THE EFFECTS OF ORTHODONTIC FORCE APPLICATION UPON THE TISSUES OF THE HUMAN DENTAL PULP

By

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Abstract

Aims: To investigate the effects of orthodontic force application on pulpal tissues.

Materials and methods: Sixteen patients were scheduled for bilateral upper premolar removal followed by fixed appliance treatment. Each had a sectional fixed appliance placed in order to extrude one premolar only for a period of 14 days. The contralateral premolar was used as a control. After 14 days both premolars were extracted and processed for MMA or paraffin embedding, and sectioning. The pulpal morphology of both control and test tissues were examined under a light microscope, and fibroblast numbers, together with blood vessel number and area, analysed on serial sections. Immunohistochemical staining for Von Willebrand's factor and CD31 was undertaken to assess angiogenesis.

Results: No statistically significant difference was found in fibroblast numbers, blood vessel number or angiogenic activity and no morphological differences were observed between the control and test tissues after the 14-day period. However the overall blood vessel area was greater ($p<0.05$) in the test tissues.

Conclusions: A 2-week application of 50g extrusive force leads to significant blood vessel dilation, but does not give rise to any significant changes in overall pulpal morphology, fibroblast number, blood vessel number, or angiogenesis.

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1.0 Introduction

Fixed orthodontic appliances have been in use since the 19th century and it is known that application of either tensile or compressive forces to teeth elicits molecular changes in the cells of the periodontal ligament, alveolar bone and the pulpo-dentine complex; the way in which each compartment responds being linked to the response elicited in the other compartments (Vandevska-Radunovic 1999).

1.1 Theories of tooth movement

There exist two, classical theories of orthodontic tooth movement:

i) The *bone bending/bioelectric theory*- without bone remodelling the bodily movement of teeth cannot be effected by orthodontic appliances. Bone is a crystalline structure, and as such, when sufficient mechanical force is applied to cause deformation, electron displacement occurs. This generates a piezo-electric current- the concave side becomes electronegatively charged, favouring osteoblastic activity, whilst the convex side becomes electropositively charged, favouring osteoclastic activity; and so remodelling occurs (Grimm 1972).

ii) The *pressure-tension hypothesis* (Oppenheim 1911; Schwarz 1932)- areas of tension and compression induced in the ligament through force application cause dilatation and compression (respectively) of blood vessels, and thus alterations in blood flow. This results in a change in oxygen tension, leading to release of chemical messengers such as cytokines and prostaglandins, which alter cellular behaviour in both the

ligament and surrounding bone, giving rise to periodontal remodelling (Proffit et al. 2007).

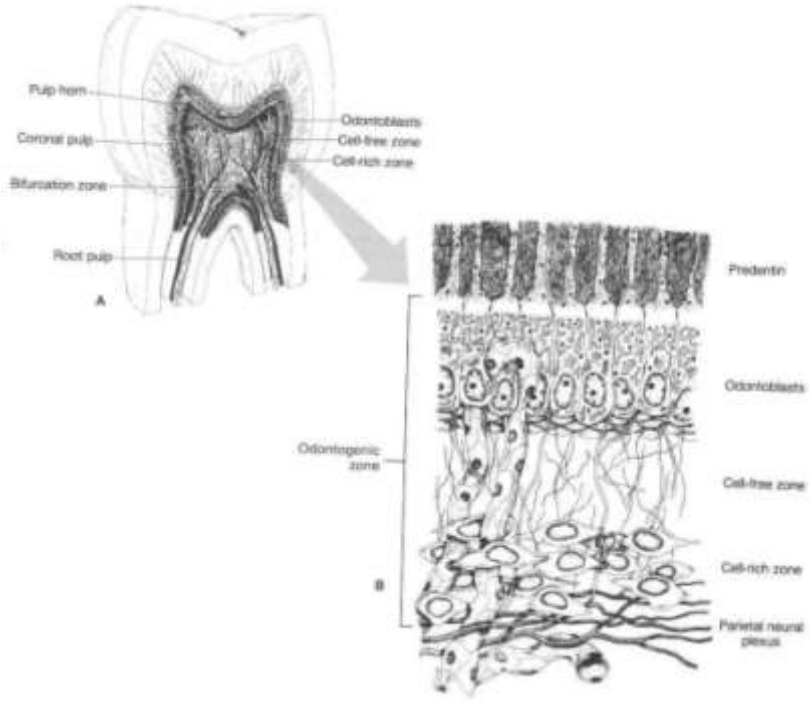
Despite the wealth of literature present, the detailed cellular and molecular mechanisms involved in periodontal ligament/alveolar bone remodelling have yet to be elucidated, with a recognised need to correlate more *in vivo* and *in vitro* data (Meikle 2006). In addition very few studies have examined the effects of tension and compression on the pulp at the molecular level, despite the potential for force transduction to this tissue.

1.2 Tooth Tissue Anatomy

Figures 1.1 and 1.2 (page 3) illustrate the anatomy of both hard and soft tissues of the human tooth. The dental pulp is a complex organ, which interacts closely with other compartments both within and outside of the tooth as a whole (Vandevska-Radunovic 1999). It can be seen that the most intimately integrated of these compartments with the pulp is the dentine layer, with the cellular processes of the layer of odontoblasts in the pulp passing into the dentinal tubules. This often results in the term ‘dentine-pulp complex’ being used (Alberts et al. 2002).

Figure1.1

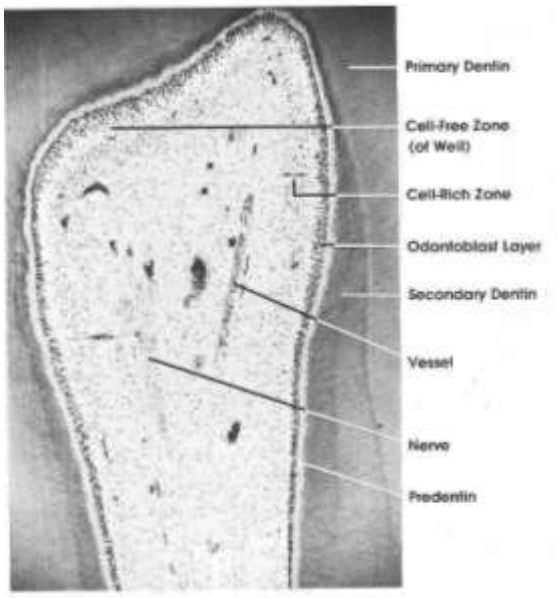
A: Diagram of pulp organ
B: Odontogenic zone of the pulp



From Avery et al. (1992)

Figure 1.2

Photomicrograph of the dentine-pulp complex



From Ten Cate (1998)

1.3 Pulpal Changes During Application of Orthodontic Forces

1.3.1 Morphological changes

A wide variety of morphological changes occurring in response to orthodontic force application have been documented throughout the literature, affecting both the inner and outer layers of the pulpal tissue. The majority of these are deleterious in nature, with vacuolization and degeneration of the odontoblast layer being a commonly observed occurrence (Stenvik and Mjör 1970; Mostafa et al. 1991; Shigehara et al. 2006; Ramazanzadeh et al. 2009); this is most pronounced coronally (Villa et al. 2005). This degeneration may, however, be compensated for by osteopontin release which stimulates differentiation of new odontoblasts (Shigehara et al. 2006), thereby helping to repair any tissue damage. These deleterious changes appear to be more marked in older patients, possibly due to a reduction in the size of the apical foramen, with concurrent constriction of apical vessels. Younger patients (the most common orthodontic patient cohort) may, therefore, be at less risk (Hamilton and Gutmann, 1999).

A change in the width of the pre-dentine layer has also been documented as a response to orthodontic force application, however this shows considerable inter-species variation. In rats an increase in the width of the pre-dentine has been observed (Nixon et al.1993;), with a reduction in width in dogs (Konno et al. 2007).Whether the pre-dentine width reduces (Stenvik and Mjör 1970),

increases (Villa et al. 2005) or remains unchanged (Kayhan et al. 2000) in the human model, however, remains unclear.

Within the core of the pulpal tissue blood vessel congestion (Mostafa et al. 1991) and haemorrhage have been observed in human teeth subject to heavy (150gm), intrusive forces (Raiden et al. 1998), together with an increase in collagen fibre number and width (Villa et al. 2005), giving rise to some fibrosis (Raiden et al. 1998; Mostafa et al. 1991). The extent of fibrosis appears to be proportional to length of time for which force is applied (Kayhan et al. 2000; Ramazanzadeh et al. 2009). Very rarely, total obliteration of the pulp space may be observed (Delivanis and Sauer 1982; Woloshyn et al 1994), with concurrent loss of vitality; this is, however, most likely to occur in teeth that have experienced previous trauma (Bauss et al. 2008).
Neuronal changes have also been documented in the rat model, with nerve-sprouting observed as a consequence of orthodontic force application (Vandevska-Radunovic et al. 1997). However, in the human, there would appear to be minimal effects (both in the long and short-term) on the nerve fibres, as demonstrated, morphologically, by Scanning Electron Microscopy (SEM) (Bunner and Johnsen 1982) and, clinically, by a continued normal response to thermal stimulation (Hall and Freer 1998).

However the afore-mentioned changes are unlikely to be sustained in the long-term: one study showed that approximately 5 years post-retention no overall morphologic differences were observed in teeth subjected to orthodontic treatment, as judged radiographically (Popp et al. 1992) . This was

corroborated by the findings of Melsen, who demonstrated that in monkeys treated with fixed appliances, over a total period of 20 weeks of active movement, there were no significant morphological pulpal changes (Melsen 1986).

1.3.2 Angiogenic changes

It has been proposed that, in order to avoid undermining bone resorption, the ideal force for tooth movement be in the order of to 35-60g for tipping movements and 70-120g for bodily movement (Proffit et al. 2007). The effects of these forces upon the pulpal blood supply are contentious, but several studies have found that under these 'ideal' conditions, orthodontic forces bring about an initial reduction in vascular flow to the pulp (McDonald and Pitt-Ford 1994; Vandevska-Radunovic et al. 1994; Brodin et al. 1996; Sano et al. 2002) that lasts for approximately 30 minutes (McDonald and Pitt-Ford 1994), with a corresponding reduction in pulpal respiration rates (Hamersky et al 1980;Unsterseher et al. 1987). This can lead to a drop in the oxygen tension and so cellular injury, with an increase in apoptosis, (Rana et al. 2001; Perinetti et al. 2004; Shigehara et al. 2006) as determined by the release of markers such as Aspartate Aminotransferase (AST) (Veberiene et al. 2009), impaired Extra-cellular Matrix (ECM) remodelling, as determined by a reduction in matrix-metalloproteinases (MMPs)(Leone et al. 2009) and inflammatory changes (Perinetti et al. 2004). These changes give rise to marked Vascular Endothelial Growth Factor (VEGF), Platelet Derived Growth Factor (PDGF), Fibroblast Growth Factor 2 (FGF2), Epidermal Growth Factor (EGF), basic Fibroblast Growth Factor (bFGF), Transforming Growth Factor

Beta (TGFβ) and Tumour Necrosis Factor Alpha (TNFα) release, initiating endothelial cell proliferation (Grünheid et al. 2007) and thus angiogenesis (Derringer and Linden 2004; Perinetti et al. 2005; Derringer and Linden 2007). The density, number and area of blood vessels then subsequently increases (Nixon et al. 1993; Wong et al. 1999; Santamaria et al. 2006) over the next 24-48hrs (McDonald and Pitt-Ford 1994) reaching a peak, in the rat model, at around seven days (Vandevska-Radunovic et al. 1994) . However, under certain conditions microvessel formation has been observed after only 5 days of force application (Derringer et al. 1996; Derringer and Linden 2003).

These vascular changes appear to be mediated by the release of neuropeptides (Parris et al. 1989; Kimberly and Byers 1988; Kvinnsland, and Kvinnsland 1990), such as substance P and Calcitonin Gene-related Peptide (CGRP) (Norevall et al. 1995), with increasing amounts of CGRP seen with increasing force levels (Caviedes-Bucheli et al. 2011). Indeed these neuropeptides have been shown to regulate angiogenic growth factor expression by pulp fibroblasts *in vitro* (El karim et al. 2009). CGRP release also accompanies an increase in nerve fibre number and density which, comparative to the vascular changes, reaches a peak at around 7 days in the rat model, before normalising once more (Vandevska-Radunovic et al.1997).

Clinically 72 hours after orthodontic force application has commenced normalisation of the blood supply occurs (McDonald and Pitt-Ford,1994). Normalisation has also been observed in the rat model between days 14 and 28 of force application (Shigehara et al. 2006), corroborating the fact that the

pulp acclimatises to force application. However if excessive force is applied the blood flow may not recover, and the resulting ischaemia will lead to tissue necrosis (McDonald and Pitt-Ford,1994).

