerves arise as branches from the superior dental plexus, which in turn comprises branches of the posterior, middle, and anterior superior alveolar nerves, all of which originate from the maxillary nerve, the intermediate division of the trigeminal nerve. In the mandible, the PDL nerves arise from the inferior dental plexus, which is derived from the inferior alveolar (dental) nerve and its incisive branch (or even extending branches of the mental

nerve, re-entering the alveolar process). These nerves are the part of the mandibular nerve, the largest division of the trigeminal nerve.

The origin of at least part of the PDL innervation is the trigeminal ganglion demonstrated by radioautographic studies in the cat (Weill et al., 1975), and the rat (Byers and Holland, 1977 ; Pimenidis and Hinds, 1977 ; Byers, 1985). Following the injection of ^{3}H-proline into the Gasserian ganglion, PDL nerves and particularly the nerve endings were found to heavily labelled. Both thick fibre bundles entering the fundus of the alveolus and ascending parallel to the root and fine fibres associated with blood vessels and of local distribution appear clearly marked, although the label density may be comparatively low in the PDL.

The nerve branches enter from two sources. In the cat, Robinson (1979) demonstrated that one pathway is via peripheral branches arising from the dental nerve prior to its entering the apical foramen and running through the PDL towards the gingiva. The other pathways involve branches of the nerves travelling through interdental and interradicular septa to penetrate through the openings (Volkmann's canals) of the alveolar bone proper and reach the PDL at various levels laterally. Both fibre groups join each other to form a nerve plexus in the PDL which comprises coarse fibre bundles running mostly 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 PDL (Bernick, 1952; Griffin and Harris, 1968; van Steenberghe, 1979; Hannam, 1982; Freezer and Sims, 1987).

Both Van Steenberghe (1979) and Hannam (1982) have concluded that the afferent sensory fibres of the PDL are between 0.5 and 1.0 μm in diameter when unmyelinated, and between 1.0 and 16 μm when myelinated. Van Steenberghe (1979) demonstrated variation in the neural ultrastructure between species.

The distribution of the terminal branches and nerve fibres (and their endings) forming the PDL plexus may not be evenly distributed throughout the whole PDL space. For example, Falin (1958), Byers and Holland (1977), Dubner et al. (1978), and Byers (1985) found most of the neural structures around the root apex and less cervically. Kubota and Osanai (1977) demonstrated, in the shrew mole, that the apical PDL innervation is much denser than that in the midroot region. In the mouse, Everts et al. (1977) reported that most, if not all, neural elements were found in the lateral, alveolar compartment of the PDL space, while the tooth-related, mostly avascular compartment was free of neural elements. Freezer (1984) investigated the lateral PDL thirds in the mouse mandibular first molar. A greater mean number of axons per unit area was found in the bone third and decreased towards the avascular tooth region. In contrast, Byers (1985) reported that in the apical third of the PDL around rat molars most of the neural elements of trigeminal ganglion origin were found in the avascular, tooth-related region.

b. Quantification

There is limited information on the quantification of the PDL axons. Recently, there have been TEM studies (Freezer and Sims, 1987, 1988, 1989; Crowe, 1989; Weir, 1990; Parlange and Sims, 1993), whereas previous information was derived from light microscopy studies (Kubota and Osanai, 1977; Byers, 1985).

Freezer and Sims (1987) investigated the neural distribution in the mouse PDL using stereological parameters. The most cervical region contained twice as many axons as the middle of the PDL, and the apical portion had an innervation three times that of the mid-root region. Unmyelinated nerves were present at all depths in the tooth, middle and bone-thirds of the PDL. They constituted 95% of the nerve fibre length. Most of them were found in the middle and bone third of the PDL. Myelinated nerve

fibres constituted 5% of the length of the PDL nerve axons and comprised 26% of the neural volume. No statistically significant variation in the neural diameters was detected along the tooth root.

Freezer and Sims (1989) performed a stereological quantification of neural structures in the mouse molar PDL. Their finding included that:

(1) 45% of all axons (myelinated and unmyelinated) were located in the PDL parenchyma, and 55% occurred in close apposition to the luminal endothelium of blood vessel walls.

(2) The alveolar annular third of the PDL had approximately twice the innervation of the middle annular third, while the tooth third of the PDL contained only 3% of the PDL innervation. By contrast, in the primate, Parlange and Sims (1993) showed that the neural volumetric density in the middle third of the controls was found to be approximately six times that of the bone third, with negligible innervation in the tooth third.

(3) A total volume density for all axons was 0.5%, excluding the subapical area, which was comparable with the marmoset incisor (Parlange and Sims, 1993).

Studies pertaining to PDL neural changes incident to orthodontic tooth movements are rare (Crowe, 1989; Weir, 1990; Parlange and Sims, 1993), although possible regeneration of PDL mechanoreceptors following tooth extraction has been studied (Linden and Scott, 1988). Specific data incident to orthodontic extrusion is lacking.

Parlange and Sims (1993) examined the neural changes in the marmoset incisor PDL after endodontic treatment, orthodontic extrusion, and a retention period of 9 weeks. Their finding included that: (1) The neural volume increased from 0.4 to 0.6%; and (2) The neural volume changed from 1:2.5 to 1:3.5 for unmyelinated and myelinated axons, respectively. Therefore, they concluded that reconstitution of the neural system was essentially complete

after a 9-week retention period in the marmoset, which approximates 1 year in the human.

2.8 SUMMARY

Effective and efficient tooth movement depends upon the application of forces to move teeth via the mediation of the periodontal tissues. Understanding the properties of the PDL and its response to orthodontic force is vital for the advancement of clinical orthodontics.

Current knowledge on the effect of orthodontic tooth movement on the PDL has a number of shortcomings. Light microscopic studies of tissue reactions incident to orthodontic tooth movement are numerous (reviewed: Moxham and Berkovitz, 1982). There is, however, a general paucity of ultrastructural information regarding the effect of orthodontic tooth movement, and extrusive forces in particular.

Studies on the effect of orthodontic tooth extrusion on the PDL vary widely, both according to species and the number of experimental animals. Huettner and Whitman (1958) used 10 rhesus monkeys; Batenhorst et al. (1974) used 2 Macaca mulatta monkeys, Rygh (1976) used 67 rats; Simon et al. (1980) used 3 dogs; Crowe (1989), Weir (1990), Parlange and Sims (1993) used 4 marmosets.

Variation in forces applied to the PDL and in methods of recording and analyzing experimental results also serve to limit the application of experimental findings to the clinical practice of orthodontics in humans.

There have been a few ultramicroscopic studies on the effects of extrusive orthodontic forces on the PDL (Crowe, 1989; Weir, 1990; and Parlange and Sims, 1993).

Parlange and Sims (1993) examined changes in the blood vessel and neural system in the marmoset incisor PDL after a 9-week retention period, which approximates 1 year in the human. They found that PDL reconstitution

was incomplete for the MVB, but essentially complete for the nerve population. However, animal tissues generally heal very rapidly. Therefore, they concluded that for human patients, retention periods in excess of 1 year were indicated for complete microvascular and neural reconstitution.

The goal of the present study was to determine whether reconstitution of blood vessels and nerves occurs after a relatively long term animal retention period of 30 weeks, which approximates 3 years in the human. The results will be evaluated to provide data which may be relevant to the clinical retention period.

CHAPTER 3
MATERIALS AND METHODS

3.1 REVIEW OF WEIR'S EXPERIMENTAL PROCEDURES

Weir (1990) used 7 male and 3 female cotton-eared marmosets (Callithrix jacchus), as a model to simulate a routine clinical regime. He finally selected the final experimental group of 4 male marmosets. The selection criteria will be explained in part 3.5. The material for the present study was obtained from his final experimental group. All of the four specimens had already been TEM processed and embedded in resin blocks, which contained the remaining non-apical segments of the maxillary incisors. The age and weight of the four male marmosets are given in Table 3.1.

Table 3.1 Birth date of the final experimental animals and their age and weight at 21/04/89

Animal number	Weight	Birth date	Age
1. 29 male	359g	14/04/86	36 months
2. 31 male	361g	19/12/86	30 months
3. 33 male	390g	22/06/87	24 months
4. 38 male	300g	11/11/87	17 months

All animals had their teeth cleaned every two days with a Tek (Johnson and Johnson) Junior tooth brush. In addition, the teeth were swabbed with a 0.02% chlorhexidine digluconate solution on a cotton bud for an average of one minute. These procedures were continued for the duration of the experiment until sacrifice.

Magnets were used as the extrusive mechanism because of the difficulty in using normal orthodontic extrusive devices in an animal of this small size.

The magnets used were of the Samarium-Cobalt type, normally used as quartz digital watch rotors (Seiko product no. 4146235). Crowe (1989) reported that the attraction force between two magnets of this type, at an intermagnet distance of 0.6 mm was approximately 0.02 N.

Under Saffan anaesthesia the left maxillary central incisor of each animal was sectioned at the gingival margin using a high speed diamond bur. 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 root 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 a chrome cobalt splint. The splint magnet was fixed 0.6 mm 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 (Figure 3.1, P.48). As soon as the magnets approximated each other (indicating tooth extrusion), the magnet in the splint was repositioned further occlusally by 0.6 mm.

A fractured human incisor, approximately 22 mm long, was usually extruded an average of 4-6 mm after a traumatic injury. A corresponding proportional movement in a marmoset incisor, approximately 6mm long, was 1.2 mm. The total amount of extrusion was 1.2 mm and took an average of two weeks. Each tooth was retained at the new position for 30 weeks prior to sacrifice, using either the opposed magnets as a retentive device, or a concise resin splint (Figure 3.2, P.48).

The animals were anaesthetised with I.M. Saffan (18mg per kg body weight). 0.04ml of Heparin (1000 units/ml, Commonwealth Serum Laboratories) was injected following the onset of anaesthesia to prevent blood clotting. The surgical preparation was carried out followed by perfusion.

Perfusion

Before perfusion fixation, a vascular washout was performed via the carotid arteries with 300 ml of millipored Ringer's solution mixed with 1% sodium nitrite until a clear exudate flowed from the drainage tube. The sodium nitrite was used as a vaso-dilator to minimise vascular shut down in the key arterial channels to the experimental and control areas (Casley-Smith, 1987).

The tissues were then fixed by vascular perfusion with 5.6% glutaraldehyde and 0.9% osmium tetroxide in 0.06M cacodylate buffer. The perfusate was delivered at a mean pressure of 160 mm Hg. In accordance with Thorball and Tranum-Jensen (1983), the flow rate was gradually increased and the perfusate was introduced for a minimum of five minutes in order to saturate the tissues.

A good perfusion was obtained when the following signs were present:

1) The jaws were locked together.

2) Osmium tetroxide was present in the facial arteries and oral mucosa (ie. they were black).

3) The tissue had lost most of its colour and the body was rigid.

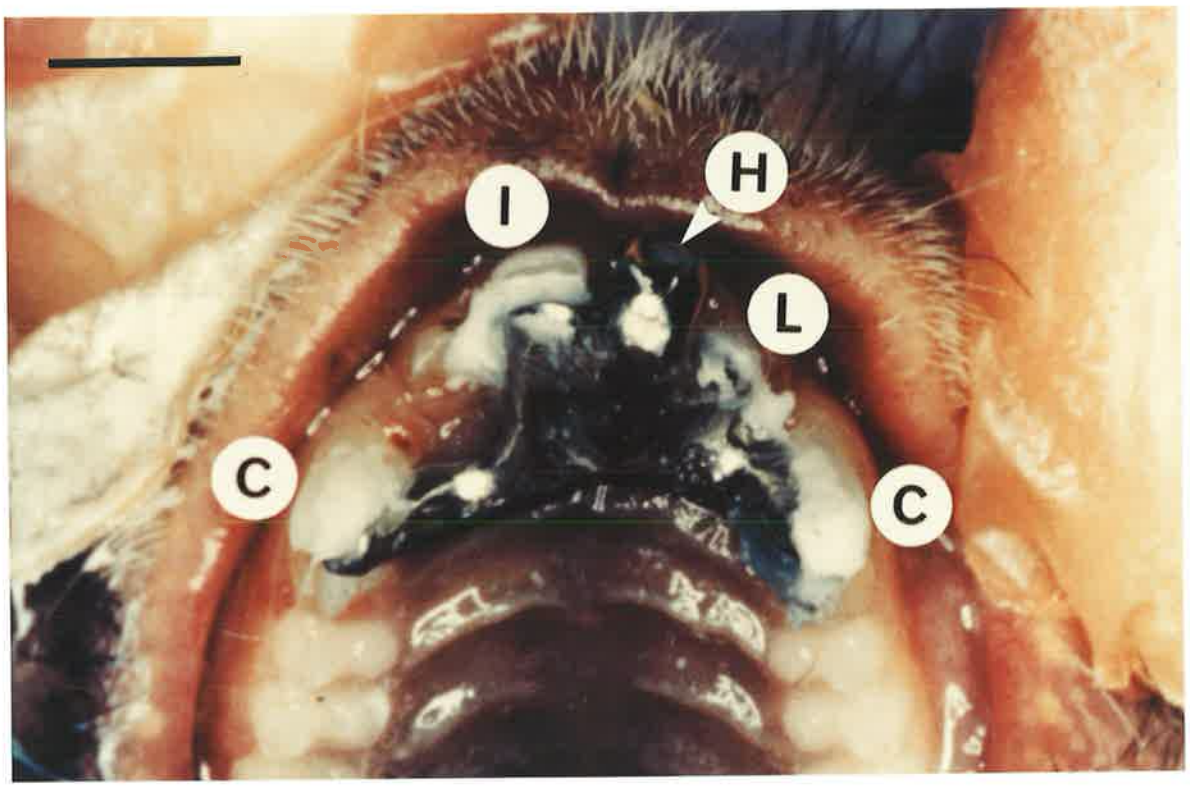


Figure 3.1 Occlusal view of extrusion device "in situ" (Weir, 1990)
C = Canine, H = Magnet Housing, I = Control Incisor,
L = Lateral incisor adjacent to the experimental tooth. Bar = 5 mm.

