Chapter 1: Introduction

Orthodontic forces are transmitted via the tooth and its root to the surrounding periodontal tissues. The generated mechanical strain is then converted to a biological response as cells participate in the remodeling of the supporting structures.[6]

Many cell types existing within the tissues surrounding the teeth are involved in this remodeling process. These include fibroblasts, endothelial cells, blood vessels and sensory nerves in the periodontal ligament as well as osteoblasts, osteoclasts and cementoblasts on the root and bone surfaces. The stress acting on the periodontal tissue during orthodontic tooth movement is believed to cause distortion of the nerve fibres and neuroreceptors within the tissue with injury and initiation of an inflammatory response. This leads to changes in the levels of expression and release of neurotrophins with effects on innervating nerves. The neurotrophins are also believed to interact with fibroblasts, endothelial and alveolar cells to initiate the process of bone resorption.[1]

Neurotrophins, including nerve growth factor (NGF), play an important role in neural cell differentiation and survival.[4] NGF is a polypeptide essential for supporting cholinergic innervation in the brain and sympathetic and sensory innervation in the peripheral tissues[7], as well as being required for the development of those neurons. Its presence allows for the neurons to grow and differentiate.[8] NGF is produced and released by target cells and reacts with specific receptors associated with the neuron and is transported to the cell body to maintain cell viability.[8]

The exact localization and function of neutrophins and neurotrophic receptors in the dento-alveolar complex remains unclear. Moreover, the identity and distribution of structures expressing neutrophins and neurotrophic receptors has yet to be fully determined.

O'Hara [5] carried out a controlled animal intervention study on 42, eight-week old male Sprague-Dawley rats. Orthodontic forces were applied to the right maxillary first and second molars. In some animals neutralising antibodies to NGF were injected into this region. The control and test maxillae were prepared for histological examination and immuno-staining to compare the relative intensities of NGF, CGRP, TrkA and p75 and focusing particularly on the region of alveolar bone remodeling. Immunohistochemical evidence of changes in the amount of NGF, NGF receptors and CGRP fibre density during the 2 weeks tissue remodelling period was observed, suggesting that transient increase in the levels of NGF synthesis and release by certain cells during the initial injury response period occurred.

An understanding of orthodontic tooth movement would be enhanced by an investigation of the cells responsible for NGF secretion within periodontal ligament (PDL), pulp and bone. In addition, knowledge of the effects that anti-NGF might have on orthodontic tooth movement, induced with a coil-spring orthodontic apparatus, would be revealing and provide a fuller appreciation of the distribution of neurotrophins and neurotrophic receptors in neural and non-neural tissue in the rat dento-alveolar complex.

Chapter 2: Aims and Hypotheses

Aims

1. To identity target cells associated with neurotrophin production and neurotrophic receptors expressed in the periodontal ligament during orthodontic tooth movement.

2. To investigate the effects of anti-NGF (antibodies) on tooth movement induced with a coil-spring orthodontic apparatus in rats.

The null hypotheses being investigated are:

1. Anti-NGF has no measurable effect upon the rate of orthodontic tooth movement.

2. Osteoclasts do not associate with NGF or NGF receptor expression.

Chapter 3: Review of the Literature

3.1 The Periodontium

3.1.1 Structure of the Periodontal Ligament

The periodontal ligament (PDL), which ranges in width from 0.15 to 0.4mm, is a fibrous connective tissue that occupies the periodontal space between the root of a tooth and its bony socket. It provides support and maintenance of the dentition and alveolar bone.[9] The PDL also serves a vital role in the transmission of neural input to the masticatory apparatus, to provide nutrition to the surrounding tissue, and to maintain alveolar bone height [9]. At the cervical region of the tooth, above the alveolar crest, the PDL merges with the gingival connective tissue. At the root apex, the PDL merges with the dental pulp. The PDL links the cementum of the tooth to the alveolar bone and provides anchorage by way of its orientated collagenous fibre bundles.[10]   The cells of the PDL form, maintain and repair the PDL as well as structures around it, such as adjacent alveolar bone and cementum.[11]   This ability to rapidly remodel forms the basis of orthodontics.