Not all studies concur with the above findings however: Barwick found that 4 minutes of intrusive force had no significant effect upon pulpal blood flow (Barwick, and Ramsay 1996), and whilst others have found that blood vessel dilatation and increase in area occur following force application, two clinical trials utilising Rapid Maxillary Expansion (RME) found no significant changes in the overall number of blood vessels (Kayhan et al. 2000; (Taşpinar et al. 2003). In the rat model early increase (rather than reduction) in blood flow (Kvinnsland 1989) has been observed, with hyperaemia (Nixon et al. 1993) and an increase in respiration rates (Labart et al. 1980).

The variation in findings documented within the literature may be, in part, due to the type of movement exerted, with some evidence that intrusive effects may be more dramatic than extrusive (Brodin et al. 1996; Ramazanzadeh et al. 2009; Li et al. 2010). The intensity of this vascular response and change in flow within the pulpal tissues is also proportionally dependent on the magnitude of the force (Nixon et al. 1993; McDonald and Pitt-Ford 1994; Sano et al. 2002) and the length of time for which it is applied (Ng et al. 1981). Speed of tooth movement is also important (Seltzer 2002) and so observed vascular changes will be affected greatly by appliance design and usage. Stenvik found only minor circulatory changes in teeth extruded orthodontically if the teeth were allowed to erupt but remained in occlusion.

Changes were more significant if the teeth erupted freely (Stenvik 1971); thus occlusal forces may also play a role in regulating vascular changes.

1.3.3 Inflammatory changes

As with the documented changes in vascularity, there is discord within the literature as to whether or not orthodontic force application brings about inflammatory pulpal changes. Studies vary from showing no significant inflammatory changes (Sübay et al. 2001) to some evidence of macrophage infiltration (Grünheid et al. 2007), through to possible pulpal injury associated with rapid tooth movement (Seltzer 2002).

Any inflammatory reaction is likely, in part, to be mediated by fibroblasts. *In vitro*, substance P, which is known to be present in the human dental pulp, (Goodis and Saeki 1997) has been shown to stimulate the production of Prostaglandin E2 (PGE2) and Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL) by cultured dental pulp fibroblasts (Kojima et al. 2006). These then have been shown to produce large amounts of Interleukins (IL): IL-1β, IL-6, and TNF-α (Yamaguchi et al. 2004) when under cyclic strain (Lee et al. 2008). Orthodontic force application does bring about strain within the pulp tissue (Dhopatkar 2006), together with upregulation of fibroblast growth factors (Grünheid et al. 2007), and so it could, in theory, give rise to cytokine production by fibroblasts.

1.3.4 Cellular changes

As a result of vascular occlusion, alterations in cell metabolism take place, followed by tissue degeneration and remodelling. The most significant changes occur in the mid and apical regions of the pulp and increase in a time dependent manner (Grünheid et al. 2007).

The odontoblast layer forms the outermost layer of the pulpal organ (fig. 1.2, page 3), and plays an important role in the functional regulation of the pulp. If the pulp of a tooth is exposed, resulting in injury to the odontoblast layer, pulpal cell proliferation may be seen, along with pulpal stromal/mesenchymal differentiation into odontoblast-like cells, thereby allowing formation of new dentine matrix (Fitzgerald and Fitzgerald 1979; Fitzgerald et al. 1990). This is a process known as reparative dentinogenesis, where, in response to noxious stimuli, newly differentiated odontoblast-like cells lay down tertiary dentine matrix in the area local to the site of injury at an accelerated rate (Lesot et al. 1994; Smith et al. 1995). If the stimulus is relatively mild (e.g. if a tooth is worn down gradually) pre-existing odontoblasts may lay down new matrix- this is known as reactionary dentinogenesis (Lesot et al. 1994; Smith et al. 1995). The compositions of the tertiary dentine formed vary between the two processes, suggesting that the activity of the odontoblast/odontoblast-like cells is modulated accordingly (Smith et al. 1995).

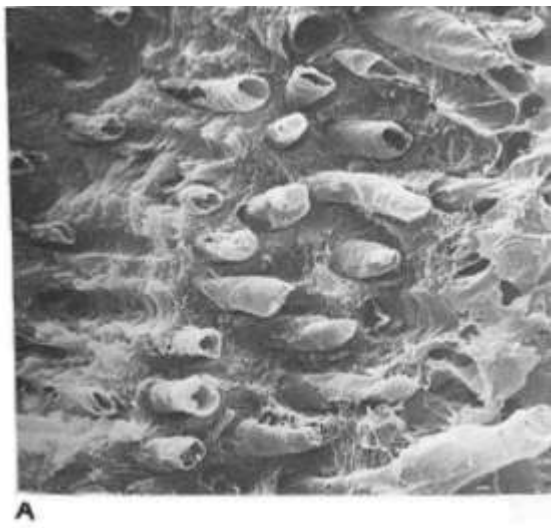
It has been shown that odontoblasts will transmit external stimuli to the pulpal cell layer beneath them, indeed they are ideally situated to enable them to act as receptors to stimuli external to the tooth, due to the localisation of their

processes within the dentinal tubules (Ikeda and Suda 2006) (fig. 1.3, page 13). It may be seen that the dentinal processes extend out from the odontoblast cell body, into the dentinal layer (fig. 1.3, page 13). This allows exogenous stimuli to be detected by the odontoblasts, which may then signal to underlying pulpal cells. In 2003, an *in vitro* experimental model for studying cellular and molecular effects brought about by compressive and tensile forces was developed (Dhopatkar 2006). Using finite element analysis of an organ culture model, evidence indicated that forces transmitted externally produce cellular effects at the pulpal core, with upregulation of genes associated with cellular proliferation such as Proliferating Cell Nuclear Antigen (PCNA) (Dhopatkar et al. 2005; Dhopatkar 2006). These effects were found to be the same, regardless of whether compressive or tensile forces were applied externally, as external tension resulted in internal compression, due to Poisson's Effect (Dhopatkar et al. 2005) (note that this contrasts with the cells of the periodontal ligament that show different molecular changes in the extracellular matrix according to whether tension or compression is applied (Meikle 2006)). Despite the fact that the bulk of cellular and molecular changes occur in the pulp core, the model demonstrated that the magnitude of the force was absorbed by the dentine (Dhopatkar 2006). This suggests strongly that a signalling pathway exists between the odontoblasts (being the only viable cells in communication with the dentine) and the core of the pulp. However finite element analysis has also shown that the dentine layer bends under loading (Middleton et al. 1990), and so it may be hypothesised that force distribution across the pulp chamber will not occur in an even manner.

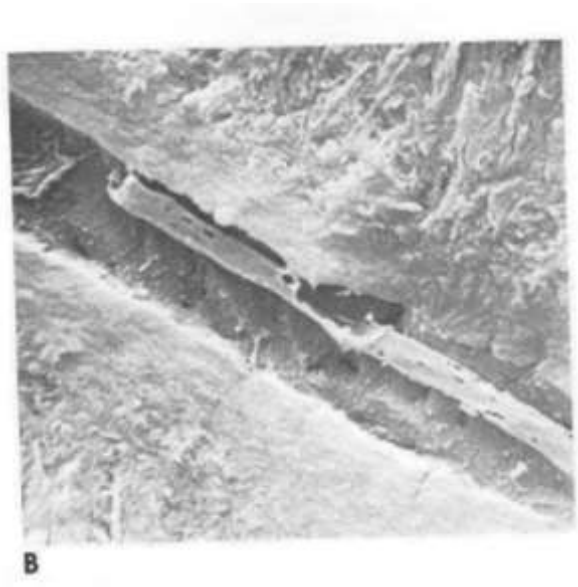
Odontoblast activity upregulation has also been observed in mice in response to orthodontic force application (Nakano et al. 2010) and the application of hydrostatic pressure has been shown to promote dental pulp stem cell differentiation, giving rise to mineralization and hard tissue regeneration for cultured rat dental pulp stem cells both *in vivo* and *in vitro* (Yu et al. 2009). Additionally application of mechanical stress, *in vitro*, to human dental pulp cells has been shown to result in an up-regulation of genes associated with the differentiation of dental pulp stem cells into odontoblasts (Lee et al. 2010). It has also been shown that, following pulpal insult, NGF (Nerve Growth Factor) production increases (Woodnutt et al. 2000), and so it has been hypothesised that NGF is produced by pulp cells, which signal to odontoblasts, altering the way they behave in a Transforming Growth Factor-beta (TGF-β) dependent manner (Yongchaitrakul and Pavasant 2007); thus, the odontoblast-pulpal signalling pathway may be reciprocal.

Figure 1.3
Scanning Electron Micrographs:

A: Dentine/pulp interface



B: 0.5 mm into the dentine



From Brannstrom et al. (1972)

1.4 Pulpal cellular signalling pathways

1.4.1 Integrins

The nature of the signalling pathways between cells of the dentine-pulp complex is not yet understood, although there is evidence to suggest that proteins such as phosphoryn-utilising integrins, could signal cell differentiation within the core (Staquet et al. 2006). Integrins are heterodimeric transmembrane glycoprotein receptors composed of two, covalently linked, α and β chains. (Hynes 2002). They are found on the surface of cells in an inactive state, 'switching on' only when they become attached to ECM molecules such as proteoglycans, laminin, fibronectin and collagen through ligand binding at the extracellular Arginylglycylaspartic acid (RGD) binding sites (Sandy 1998; Kerrigan et al. 2000; Meikle 2006). The intracellular tail of the β subunit can bind, via anchor proteins, to actin filaments and so ligand binding results in cytoskeletal reorganisation (Alberts et al. 2002), cytokine release, activation of ribosomes and gene transcription. Thus, when bound to the extracellular matrix they form a link between it and the actin component of the cytoskeleton (Alberts et al. 2002; Hargreaves and Goodis 2002; Meikle 2006) and so are said to control 'outside-in' changes in cell physiology (Wang et al. 1993). They also mediate cell-cell binding in addition to the cell-matrix binding and thus have important roles in cell adhesion, cell signalling (Flier and Sonnenberg 2001), and, (together with other signalling molecules such as Mitogen-activated Protein Kinases (MAPKs), they control cell differentiation, proliferation, growth and apoptosis (Flier and Sonnenberg 2001; Hynes 2002; Alberts et al. 2002). Integrins can signal to other integrins and affect their behaviour and may also be able to organise the location of a variety of signalling

molecules (Flier and Sonnenberg 2001). They therefore play a vital role in many biological processes.

It has previously been demonstrated that odontoblasts express αv, α6, β1, β5 and β8 integrin mRNA, with αv and β1 most strongly expressed (Zhu et al. 1998). The type, location and expression of integrins is vital in determining the differentiation patterns of odontoblasts, with newly differentiated root odontoblasts expressing β1, and αv being primarily expressed by mature odontoblasts, with a sharp increase in both of these subunits marking maturation (Staquet et al. 2006). They also play an important role in inter-odontoblast adhesion and adhesion between odontoblasts and the pre-dentine/dentine/pulp matrix (Lucchini et al. 2004; Staquet et al. 2006). Because of this, it has been speculated that integrins may stimulate post-caries repair, maintain homeostasis and generally exchange information between odontoblasts, through their interaction with the ECM and growth factors (Staquet et al. 2006), (Lesot et al. 2001). However, it is still unknown which integrins specifically regulate function (Staquet et al. 2006), or what is their precise role in odontoblast signalling .

Pulp cells, such as fibroblasts and endothelial cells, are also known to express α1, α3, α5, α6, αv and β1 integrin subunits (Hargreaves and Goodis 2002). Because the β1 subunit is extremely important in adhesion of dental pulp cells to laminin it is thought that integrins may play a role in their migration (Zhu et al. 1998). It would seem reasonable therefore that integrins (along with other cell adhesion molecules) may be able to regulate the pathway of communication between odontoblasts and the core of the pulp.

1.4.2. MAPKs

There exist many different intracellular signalling pathways, utilising numerous different receptor and signalling proteins, but one which is well documented and known to interact with integrin signalling, is the MAPK (Mitogen Activated Protein Kinase) pathway (Fig 1.4, page 18). The MAPK pathway is, in fact, a complex network of interlinking pathways containing many different proteins. MAPKs are one of a group of three serine/threonine kinases which form the main part of a cascade that leads to the phosphorylation of proteins, other kinases and gene regulatory proteins, resulting in a significant alteration of gene expression within the cell nucleus (Alberts et al. 2002; Miyamoto et al. 2005). MAPKs therefore play an important role in cell cycle regulation (Alberts, Johnson et al. 2002), with this pathway being involved in the signalling in most cells (Miyamoto et al. 2005).