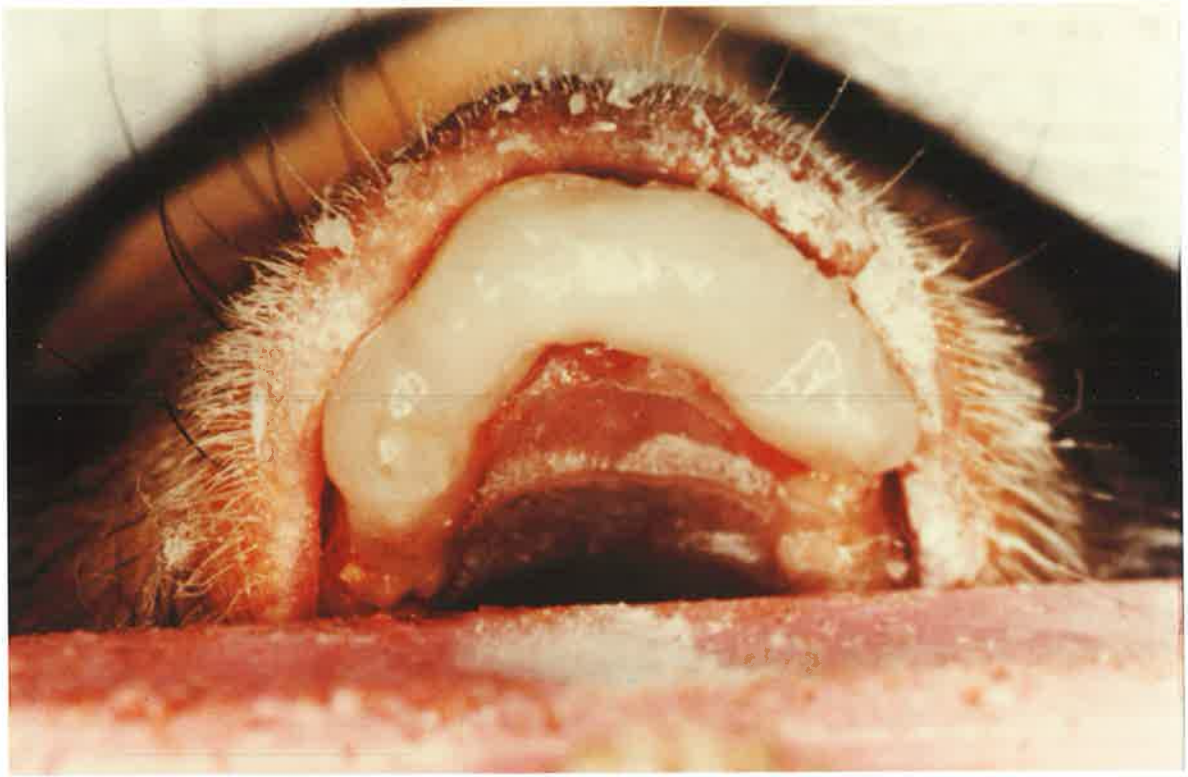


Figure 3.2 Resin retaining device (Weir, 1990)

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 7.1, P. 96). The four specimens thereafter required the following procedures :

3.2 TRIMMING

A razor blade was used to make bilateral diagonal cuts in the block from the mesial of the canines, and running medially to intersect in the midline of the palate. This separated the premaxilla from the maxilla proper. During this procedure the tissue was kept moist in 0.06M cacodylate buffer (Appendix 7.2, P. 96). The blocks containing the incisor teeth were embedded in alginate and a para-sagittal cut was made through the mesial third of the lateral incisors to reduce block size. A mid-sagittal cut was then carefully made to separate the experimental from the control blocks. In both cases the cuts were made with a new razor blade (Blue Strike, U.S.A). The blocks containing the control and experimental central incisors were then embedded in alginate. These teeth were cut mesio-distally. This procedure provided two experimental and two control blocks per animal. Trimming was facilitated by the use of a dissecting stereo-microscope. The blocks were numbered, and diagrams drawn to facilitate orientation during embedding.

3.3 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 7.2, P. 96).

2. *Post-fixation*

   4% OsO_4 in double distilled (d.d.) water for 1 hour (Appendix 7.3, P. 96).

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 7.4, P. 97).

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 7.7, P. 98)

   20 hours in agar 100 embedding resin (Appendix 7.7, P. 98)

3.4 EMBEDDING

Fresh Agar 100 (Appendix 7.7), 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 1 ml. of agar resin added. The resin filled moulds were incubated at 37° for 48 hours, and then at 60°c for a further 48 hours, before being coded and stored at room temperature.

3.5 SAMPLE SELECTION

Weir (1990) used 7 male and 3 female marmosets for his study. He finally selected 4 male marmosets due to the following criteria:

1. **Sex**: Animals selected were to be of the same sex, to minimise effects of any hormone-induced changes on experimental results and provide a more homogenous experimental group.

2. **Retention of the extrusion device**: Of the four animals chosen, three retained their extrusion devices throughout the experimental period, while the fourth animal dislodged its extrusion device after 22 weeks of retention. An immediate composite resin retaining device was then employed in this animal for the remaining eight weeks of retention until sacrifice. It was considered that, owing to rapid replacement of the extrusion device with a bonded retainer, retention of the extruded teeth could be effectively considered the same for all animals in the final group.

3. **Evaluation of tissue perfusion**: Quality of perfusion was visually assessed at the time of perfusion by the rapidity and evenness of spread of osmium tetroxide/glutaraldehyde through the tissues, as indicated by black discolouration of the soft tissues of the oral mucosa, nose and face. All experimental animals in the final group were judged to have been excellently perfused.

4. **Health of the periodontal tissues**: Clinical assessment throughout the experimental period and radiographic assessment after sacrifice enabled elimination of those animals from the final experimental group where evidence of periodontal pathology existed.

5. **Adequacy of the root canal filling**: Experimental teeth with radiographic evidence of over- or under-extended root filling were excluded from the final experimental group.

The specimens used in the present study were obtained from the Weir (1990) final experimental group. The paired blocks containing the mesial PDL from the control and the experimental sides were used.

3.6 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 d.d. 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 7.5, P. 97). Sections were rinsed with millipored d.d. water and dried on the hotplate. The tissue was examined microscopically and appropriate orientation photographs were taken on Polaroid Type 107 black and white land film. The zero level was defined when a "streaming" effect of the PDL was detected across the mesial aspect of the tooth e.g. collagen fibres and cells orientated perpendicular to the tooth surface.

The final trapezoidal mesa was outlined to cover the PDL at the most mesial part of the incisor (Figure 3.3, P. 58). Bone and tooth substance were included at the base and top of the trapezium, respectively. This not only gave better support for the section and allowed for orientation during TEM viewing, but also gave better sampling. From each block, thin sections (in the silver/gold interference range: 60-80nm) were cut with a Diatome diamond knife. At each level 36 sections were taken to provide 3 sections on each grid. On the basis of previous investigations (Parlange and Sims, 1993), the levels were at 150 micron intervals (Figure 3.3, P. 58).

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.

3.7 END LEVEL

The tissue blocks were sectioned at 150 µm intervals down to the apex and provided sampling from approximately 10 to 12 levels (1,500 µm - 1,800 µm). The end level was determined by the appearance of a small circular area of cellular cementum.

3.8 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 7.6, P. 98) 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 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°.

The grids were stained with lead citrate for 4 minutes by floating them in microfiltered droplets of freshly prepared modified Reynolds' lead (Appendix 7.6, P. 98) 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 minimise the formation of lead carbonate precipitated on the tissue sections.

The tissue was rinsed again in fresh d.d., 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 TEM.

3.9 TRANSMISSION ELECTRON MICROSCOPY

A Joel 100S transmission electron microscope (Appendix 7.9, P. 100) was used to examine the tissue sections. Grids were placed tissue side down in the vacuum column of the microscope and conditioned in the electron beam before being examined.

3.10 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. An orientation micrograph of the whole area was taken at a magnification of 500x (Figure 3.4, P. 59), which was used as an aid in the identification of the different types of blood vessel. A replicating

graticule (2190 lines/mm) was also exposed at a magnification of 3,000x and 500x at the end of each TEM session.

Ultramicrographs were obtained with Kodak Electron Microscope film using the built-in photographic equipment of the Joel 100S. This equipment had an automatic aperture setting. An exposure time of 3 seconds and 1 second was 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.

3.11 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 deionised 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 Durst Laboratory 54 enlarger. Replicating graticules were used to ensure that variation in magnification between the 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.

3.12 POINT COUNTING PROCEDURES

Each 8,500x photomicrograph print was masked to leave an area of 170 × 238 mm uncovered as this corresponded to an area of 20 × 28 μm of the PDL. A 140 point square lattice grid, with adjacent points spaced 17 mm apart, equal to 2 μm, was placed over the micrograph (Figure 3.5, P. 60). Profiles underlying each point were scored in accordance with the categories

listed in Appendix 7.11, P. 102. 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 (Gundersen et al., 1988). The number of profiles of blood vessels and nerves on each photomicrograph was counted using the convention of forbidden lines (Figure 3.6, P. 60), and the data was tabulated for stereological analyses. The analyses were made using the equations presented in Appendix 7.10, P. 100.

3.13 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 modifications including pericytic and non-pericytic postcapillary-sized venules were included from Freezer (1984), Crowe (1989), and Cooper and Sims (1990). The luminal diameter and the structure of the blood vessel walls formed the main criteria for vessel identification.

Blood vessel luminal diameters were measured across the narrowest diameter of the vessels according to stereological principles. 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 (Parlange, 1991).

b. Nerve Identification

The classification used for unmyelinated and myelinated nerves was based upon the location in the PDL and morphological features described by Freezer (1984), Freezer and Sims (1989), Crowe (1989), and Parlange and Sims (1993). Nerves were located in the parenchyma of the PDL or in close

apposition to the endothelium of the blood vessel walls. However, the present study was focussed on the presence of nerves within 1μm of vessel walls.

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

3.14 STATISTICAL ANALYSIS

The data were entered into a Sun computer at the University of Adelaide. For each vessel type, an analysis of variance was performed for the luminal diameter, abluminal diameter, and wall thickness, using the statistical package Minitab version 7. Differences between control and experimental groups, and 3 regions were tested (adjusted for animal variation).

The data for vessel volumes were considered to come from the binomial distribution (number of grid points covered by a particular vessel, out of a total possible grid count). However, the variance was greater than what we would expect for a binomial distribution, hence the data are overdispersed. Log-likelihood ratio statistics were calculated using a generalised linear model (McCullagh and Nelder, 1983) to test for:

1. Differences in blood vessel volumes between control and experimental groups (over regions, adjusted for animals).
2. Differences in blood vessel volumes between regions (over treatment group, adjusted for animals).
3. Interaction between regions and treatment group (adjusted for animals).

All analyses of blood vessel volumes were done by using the statistical package Genstat version 5, release 2.2.

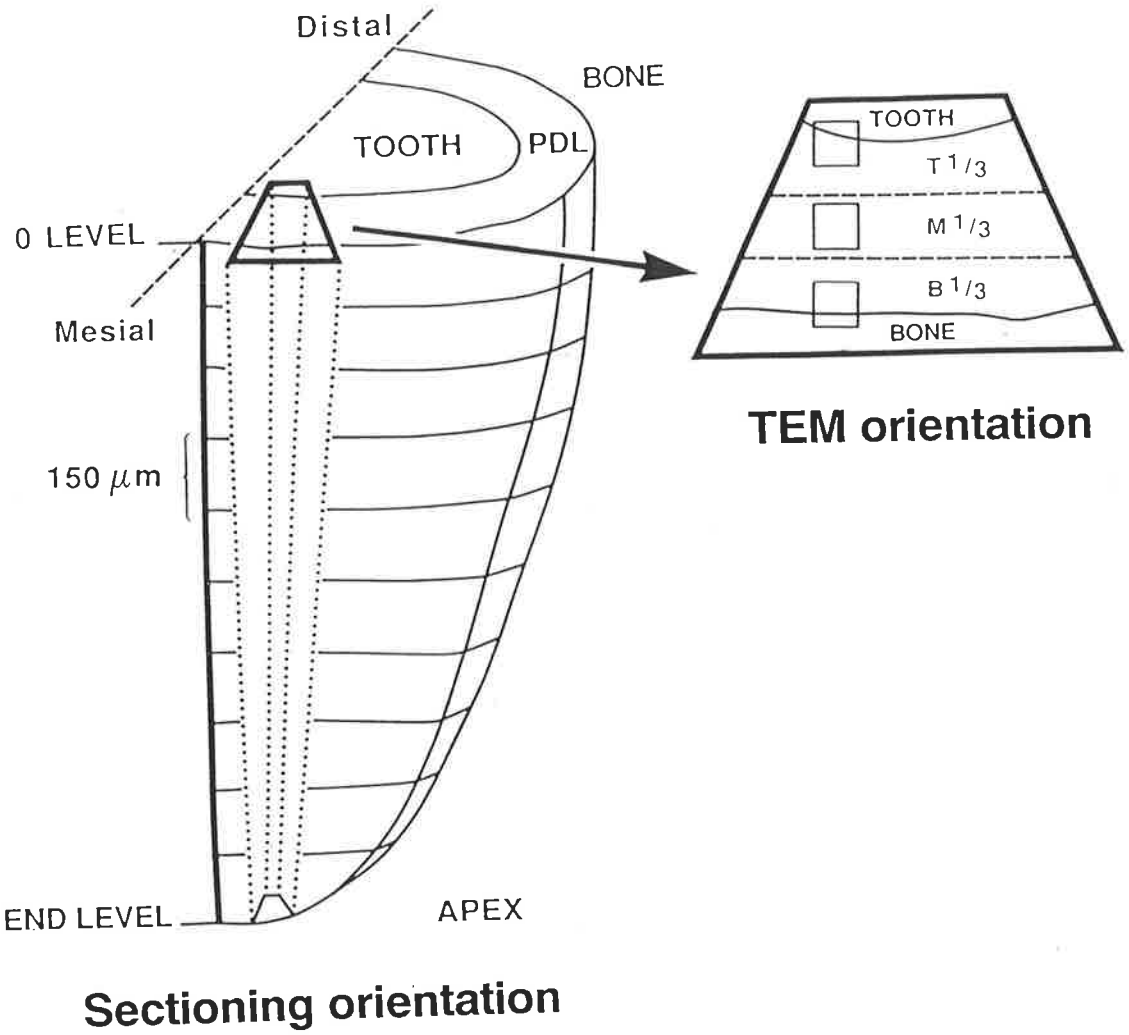


Figure 3.3 Diagram illustrating the sectioning orientation and region of the PDL examined. Ultra-thin sections were collected at 150 μm intervals. The TEM orientation illustrates the site selection of quadrats in the tooth, middle and bone thirds of the PDL.