Orthodontic treatment is based on the principle that if prolonged pressure is applied to a tooth, tooth movement will occur as the surrounding bone remodels.[12] Bone is selectively removed in some areas and added in others. In essence, the tooth moves through the bone carrying its attachment apparatus with it, as the socket of the tooth migrates.[13]   Since the bony response is mediated by the PDL, tooth movement is primarily a periodontal ligament phenomenon.[14]

3.1.2 Cells of the periodontal ligament

The PDL contains cellular and non-cellular components. It comprises fibroblasts (43-55 percent of periodontal ligament connective tissue in rodents), vascular-related cells, nerve-related cells, inflammatory cells, undifferentiated mesenchyme cells, and the epithelial rests of Malassez.[9] At the periphery of the periodontal ligament, cementoblasts and osteoblasts line

hard tissue surfaces, and osteoclasts and odontoclasts may be evident in areas of resorptive activity.[15]

3.1.2.1 Fibroblasts

The fibroblast is a common and functionally important cell within the PDL. In health, fibroblasts regulate PDL tissue integrity and maintain its homeostasis.[16] It lies between collagen fibres and their appearance is governed by the surrounding matrix. They take the form of flattened irregular discs approximately 30 micrometers in diameter.[17] Fibroblasts contain high numbers of organelles and secrete structural proteins such as collagen and elastin, as well as PDL ground substance, in the form of glycosaminoglycans and glycoproteins. Fibroblasts are also capable of producing matrix-degrading enzymes such as collagenase and other matrix metalloproteinases.

3.1.2.2 Cementoblasts

Cementoblasts are cells that secrete the organic (mainly collagenous) matrix of cementum. Their appearance is dictated by activity and when active they appear as a distinct layer on the tooth root surface. A prominent feature is the accumulation of numerous glycogen granules in the cytoplasm. However, identification of them is often difficult as their appearance is often indistinguishable from fibroblasts of the PDL.[18]

Cells from the dental follicle differentiate into cementoblasts at the same time as root dentine formation occurs. Cementoblasts trapped within the mineralised cementum are called cementocytes and are responsible for the turnover of collagen and ground substance of the cementum. The resorption of cementum is undertaken by cementoclasts and odontoclasts, which are multinucleated cells derived from the monocyte/macrophage cell line. Brudvik and Rygh[19] suggested that mononuclear macrophage-like cells may also be involved. However, resorption only occurs under a functional stimulus, which is greater than that required for bony resorption .[10]

3.1.2.3 Epithelial Cells and undifferentiated mesenchymal cells

The epithelial rests of Malassez (ERM) within the PDL represent the remains of Hertwig's epithelial root sheath that may be histologically seen as strands of cells near the cementum surface.[10] They secrete enamel-like proteins onto the root surface. They are closely packed cuboidal cells located closer to the cementum than alveolar bone, stain deeply and are completely surrounded by connective tissue cells.[20] They are contained within an almost complete basal lamina, have a high nuclear to cytoplasmic ratio. They exhibit a prominent nucleus that contains condensed heterochromatin and often show invaginations. With age, the number of epithelial cells reportedly decreases.[21] The function of these epithelial cells is unknown but it has been suggested that they may play a significant role in the maintenance of homeostasis in the tooth supporting structures.[22] Epithelial cells may also be involved in repair cementogenesis and in the maintenance of the periodontal ligament space.[23] A further more detailed description of ERM is in the text to follow.

Undifferentiated mesenchymal cells, found within 5μm of the blood vessels, are believed to provide new cells for the ligament .[10]

3.1.2.4 Non-cellular components

Collagen fibres, which are arranged in bundles, together with small proportions of oxytalan and elastin fibres form the non-cellular part of the PDL. The collagen is aggregated into bundles of tough fibre groups orientated in specific planes, which can be distinguished histologically. The fibre bundles extend across the width of the periodontal ligament space, attaching to the cementum on the tooth side and inserting into the cribriform plate of the alveolar bone as Sharpey's fibres.[9] It has also been suggested that individual collagen fibres do not necessarily extend the full width of the PDL, an immediate plexus sometimes exist near the apical one-third of the PDL.[9] Elastin is generally found associated with the blood vessels.[10] Oxytalan fibres are distributed extensively in the periodontal ligament to form a network that

surrounds the root and are associated with the neural elements. These fibres are thought to regulate vascular flow in relation to tooth function .[10]

3.1.3 Blood Supply of the Periodontal Ligament

The periodontal ligament is highly vascularized connective tissue [9, 10]. The vasculature in the periodontal ligament is adapted to enable it to withstand high intermittent pressures during mastication, and responds to continuous pressure during orthodontic tooth movement. It also provides for substrate and metabolite exchanges between blood and periodontal tissues, including dentine.[9, 10]

The major blood supply of the ligament is derived from the superior and inferior alveolar arteries as branches of the maxillary artery.[10] These arteries course through the alveolar bone and give off branches that penetrate the cribriform plate of the alveolus to enter the periodontal space. The vessels course in an apical-occlusal direction and have extensive transverse connections and arteriovenous anastomoses. Venous drainage occurs in an apical direction towards larger diameter venules. Lymphatics vessels drain similarly.[10]