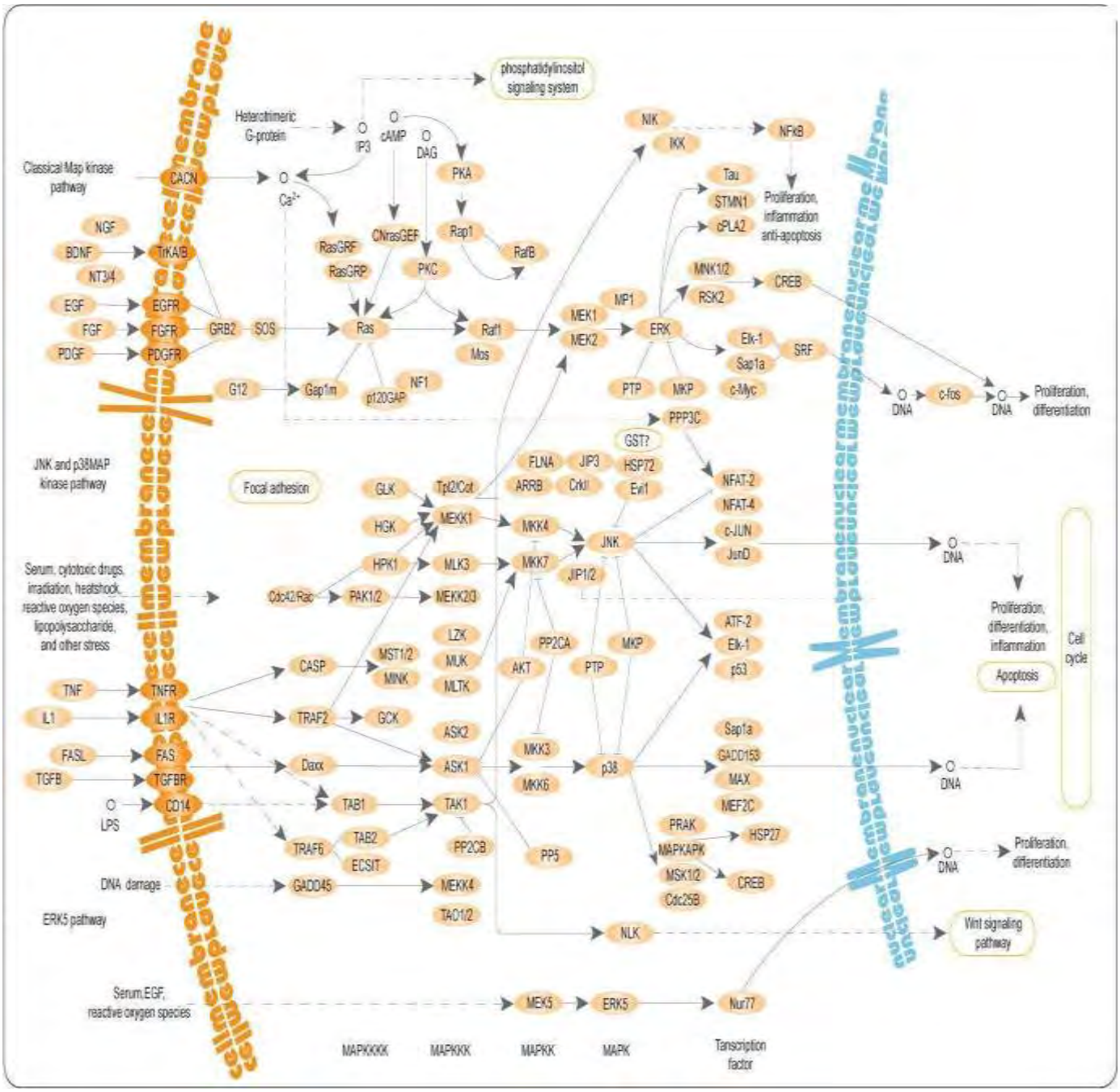
Dental pulp fibroblasts are known to express MAPKs (Gruber et al. 2004) and human dental pulp cells receiving stress have been demonstrated to show MAPK/ERK (extra-cellular signal-regulated protein kinase) activation (Miyata et al. 2006). Other stresses, which cause neurogenic inflammation (e.g. orthodontic stresses), give rise to activation of the p38 MAPK pathway within dental pulp fibroblasts, which is important for Nuclear Factor Kappa-B (NFκB)-independent neurogenic inflammation regulation (Tokuda et al. 2005). Lysophosphatidic Acid (LPA), a potent mitogen for pulpal fibroblasts under *in vitro* conditions, requires signalling via Extra-cellular Receptor Kinase (ERK), p38 MAPK and C-Jun N-terminal kinases (JNK- MAPKs that respond to stress) for its mitogenic activity (Gruber et al. 2004). This evidence

would suggest that in addition to integrins, MAPKs might be involved in signalling pathways between odontoblasts and the pulpal core.

1.4.3. Gap junctions

The odontoblast/pulpal signalling pathway also appears to be, at least in part, dependent upon gap junctions (Ikeda and Suda 2006). When Lucifer yellow dye (a fluorescent dye used to visualise cells under a microscope) was applied to the etched enamel of feline teeth, rapid penetration of the dye into the odontoblasts and the pulp cells beneath them was observed (Ikeda and Suda 2006). In addition when the same dye was injected into the odontoblasts alone, dye penetration into the surrounding pulp cells occurred (Ikeda and Suda 2006). These experiments give strong evidence for functional coupling of odontoblasts and pulp cells (Ikeda and Suda 2006). In addition gap junctions existing between odontoblasts themselves, and between odontoblasts and their neighbouring pulp cells, are known to be important in both tooth development, cellular differentiation and functional regulation (Fried et al. 1996). Connexin-43 (Cx43- a known gap junction protein) levels in young dental pulps are high, suggesting abundant gap junctional activity, however Cx43 does dramatically decrease with age (Muramatsu et al. 2004). There is a large body of evidence demonstrating their vital role in bone cell signalling. This may be used to form a model for odontoblast/pulp cell signalling.

Figure 1.4
The MAPK Pathway



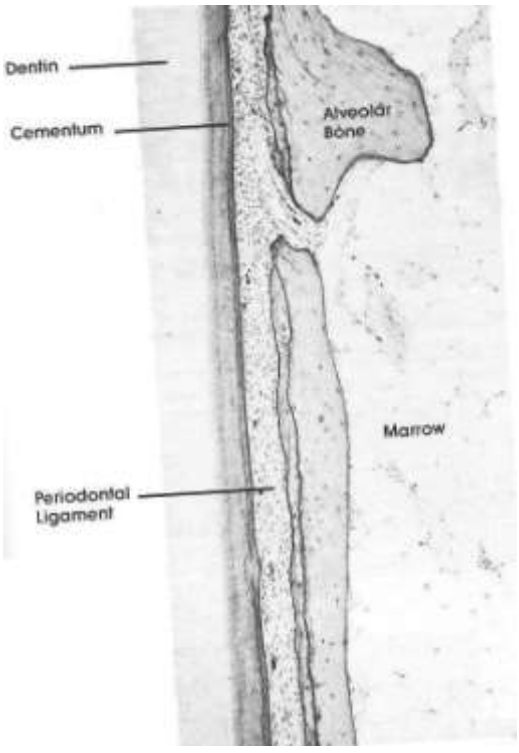
From Gramatikoff (2007)

1.5 Cellular/molecular mechanisms of force transduction

As has already been seen, there is relatively little data in the literature regarding the cellular responses to orthodontic tooth movement. A significant amount of research has, however, been performed, into the cellular responses of the tooth supporting tissues, i.e. the periodontal ligament (PDL) and alveolar bone, in response to mechanical force.

Figure 1.5

The PDL in longitudinal section



From Ten Cate (1998)

1.6 The Periodontal Ligament

Fig 1.5 (page 19) illustrates the complex inter-relationship between the PDL and the surrounding alveolar bone. The periodontal ligament is a connective tissue, consisting of collagen fibres embedded in an extracellular matrix which contains a variety of different cells. The fibres of the ligament insert into both the cementum of the tooth and the alveolar bone (through extensions of the fibres known as Sharpey's fibres), with osteoblasts functionally associated with bone, lying on its external surface, within the ligament (Ten Cate 1998). This intimate relationship between two different somatic compartments results in changes to one (such as pressure arising in the ligament through orthodontic force application) giving rise to cellular and molecular changes in the other.

The periodontal ligament is one of the most highly metabolically active tissues in the human body (Sodek 1977). Its remodelling in response to orthodontic force application is reliant partially on the initiation of an inflammatory process (Low et al. 2005; Krishnan and Davidovitch 2006) . In the first few seconds of force application, fluid is expelled from the PDL and the tooth moves within the ligament space, setting up tension within the ligament on one side and compression on the other (Proffit et al. 2007). This change in tension/pressure strains ligament fibres, cells and their extracellular matrices, as well as compressing and dilating blood vessels. Over the next few minutes, the resultant changes in blood flow within the ligament alter its oxygen tension, giving rise (over the following hours) to release of cytokines, growth factors, colony-stimulating factors and vasoactive neurotransmitters (Davidovitch et

al. 1988; Davidovitch 1991; Proffit et al. 2007). About four hours after force application, the vascular changes within the PDL bring about leukocyte migration from the capillaries, with subsequent production of neurotransmitters, cytokines, growth factors, colony-stimulating factors and arachidonic acid metabolites, resulting in interactions between the native paradental cells. There is thus an overall recruitment of osteoclastic and osteoblastic progenitors as well as chemo-attraction of inflammatory cells, with proliferation and differentiation of osteoblasts (Roberts and Chase 1981; Krishnan and Davidovitch 2006). Thus, the surrounding alveolar bone begins to remodel and about two days after initial force application, tooth movement commences (Proffit et al. 2007). Some trauma will always occur to the ligament as a result of orthodontic force application (Storey 1973), but excessive forces (greater that 20-25g/cm^2 (Schwarz 1932)) will compress blood vessels to a degree sufficient to occlude flow; this leads to areas of ischemia such that cellular necrosis and apoptosis occur within the ligament (Storey 1973; Rana et al. 2001); such changes may be very difficult to recover from (Liu et al. 2007).

1.7 Summary

There is a vast wealth of literature about the effects of orthodontic force application on the periodontal ligament, but relatively little work has been done to investigate the effects upon the pulpal tissues. From the available evidence it would seem that there are many different effects, including morphogenic, angiogenic, inflammatory and cellular-level changes, which are mediated by a variety of signalling molecules and pathways. It is almost impossible to compare the evidence directly however, as the various studies use a variety of different force- appliance systems, with a large range of forces being employed over vastly differing time periods, to effect very different

three-dimensional movements. In addition one has to question the applicability of animal studies to the clinical environment.

What is known is that it is unlikely that any changes elicited are maintained in the long-term: given adequate time most pulps usually recover from the physical insult, provided it has not been too severe (Unsterseher et al. 1987).

1.8 Aims and objectives

1. To investigate the effects of orthodontic force-application upon the overall morphology of the pulpal tissues
2. To investigate the effects of orthodontic force-application upon pulpal fibroblast number.
3. To investigate the effects of orthodontic force-application upon the vasculature of the pulpal tissues, as determined by blood vessel area and number.
4. To investigate the effects of orthodontic force-application upon angiogenesis within the pulpal tissue, as determined by immuno-histochemical staining.

Methodology

Prior to trial commencement, ethical approval was gained from the West Midlands Research Ethics Committee (reference 09/H1208/61). South Birmingham PCT granted research and Development approval. For the purposes of this trial, specialist statistical advice was sought and a power calculation was deemed unnecessary, since the trial was classified as a pilot study. Potential participants were identified from new patient assessments undertaken on the department.

2.1 Inclusion and exclusion criteria

The following inclusion and exclusion criteria were applied:

Inclusion criteria:

- Participants had to be between 11 and 18 years of age,
- Participants had to be free from any oral or systemic disease and not taking any medication.
- Participants were not to take any anti-inflammatory drugs during the 2-week test period.
- All participants had to be patients who required bilateral symmetrical upper premolar extractions as part of their orthodontic treatment, followed by fixed appliance treatment, involving at least the upper arch.
- The teeth to be extracted had to be sound, i.e. free from caries and restorations.
- Participants should not have any tooth malformations.

Exclusion criteria

- Any patient who fell outside of the 11-18 year age group;
- Any patient who was systemically unwell and/or taking systemic medication;
- Any patient who had periodontal disease or caries/restorations in their upper premolars
- Any patient whose treatment-plan did not include bilateral symmetrical premolar extraction followed by fixed appliance therapy.
- Any patient who had tooth malformations.