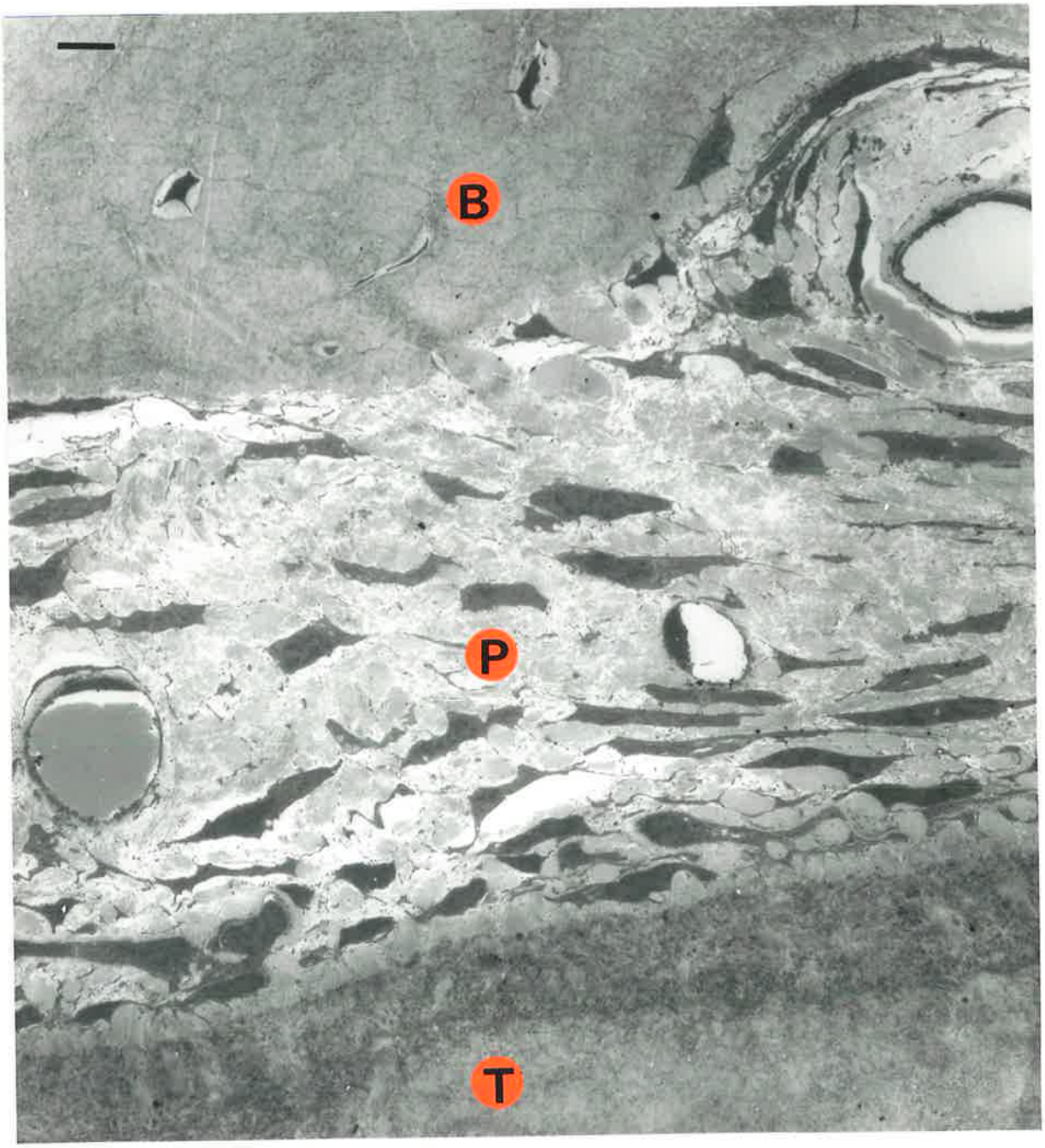


Figure 3.4 Orientation micrograph of the control block across the width of the periodontal ligament. (T) tooth; (P) periodontal ligament; (B) Bone.

Print Magnification x1,415

Bar = 6 µm

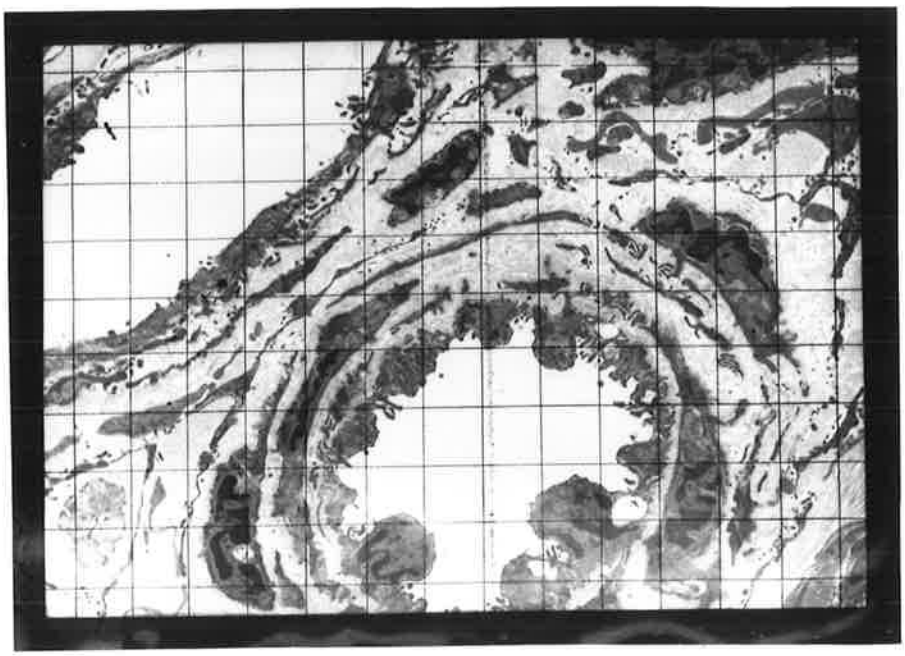


Figure 3.5 A 140 point square grid superimposed on a photomicrograph. Original print magnification : x8,500.

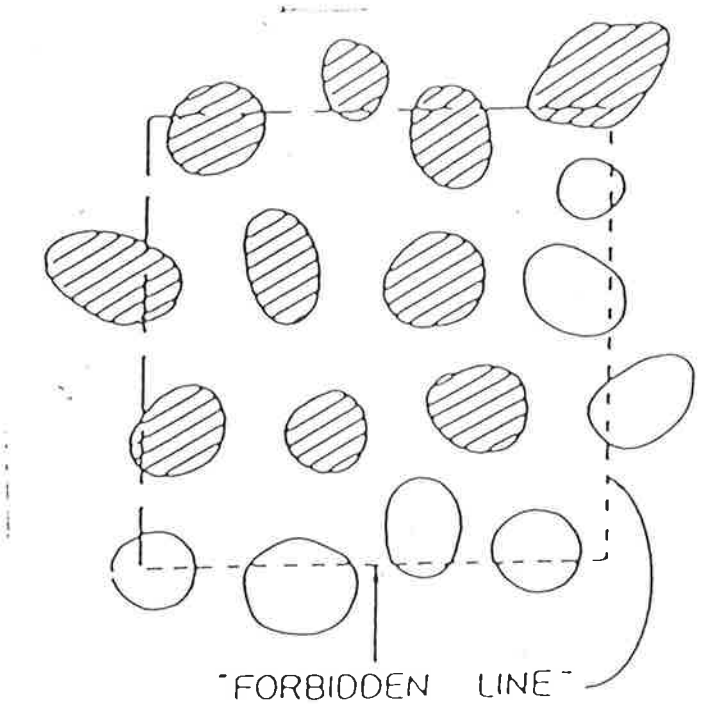


Figure 3.6 The convention of forbidden lines (Weibel 1979) modified according to Casley-Smith (1984). Profiles that intersect both the "accept" and "forbidden" lines are accepted only when the intersection is greater along the "accept" than the "forbidden" line. If the intersection is greater along the "forbidden" line, then the profile is not counted. Consequently, the shaded profile in the upper right cornor is counted but the one in the lower left cornor is not.

CHAPTER 4
FINDINGS

4.1 MORPHOLOGICAL FINDINGS

4.1.1 BLOOD VESSEL TYPES AND MORPHOLOGY

Seven types of vessel profile within the PDL were identified:

1) Pericytic venous capillaries,
2) Apericytic venous capillaries,
3) Pericytic postcapillary-sized venules,
4) Apericytic postcapillary-sized venules,
5) Collecting venules,
6) Arterial capillaries, and
7) Terminal arterioles.

1) Pericytic venous capillaries (pVC)

The vessel luminal wall ranged in thickness from 4-10 μm, consisting of a layer of endothelium and a pericytic layer. The pericytes were elongated cells with minimal cytoplasmic branching and a basement membrane. (Figure 4.1, P. 63; Figure 4.2, P. 64)

2) Apericytic venous capillaries (aVC)

The vessel lumen varied in thickness from 4-10 μm, and consisted of a single layer of endothelium. This vessels had no pericytic layer.

3) Pericytic postcapillary-sized venules (pPCV)

These vessels ranged from 10-30 μm in luminal diameter. Generally, this vessel had an incomplete pericytic layer. pPCVs were more common than apericytic postcapillary-sized venules (aPCVs). (Figure 4.2, P. 64)

4) Apericytic postcapillary-sized venules (aPCV)

The vessels ranged from 10-30 μm in luminal diameter. These vessels had no pericytic layer. aPCVs were less common than pPCVs.

5) Collecting venules (CV)

Collecting venules had the largest luminal diameter ranging from 30-80 μm. The vessel wall consisted of a single endothelial layer. (Figure 4.3, P. 65)

6) Arterial capillaries (AC)

The vessel wall varied in thickness from 3-5 μm. The endothelial cell bulged into the lumen and was thicker and more lobulated than its venous counterpart. Microvillous projection were often noted along the luminal surface in the region of the endothelial junctions. (Figure 4.4, P. 66)

7) Terminal arterioles (TA)

Terminal arterioles had the thickest cell wall, ranging between 3-6 μm. The vessel walls were characterised by one to three layers of smooth muscle cells which encircled the luminal endothelium. 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. (Figure 4.5, P. 67)

4.1.2 NERVE TYPES AND MORPHOLOGY

Myelinated and unmyelinated nerves were categorised according to location within the PDL:

a) within the parenchyma of the PDL;

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

1) Myelinated nerves

Most myelinated nerves were located in the parenchyma of the PDL and were surrounded by a thin capsular wall. (Figure 4.6, P. 68)

The apical third of the PDL demonstrated neural morphology which differed from the middle and cervical regions. The apical-third tissues were distinguished by the presence of numerous myelinated nerves arranged in encapsulated bundles, surrounded by a thin, multi-layered capsule (Figure 4.6, P. 68).

2) Unmyelinated nerves

Unmyelinated nerves were present as groups of nerves or single nerve, which were frequently related to extensions of the Schwann cell cytoplasm. (Figure 4.6, P. 68)

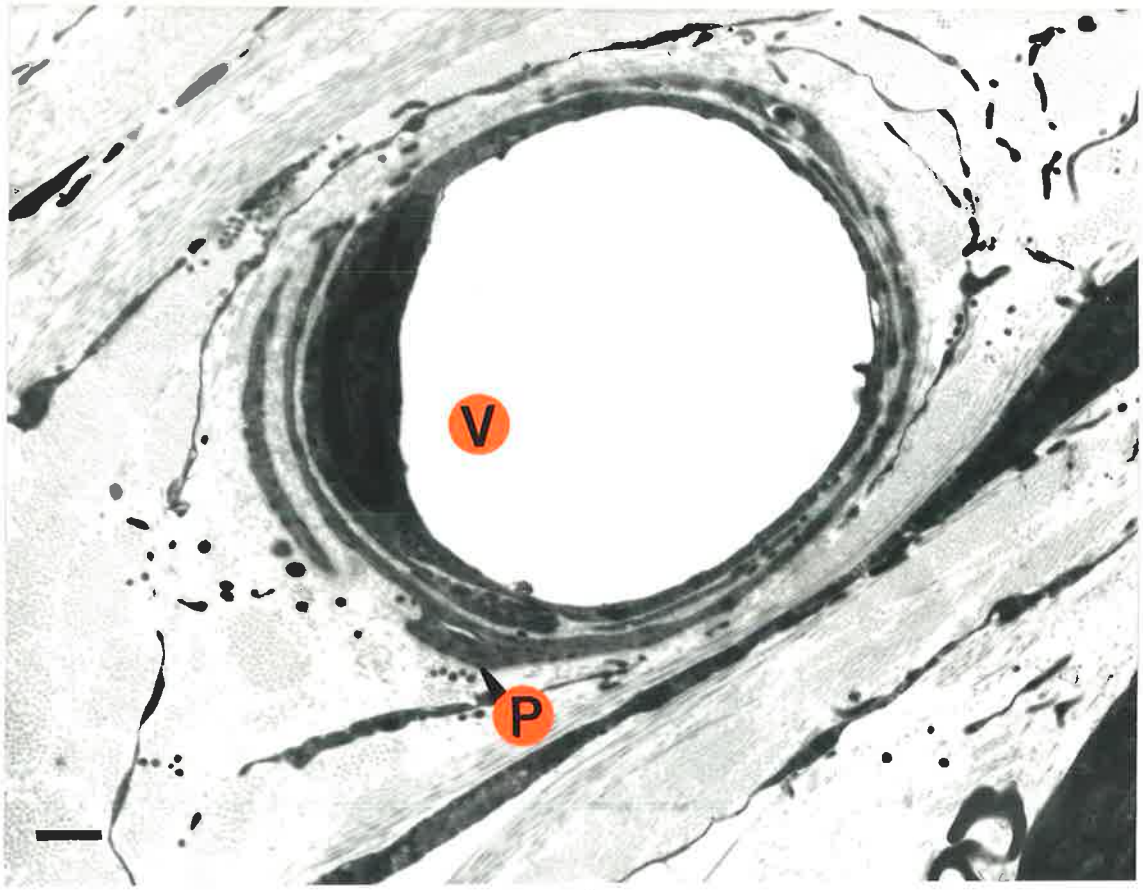


Figure 4.1 A pericytic venous capillary (V) with a single endothelial layer, partially surrounded by a pericytic cell (P).

Print Magnification x8,500.