Blood flow in the periodontal ligament has been suggested to be lower than in alveolar bone marrow due to the smaller sized periodontal ligament capillaries. However, blood flow through the periodontal ligament of rats has been reported to increase during orthodontic tooth movement, and was attributed to the increase in cellular activity during bone apposition and resorption processes.[22]

3.1.4 Nerve Supply of the Periodontal Ligament

The nerve supply of the periodontal ligament arises from the fifth cranial nerve, the trigeminal nerve, which emerges from the ventral surface of the pons, near its upper border, as a large sensory and a smaller motor root.[24] This provides the sensory supply to the face, scalp, nasal mucosa, oral

tissues, as well as the motor supply to the muscles of mastication. The trigeminal nerve innervates the PDL from either its maxillary nerve or inferior alveolar nerve branches. Nerve fibres supplying the PDL pass through foraminae in the alveolar bone to enter the PDL space close to the tooth apex, while others enter via the lateral aspect of the alveolar wall.[24, 25] Nerve fibres and associated blood vessels run parallel to the long axis of the tooth. They are mainly found in the two thirds of the ligament space adjacent to the alveolar bone.[26] Single myelinated and unmyelinated fibres have been observed near the avascular cementum region of the ligament.[27]

There is no confirmed evidence regarding the presence of the parasympathetic nerve supply within the PDL, while the postganglionic fibres of the sympathetic autonomic system arise from the superior cervical ganglion.[24] The sensory innervation subserves touch, pressure and pain as well as proprioceptive function; however, the majority of neurones also respond to mechanical stimulation by mechanoreceptors.[28] The cell bodies of these mechanoreceptors are located in either the trigeminal ganglion or in the mesencephalic trigeminal nucleus. Sensory neurons are usually classified physiologically according to their axonal conduction velocity and their adequate stimuli.[29] Mechanoreceptors within the periodontal ligament are likely to be the large diameter fibres (Aβ fibres), which respond to very low-threshold stimuli, such as forces applied to teeth and supporting structures.[29] Small myelinated Aδ fibres are slow conducting and are usually high-threshold mechanoreceptors and respond to noxious stimuli.[29] Unmyelinated C fibres form the majority of sensory neurons and they respond to noxious stimuli, as well as to crude touch and temperature.[30]

Freeman[10] described four types of neuro-receptors found in association with the periodontal ligament, namely; free nerve endings, Ruffini-like corpuscles, coiled endings and spindle-like endings. The free nerve endings have a tree-like configuration and are found throughout the length of the root, extending into the cementoblast layer. They originate from unmyelinated

fibres but the terminal ends carry a Schwann cell envelope. These neuro-receptors are believed to function as nociceptors [10, 31] and mechanoreceptors.[10] All nociceptors are free nerve endings but not all free nerve endings are nociceptors as some may respond to temperature and touch.[30] Ruffini-like receptors are found mainly in the apical segment of the periodontal ligament. They have a dendritic appearance and are ensheathed in Schwann cells. The two patterns that may be distinguished are a simple form consisting of a single neurite, and a compound form consisting of several terminations. These mechanoreceptors have finger-like processes that are anchored in the nearby collagen bundles that serve to increase the receptive field of the nerve terminals.[9, 26] The Ruffini-like terminals are able to monitor deformation of the adjacent collagen bundles, which leads to opening of the ion channels in the receptor membrane, thus allowing ions to pass leading to depolarization of the receptor. A coiled form of neuroreceptor whose function is unknown, is found in the middle section of the periodontal ligament.[10] Spindle-like terminals surrounded by a fibrous capsule are found near the root apex.[10] Oxytalan fibres in close association with blood vessels and nerves have been suggested to contribute to mechanoreception in the periodontal ligament.[32]

The distribution of nerves in the mouse periodontal ligament has been demonstrated by Freezer and Sims.[33] The apical third of the PDL was shown to have three times more nerve fibres than the middle third. 95% of the nerves were unmyelinated, with 64% concentrated near the alveolar region and only 3% near the tooth. These unmyelinated nerves were usually found associated with venous capillaries.

3.2 Tooth Movement

Orthodontic tooth movement relies on the transmission of mechanical stress from the dental roots to the surrounding tissues, where cells are stimulated to remodel the matrices that engulf them. Observations of the

histology of the stress-induced periodontal ligament show extensive cellular activities within the investing paradental tissues.

Reitan described several phases associated with tooth movement.[34] With light forces, tooth movement occurs in the first five days by the compression of the PDL. This period is shorter with excessive loads. This is then followed by a period of 2-3 weeks, during which only minor changes in tooth position are seen. A secondary period of tooth movement occurs after undermining of bone.