2.2 Participant recruitment

When a patient was identified as being suitable for the trial both the patient and parent/legal guardian were given verbal and written information about the trial. They were then given up to 2 weeks to decide if they wished to participate. If they decided to participate, prior to any active treatment, written consent was gained from, the patient for those aged 16-18, or from the parent/legal guardian for those aged 11-15, as stipulated by the Research and Ethics Committee.

2.3 Appliance placement

All treatment was carried out in the Orthodontic Department of Birmingham Dental Hospital. Each patient attended the Orthodontic Department 2 weeks prior to having his or her upper premolars extracted. They then had a sectional fixed appliance placed from the upper lateral incisor to the upper first molar, with the side of the arch chosen

according to ease of access to the test tooth. The following bonding protocol was used for every patient in order to standardize the bonding procedure:

- The teeth were cleaned with oil-free prophylactic paste on a brush in a slow handpiece, to remove the plaque pellicle.
- The teeth were then isolated and self-etching primer (Transbond™ Plus Self Etching Primer, 3M Unitek, Monrovia, USA) was applied to the buccal face, in the desired area of bonding.
- The self-etching primer was agitated for ~5 sec.
- Each tooth was air-dried to evaporate off any excess solvent
- Pre-adjusted straight-wire brackets (Adhesive Pre-coated (APC) Victory™ Brackets, McLaughlin Bennett Trevisi (MBT) prescription: 3M Unitek, Monrovia, USA) were then placed, and excess composite removed with probe.
- The bracket on the test premolar was placed more gingivally, in such a position as to give 50g of extrusive force when a 0.014" superelastic Nickel Titanium (NiTi) archwire was engaged into the bracket slot, as determined using a Correx Gauge (Haag-Streit, Bern, Switzerland).
- The composite was then cured with a Light Emitting Diode (LED) curing light for 10 sec.
- The 0.014" NiTi archwire was placed and secured with grey-coloured elastomeric modules (TP Orthodontics Inc, Leeds, UK).

Each test tooth was subject to 14 days of force application. Superelastic NiTi was chosen in order to try and maintain a more continuous force upon the tooth, than that induced by other varieties of NiTi. It is recognised however that the force upon the

tooth can not be truly continuous, as the wire must undergo stress-induced phase transformations, which will alter the load applied to the tooth, with the unloading force of the wire likely to be less than the initial loading force (Ireland and McDonald 2003). Additionally consumption of hot or cold food substances can alter the crystal structure of the wire, again changing the level of force applied (Ireland and McDonald 2003). Day-to-day masticatory forces will also easily exceed the 50g placed upon the teeth premolar at the initial wire-ligation.

During the test period no anti-inflammatory medication was taken, and following this both upper first premolars were extracted atraumatically under local anaesthesia and placed directly into two separate pots of formalin. No formal patient identifiers were retained.

2.4 Tissue preparation and processing

In order to improve tissue fixation for all teeth, a 3mm portion of the root was sectioned off using a rotary saw, and both pieces were then immersed in 10% neutral buffered formalin for 48 hours.

3 test-control pairs were then processed for methylmethacyrlate (MMA) resin embedding, each according to the stepwise protocol shown in **appendix 1**.

From the resin blocks horizontal ground sections were produced and stained with haematoxylin and eosin (H&E) for use in morphological assessment and blood vessel analysis.

The 13 remaining test-control pairs were first decalcified in either 10% formic acid, or 10% Ethylene Diamine Tetra-acetic Acid (EDTA): decalcification end-point was determined using radiographic and physical testing. The pairs were then processed through graded alcohols and xylene, prior to being embedded in paraffin, in accordance with the stepwise protocol shown in **appendix 2**.

5μm serial horizontal or longitudinal sections were then taken on a semi-automated rotary microtome (Leica microsystems, Heerbrugg, Switzerland) and either stained with haematoxylin and eosin (H&E), for use in morphological assessment and fibroblast analysis, or used for immunohistochemistry.

2.5 Assessment and analysis

2.5.1 Morphological analysis

Both the non-decalcified ground sections and paraffin sections were examined at low and high-power magnification under a light microscope and the appearance of the odontoblast layer, predentine layer, blood vessels and overall pulp morphology noted for both the test and control groups.

2.5.2 Fibroblast analysis

Fibroblast numbers were assessed by counting the total number of fibroblasts (defined as spindle shaped cells) within a 20x20μm grid, superimposed over 4 different pulpal areas: apical, coronal, mesial and distal for longitudinal sections, and mesial, distal buccal and lingual for horizontal sections, at x400 magnification. This was repeated for each section, for a total of 128 sections.

2.5.3 Blood vessel analysis

Blood vessel analysis was undertaken on photomicrographs of serial ground sections using ImageJ software (National Institutes of Health, Bethesda, USA). Each section was first converted to an 8-bit grayscale image and then the binary contrast was adjusted manually (a process known as thresholding) to define the limits of each blood vessel perimeter. Each blood vessel then was outlined with the wand (tracing) tool and added into the Region of Interest manager. The collated information was then used to calculate the number of blood vessels and overall blood vessel area, as a percentage of the total pulp area, for each individual section.

2.6 Immunohistochemistry

2.6.1 Tissue staining

In order to enhance the immunoreactivity of the prepared tissue sections antigen retrieval was first undertaken on de-paraffinised serial sections: Pre-prepared proteinase-K (Sigma-Aldrich, St Louis, USA) solution was applied directly to the serial sections and left undisturbed for 6 minutes at room temperature.

The sections were then stained for Cluster of Differentiation 31/von-Willebrand Factor (CD31/VwF) immunoreactivity using a blood vessel staining kit, containing all necessary reagents for the complete staining process (Merck Millipore, Darmstadt, Germany), according to the step-wise protocol shown in **appendix 3**.
For every incubation a negative control was taken, incubating with TBS instead of the primary antibodies.

2.6.2 Analysis

Photomicrographs of the pulpal tissue of each section were captured using ProgRes® Image Capture Software (Jenoptik, Jena, Germany) on a computer connected to a light microscope (Carl Zeiss Microscopy LLC, New York, USA). An ImageJ (National Institutes of Health, Bethesda, USA) macro was developed by Professor G. Landini (The University of Birmingham, School of Dentistry) to remove erroneous background staining and create a binary image of the stained tissue; this stained area of immunoreactive tissue was then calculated as a percentage of the total pulpal area to allow for quantitative analysis of immunoreactivity, and thus angiogenic activity.

For all quantitative measurements, test and control pairs were compared using a paired Student's T-test and a significance level of $p < 0.05$. All statistical tests were performed using SPSS (Mac).

Results

3.1 Morphological observations

Vacuolation and degeneration of the odontoblast layer was a commonly observed occurrence. In Figures 3.1, 3.2 and 3.3 (pages 31 and 32) vacuolation may be seen, with distortion of the odontoblast layer and separation and elongation of the odontoblasts themselves. These degenerative phenomena were observed in both the test and control groups.

Figure 3.4 (page 32) is a 5μm paraffin section taken from a test tooth: Within this figure it may be seen that the pre-dentine layer is of a relatively uniform width, conversely figure 3.5 (page 33) is again a test specimen, but here the predentinal layer is significantly thicker in the upper part of the section. These inconsistencies were also observed in the control specimens, with some sections having a regular predentine layer (fig.3.6, page 33), whilst the pre-dentinal layer in other control sections was highly irregular (fig.3.7, page 34.

Blood vessel congestion and/or haemorrhage were not observed in any of the test (fig.3.4, page 32) or control (fig.3.8, page 34) subjects.

Pulp stones (fig.3.9, page 35) were also observed occasionally in both test and control subjects. The occurrence of pulp stones was too infrequent however to allow their inclusion in this study.

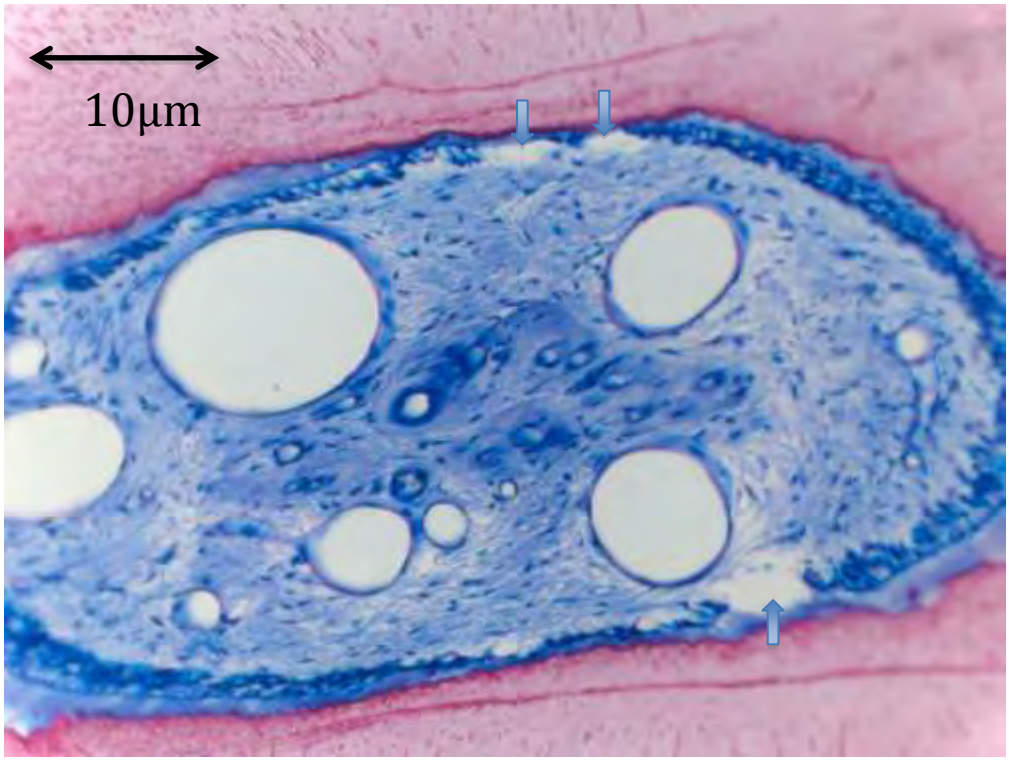


Figure 3.1: Vacuolation and distortion of the odontoblast layer (H&E section x200). Vacuoles seen within layer, indicated above.

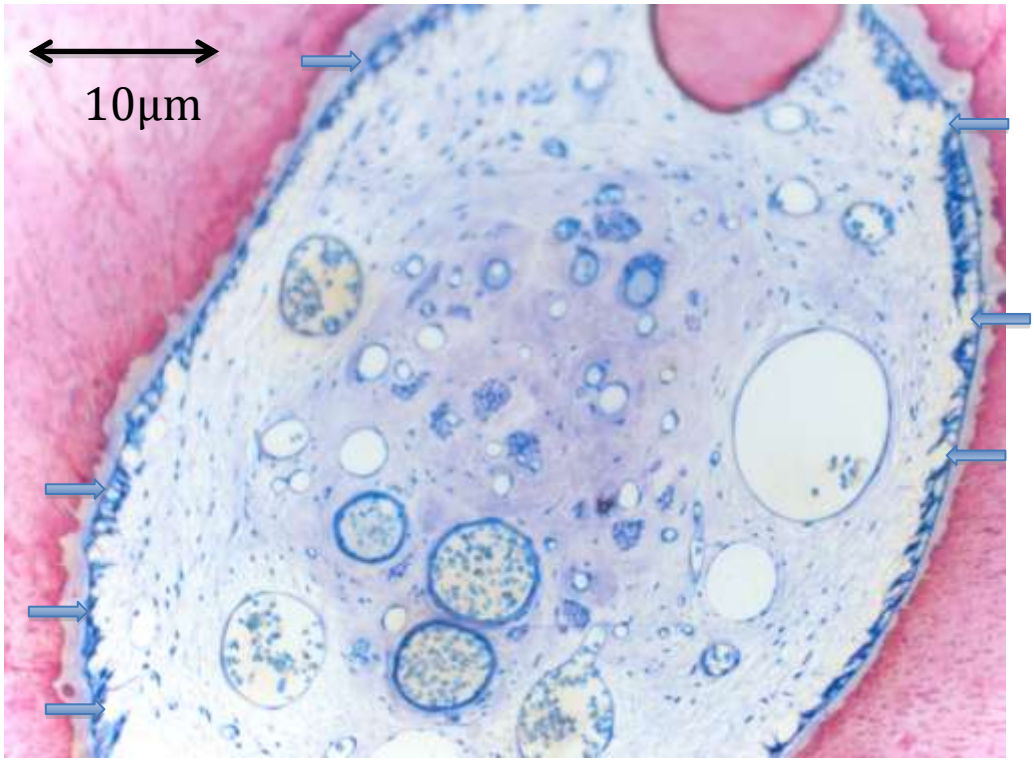


Figure 3.2: Separation and distortion of the odontoblast layer (H&E section x200). Separation seen at several points, shown above.