Bar = 1 μm

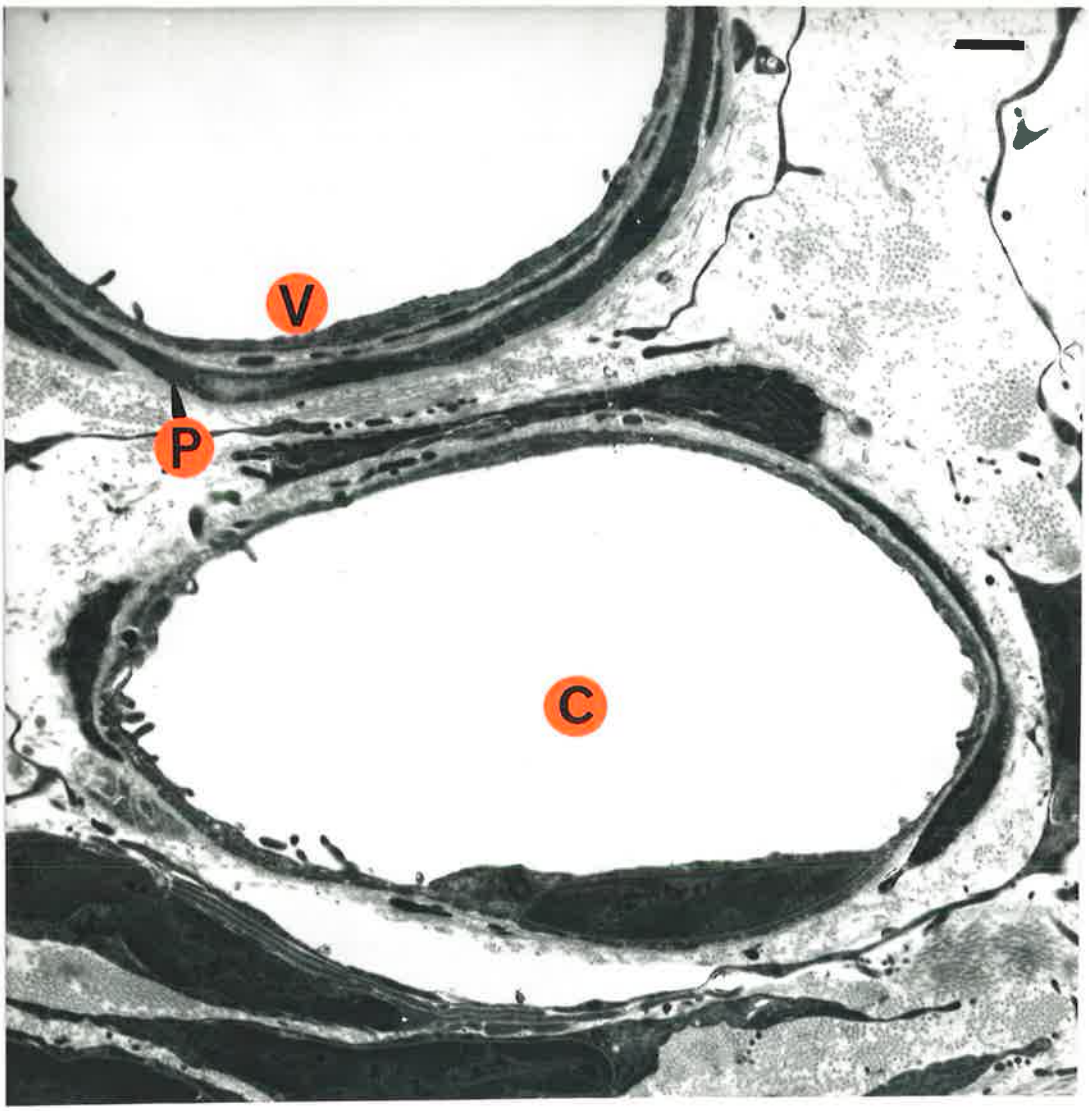


Figure 4.2 A pericytic postcapillary-sized venule (V) with a single endothelial layer, surrounded by pericytic cells (P). A pericytic venous capillary (C).

Print Magnification x8,500

Bar = 1 μm

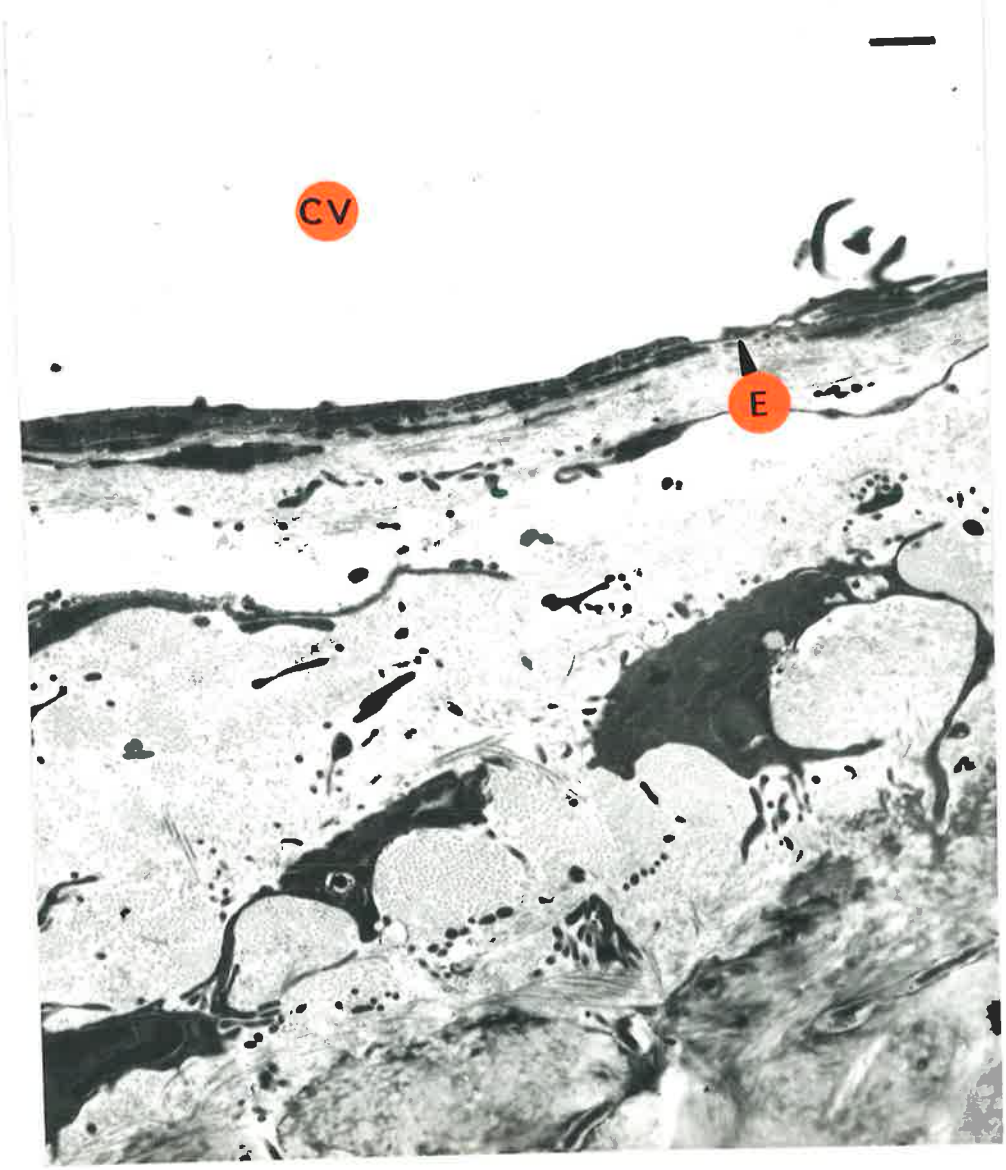


Figure 4.3 Segment of a collecting venule (CV) with a thin endothelial wall (E).

Print Magnification x8,500

Bar = 1 μm

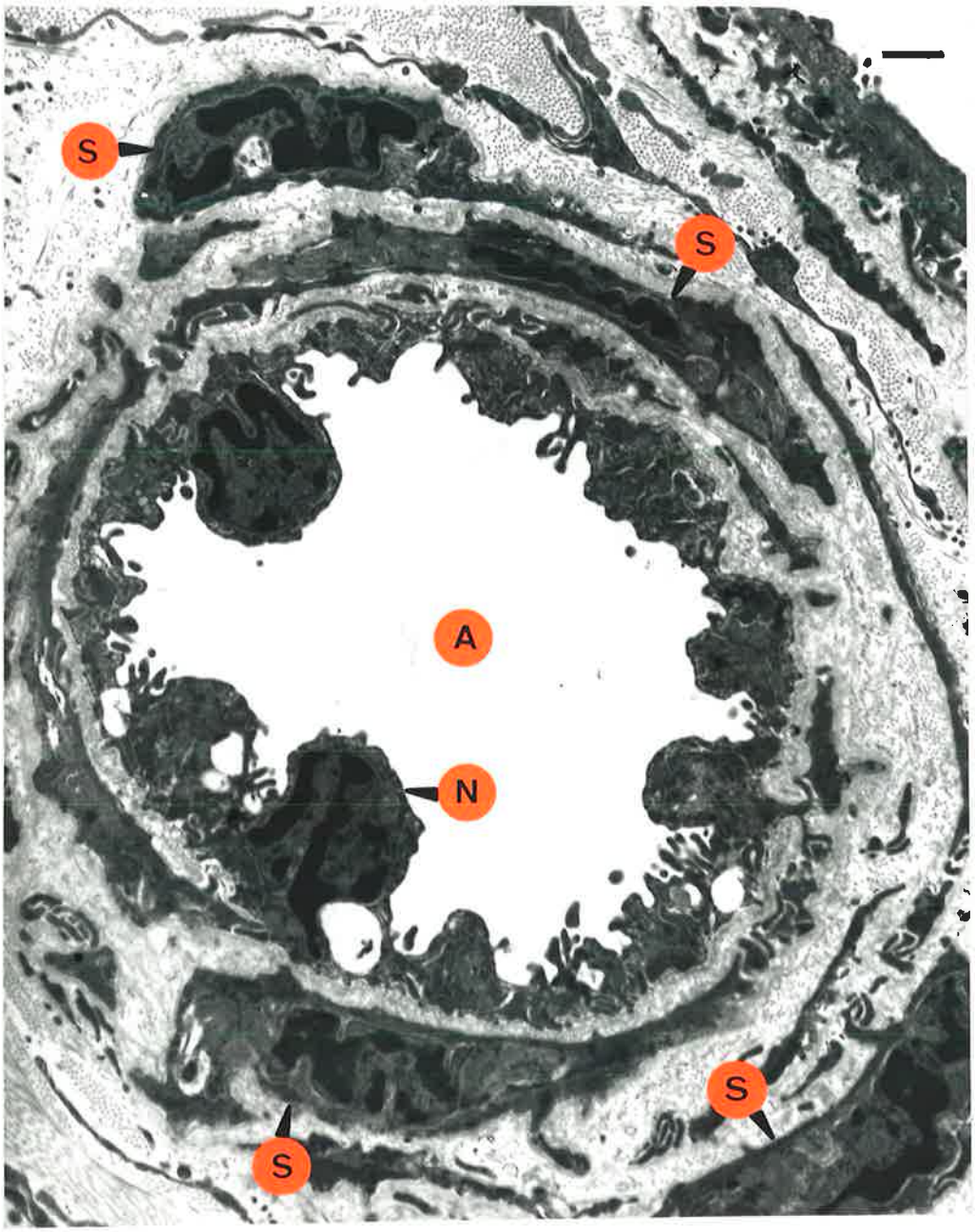


Figure 4.4 An arterial capillary (A) with lobulated endothelial nuclei (N). Smooth muscle cell investment (S) adapted to the endothelial layer. Numerous luminal microvillus extensions present.

Print Magnification x8,500

Bar = 1 μm

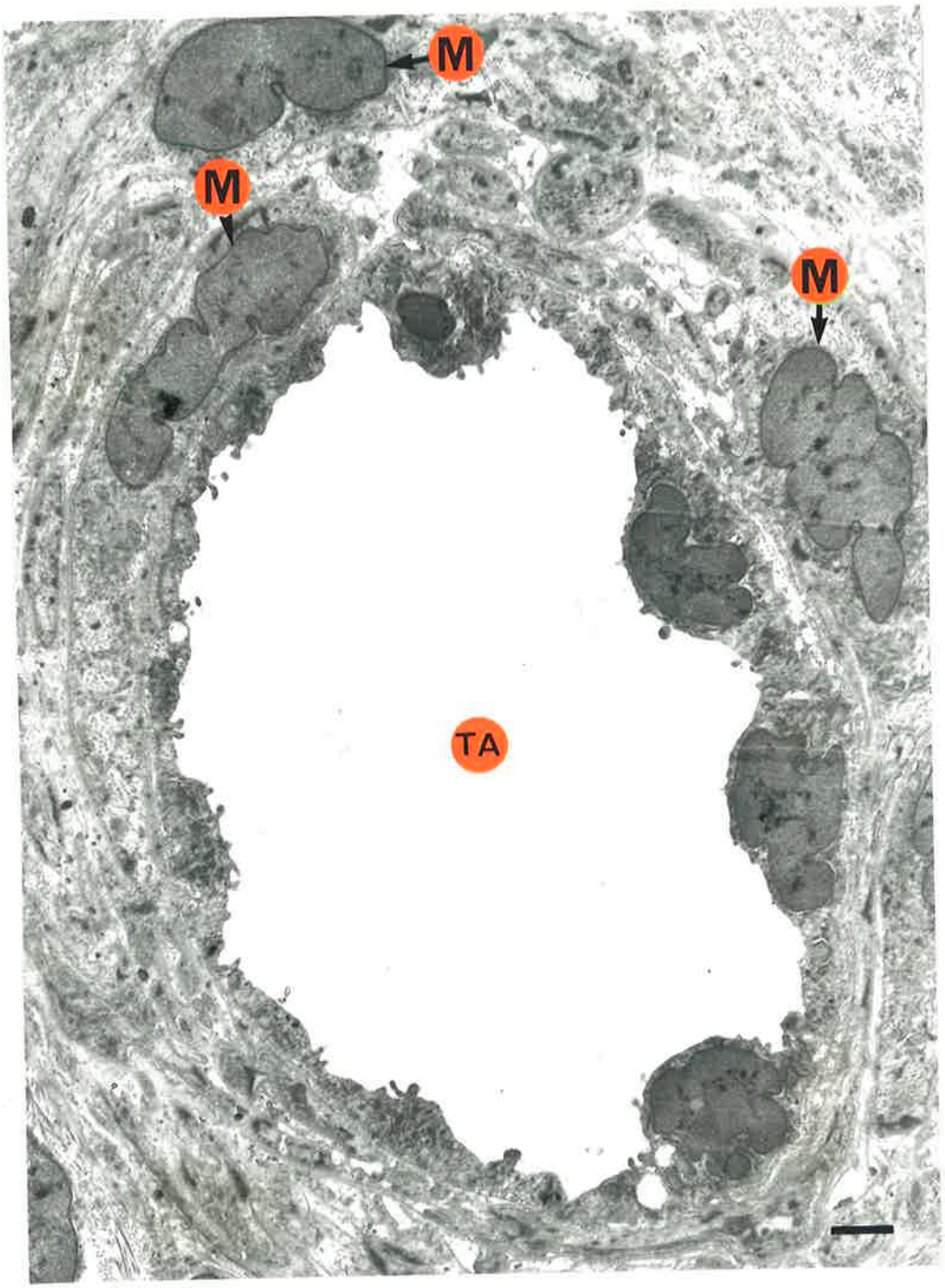


Figure 4.5 A terminal arteriole (TA) with triple layer of smooth muscle cells (M).