The pressure- tension theory initially described by Oppenheim in 1911,[35] relates the alteration in blood flow within the periodontal ligament in response to compression and stretching with tooth movement to cellular changes produced by a series of chemical messengers. This process mediates bone remodelling where osteoclasts resorb bone on the pressure side and osteoblasts deposit bone in areas of tension.

Waldo and Rothblatt devised a simple method for the study of tissue reaction to mechanical stress associated with tooth movement on the molar teeth of laboratory rats.[36] A specifically designed instrument was used to place an orthodontic elastic between the first and second maxillary molar. The ends of the elastic were subsequently cut, leaving an interproximal segment of elastic, which was allowed to return to original dimensions, thereby inducing tooth movement (Figure 1).

NOTE: This figure is included on page 25 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1: Distribution of stress on maxillary rat molars following placement of rubber band between first and second molars.[36]

A tipping movement is created, with the location of the fulcrum near the apical third of the molar roots. Separation of molars was achieved in 24 hours, and reached a maximum at 3 days with accompanying destruction of the interdental papilla.

Twenty-four hours after placement of the elastic, the PDL appeared narrower in pressure zones, wider in areas of tension, and an increase in inflammatory cells in the alveolar bone and PDL vascularity were also observed.[36] After three days, haemorrhage, PDL compression and undermining resorption with large numbers of multinucleated osteoclasts were seen in association with the first and second molars. The intensity of the response was reduced around the maxillary third molar.

By 3 to 5 days movement of the teeth appeared to be due to a combination of tipping and bodily movement, with the fulcrum closer to the apex, although, occasionally nearer to the alveolar crest.

After 5 days, the disparity in PDL widths associated with pressure and tension sides were even more marked. Root and bone resorption associated with clastic cell activity was observed in areas of compression. Small Howship's lacunae were seen as scalloped out craters in the bone on the pressure side. Thin strands of well demarcated new bone were seen on the tension side.

3.2.1 Responses of the periodontal ligament on tension side

As tooth movement commences, the periodontal space on the tension side becomes wider as it is drawn away from the alveolar bone.[13] Collagen bundles are stretched, and the alveolar crest is pulled in the direction of movement. After 30-40 hours, cell proliferation along the socket wall occurs, and osteoid tissue is deposited.[37] Whilst the superficial layer remains uncalcified, the deeper layers undergo calcification to produce bone within 3

to 5 days after force application. Osteoclasts are recruited from the PDL and aligned on the surface of the alveolar bone.

As tooth movement continues, blood vessels become distended, and fibroblasts become aligned with the principal fibres of the PDL. The volume of collagen fibres reduces as the volume of blood vessels increases, although an increase in non-oriented fibrils and elastic fibres increases from 2 to 14 days.[38] Increased levels of endoplasmic reticulum also indicate a high degree of cell activity.[39, 40] It has been suggested that the breakdown of collagen is mainly extra-cellular and due to collagenases produced through a macrophage and fibroblast interaction. In areas of tension, with heavy loads, the blood vessels of the region may become occluded resulting in cell death between stretched fibres. Resorption of parts of the alveolar bone may occur from both sides.[41]

Leukocytes, proteins and fluids transport and migrate into the PDL from the blood vessels in the early stages of tooth movement.[13, 39] The leukocytes are not only involved in a phagocytic role but produce a cascade of signalling pathways which are involved in the inflammatory processes required for tooth movement.[42]

3.2.2 Responses of the periodontal ligament on pressure side

It was suggested by Bien in 1966, that the initial effect seen in orthodontic tooth movement may be due to interstitial fluid movement and the tightening of randomly orientated fibers in contact with interlacing blood vessels.[43] This results in a relative resistance to PDL compression with heavy instantaneous forces but a more readily compressed PDL with light forces of long duration.[43]

Rygh and Brudvik[40] categorized responses to orthodontic tooth movement into "direct resorption" where pressure is light, and "hyalinisation" where greater pressures produce degenerative changes.