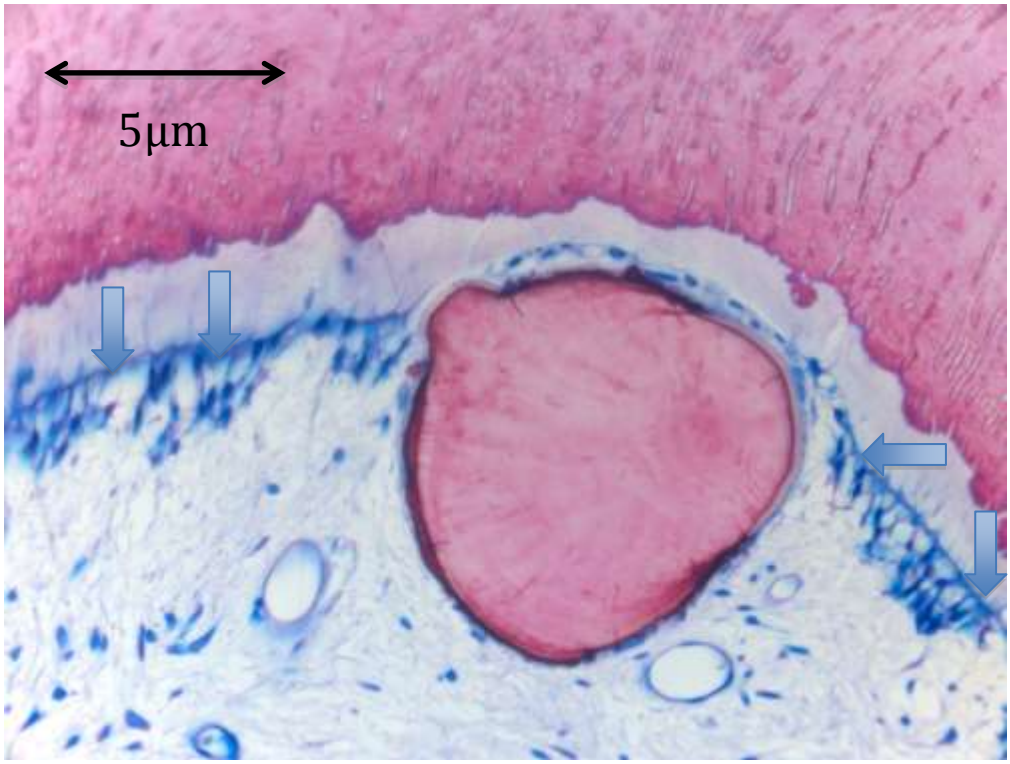


Figure 3.3: Separation and distortion of the odontoblast layer (H&E section x400). Areas of separation seen at several points within layer, as highlighted above.

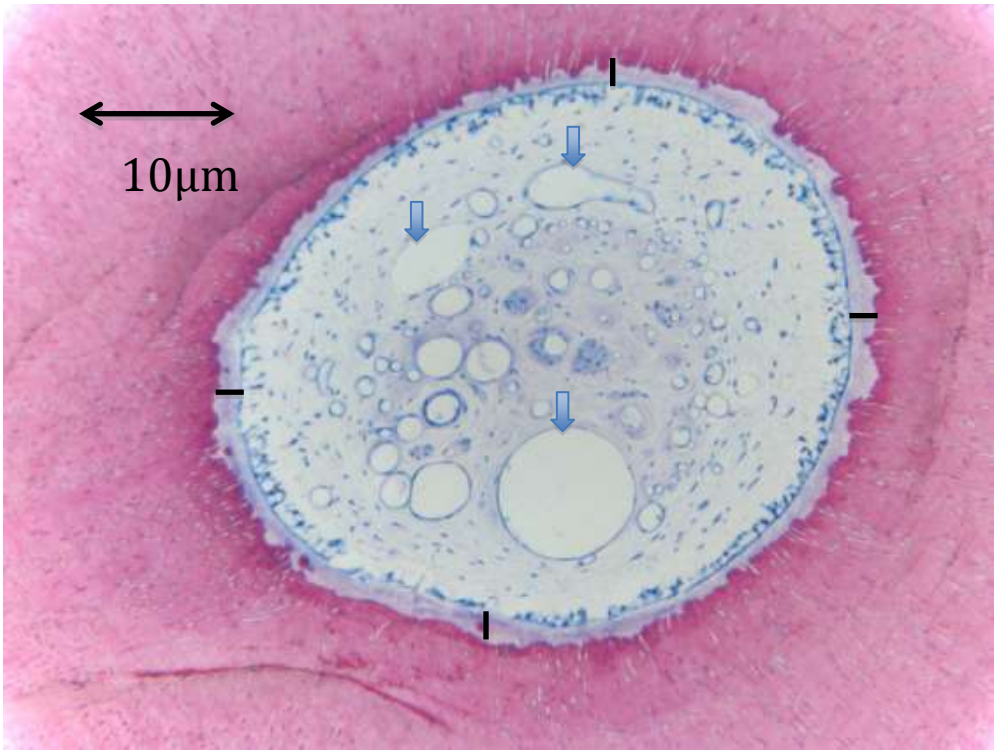


Figure 3.4: H&E section x160, demonstrating a pre-dentine layer of uniform thickness, with no vascular congestion. Test specimen.
It may be seen that the blood vessels are empty and the pre-dentine layer is relatively uniform around the whole specimen.

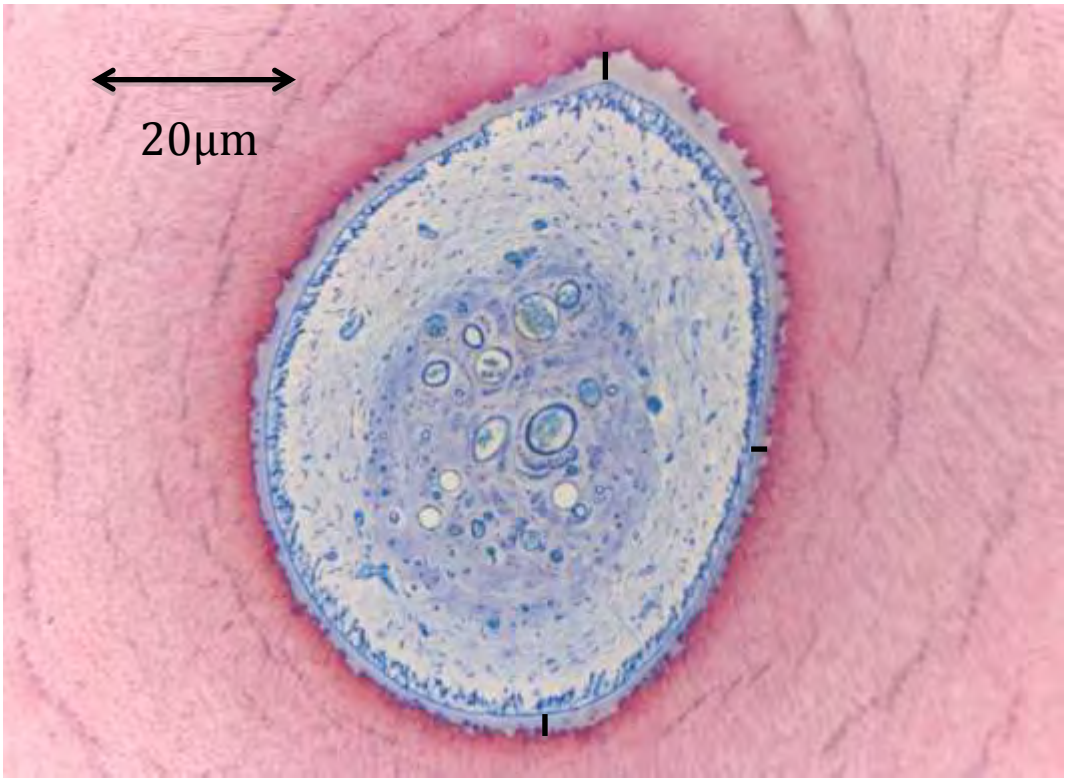


Figure 3.5: H&E section x100, demonstrating a pre-dentine layer that is not of uniform thickness. Test specimen. Variations in the width of the layer are shown above

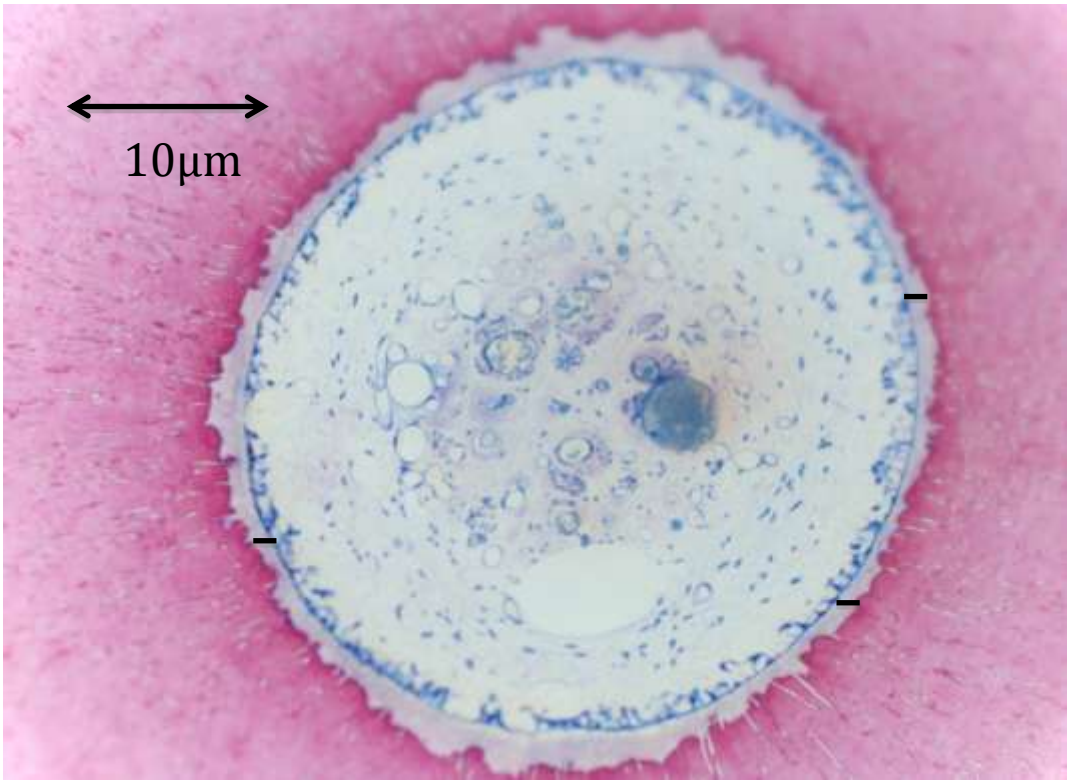


Figure 3.6: H&E section x200, demonstrating a pre-dentine layer of uniform thickness. Control specimen. The layer, whilst not perfectly even, is of a more uniform width than that seen in fig. 3.5.