Print Magnification x5,670

Bar = 1.5 μ

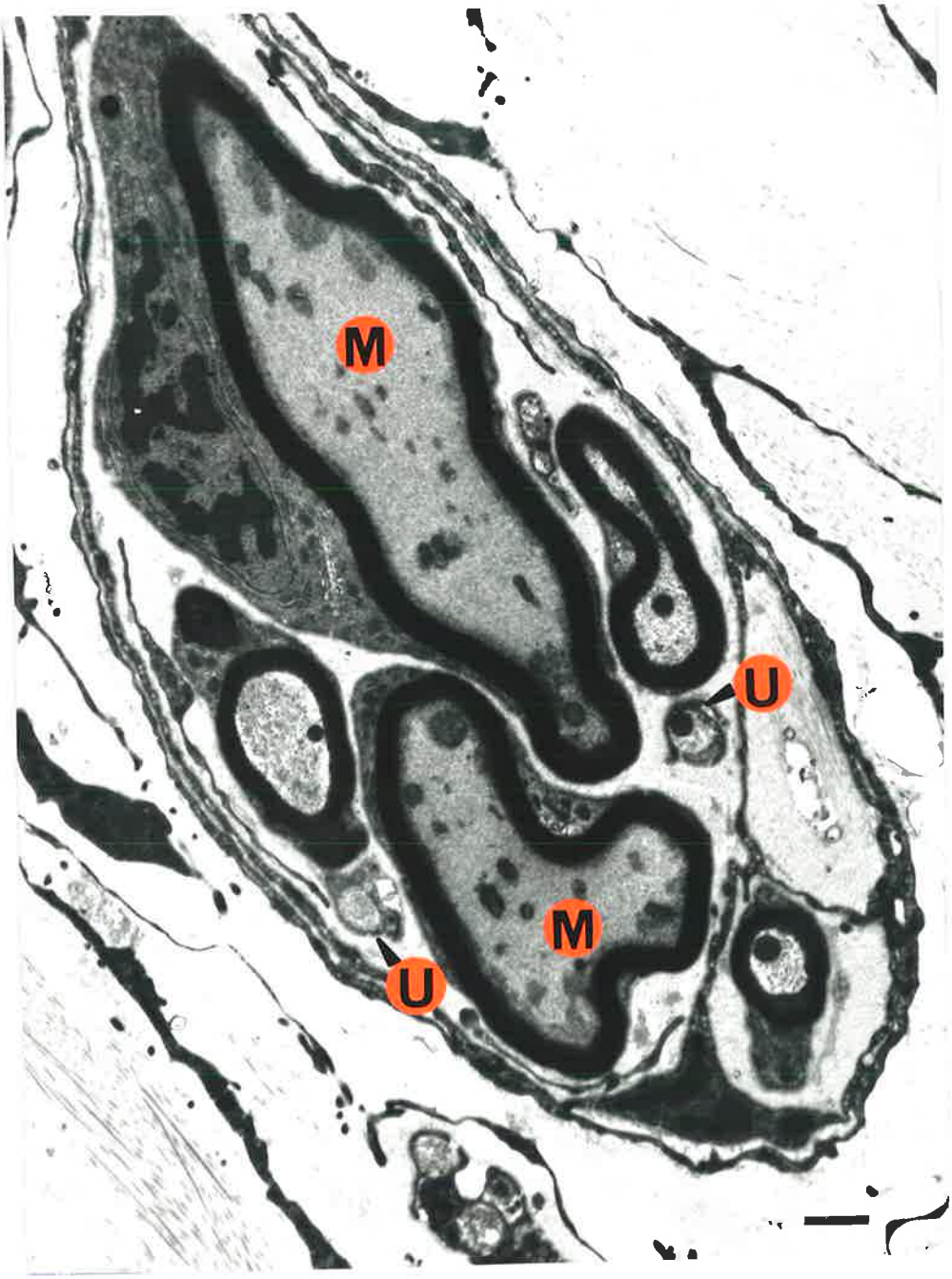


Figure 4.6 A bundle of myelinated (M) and unmyelinated (U) nerves enclosed by a thin, multi-layered capsule (taken from the apical-third region of the PDL)

Print Magnification x8,500

Bar = 1 μm

4.2 BLOOD VESSEL QUANTIFICATION

4.2.1 Vascular Volume

a. Total Vascular Volume

The total luminal volume was 10.79% and 12.60% of the total PDL volume in the control and experimental PDL, respectively (Table 4.1). The overall abluminal volume was 13.37% and 15.13% in the control and experimental PDL when the blood vessel wall thickness was included. In other words, the volumes of endothelia and perivascular cells in the control and experimental groups were 2.58% and 2.53%, respectively. No significant differences in total luminal and abluminal volumes were found between the control and experimental teeth as shown in Table 4.1. The overall luminal and abluminal volumes are presented graphically in Figure 4.7, P. 70.

Table 4.1 Total luminal and abluminal volume densities (V_V) as a percentage of total PDL volume: V_V (%) ± approx. S.E.

Volume	Control	Experimental	
	V_V ± S.E.	V_V ± S.E.	
Luminal	10.79 ± 3.17	12.60 ± 4.35	NS
Abluminal	13.37 ± 3.82	15.13 ± 5.20	NS

NS = No significant difference between control and experimental groups ($P > 0.05$)

b. Vascular Volume of Each Blood Vessel Type

Luminal and abluminal volumes of different blood vessel types are given in Table 4.2 and 4.3, respectively. No statistically significant differences were present in luminal and abluminal volumes of each blood vessel type between the experimental and control PDL after adjusting for possible differences due to animals. The mean luminal volume of each blood vessel type is demonstrated graphically in Figure 4.8, P. 71.

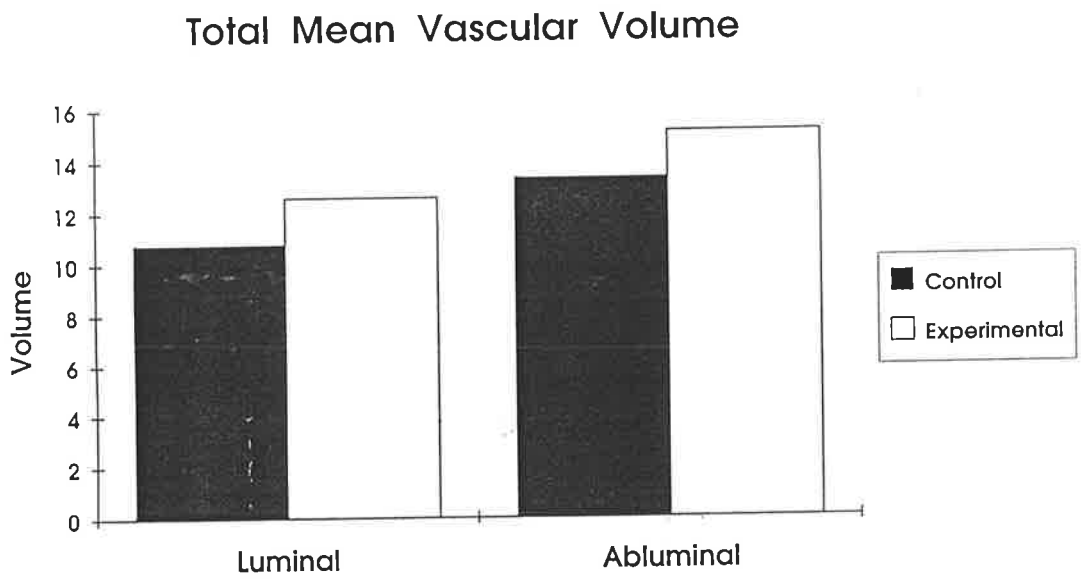


Figure 4.7 Mean total luminal and abluminal volume densities (%) in the control and experimental PDL.

Table 4.2 Luminal volume density (V_V) as a percentage of total PDL volume: V_V (%) ± approx. S.E.

	Control	Experimental	
	V_V ± S.E.	V_V ± S.E.	
Venous capillaries	0.82 ± 0.31	2.07 ± 0.67	NS
Postcapillary-sized venules	7.13 ± 2.32	8.13 ± 3.19	NS
Arterial capillaries & Terminal arterioles	0.64 ± 0.20	0.04 ± 0.07	x
Collecting venules	2.23 ± 0.97	2.32 ± 1.12	NS
Total	10.79 ± 3.17	12.60 ± 4.35	NS

x = No statistical analyses performed due to inadequate observations.
NS = No significant difference between control and experimental groups ($P > 0.05$).

Table 4.3 Abluminal volume density as a percentage of total PDL volume: % ± approx. S.E.

	Control	Experimental	
	volume ± S.E.	volume ± S.E.	
Venous capillaries	1.22 ± 0.43	2.83 ± 0.91	NS
Postcapillary-sized venules	8.26 ± 2.67	9.39 ± 3.65	NS
Arterial caplilllaries & Terminal arterioles	1.30 ± 0.41	0.16 ± 0.21	x
Collecting venules	2.65 ± 1.15	2.64 ± 1.35	NS
Total	13.37 ± 3.82	15.13 ± 5.20	NS

x = No statistical analyses conducted due to insufficient observations.
NS = No significant difference between control and experimental groups ($P > 0.05$).

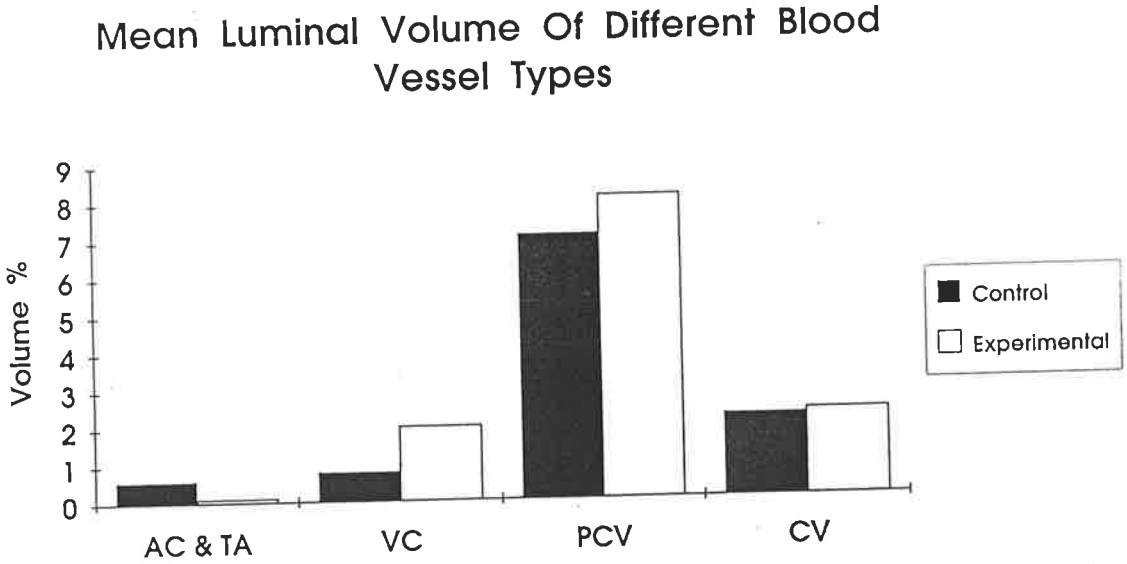


Figure 4.8 Mean luminal volume of each blood vessel type

c. Regional Variation and Total Vascular Volume

The luminal and abluminal volumes in the circumferential thirds of the PDL were analysed to test for differences due to regions, and interactions

between treatment group and regions. Tables 4.4 and 4.5 give the estimated V_V (%) for luminal and abluminal, in different regions of the control and experimental teeth. No significant differences were found in the total luminal and abluminal volumes between the control and experimental teeth. There were significant differences in volume due to 3 regions but no significant interactions between regions and treatment group. Very few blood vessels were present in the tooth-third of PDL. Most of them were located within the middle-third region. Luminal volume density in different regions of PDL is illustrated graphically in Figure 4.9, P. 73.

Table 4.4 Luminal volume density (V_V) as a percentage of total PDL volume in different regions: V_V (%) ± S.E.

Region	Control	Experimental	(1)	(2)	(3)
	Mean ± S.E.	Mean ± S.E.			
Tooth	0.10 ± 0.17	0.80 ± 0.66	NS	*	NS
Middle	23.47 ± 2.56	20.75 ± 3.14	NS	*	NS
Bone	8.89 ± 1.74	16.45 ± 2.88	NS	*	NS

(1) No significant difference ($P > 0.05$) between control and experimental groups.
(2) Significant difference ($P < 0.05$) between 3 regions.
(3) No significant interaction between regions and treatment group.

Table 4.5 Abluminal volume density as a percentage of total PDL volume in different regions: % ± S.E.

Region	Control	Experimental	(1)	(2)	(3)
	Mean ± S.E.	Mean ± S.E.			
Tooth	0.17 ± 0.27	1.10 ± 0.89	NS	*	NS
Middle	28.40 ± 3.08	24.96 ± 3.82	NS	*	NS
Bone	11.67 ± 2.22	19.59 ± 3.52	NS	*	NS

(1) No significant difference ($P > 0.05$) between control and experimental groups.
(2) Significant difference ($P < 0.05$) between 3 regions.
(3) No significant interaction between regions and treatment group.