3.2.2.1 Direct resorption

During orthodontic tooth movement, the pressure side of the periodontal space becomes narrower and vascular activity is low. The blood vessels in the area of compression are occluded, but the blood supply to areas with osteoclastic activity are increased. After 30-40 hours, osteoclasts differentiate and line the alveolar wall and result in alveolar bone resorption.[37] Two patterns of resorption have been described, depending on the magnitude of the applied force. Light forces, below 25 g/cm^2, allows for the blood vessels to remain patent under compression.[34] This facilitates osteoclasts present in the ligament to begin resorption of the alveolar bone adjacent to the compressed ligament. This process is called 'frontal resorption'. Using electron microscopy, the ruffled border of osteoclasts has been shown to have close contact with resorbing alveolar bone surface, with crystals and collagen fibres being found between cell processes.[40, 44, 45]

3.2.2.2 Hyalinisation

Under application of high forces, compression in focal areas impedes vascular circulation and cell differentiation causing degradation of cells, to produce an area of sterile necrosis, termed hyalinisation.[34]

In an investigation into the response of rat periodontal ligament cells to orthodontic force in a bone formative section, Yee[46] found that there was an increase in cell mitosis 24 hours following stimulation, primarily in the central portion of the PDL. The dividing cells lacked cell processes, had extensive rough endoplasmic reticulum (RER), and many of the cells contained intracellular collagen containing vesicles which were characteristics suggestive of ligament fibroblasts. This implied that mitotic activity might be the mechanism by which functional connective tissue cells are generated. Between 48 and 120 hours following stimulation, newly generated osteoblasts and active bone formation were seen associated with the alveolar bone surface.

Within a few hours of application of force there is a retardation of blood flow followed by disintegration of blood vessels and other structures within the PDL. In addition, cell rupture leading to isolated nuclei being distributed between compressed fibrous elements occurs, and has been termed pyknosis.[47-50] Rygh,[51] found that the hyalinized compressed tissue was ingested by invading cells and blood vessels from adjacent undamaged PDL.

Tooth movement stops until the adjacent alveolar bone is resorbed and the hyalinized tissue is removed. This undermining resorption occurs as phagocytic cells from the periodontal ligament at the periphery of the hyalinized area and from bone marrow spaces remove bone and necrotic tissue.[40]

3.2.3 Summaries of Tooth Movement

The physiological response to sustained pressure against teeth is summarised by Proffit[52]

Table 1: Physiologic response to sustained pressure against a tooth (from Proffit[14] page 301)

> NOTE: This table is included on page 30 of the print copy of the thesis held in the University of Adelaide Library.

Roberts,[53] in an explanation of orthodontic tooth movement, divided the process into three phases; initial strain, lag phase and progressive tooth movement. (Figure 2)

Figure 2: Tooth movement graph. (from Roberts[53], page 231-237)

1. The initial strain occurs over the first week and ranges from 0.4mm to 0.9mm. It is a result of PDL displacement, bone strain and extrusion. Initial tooth displacement occurs within seconds, but compression of the PDL may take hours and it may be days until tooth movement stops.

2. During the lag phase there is no tooth movement. This phase coincides with the time taken for hyalinized tissue to be eliminated, and for the cortical bone to be resorbed from the bone marrow side. The time required for this is variable and may take from 2 to 10 weeks.

3. Progressive tooth movement is the third phase described. The PDL is widened and bone resorption occurs over a wider area and via frontal resorption if forces are kept light. Bone formation occurs on the tension side simultaneous to the changes on the pressure side.

Diurnal variation in tooth movement in response to orthodontic forces has been investigated by Miyoshi et al.[54] Six week old, male, Wistar rats were divided into one control group without any application of force, and three experimental groups, including; all-day, daytime- only, and nighttime only force application. Initial tooth movement was similar in the three experimental groups up until the third day. Thereafter, the all-day group showed a distinct lag phase followed by a phase of progressive tooth movement. The daytime-only group showed no lag phase but a continued tooth movement and was

significantly greater than the all-day group at days 7, 10 and 14 days. In the nighttime group tooth movement was significantly less, and at 21 days was half that of the other two groups. Formation of new bone on the tension side and osteoclast numbers on the pressure side were greater in the all-day group and the daytime only group. The day-only group showed less hyalinisation than the other groups. Root resorption was least in the night-only group. These results suggested that there is considerable variation in response to orthodontic tooth movement at different times of the day and that diurnal rhythms in bone metabolism have important implications in orthodontic treatment.

3.2.4 Osteoclasts

The appearance of clastic cells is an initial step observed in the process of orthodontic tooth movement, necessary for removal of hyalinized tissue and alveolar bone in order that tooth movement may proceed.

Rody et al[55] investigated the origin of osteoclasts involved in sites of compression during orthodontic tooth movement in 30 day old male Sprague-Dawley rats. Progenitor cell DNA was labelled with 5-bromo-2-deoxyuridine (BrdU) at time of orthodontic force application. BrdU is incorporated into the nuclei of cells in the S-phase of the cell cycle. Committed pre-osteoclasts and mature osteoclasts in the PDL are post-mitotic; therefore, the appearance of BrdU in these cells would suggest recruitment from precursor pools after application of orthodontic force. The significant increase in number and percent of BrdU positive pre-osteoclasts in the PDL at day 3 would suggest that osteoclasts in the PDL originate from the haemopoietic tissue rather than from local PDL cells.