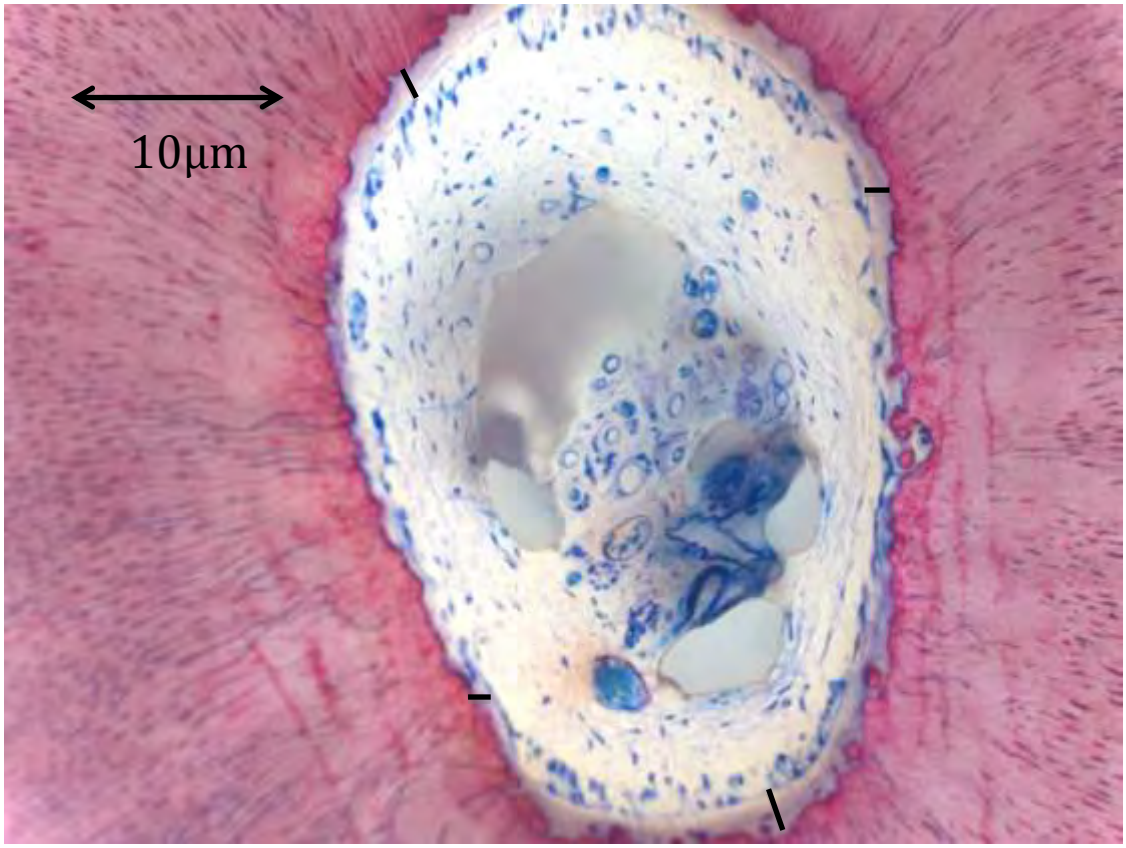


Figure 3.7: H&E section x50, demonstrating a grossly irregular pre-dentine layer. Control specimen. The layer is of widely varying thickness, as shown above.

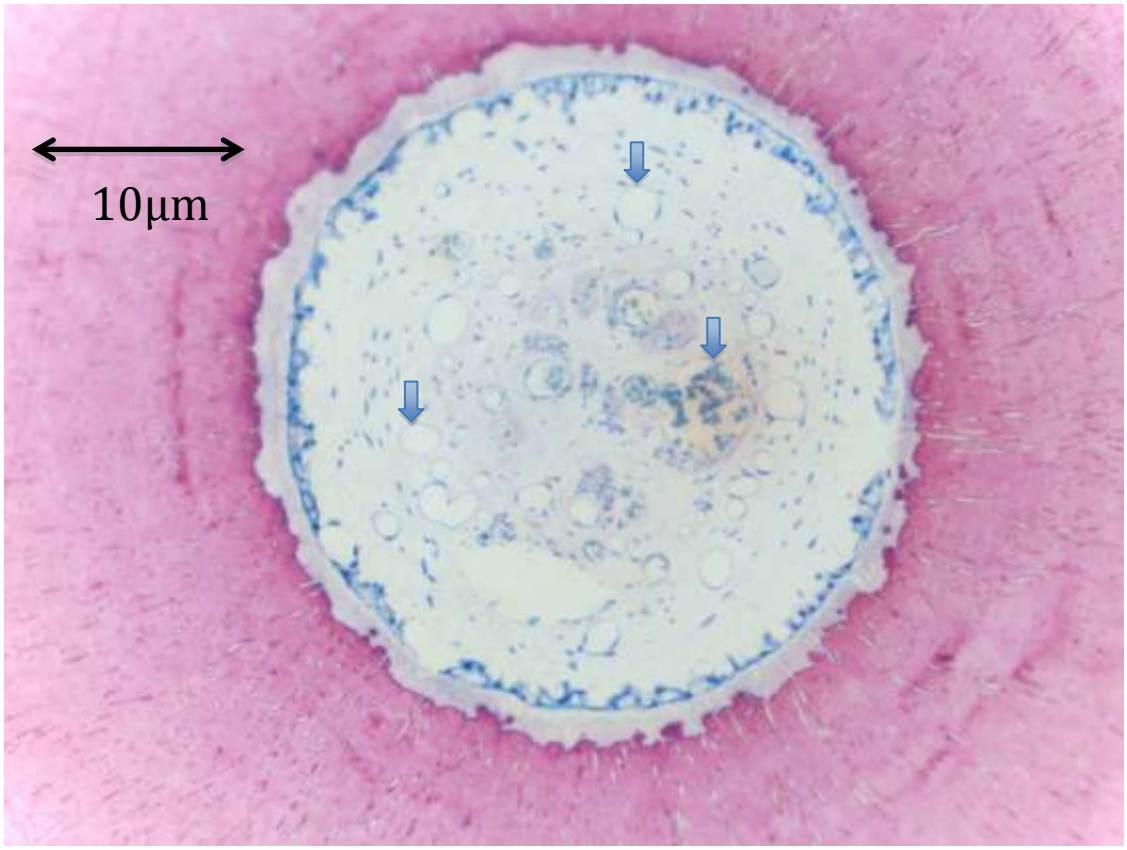


Figure 3.8: H&E section x50: control specimen with no vascular congestion or haemorrhage. The vessels may be seen to be largely empty, with a few red blood cells contained within the lumen, but not within the ECM.

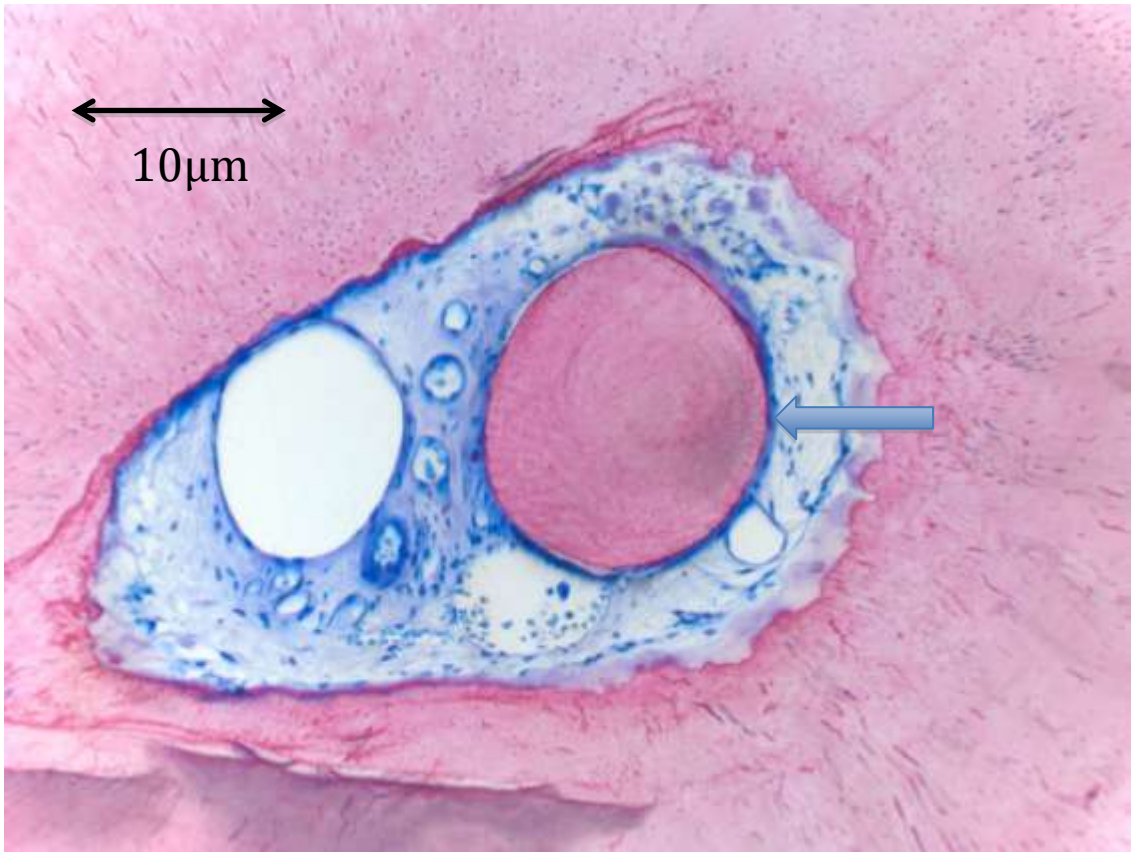


Figure 3.9: H&E section x200 with large pulp stone. The stone is stained a dense pink with haematoxylin, and occupies a large area within the pulp chamber.

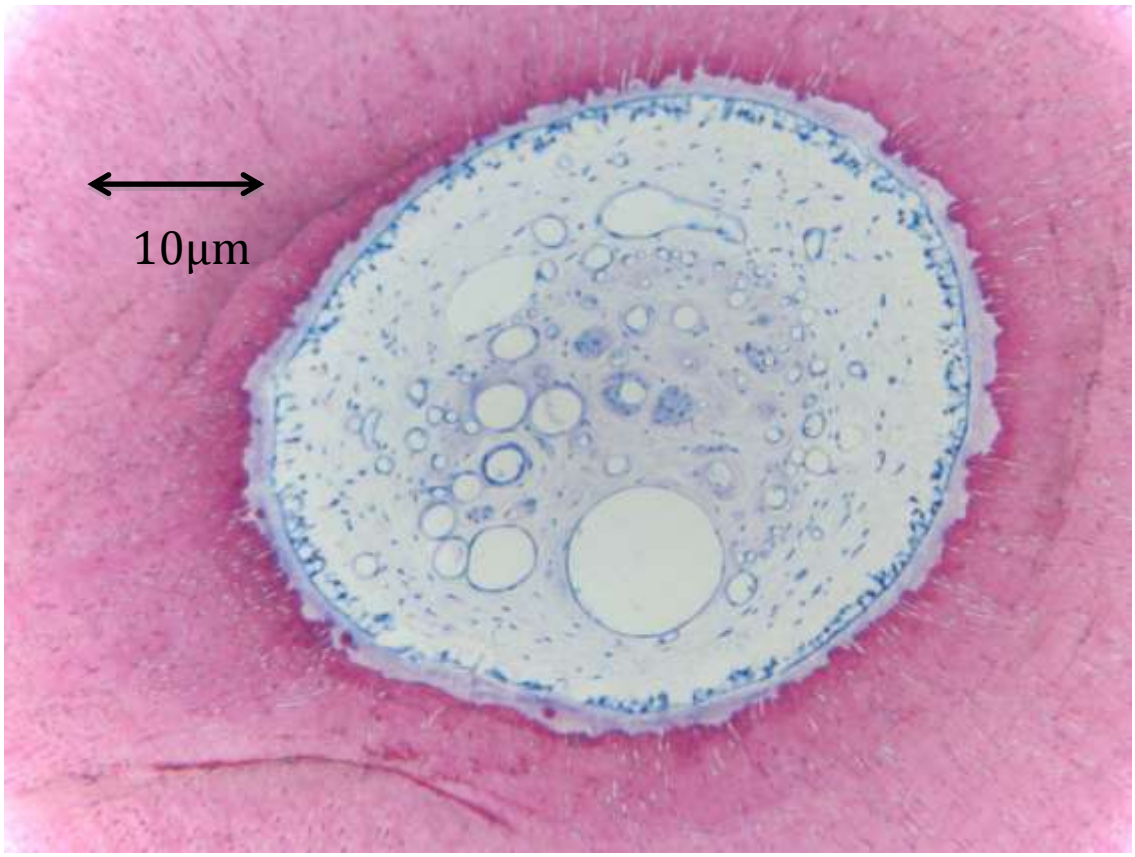


Figure 3.10: A representative horizontal section of a MMA embedded specimen (x200 magnification)

3.2 Fibroblast numbers

Counts were performed for a total of 728 areas. The mean counts for each specimen are shown in the table below:

Table 3.1: Mean fibroblast counts per 400µm^2 for paired test and control specimens

Specimen Number	Test (mean)	Control (mean)
1	70.29	86.39
2	75.39	84.14
3	75.29	81.82
4	93.21	93.86
5	104.82	116.82
6	56.54	54.40
7	72.89	78.18
8	110.89	98.57
9	95.67	103.50
10	112.07	99.54
11	109.79	140.54
12	121.25	133.00
13	80.18	79.14

The results were analysed using a paired samples T-test (SPSS Statistics, Mac, IBM, New York, USA):