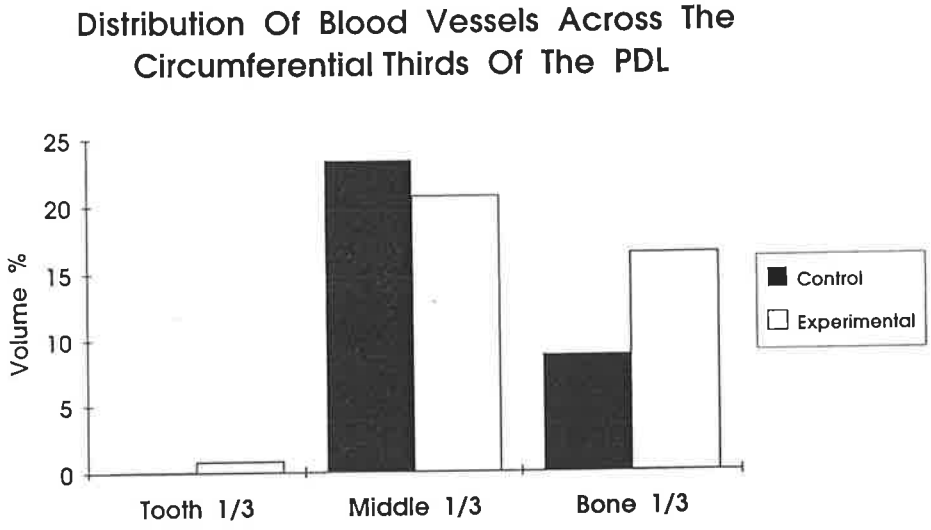


Figure 4.9 Mean luminal volume density (V_V) as a percentage of total PDL volume in different regions in the control and experimental PDL.

d. Regional Variation and Blood Vessel Type

For venous capillaries and collecting venules the only differences in luminal volume density (V_V) were due to the 3 regions (Tables 4.6 and 4.7). However, for postcapillary-sized venules, there was a significant interaction between region and treatment (Table 4.8). This finding is presented graphically in Figure 4.10, P. 74, where the volume in the middle third was greater in the control teeth than the experimental teeth, but less than the experimental teeth in the bone third. All 3 regions did not behave in a similar manner.

Table 4.6 Luminal volume density (V_V) of *venous capillaries* as a percentage of total PDL volume in different regions: V_V (%) ± S.E.

Region	Control	Experimental	(1)	(2)	(3)
	Mean ± S.E.	Mean ± S.E.			
Tooth	0.00 ± 0.00	0.00 ± 0.57	NS	*	NS
Middle	0.94 ± 0.45	1.98 ± 0.90	NS	*	NS
Bone	1.55 ± 0.58	3.45 ± 1.19	NS	*	NS

(1) No significant difference ($P > 0.05$) between control and experimental groups.
(2) Significant difference ($P < 0.05$) between 3 regions.
(3) No significant interaction between regions and treatment group.

Table 4.7 Luminal volume density (V_V) of *collecting venules* as a percentage of total PDL volume in different regions: V_V (%) ± S.E.

Region	Control	Experimental	(1)	(2)	(3)
	Mean ± S.E.	Mean ± S.E.			
Tooth	0.00 ± 0.01	0.00 ± 0.01	NS	*	NS
Middle	4.31 ± 1.86	4.89 ± 2.32	NS	*	NS
Bone	2.41 ± 1.41	2.14 ± 1.55	NS	*	NS

Table 4.8 Luminal volume density (V_V) of *postcapillary-sized venules* as a percentage of total PDL volume in different regions: V_V (%) ± S.E.

Region	Control	Experimental	(1)	(2)	(3)
	Mean ± S.E.	Mean ± S.E.			
Tooth	0.10 ± 0.16	0.10 ± 0.22	NS	*	*
Middle	17.52 ± 2.24	13.41 ± 2.57	NS	*	*
Bone	3.78 ± 1.13	11.03 ± 2.38	NS	*	*

(1) No significant difference ($P > 0.05$) between control and experimental groups.
(2) Significant difference ($P < 0.05$) between regions.
(3) Significant interaction between regions and treatment group.

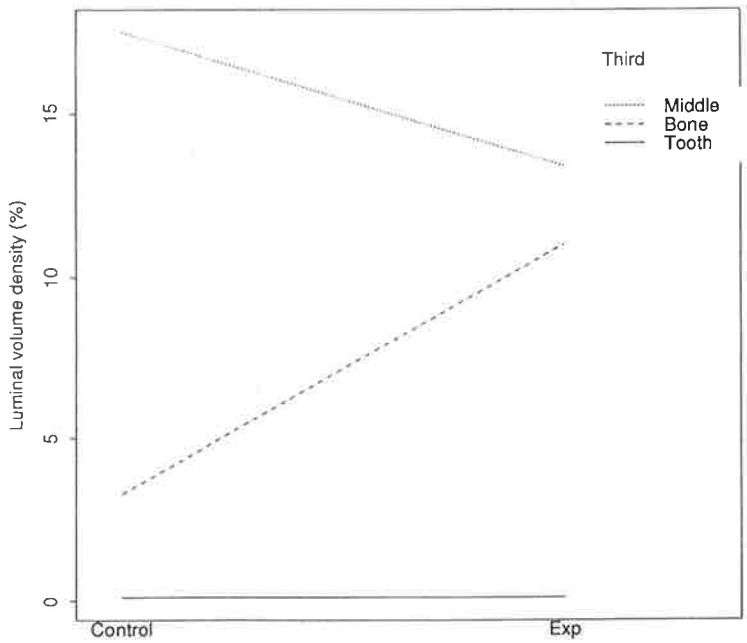


Figure 4.10 Mean luminal volume of postcapillary-sized venules in different regions. Note interaction between regions and treatment group.

4.2.2 Blood Vessel Diameter

Luminal and abluminal diameters are given in Tables 4.9 and 4.10. No statistically significant changes occurred from the treatment for the mean luminal and abluminal diameters. Note that no statistical analyses were conducted for arterial capillaries and terminal arterioles due to insufficient observations. The mean luminal diameter of each blood vessel type is shown graphically in Figure 4.11, P. 76.

Table 4.9 The mean caliper luminal diameter of different blood vessel types: μm ± S.E.

	Control	Experimental	
	Mean ± S.E.	Mean ± S.E.	
Venous capillaries	6.68 ± 0.50	7.06 ± 0.39	NS
Postcapillary-sized venules	17.02 ± 0.77	16.23 ± 0.89	NS
Arterial capillaries & Terminal arterioles	8.57 ± 2.11	20.00 ± 0.00	x
Collecting venules	38.08 ± 2.49	30.02 ± 4.60	NS
Total	16.63 ± 1.11	14.18 ± 1.27	NS

Table 4.10 The mean caliper abluminal diameter of different blood vessels types: μm ± SE.

	Control	Experimental	
	Mean ± S.E.	Mean ± S.E.	
Venous capillaries	8.36 ± 0.58	8.61 ± 0.45	NS
Postcapillary-sized venules	19.20 ± 0.76	17.95 ± 0.87	NS
Arterial capillaries & Terminal arterioles	14.30 ± 2.57	25.90 ± 0.00	x
Collecting venules	39.72 ± 2.56	31.95 ± 4.73	NS
Total	18.96 ± 1.10	15.91 ± 1.26	NS

NS = No significant difference between control and experimental groups (adjusted for animals, $P > 0.05$).
x = No statistical analyses conducted due to inadequate observations.

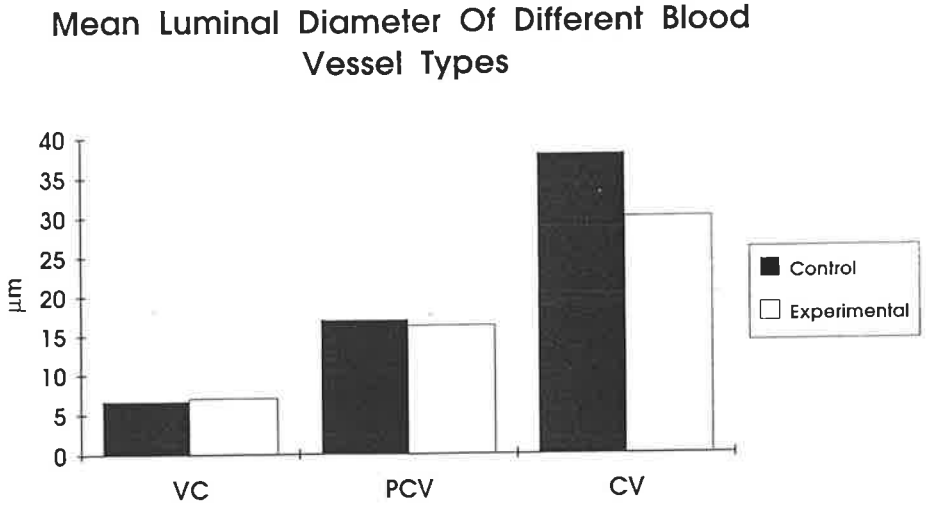


Figure 4.11 Mean luminal diameter (µm) of different blood vessel types in the control and experimental PDL.

4.2.3 Blood Vessel Wall Thickness

The wall thickness of different blood vessel types is presented in Table 4.11. No statistically significant differences were found in wall thickness of venous capillaries and collecting venules. However, postcapillary-sized venules in the experimental PDL had significantly thinner blood vessel wall than those in the control PDL ($P < 0.05$). Similarly, the total wall thickness in the experimental PDL is significantly thinner than that in the control PDL ($P < 0.05$). The graphical representation of Table 4.11 is shown in Figure 4.12, P.77).

Table 4.11 The wall thickness of different blood vessel types: µm ± S.E.

	Control	Experimental	
	Mean ± S.E.	Mean ± S.E.	
Venous capillaries	1.67 ± 0.30	1.55 ± 0.23	NS
Postcapillary-sized venules	2.18 ± 0.14	1.72 ± 0.16	*
Arterial capillaries & Terminal arterioles	5.72 ± 0.65	5.90 ± 0.00	x
Collecting venules	1.64 ± 0.14	1.93 ± 0.26	NS
Total	2.33 ± 0.15	1.73 ± 0.17	*

* = Significant difference between control and experimental groups (adjusted for animals, $P < 0.05$)

x = No statistical analyses performed due to insufficient observations.

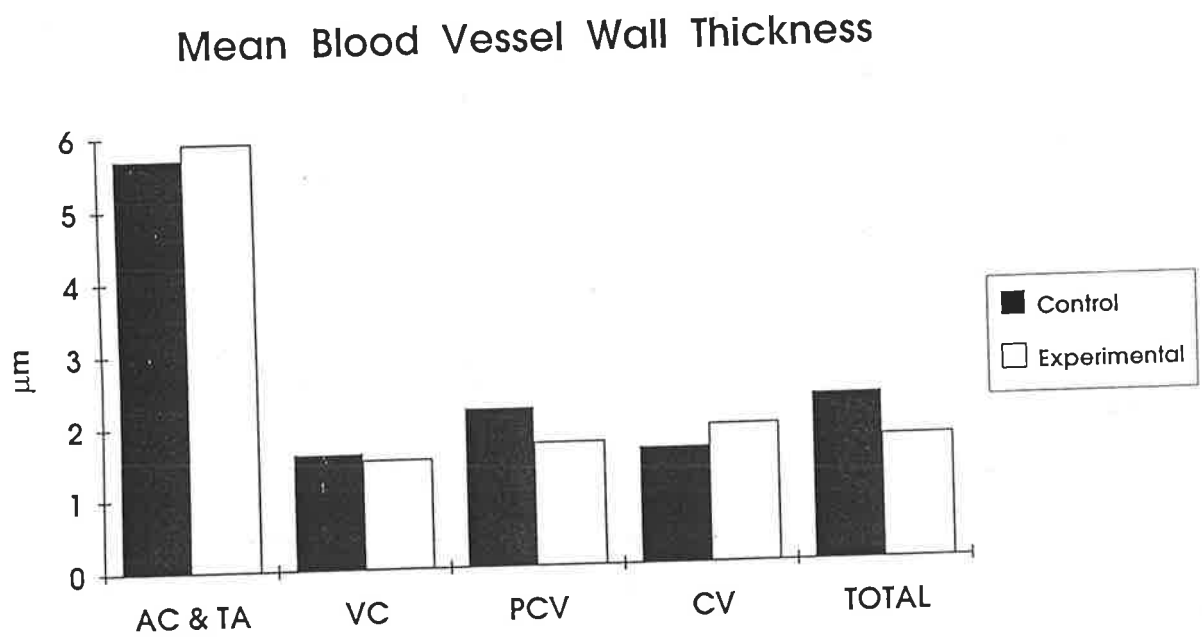


Figure 4.12 Mean wall thickness (µm) of different blood vessel types and the total mean wall thickness (µm) in the control and experimental PDL.

4.2.4 Surface Density (S_V)

The mean luminal surface densities (S_V) were 259.53 and 355.43 cm^2/cm^3 in the control and experimental PDL, respectively (Table 4.12). Postcapillary-sized venules were the major contributor to the total luminal surface density in the control and experimental PDL with 64.2% and 56.4%, respectively.

Table 4.12 Luminal surface density (S_V) of blood vessels: S_V (cm^2/cm^3) ± S.E.

	Control	Experimental
	Mean ± S.E.	Mean ± S.E.
Venous capillaries	49.06 ± 14.88	117.23 ± 36.41
Postcapillary-sized venules	166.58 ± 46.64	200.37 ± 67.60
Arterial capillaries & Terminal arterioles	29.87 ± 1.98	0.80 ± 1.40
Collecting venules	23.42 ± 8.65	30.91 ± 10.11
Total	259.53 ± 58.90	355.43 ± 90.80

4.2.5 Length Density (Lv)

Blood vessel length densities (L_V) were 49.68 x 10^3 and 79.79 x 10^3 cm/cm^3 in the control and experimental PDL, respectively (Table 4.13). Postcapillary-sized venules were the major contributor to the total luminal length in the control PDL with 62.3% and 49.2%, respectively.