The mechanism by which osteoclasts are cleared from the PDL and alveolar tissue during orthodontic tooth movement is probably by cell death.[56] Two types of cell death described include necrosis and apoptosis. Necrosis is characterised by cellular swelling and rupture of the cell membrane. It usually affects layers of cells and results in release of chemotactic substances that

lead to inflammation. Apoptosis, a programmed cell death, is associated with condensation of chromatin around the nuclear membrane, loss of cell surface microvilli, and fragmentation of cells into membrane-bound particles. It is an essential process in development and turnover of tissues.[56]   Rana et al [56] used deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) to detect occurrence of apoptosis during orthodontic tooth movement in adult male Sprague-Dawley rats. They observed maximum apoptotic activity 3 days after application of force. Apoptotic cells were not observed at the one or two-week time points, possibly due to removal by macrophages.

Noxon et al used ApopTag staining (TdT mediated dUTP-biotin nick 3' end labeling and tartrate-resistant acid phosphatase (TRAP) to identify osteoclasts and committed pre-osteoclasts and to distinguish apoptotic from non-apoptotic nuclei during orthodontic tooth movement in rats. A highly significant increase in the percentage of TRAP-positive cells undergoing apoptosis in the periodontal ligament, on the bone surface, and in the marrow spaces was observed at days 3, 5, and 7. [57]   The greatest percentages of apoptotic cells were observed at 5 and 7 days, and coincided with a return to baseline levels of osteoclast numbers. This suggested that osteoclasts recruited for orthodontic tooth movement are removed, in part, by apoptosis.

3.2.5 Epithelial Rests of Malassez

Epithelial rests of Malassez are believed to be remnants of Hertwig's root sheath. Although several possible functions of them have been suggested, the exact nature of their role within the PDL remains unclear.

Reitan examined epithelial rests in the PDL of human teeth, extracted in orthodontic cases and compared to adult and certain animal Malassez epithelium.[58]   On the tension side, epithelial cells were observed to be slightly compressed between stretched fibre bundles, and to have moved a small distance from the tooth surface. On the pressure side, during direct bone resorption, the epithelial cells were situated more towards the root surface. In

areas of hyalinisation, the epithelial cells were observed to disappear within a few days. Reitan stated that "The epithelial cells of formerly hyalinized tissue do not reappear." He also concluded that an area with no epithelial rests of Malassez is an indication of reorganised, formerly hyalinized periodontal tissue.

Gilhuus-Moe et al found a reduction in number of epithelial rests on the pressure side of rat molars subjected to orthodontic tooth movement. On the tension side there is a proliferation of epithelial cells compared to the pressure side and control teeth. The cells appeared to be transferred from a resting state to a more proliferative state.[59]

Brice et al. [59] found epithelial cells in areas of repairing root resorption bays but not within actively resorbing bays, subsequent to tooth movement. Using a transmission electron microscope, ultra-structural detail was observed and cell identification was established. The clusters of cells were encircled by a basement membrane and confirmation of their epithelial nature was the presence of desmosomes and tonofilaments. Epithelial rests of Malassez adjacent to root surface that had not undergone root resorption consisted of a dark-staining type of cell, with many free ribosomes, polyribosomes and tonofilaments, and a light-staining type of cell. These cells made contact with the basement membrane but a darker-staining type of cell was found within epithelial clusters associated with repairing root resorption.[23]

Kittel and Sampson [60] in a 3-D reconstruction of root resorption and repair showed that the epithelial rests of Malassez were not separate islands but consisted of strands that branched and were interconnected. They observed that blood vessels were found between the tooth surface and epithelial cells in actively resorbing bays, whereas in repairing resorption bays there were no blood vessels between tooth surface and epithelial cells. Repair of the resorption bays was mostly observed in the centre of the bay whereas active resorption was seen at the periphery, where it often undermined surface cementum.[60]

Lambrichts et al. using electron microscopy, observed an intimate association between basal lamina of the epithelial rests of Malassez and Ruffini-like and free nerve endings. [61]

Kvinnsland et al investigated Malassez epithelium in cats for the presence of neuroendocrine cells.[62] Using immunohistochemical techniques, they found single cells within Malassez epithelial strands that expressed calcitonin gene related peptide (CGRP) and vasoactive intestinal polypeptide (VIP). These cells were closely situated to the root surface. The expression of substance P was weak. However, the expression of the three neuropeptides was similar to that seen in the basal epithelial cell layers in gingival rete pegs. It was, therefore, suggested that Malassez epithelium might have biological functions and significance in maintaining homeostasis in the tooth supporting structures, and that the neuropeptide content might indicate some endocrine function of the cell.[62]

In an ultrastructural investigation of epithelial rests of Malassez, Yamasaki found differences between cells in a resting state compared to proliferating cells.[63] The resting cells appeared in small islands or strands and had scant cytoplasm with poorly developed organelles. The proliferating cells were associated with increases in rough endoplasmic reticulum and free ribosomes, formation of actin microfilaments, and less prominent tonofilaments, desmosomes and loss of gap junctions. There was a difference between the outer cells on the periphery of clusters, and the inner cells described as tonofilament-rich prickle-like cells, suggesting differentiation and desquamation centripetally.