Table 3.2 Fibroblast counts: paired samples statistics

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	Test	90.6369	13	20.15683	5.59050
	Control	96.1462	13	23.50962	6.52040

Table 3.3 Fibroblast counts: paired samples test

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Test - Control	-5.50923	11.60326	3.21816	-12.52101	1.50255	-1.712	12	.113

3.3 Blood vessel analysis

Figure 3.10, page 35, is a representative MMA ground section. The mean % blood vessel areas for paired test and control specimens as determined on serial MMA sections are outlined in Table 3.4 below:

Table 3.4: Blood vessel area as a % of total pulp tissue area

Specimen pair	Mean blood vessel area: Test	Mean blood vessel area: control
1	7.81	5.98
2	12.72	11.35
3	11.63	9.25

The results were analysed using a paired samples T-test (SPSS Statistics, Mac, IBM, New York, USA):

Table 3.5: Blood vessel area: paired samples statistics

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	Test	10.7200	3	2.57839	1.48863
	Control	8.8600	3	2.70616	1.56240

Table 3.6: Blood vessel area: paired samples test

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Test - Control	1.86000	.50567	.29195	.60385	3.11615	6.371	2	.024

The mean number of blood vessels per pulpal area for paired test and control specimens as determined on serial MMA sections are shown in Table 3.7 below:

Table 3.7: Mean blood vessel number per pulpal area

Specimen pair	Mean blood vessel number: Test	Mean blood vessel number: control
1	133.63	125.75
2	79.33	117.78
3	125.10	128.10

The results were analysed using a paired samples T-test (SPSS Statistics, Mac, IBM, New York, USA):

Table 3.8: Mean blood vessel numbers: paired sample statistics

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	Test	112.6867	3	29.20087	16.85913
	Control	123.8767	3	5.40903	3.12291

Table 3.9: Mean blood vessel numbers: paired samples test

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Test - Control	-11.19000	24.22652	13.98719	-71.37201	48.99201	-.800	2	.508

3.4 Immunohistochemistry

Representative horizontal (Figure 3.11) and longitudinal (Figure 3.12) sections stained with VwF and CD31 are shown on page 42 overleaf:

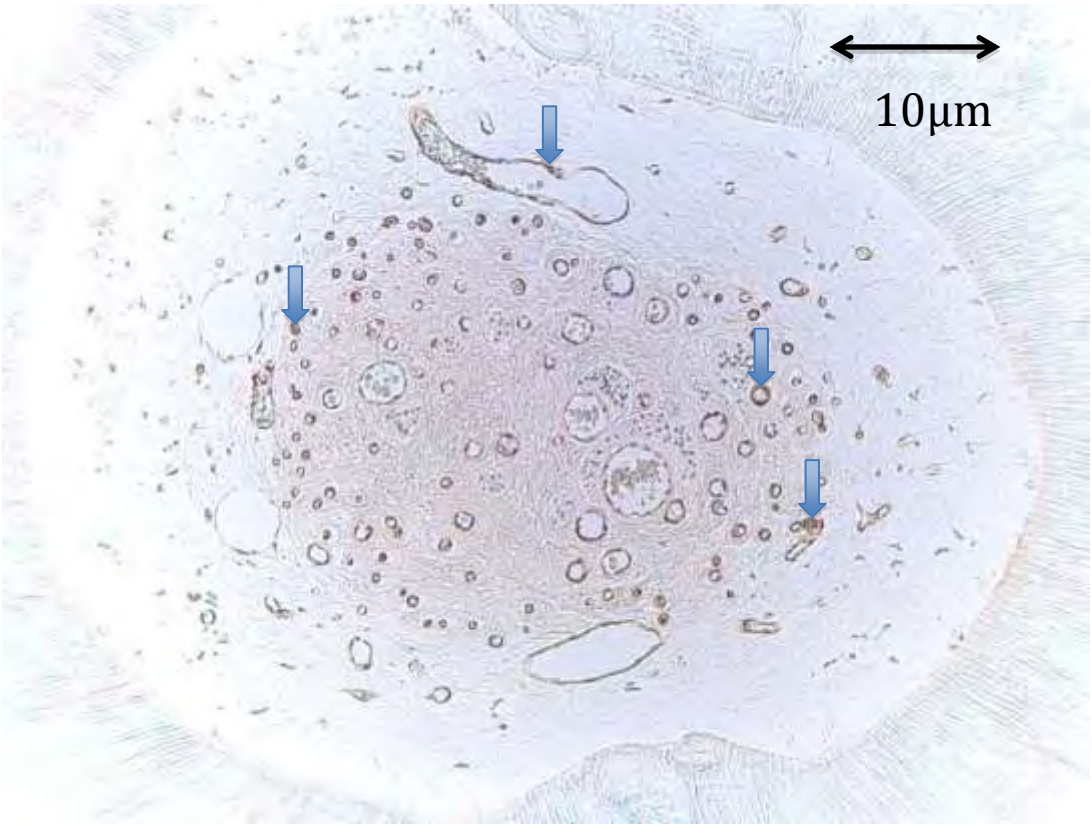


Figure 3.11: Horizontal section of a decalcified, paraffin embedded specimen stained with VwF and CD31 (x160). The areas of VwF/CD31 expression are stained brown with DAB, and represent areas of angiogenic activity.

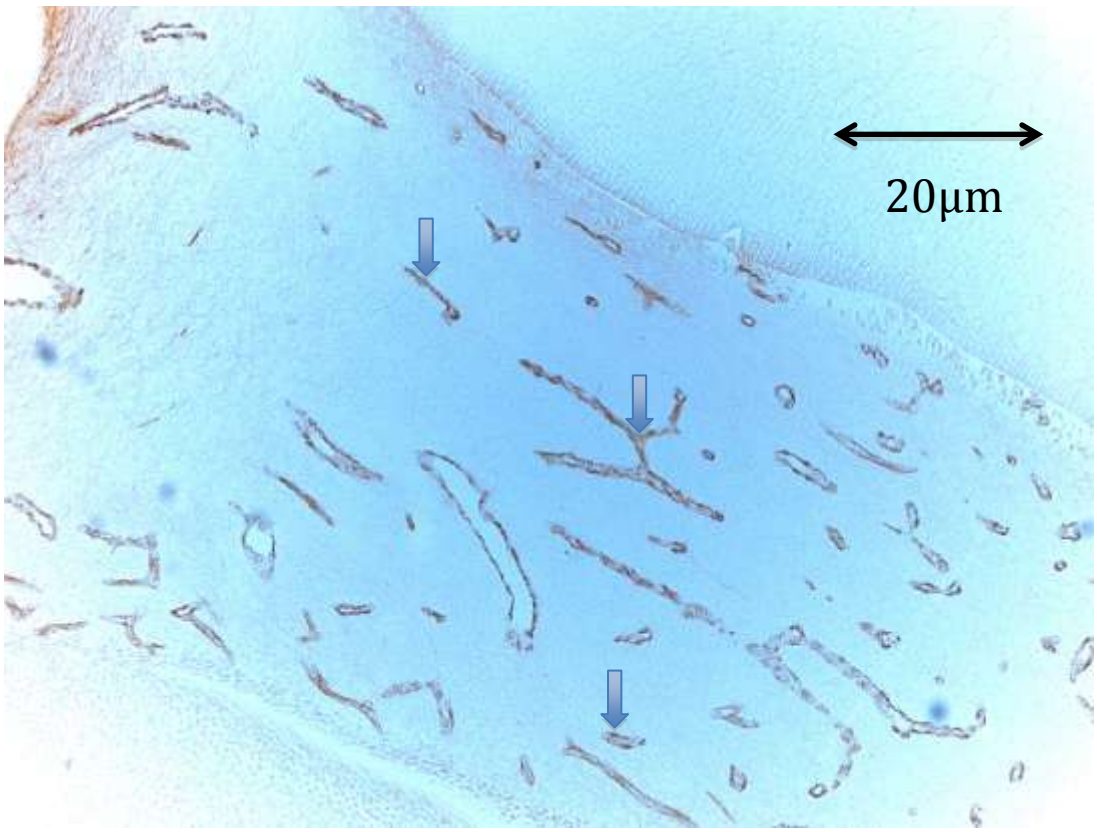


Figure 3.12: Longitudinal section of a decalcified, paraffin embedded specimen stained with VwF and CD31 (x100). The areas of VwF/CD31 expression are stained brown with DAB, and represent areas of angiogenic activity.

338 sections were analysed in total. For each test-control pair the mean percentage of stained tissue was calculated from the serial sections: the results are shown in Table 3.10 below:

Table 3.10: CD31/VwF stained tissue as a % of overall pulp tissue area

Specimen pair	% stained tissue: Test	% stained tissue: control
1	2.51	4.87
2	6.31	16.02
3	2.40	2.74
4	5.06	7.34
5	1.96	2.22
6	5.43	5.23
7	2.73	2.22
8	3.10	3.05
9	3.08	2.74
10	5.20	3.07
11	4.54	3.66
12	4.96	2.90
13	3.92	2.78

The results were analysed using a paired samples T-test (SPSS Statistics, Mac, IBM, New York, USA):

Table 3.11: % CD31/VwF stained tissue: paired samples statistics

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	Test	3.9385	13	1.39421	.38669
	Control	4.5262	13	3.74611	1.03898

Table 3.12: % CD31/VwF stained tissue: paired samples test

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Test - Control	-.58769	3.05530	.84739	-2.43399	1.25860	-.694	12	.501

Discussion

4.1 Morphological observations

4.1.1 Vacuolation and degeneration of the odontoblast layer

Many authors have concluded that orthodontic force application gives rise to vacuolation and degeneration of the odontoblast layer (Stenvik and Mjör 1970; Mostafa et al. 1991; Shigehara et al. 2006; Ramazanzadeh et al. 2009), with high-quality photomicrographs seemingly corroborating their findings. However in the present study these phenomena were observed in both test and control subjects (Figures 3.1, 3.2 and 3.3, pages 31 and 32).

Odontoblasts are sensitive cells, and as such are easily disturbed by changes in their external environment; it is entirely possible that there may have been insufficient fixation speed or that it is the trauma induced by tooth-extraction, combined with the drying effects of graded alcohols and xylenes on the pulpal tissue that give rise to the reported histological deterioration of the odontoblast layer, rather than orthodontic force application itself. During tooth extraction the tooth is lifted out of its socket; as the pulp is enclosed, with only an opening at the apex, it is conceivable that the stretching of apical tissue could create a vacuum-effect upon the pulp, leading to the formation of vacuoles within this tissue. If this is the case it would mean that the vacuolation of the odontoblast layer, observed in previous studies, is attributable to the process of tooth-extraction rather than an effect elicited by orthodontic force application.

4.1.2 Changes in the width of the pre-dentinal layer

It is interesting to note that in the current study variation in the pre-dentinal width was observed: a finding documented in previous studies (Stenvik and Mjör 1970; Nixon et al.1993; Villa et al. 2005; Konno et al. 2007). However in the current study changes in pre-dentinal width were observed in both test and control subjects; this may be, at least in part, explained by section orientation. For example a closer examination of figure 3.5a & b (page 47) reveals that, whilst the pre-dentine layer appears thicker in the upper part of the section, in fact the dental tubule orientation has also altered, indicating a change in the plane of sectioning. The literature regarding changes in pre-dentinal thickness is heavily conflicted, and it may be that this is due to inconsistencies in the plane of sections taken for examination, thus giving rise to artificial variations.

Figure 3.5a: Upper portion of the MMA section. Here the tubules have been sectioned transversely and appear round in cross-section.