Table 4.13 Luminal length density (L_V) of different PDL blood vessel types: L_V (10^3 cm/cm^3)

	Control	Experimental
	Mean ± S.E.	Mean ± S.E.
Venous capillaries	23.36 ± 5.34	52.83 ± 11.21
Post-capillary-sized venules	30.97 ± 7.26	39.30 ± 11.10
Arterial capillaries & Terminal arterioles	11.10 ± 1.99	0.13 ± 0.22
Collecting venules	1.96 ± 0.59	3.28 ± 0.57
Total	49.68 ± 7.95	79.79 ± 13.22

4.2.6 Mean Number (N_A)

The total mean number (N_A) of blood vessel/560 μm^2 in the control PDL (0.11/560 μm^2) closely matched with that in the experimental PDL (0.14/560 μm^2). Postcapillary-sized venules were the main contributor to the total mean number of blood vessels in the PDL: 63.63% and 57.14% in the control and experimental PDL, respectively. (Table 4.14)

Table 4.14 The mean number of blood vessel types/560 μm^2 of PDL tissues: N_A (/560 μm^2)

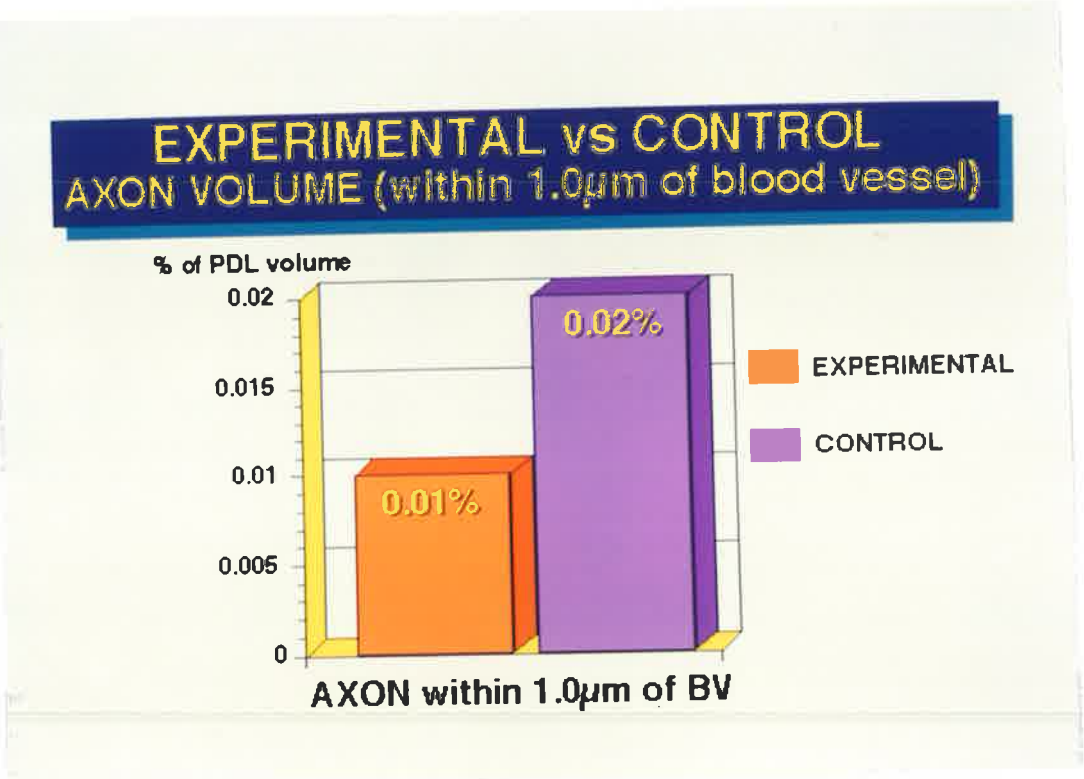
	Control	Experimental
Venous capillaries	0.01	0.03
Postcapillary-sized venules	0.07	0.08
Arterial capillaries & Terminal arterioles	0.01	0.00
Collecting venules	0.02	0.03
Total	0.11	0.14

4.3 NERVE QUANTIFICATION

Nerve (within 1.0 μm of blood vessel) volumes in the experimental and control PDL are shown in Figure 4.13.

Nerve volumes in the experimental and control PDL were 0.01% and 0.02%, which were minimal percentage due to insufficient data. Therefore, statistical analyses were not performed. General speaking, no significant difference was found in nerve volumes between the experimental and control groups.

Figure 4.13 Nerve volume (within 1.0 μm of blood vessel) as a percentage of total PDL volume.



CHAPTER 5
DISCUSSION

5.1 THE MARMOSET AS AN EXPERIMENTAL ANIMAL

The marmoset PDL has been examined in some detail by Dreizen et al. (1967), Levy and Bernick (1968a, b), Bernick and Levy (1968a, b), Levy et al. (1970), Skougaard et al. (1972), Page et al. (1974), Levy (1976), Bernick et al. (1977), Douvartzidis (1984), Crowe (1989), Weir (1990), and Parlange and Sims (1993). This non-human primate has been found to provide an acceptable biological analogue for the study of human PDL, owing to its similar histological and ultrastructural features. The present study revealed that the PDL luminal blood volume was 10.8% in control maxillary incisors. Comparably, Foong (1994) found that the total luminal volume was 9.5% in the human mandibular distal premolar PDL of non-functional teeth. Therefore, it would appear that on the basis of comparable vascular systems the marmoset is suitable for dental research as an analogue of man. Other criteria including vascular wall thickness and diameter, distribution of blood vessels in the circumferential thirds, and nerve volume will be considered later in the discussion.

According to Weir (1990), however, the small size of the animal did pose problems for experiments of the type required in his project on the dentition. Access to the dentition, the isolation of teeth, and moisture control during experimental procedures were all difficult.

5.2 EFFECTS OF EXPERIMENTAL PROCEDURES

As noted by Weir (1990), no adverse responses were found in any of the experimental procedures, with the exception of minor temporary weight loss noted in some of the animals following appliance insertion. All animals

adapted to the appliances within the first two weeks and weight gains were noted.

5.3 ORAL HYGIENE

Crowe (1989) reported that the tri-weekly oral hygiene regime undertaken had little effect upon the overall gingival condition of the animals. Those animals with initially good periodontal health remained relatively free from signs of inflammatory periodontal breakdown, while those with initially poor periodontal condition showed little improvement. Weir (1990) noted that more frequent cleaning was impractical owing to time constraints. Moreover, daily cleaning damaged gingival tissues (Crowe, 1989).

5.4 ENDODONTIC THERAPY

The root canal fillings were radiographically assessed after perfusion, and appeared adequate in all animals. A minor overfilling was noted in one animal (No. 36, a female). This animal was excluded from the final experimental group.

5.5 EXTRUSION DEVICES AND THEIR RETENTION

Weir (1990) found that the splint design (Figure 3.1, P. 48) was successful during extrusion. The splint allowed extrusion of the experimental tooth without movement of the control incisor and minimised interference with the animals' masticatory ability during the experiment because the splint did not extend to the posterior maxillary teeth. However, Weir (1990) reported that six of the ten animals dislodged the devices after extrusion was completed because a retention period of 30 weeks was relatively long and all of the marmosets habitually chewed their wooden perches. These were broomstick handles placed two to a cage. Therefore, he replaced six lost devices during retention with Concise bonded composite resin splints (Figure 3.2, P. 48).

This method of retention proved quite resistant to dislodgment, although the self-curing resin retaining splints were constructed intraorally, under less than ideal working conditions. Prefabricated resin splints may be a better choice of design to improve retention and oral hygiene in experimentation requiring long term wear. However, these relatively large plastic splints could prevent functional stimulation on the PDL.

5.6 FIXATION PROCEDURES FOR THE T.E.M.

Since osmium tetroxide (OsO_4) was introduced as a fixative for electron microscopy, attempts have been made to improve final image quality through manipulation of fixation times, temperature, pH, osmolarity, specific ionic composition, and mode of application of the fixative solution. When OsO_4 is used alone, however, it appears to be an imperfect protein fixative. (Trump and Bulger, 1966)

Glutaraldehyde fixative renders many protein structures stable to OsO_4 post-fixation, with preservation of continuity of the delicate intracellular membranes. Glutaraldehyde used as a primary fixative, followed by secondary OsO_4 fixation, has undesirable side effects including uneven fixation of tissue blocks, cell shrinkage, mottled extraction or swelling of mitochondria, and marked clumping of nuclear chromatin (Sabatini et al., 1963 ; Trump and Bulger, 1966).

Trump and Bulger (1966) were the first to demonstrate a mixture of OsO_4 and glutaraldehyde as a fixative for electron microscopy. Combination of these two fixatives has proved helpful in minimising artifacts seen with either fixative alone and in the identification of fine cell structure.

Franke et al. (1969) reported that this combined fixation method was superior with respect to :

1. Contrast distinctiveness of lipid containing structures including membranes;

2. Preservation of polyribosomes, chromatin strands and other nucleoprotein containing structures;
3. Preservation of cytoplasmic microtubules;
4. High staining of polysaccharide-containing structures, and
5. The elimination of precipitation contaminants frequently caused by fixation were also noted to be eliminated.

The combined OsO_4/glutaraldehyde fixation method was reported by Tzeng et al. (1981) to enhance preservation of tonofilaments in rat epidermis. The presence of tonofilaments is an identifying feature of periodontal epithelial cells (Lester, 1969), so adequate fixation preservation is necessary.

Fixative osmolarity is important when glutaraldehyde fixatives are used alone, as retention of some osmotic activity following glutaraldehyde fixation has been reported (Jard et al., 1966; Bone and Denton, 1971; Wangensteen et al., 1981). Primary fixation with OsO_4, however, has been reported by Tormey (1964), Glauert (1978) and Wangersteen et al., 1981 to cause cell membranes to become freely permeable and lose their osmotic properties. Bone and Ryan (1972) concluded that osmolarity of the fixative vehicle is very important when glutaraldehyde is the fixing agent, but is of less importance when OsO_4 alone, or combined with glutaraldehyde, is used as the fixing agent.

The inclusion of dextran in the fixative has two major effects :

1. It acts as a plasma expander, preventing artificial enlargement of extravascular tissue spaces by helping to balance hydrostatic pressure between blood vessels and extravascular tissues (Casley-Smith, 1987);
2 It improves preservation of the myelin sheath when used with glutaraldehyde fixatives (Hayat, 1981).

Millonig (1962) determined the normal pH of animal tissues to be 7.4. The pH of the fixative solution in the present study was adjusted accordingly, using 1N HCL (Weir 1990).

Palade (1952) first introduced the practice of buffering the fixative to control pH, providing more uniform tissue preservation. Wood and Luft (1965) noted that arsenate buffers such as the 0.06M sodium cacodylate buffer used in the present study have the advantage of producing improved tissue stainability following fixation. Other advantages of sodium cacodylate buffers are reported as :

1. Greater resistance to bacterial contamination (Hayat, 1981), and
2. Improvement of stability and fixative effects of glutaraldehyde with OsO_4 (Takahashi, 1980).

5.7 PERFUSION FIXATION

OsO_4 will rapidly and uniformly preserve tissue to a depth of 0.25m, whilst glutaraldehyde will preserve tissue to a depth of 0.5mm (Glauert, 1978 ; Hayat, 1981). Immersion fixation with these substances is, therefore, inadequate for PDL tissues.

Perfusion fixation is excellently suited for tissue preservation for electron microscopy (Veerman et al., 1974). Bernick (1962) was the first to use this technique to preserve periodontal tissue.

Gil and Weibel (1969) found that, while steady perfusion rates were associated with substantial tissue edema in lung alveoli, pulsatile (peristaltic) perfusion pressures were not associated with this problem. There is, however, no convincing evidence of the need for pulsatile perfusion of other tissues. Gannon (1978) suggested that the problem of interstitial edema may be overcome by the use of plasma expanders such as dextran (used in the present study), or polyvinyl pyrollidone (PVP). This view is supported by the results obtained by using steady perfusion rates by Crowe (1989) and Weir (1990).

Washout pressure is an important variable (Lew, 1986). Insufficient pressure may result in incomplete blood clearance, while excessive pressure may cause capillary rupture. A non-pulsatile pressure of 80-100mm Hg as

used with good results by Mathieu-Costello (1987) to perfuse rats for TEM and light microscopic examination of striated muscles.

Charnock et al. (1987) reported the normal systolic blood pressure of the marmoset to approximate 100mm Hg. Weir (1990), however, followed the method of Crowe (1989) in using washout and perfusion pressures of 160mm Hg. Using the remaining segments of Weir's (1990) specimens, there was no evidence of incomplete blood clearance or capillary rupture found in the present study.

In a study of vascular reactions to perfusion fixation, Thorball and Tranum-Jensen (1983) concluded that reliable fixation of vascular beds can be obtained by providing the strictly controlled hydrodynamic parameters. They further noted that, in optimally perfused preparations, 5-10 minutes are required to fully saturate the glutaraldehyde binding capacity of the tissue. All of the animals used in this study were perfused for a minimum of 10 minutes (Weir, 1990). Excellent perfusion of periodontal tissues using a similar technique was demonstrated by Beertsen and Everts (1977) and Crowe (1989).

5.8 DIMENSIONAL CHANGES DURING PROCESSING

According to Weibel and Knight (1964), the influence of fixing and dehydrating and embedding agents may introduce some dimensional changes in the progression from the fresh to the processed specimen.

In a study on the effect of different demineralising agents on oral mucous membrane, Fejerskov (1971) determined that, of the agents studied, EDTA allowed the best preservation of cytological detail. Further, demineralisation at 4°C was found to reduce the amount of epithelial cell desiccation. In accordance with these findings, EDTA demineralisation at 4°C was used in the present study.

Bahr et al., (1957), Kushida (1962) and Weibel and Knight (1964) all reported cell shrinkage during tissue dehydration. Weibel and Knight (1964),

while demonstrating cellular shrinkage following dehydration in alcohol schedules starting with 50% ethanol, found that dehydration started with 70% ethanol minimised dimensional changes.

Kushida (1962) found that cellular shrinkage of 0.6% occurred during polymerisation of the embedding medium Epon 812. The embedding medium used in the present study, Agar 100, belongs to the same family as Epon 812 and, therefore, similar results can be expected from it. Weibel and Knight (1964) also attributed little dimensional change to embedding in Epon 812. Williams (1977) noted that tissues for ultramicroscopic study should be embedded in epoxy or polyester resins, because of the minimal dimensional changes during polymerisation and the stability of ultrathin sections in the electron beam.

The use of heavy metal block stains with uranyl acetate, followed by lead stains, was recommended by Williams (1977) to ensure the best obtainable contrast, since low contrast can result in serious underestimation of some tissue components. The staining regime used in the present study followed these recommendations, with sections stained in modified Reynolds' lead and uranyl acetate (Weir, 1990). This tissue staining does not affect tissue dimensions (Crowe, 1989).

As noted by Crowe (1989) and Weir (1990), reports of dimensional changes in the PDL using the fixation and tissue processing regimes used in the present study are not available. Direct extrapolation from other studies is invalid as dimensional changes during fixation and tissue preparation are tissue and methodology dependent (Bahr et al., 1957; Kushida, 1962). Consideration of the results by Kushida (1962) and Weibel and Knight (1964) leads to the conclusion that changes in the present study were likely to be less than 1% linearly and 2% volumetrically. Tissue changes during TEM processing are minimal and can therefore be neglected in assessing their impact on overall dimensional change (Hayat, 1981).

5.9 GRID SELECTION AND TISSUE STAINING

The bars of the slotted grids interfered less with the viewing of a combined area of tooth, PDL 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 should be assessed to overcome this problem.