3.3 Cell biology associated with tooth movement

The initial effect of orthodontic tooth movement is physical, consisting of gradual movement of tissue fluids,[43] accompanied by increasing distortion of extracellular matrix and cells.[13] This distortion evokes a rapid cellular response by all mechanosensitive cell types.[42]

Storey[13] considers bone and periodontium as the supporting structures of the teeth, to display bioelastic and bioplastic properties related to the functioning of the dentition. Orthodontic forces exceeding bioelastic limits of the supporting structures results in translation of the tooth leading to connective tissue and vascular changes which, in turn, produce adaptive cellular proliferation and tissue remodeling. Forces exceeding bioplastic limits result in biodisruptive deformation, which leads to the interruption of nutrients, ischemia, cell death, inflammation and connective tissue rupture.[13]

3.3.1 Cellular Detection of Mechanical Strain

3.3.1.1 Cell Matrix Interactions

Extracellular matrix (ECM) and cell surface proteins are linked to the cytoskeleton. Orthodontic forces producing distortion can activate ion channels and enzymes. Osteoblast-like cells show an increase in Ca^{2+} for the duration of strain. Shortly after initiation of orthodontic force, phospholipase A is activated. This acts on arachidonic acid, by cleavage resulting in PGE2 which is subsequently followed by significant elevations in levels of cAMP and cGMP, which in turn act as second messengers, eliciting cell responses.[42]

3.3.1.2 Force Transduction by Cytoskeleton

Integrins are a family of adhesion receptors, which may have some mechanotransducer function. They are anchored in the plasma membrane and connected to the ECM by fibronectin and to cytoplasm microfilaments by talin, vinculin and α-actinin. Force is transduced to biochemical signals in anchorage dependant cells. Changes in the shape of a cell can affect the attachment mechanism and phenotypic expression of the cell. [42, 64]

3.3.1.3 Mechanosensitive Ion Channels

Most cells have ion channels regulating cell function and open on stress at the plasma membrane to allow the passage of cations including

calcium and potassium. Application of stress can activate stretch-activated ion channels and raise intra-cellular calcium.[42, 64]

3.3.1.4 Force- Cell Function Interactions

Mechanical forces can deform the cell or ECM leading to polymerization of cytoskeletal elements and modification of receptor availability, thereby affecting cellular function.[42]

3.3.1.5 Bioelectric signals

It has been suggested that bone is surrounded by an electric layer in which electric charges flow due to stress-related fluid flow. As bone is strained, interstitial fluid flows through the canalicular network, producing streaming potentials as mobile ions are displaced relative to negatively charged proteoglycans. This stress-generated potential may affect the charge of cell membranes. Fluid movement can generate shear stress, which may directly stimulate some cells.[42, 64]

3.3.1.6 Cellular translation of mechanical strain

A signalling cascade ensues the cellular detection of mechanical strain. Receptor activation leads to an increase in levels of the secondary messengers identified as cyclic adenosine monophosphate (cAMP) and inositol triphosphate (IP3). The signal reaches the nucleus via a series of kinase reactions, which initiates the transcription of immediate early genes (IEG), and the production of activator protein-1 (AP-1). AP-1 modulates activity of particular genes, and depending on various conditions, can produce cellular proliferation or differentiation.[64]

3.3.2 Signal molecules

As ECM fluids in the mechanically stressed PDL shift, sensory nerve endings release their contents, including VIP, SP, CGRP. SP stimulates bone resorption, increases vasodilation and capillary permeability, and increases

concentrations of cAMP and PGE2. VIP stimulates bone resorption, independent of PGE2, while CGRP increases blood flow.[42, 65]

Leucocytes in the PDL not only phagocytose necrotic tissue, but also produce numerous signal molecules. These products may be classified into cytokines, growth factors, colony stimulating factors, and cell adhesion molecules. Osteoblasts, fibroblasts, epithelial cells, endothelial cells and platelets can also synthesise many of these factors.[42, 65]