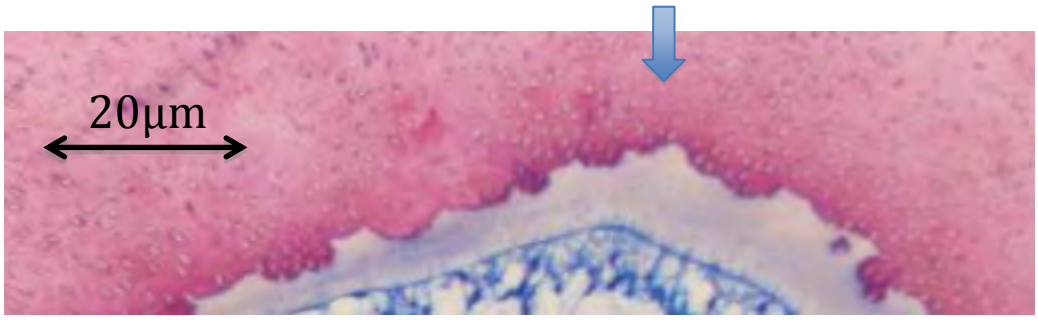
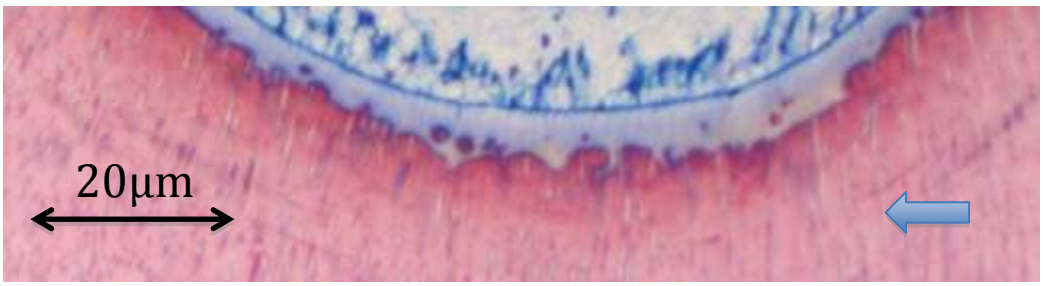


Figure 3.5b: Lower portion of the MMA section. Here the tubules have been sectioned longitudinally and appear rectangular in cross-section.



4.1.3 Vascular congestion and haemorrhage

In the present study no evidence of blood vessel congestion and/or haemorrhage was found in either test or control subjects (figs. 3.4 and 3.8, pages 32 and 34). These findings have been associated previously with the use of heavy, intrusive forces in the order of 150 grams (Raiden et al. 1998). Approximately 10g of force is considered to be appropriate for the intrusion of teeth *in vivo* (Proffit et al. 2007); this level of force is sufficient to stimulate bone remodelling without causing damage to the apical vessels. It may be hypothesised therefore, that the use of very heavy forces for intrusion leads to vascular damage and thus haemorrhage. In this study light (50gm), clinically relevant, extrusive forces were used. Light forces allow for periodontal remodelling and tooth movement, without damage to the apical vessels. The use of light forces and extrusive movements in this study may thus account for the lack of vascular congestion and haemorrhage observed.

It is also important to note that in the current study the test teeth were extruded, but remained in occlusion. Stenvik found that circulatory disturbances were more pronounced in teeth that were left to erupt unopposed (Stenvik 1971). It may be that if the study were to be repeated with the teeth taken out of occlusion, that more significant vascular changes would be seen.

4.2 Fibroblast numbers

Earlier studies have demonstrated an increase in fibroblast number (Dhopatkar 2006) associated with sustained orthodontic force application, which may account for previous observations of increased collagen fibre number and width (Villa et al. 2005), together with fibrosis (Raiden et al. 1998; Mostafa et al. 1991). In this study no such increase in fibroblast number was observed however: there was no statistically significant difference (p=0.113) between test and control paired specimens for number of fibroblasts per $400\mu m^2$ pulpal area (tables 3.2 and 3.3, page 37). The discrepancy between the current study and those previously mentioned may be due to the relatively short time frame of the study, together with the use of light forces. Previous studies have used heavier forces and/or longer periods of force application: it has been shown that the extent of pulpal fibrosis is proportional to the time for which force is applied (Kayhan et al. 2000; Ramazanzadeh et al. 2009). Thus with longer periods of force application and/or the use of heavier forces changes in the fibroblast population may be seen.

4.3 Vascular changes

4.3.1 Blood vessel area and number

In this study there was found to exist a statistically significant difference in blood vessel area between test and control teeth (tables 3.5 and 3.6, page 39): The overall percentage blood vessel area was significantly greater in the test specimen group (10.72% in the test group and 8.86% in the control group; p=0.024). This finding is corroborated by other studies (Nixon et al. 1993; Wong et al. 1999; Santamaria et al. 2006). The increase in blood vesel area can not, however, be attributed to blood vessel

growth as there was no statistically significant difference in blood vessel number per pulpal area between test and control pairs (p=0.508) (tables 3.8 and 3.9, page 41); if there had been growth of new vessels then there would have been a concurrent increase in the numbers of vessels observed. Thus the increase in blood vessel area observed in the test specimens must be due to dilatation of the vessels.

Initial application of orthodontic force has been shown to cause vascular constriciton, with the concurrent drop in oxygen tension leading to cellular injury, apoptosis (Rana et al. 2001; Perinetti et al. 2004; Shigehara et al. 2006), reduced ECM turnover (Leone et al. 2009), and inflammation (Perinetti et al. 2004). Blood vessel dilatation would bring about a corresponding increase in blood flow, allowing for delivery of growth factors and nutrients, promoting and enhancing tissue repair.

The dental pulp is not a closed system, but is linked intimately through apical vessels to the surrounding periodontal ligament, and is thus subject to the same inflammatory processes as other somatic tissues. Orthodontic force application has been shown to bring about strain within the pulp tissue (Dhopatkar 2006) and with application of force CGRP is released, with levels rising as the force is increased (Caviedes-Bucheli et al. 2011). The blood vessels within the dental pulp are highly innervated, and have been shown to undergo dilatation in response to CGRP release from afferent nerve endings (Ten Cate 1998). Thus it may be that as force is applied through the use of orthodontic appliances CGRP is released, stimulating blood vessel dilatation. It has also been shown that cultured dental pulp fibroblasts will produce large amounts of IL-1β, IL-6, and TNF-α (Yamaguchi et al. 2004) when under cyclic strain (Lee et al. 2008). Thus the force applied by orthodontic appliances could lead to to cytokine

production by fibroblasts; these cytokines may then act upon cell adhesion molecules (such as integrins) to intiate vascular dilatation.

Thus blood vessel dilatation may be arising in a neuropeptide and cytokine mediated fashion in order to facilitate repair of tissue damage caused by orthodontic force application.

4.3.2 Angiogenesis

Both CD31 and VwF are used commonly to assess tumour angiogenesis, staining blood vessels that may not be observed on plain H&E sections (Kerbel 2000). CD31 is a highly sensitive marker for endothelial cells (Gasparini and Harris1995) and other angiogenic cells (Kim et al. 2010), its action being critical to angiogenesis. VwF is produced by endothelial cells and, in tumour tissues, as angiogenesis takes place, VwF mRNA expression increases (Zanetta et al. 2000) due to endothelial cell proliferation (Gil-Bazo et al. 2003). Thus VwF is also used routinely for blood vessel staining. For the above reasons CD31 and VwF were chosen for angiogenesis assessment in this study.

There was found to be no statistically significant difference in the percentage of stained tissue area for VwF/CD31 staining (P=0.501) between test and control specimens (tables 3.11 and 3.12, page 44). This, together with the lack of change in blood vessel number, would indicate that angiogenesis is not occurring. This conflicts with findings of previous studies (Derringer and Linden 2004; Perinetti et al. 2005; Derringer and Linden 2007). The current study however, represents a relatively short

period of time, and thus conclusions can only be drawn for a 14-day time frame, with prolonged force application perhaps angiogenesis would be stimulated.

Whilst the current study has used a fixed appliance that is in routine use, previous studies have not always done so; this may account for some of the disparity between the results published by other authors. It is possible that with different force levels angiogenic changes may be seen, as use of greater forces would be more likely to elicit tissue damage and thus generate a need for angiogenesis in order to promote tissue remodelling and repair.

Conclusions

5.1 Conclusions

The present study has shown that a 14 day period of 50g of extrusive orthodontic force application:

- Does not lead to any significant change in the overall morphology of the pulpal tissues
- Does not give rise to a change in pulpal fibroblast number.
- Does not bring about a change in blood vessel number.
- Does not give rise to a change in angiogenesis within the pulpal tissue, as determined by immuno-histochemical staining.

However a 14-day period of 50g of extrusive orthodontic force application does:

- Give rise to significant blood vessel dilatation

Thus overall it seems that the application of 50g of extrusive orthodontic force to the teeth, over a 14 day time period, does not seem to cause any deleterious effects upon the pulp.

5.2 Recommendations for further study

The present findings conflict with some of those documented in previously within the literature. However it is possible that some of the effects documented, such as odontoblast vacuolation and degeneration, could have arisen due to the trauma associated with tooth extraction, or could represent damage arising from tissue processing (e.g. due to insufficient fixation speed). Great care must be taken therefore in the removal of teeth, their handling and processing if such artefacts are to be minimized. The effects of tooth extraction and tissue processing on histological findings could, therefore, warrant further investigation.

The mechanisms underpinning blood vessel dilatation are hypothesised upon in the present study, but further investigation, utilising immunohistological techniques would be required to pinpoint them.

Whilst care has been taken to ensure these findings are as clinically relevant as possible, i.e. using ideal force levels and an appliance that is in use commonly; this still represents a very specific set of clinical conditions. 50g of force was applied, and whilst this is recommended for extrusive movements, much higher forces (70-120g) are recommended in order to elicit bodily movement, conversely much lower forces (10g) are recommended for intrusion. Whilst the appliance system used is commonplace there are a myriad of appliances available for the treatment of malocclusion. In addition an average course of orthodontic treatment would take far longer than 14 days. Thus whilst every effort has been made to ensure that the study is clinically relevant, the findings can only be taken in the context of the present study.

Further work is therefore required in this area, to include investigation of the effects of differing levels and time periods of force application upon the pulp.

Appendix 1

Stepwise protocol for MMA embedding

- Step 1: Washed overnight in running tap water
- Agitated at room temperature in:
    - Step 2: Ethanol 40%: 3 days
    - Step 3: Ethanol 70%: 3 days
    - Step 4: Ethanol 96%: 3 days
    - Step 5: Ethanol 100%: 3 days
    - Step 6: Fresh ethanol 100%: 3 days
    - Step 7: Fresh ethanol 100%: 3 days
    - Step 8: Xylene: 3 days
    - Step 9: Fresh xylene: 3 days
- Step 10: Immersed in MMA I (80% methylmethacrylate and 20% dibutylphthalate) for 3 days at 4^{o}C, in the dark.
- Step 11: Immersed in MMA II (80% methylmethacrylate, 20% dibutylphthalate and 0.5% perkadox) for 3 days at 4^{o}C, in the dark.
- Step 12: Immersed in MMA III (80% methylmethacyrlate, 20% dibutylphthalate and 1% perkadox).
- Step 13: Agitated briefly on a magnetic stirrer.
- Step 14: Samples left to rest at 4^{o}C, in the dark.
- Polymerisation was completed within 7 days**.**

Appendix 2

Stepwise protocol for paraffin embedding

- Immersed totally and agitated at room temperature in:
    - Step 1: 35% IMS 99: 1 hr
    - Step 2: 50% IMS 99: 1 hr
    - Step 3: 70% IMS 99: Overnight
    - Step 4: 95% IMS 99: 1 hr
    - Step 5: 100% IMS 99: 1 hr
    - Step 6: Fresh 100% IMS 99: 2 hr
    - Step 7: Fresh 100% IMS 99: 2 hr
    - Step 8: Fresh 100% IMS 99: Overnight
    - Step 9: Xylene: 2 hr
    - Step 10: Fresh xylene: 2 hr
    - Step 11: Fresh xylene: 2 hr
- Step 12: Immersed in paraffin wax at 60^0C: Overnight
- Step 13: Immersed in paraffin wax under vacuum at 60^0C: 4 hr
- Step 14: Tissues embedded.

Appendix 3

Stepwise protocol for CD31/VwF immunohistochemical staining

- Step 1: Sections immersed in 3% H_2O_2 (made with water) to block exogenous staining: 20m
- Step 2: Sections rinsed with Tris Buffered Saline (TBS) (20x, pH 7.6).
- Step 3: Pre-prepared blocking reagent (normal goat serum in phosphate buffered saline, with carrier protein) applied directly to sections to block exogenous staining: 22m
- Step 4: TBS rinse
- Step 5: Sections incubated with 2 primary antibodies; mouse anti-CD31 monoclonal antibody and rabbit anti-vWF polyclonal antibody: 2 hours at room temperature, in the dark.
- Step 6: TBS rinse
- Step 7: Sections incubated with pre-prepared biotinylated goat anti-rabbit immunoglobulin (IgG) and biotinylated goat anti-mouse IgG secondary antibodies (in phosphate buffered saline with carrier protein), at room temperature, for signal amplification: 12m 30s.
- Step 8: TBS rinse
- Step 9: Pre-prepared streptavidin-horseradish peroxidase (HRP) solution (diluted in TBS) applied directly to sections: 12m 30s
- Step 10: Diaminobenzidine (DAB) chromogen applied to sections and left in the dark: 10m
- Step 11: Sections rinsed in running tap-water for 7m 30s
- Step 12: Light-green counterstain applied to all sections.

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