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

5.10 T.E.M. 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.

Gundersen (1989) considered the lack of a need for vertical sectioning for stereological analysis of tubular structures e.g. blood vessels and nerves. This analysis was based on the branching and irregularity in distribution of blood vessels and nerves, 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 a section is inversely proportional to the section thickness. The compression and distortion effects in the present study were kept to a minimum by cutting sections in the silver-gold interference range (60-80nm) using a new diamond knife. Additionally, sections were expanded with chloroform vapour before collection.

Morphometry provides results which are true for the particular section studied, but not necessarily true for the tissue as a whole. However, Weir (1990) due to time constraints imposed by having to undertake the animal experiments as well as the TEM was only able to quantify the apical region. In the present study sections were taken down the whole length of the PDL.

b. Point counting

A TEM micrograph magnification of 3,000x was adequate for the recognition of cell and blood vessel types, but not suitable for the identification

of small segments of vessels at the periphery of the negative. Therefore, the lower magnification of 500x of the total PDL were used to identify these vessel segments when point counting was performed. All micrographs for point counting were printed at a final magnification of 8,500x. The magnification was standardised using a replicating graticule.

Weibel (1979) concluded that systematic point counting was the most accurate procedure in comparison to a number of other methods available for measuring volumetric proportion. This method is also good for estimating areal fractions (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 placed 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 a 64 point grid 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 this disadvantage can be overcome by using a large enough number of micrographs. In the present investigation, a 140 point grid was used to fulfill the requirements for point counting both vessels and nerves.

The convention of forbidden lines (Figure 3.6, p. 60) 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). He suggested the use of more appropriate system of ascribing the value of 1/4 for each side of the micrograph print that intersected with a blood vessel.

5.11 BLOOD VESSELS

The present study revealed that the vascular luminal volume was 10.79% and 12.60% of the marmoset maxillary incisor PDL volume in the control and experimental groups, respectively. Crowe (1989) found that 11% of the marmoset apical PDL volume was occupied by the luminal volume of the vascular component. Weir (1990) investigated the subapical vasculature of the material used in the present study and reported a vascular luminal volume of 10.76%. Parlange and Sims (1993) found the non-apical marmoset maxillary incisor luminal volume to be 11.26%. Therefore, this study confirms that the luminal volume of the marmoset maxillary incisor PDL ranges from 10% to 12%.

Gould et al. (1977) noted that in the mouse molar 73.4% of vessel volume was located in the bone half of the PDL while McCulloch and Melcher (1983) concluded that the bone half was four times more vascular than the tooth half. However, both studies reflect limited sampling from a single animal. Freezer and Sims (1987) reported that in the mouse molar PDL the vessels occupied 0.2%, 6.2%, and 15.8% of the tooth, middle, and bone circumferential third volumes. The present study found that in the marmoset incisor the MVB held 0.1%, 23.5%, and 8.89% of the tooth, middle, and bone circumferential third PDL volumes. In the mouse, the Freezer and Sims findings (1987) were consistent with those of Douvartzidis (1984) for marmoset maxillary molars, but differed from the present study and the Parlange and Sims investigation (1993), where marmoset maxillary incisor vascular volume was greatest in the middle third of the PDL. These different results were possibly indicative of tooth and functional differences.

When wall thickness is taken into account, it adds significantly to the vascular volume percentage of the PDL. For example, the control PDL luminal volume of 10.79% compared with an abluminal volume of 13.37%. Previous studies (Freezer and Sims, 1987; Sims, 1987) have not estimated

vessel wall volume which comprised 19.30% of the marmoset total incisor MVB volume. The marmoset incisor PDL had a predominantly postcapillary-sized vascular bed comprising 86.8% percent of the total luminal vascular pool, which is comparable with the Freezer and Sims (1987) result of 88% in mouse, and the Parlange and Sims (1993) outcome of 88.2% in marmoset. These conclusions confirmed that the MVB in the PDL is a predominantly venous vascular bed in nature.

No statistically significant differences were found in the total luminal and abluminal volumes, and diameters between the control and experimental groups. In different blood vessel types, no statistically significant differences were present in luminal and abluminal volumes. Also, there were no differences in luminal and abluminal volumes across circumferential thirds of the PDL, meaning that the distribution of microvascular volume across circumferential thirds in both groups was comparable. All these findings suggested that re-establishment of the total PDL vascular volume, and vessel diameter had occurred after extrusion and a retention period of 30 weeks.

If each blood type was analysed, only postcapillary-sized venules, the major contributor to the total vessel volume, length, and surface, had a statistically significant reduction in wall thickness after extrusion, therefore causing a significant decrease in total mean vessel wall thickness. Nevertheless, a significant decrease in wall thickness of these vessels did not statistically induce consequential changes in total luminal and abluminal volumes between the control and experimental teeth. This outcome could be explained in that while the physiological re-establishment of vascular system had occurred, the morphological reconstitution of postcapillary-sized venules was still incomplete or they had undergone a permanent change. This conclusion supports the views of Lew et al. (1989) and Clark et al. (1991) that postcapillary-sized venules appear to be the most sensitive vascular

component. Overall, the present investigation indicates that the MVB revascularisation was essentially complete at the end of the retention period.

Parlange and Sims (1993) found that complete reconstitution of the neural system occurred in the marmoset maxillary incisor PDL after orthodontic extrusion, and a retention period of 9 weeks, which approximates 1 year in human terms. However, they reported incomplete re-establishment of luminal volume and diameter of postcapillary-sized venules. On the contrary, the present study showed complete reconstitution of luminal volumes of all blood vessel types after a retention period of 30 weeks, which approximates 3 years in human terms. However, there was still a significant reduction in wall thickness of postcapillary-sized venules.

In the present study, no statistical analyses were performed for arterial capillaries and terminal arterioles due to insufficient observations. However, on the basis of this and earlier studies (Freezer and Sims, 1987; Tang and Sims, 1992; Parlange and Sims, 1993), it is again demonstrated that the PDL microvascular bed is predominantly venous in nature.

The present study showed that, for postcapillary-sized venules, there was a significant interaction between region and treatment. That is, the mean luminal volume of these vessels in the bone third of experimental teeth was three times greater than that of control teeth. Sims (1983) reported that the highest incidence of the fenestrae in all vessels was found in postcapillary-sized venules in the mouse PDL. The fenestrae were described as 30-50 nm openings in the endothelium (Corpron et al., 1976; Lew et al., 1989; and Clark et al., 1991). Casley-Smith et al. (1975) suggested that the fenestrae may be of greater importance in the establishment of a local extracellular capillary circulation of macromolecules. Therefore, the significant increase of three times in the luminal volume of postcapillary-sized venules in the bone third of the experimental PDL may be related to the extracellular exchange of metabolites, and bone remodeling during tooth movement.

5.12 NERVES

In the present study the principal location of unmyelinated axons occurred within the parenchyma. This distribution contrasts with the observations of Freezer and Sims (1989) who reported that the majority of mouse molar unmyelinated axon endings occurred in close apposition to the abluminal endothelium. Nevertheless, the marmoset incisors did demonstrate unmyelinated axons forming a close relationship to postcapillary-sized venules, terminal arterioles, and collecting venules.

Freezer and Sims (1989) reported a total nerve volume of 0.5%, but did not include the subapical PDL area. Parlange and Sims (1993) found a total nerve volume of 0.43% and 0.56% in the control and experimental marmoset non-apical PDL, respectively. The present study revealed that the axon volumes (within 1.0μm of blood vessels) in the experimental and control groups were 0.01% and 0.02%, respectively. No significant difference in axon volume was found between the control and experimental PDL. The axon volume needs to be reassessed with a coefficient of variation at the 5% level.

The present study has orthodontic implications in that the retention period should be determined by reconstitution of the ultrastructure of the PDL including cells and collagen turnover (Reitan, 1967), blood vessels and nerves (Crowe, 1989; Weir, 1990; Parlange and Sims, 1993), and tissue channels (Tang and Sims, 1992). This investigation was focussed on re-establishment of the MVB and neural systems. Ultimately both components need to be reconstituted if any physiological impairment results from orthodontic tooth movement. Thus, a retention period of between 1 and 3 years is suggested on the basis of this animal model in order to maximise orthodontic reconstitution of both blood vessels and nerves to minimise relapse.

CHAPTER 6
CONCLUSIONS

1. From a laboratory point of view, the marmoset proved to be an adequate experimental model for the analysis of the PDL above the subapical region because of its similar histological and ultrastructural features similar to the human PDL.

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

3. The present investigation is the first TEM stereological study to quantify the post-treatment effects on the marmoset PDL blood vessels and nerves *above the subapical region*, following endodontic therapy, orthodontic extrusion and *a relatively long-term primate retention period of 30 weeks*.

4. The total mesial luminal vascular volume in the marmoset maxillary incisor PDL was 10.79% ± 3.1%. This finding agrees with the studies of Crowe (1989), Weir (1990), and Parlange and Sims (1993). It is concluded that the mean luminal volume in the marmoset maxillary incisor PDL ranges from 10-12%.

5. No significant differences were found in luminal volume, mean luminal diameter, and nerve volume between the control and experimental PDL. These findings suggested that essentially complete reconstitution of blood vessels and nerves occurred after an animal retention of 30 weeks, which approximates 3 years in human terms.

6. The present study has clinical implications in that determination of the retention period is orthodontically important. Recommended orthodontic retention periods in the literature are rarely based on biological data (Shapiro and Kokich, 1981). As far as complete re-establishment of the PDL blood vessels and nerves is concerned, a retention period of between 1 and 3 years in human terms is suggested on the basis of this animal model in order to maximise orthodontic reconstitution of both systems, and minimise relapse.

7. Although essentially complete reconstitution of luminal microvascular and neural systems had occurred after 30 weeks, a significant decrease in wall thickness of postcapillary-sized venules was still present. This finding indicated that morphological re-establishment of postcapillary-sized venules was incomplete or represented a permanent or longer term change.

CHAPTER 7
APPENDICES

7.1 DECALCIFYING SOLUTION

SOLUTION : 0.1M EDTA in 2.5% glutaraldehyde

PREPARATION : 74.45g EDTA (Ethylenediaminetetraacetic 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.

7.2 CACODYLATE BUFFER (Sodium cacodylate; BDH Chemicals Ltd., Poole, U.K.)

SOLUTION : 0.06M sodium cacodylate.

PREPARATION : 25.68g sodium cacodylate in 2,000ml d.d water. Adjust to pH 7.4 using 1N HCL at 20°C.

SHELF LIFE : 7 days at 4°C.

7.3 4% OSMIUM TETROXIDE (OsO_4) SOLUTION

SOLUTION : 4% Osmium Tetroxide.

PREPARATION : Place 2g of osmium tetroxide in 50ml 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.

7.4 BLOCK STAIN

SOLUTION : 1% uranyl nitrate in 70% alcohol.

PREPARATION : 1g uranyl nitrate.
70ml ethyl alcohol.
30ml d.d. water.

SHELF LIFE : 7 days at room temperature.

7.5 LIGHT MICROSCOPE STAINS

a) 0.05% TOLUIDINE BLUE

SOLUTION : 0.05% Toluidine Blue in d.d water.

PREPARATION : 0.05g Toluidine Blue.
100ml 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 : 1g of Sodium Thiosulphate (Borax).

100ml of d.d water.

Dissolve by stirring.

SHELF LIFE : 6 months at room temperature.

PRECAUTION : Avoid skin contact.

7.6 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.

SHELF LIFE : 3 months at room temperature.

b) REYNOLDS' LEAD

SOLUTION : Modified Reynolds' lead.

PREPARATION : (i) 1.33g sodium citrate.

1.76g sodium citrate.

30ml d.d water.

(ii) 8ml 1N sodium hydroxide

Vigorously shake (i) and allow to stand for 30 minutes : add d.d water, mixing by inversion.

SHELF LIFE : 30 days at 4°C. Discard if pH drops below 11.

7.7 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 = 10.00ml
NMA = 8.59 ml
18.59ml

Total A + B = 30.01ml

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 = 90.04ml

Therefore : BDMA = 0.14/10ml = 1.26ml

PRECAUTIONS : Agar 100 is a suspected carcinogen and should therefore 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.

7.8 RADIOGRAPHIC EQUIPMENT

Kodak periapical ultraspeed film : 22 x 35 mm, Siemens Heliodent machine.

VOLTAGE : 50kv.

CURRENT : 7mA

EXPOSURE TIME : 0.1sec

7.9 TRANSMISSION ELECTRON MICROSCOPE

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

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

7.10 STEREOLOGICAL EQUATIONS

$N_A = N/560$

Adapted from Atherton, Cabric and James, 1982

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

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

$S_V = \bar{d} \P L_V$ Gundersen, 1989

Symbols used in stereological equations

V_V	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).
N_A	Number of profiles per unit area.
$\bar{d}$	Mean caliper diameter of a component.
V_V	Volume density.
S_V	Surface density.
L_V	Length density.
	- Compartment of interest (i)
	- Reference compartment (ref)

7.11 RECORDING SHEET

RECORDING SHEET

MICROGRAPH NUMBER	
ANIMAL NUMBER	
SIDE NUMBER (right=1, left=2)	
DEPTH (microns)	
REGION (tooth=1, middle=2, bone=3)	

PROFILE			COARSE GRID	FINE GRID	NUMBER (N)	INTERSECTS (I)	TRANSECTS (Q)	JUNCTIONS	BV INTERSECTS Lumenal	BV INTERSECTS Ablumen
TOTAL										
FIBROBLASTS										
FIB. NUCLEI										
COLLAGEN										
VESSEL LUMEN	(1)	Pericytic Capils								
	(2)	NON pericytic Capils								
	(3)	Pericytic PCVs								
	(4)	NON pericytic PCVs								
	(5)	Arterial Capillaries								
	(6)	Collecting Venules								
	(7)	A-V Anastomoses								
UNMYELINATED NERVES	(1)	in Collagen								
	(2)	in BV walls								
MYELINATED NERVES	(1)	in Collagen								
	(2)	in BV Walls								
ENDOTHELIAL CELLS										
PERICYTES										
VEIL CELLS										
SCHWANN CELLS										
MAST CELLS										
K-CELLS										
EPITHELIAL CELLS										
MESENCHYMAL CELLS										
OSTEOPROGENITOR CELLS										
OSTEOBLASTS										
OSTEOCLASTS										
CEMENTOBLASTS										
MACROPHAGES										
OTHER CELLS (incl. unknowns)										
OXYTALAN FIBRES	(1)	adj. to CELLS								
	(2)	within COLLAGEN								
	(3)	in BV WALLS								
	(4)	adj. to NERVES								
	(5)	CELL enclosed								
CEMENTICLES										
CONNECTIVE TISSUE SPACES										
GROUND SUBSTANCE										

CHAPTER 8
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