Cytokines found to affect bone metabolism are interleukin-1 (IL-1), IL-2, IL-3, IL-6, tumour necrosis factor- α (TNF- α), and gamma interferon (IFN-γ). Among these, IL-1α is the most potent stimulator of bone resorption. Other cytokines, bacterial products, neurotransmitters, and mechanical forces trigger its secretion. IL-1 acts to attract leucocytes, stimulate fibroblasts and endothelial cells, and enhance bone resorption. Osteoblasts are the target cells for IL-1α, which convey a message to osteoclasts to resorb bone. IFN-γ is a cytokine which appears to limit inflammation and favour tissue repair.[42, 65]

Growth factors including TGF-β (transforming growth factor β), PDGF (platelet derived growth factor), FGF (fibroblast growth factor) and IGF (insulin-like growth factor), colony stimulating factors such as G-CSF (granulocyte-colony stimulating factors) and M-CSF (macrophage-colony stimulating factors), parathyroid hormone related peptide (PTHrP) and prostaglandins including PGE2 have all been found to act as regulatory molecules in the tissue changes evoked by orthodontic forces.

Nitric oxide has also been implicated as an important regulatory molecule in bone formation and resorption. Hayashi et al administered N-nitro-L-arginine methyl ester-HCl (L-NAME), an inhibitor of nitric oxide synthases, throughout tooth movement in 9-week-old male Wistar rats. A significant reduction in tooth movement was observed associated with administration of L-NAME, leading to the conclusion that nitric oxide is an important biochemical mediator in the response of the periodontal tissue.[66]

3.4 Neurotransmitters

3.4.1 Protein Gene Product (PGP) 9.5 and Calcitonin gene-related peptide, CGRP

Protein gene product (PGP) 9.5 belongs to the family of ubiquitin carboxyl-terminal hydrolases, it is a general cytoplasmic marker of neurons and neuroendocrine cells and permits visualisation of the entire tissue innervation.[67] Calcitonin gene-related peptide, CGRP, is a 37 amino acid neurotransmitter derived from precursor proteins in nerve cell bodies.[68] It is found in the hypothalamus, thalamus and hippocampus of the central nervous system, and within the sensory, motor and autonomic fibres of the peripheral nervous system.[69] It is produced in small diameter neurons which give rise to unmyelinated C and thinly myelinated Aδ fibre, primary afferent fibres subserving the sensation of pain and temperature, as well as noxious stimuli.[69]

CGRP is involved in the inflammatory process by having a number of effects including; a vasodilatory effect, regulation of immune cells, proliferation of endothelial cells and an inhibitory action on bone resorption and a stimulatory effect on bone formation. CGRP and PGP 9.5 immunoreactive nerve fibres present in concentrated numbers in the PDL adjacent to the tooth root apex, are associated with, and follow blood vessels. Only some fibres approximate root surface and cellular cementum.[2] It has been demonstrated that changes in neural and blood vessel density, morphology, and distribution correspond to the sequence of connective tissue changes occurring with tooth movement. After 7 days of tooth movement, a marked increase in CGRP and PGP 9.5 labelled nerve fibres adjacent to the alveolar bone in the PDL as well as in the dental pulp were seen.[2] Periodontal and pulpal blood flow was also at its greatest after 7 days of tooth movement. Nerve fibres were not associated with root resorption areas until after 21 days. After 7 days enlarged clusters of epithelial cells, mainly in the furcation region and in the tension zone of the PDL, were observed with occasional expression of PGP 9.5.[70]

During tooth movement in rats, Kvinnsland and Kvinnsland found an increase in the number of immunoreactive nerve fibres in the coronal pulp as well as periapical tissues after 5 days of molar movement.[3] This suggested that CGRP immunoreactive nerve fibres might be involved in tissue responses during tooth movement.[3]

Saito et al observed CGRP immunoreactive nerves associated with blood vessels in the PDL. These nerves reached maximum density and intensity by three days, and returned to control levels by 7 days. It was suggested that CGRP may be directly involved in the remodelling process associated with tooth movement as well as its function as a neurotransmitter.[71]

During orthodontic movement, it has been demonstrated that there is a significant increase in numbers of pulpal microvessels, indicating an increase in angiogenic factors, which appear to be diffusable.[72] It is possible that there are pulpal substance P-expressing pain fibres and CGRP expressing fibres which provide a vasodilatory effect. No nerves have been shown to provide a vasoconstrictor action.[73]

CGRP and substance P immunoreactive nerves were found to increase in number from 24 hours to 28 days in the pulp and PDL of rat teeth subjected to orthodontic tooth movement compared to controls. Contralateral teeth not exposed to force also showed increases in CGRP and substance-P expressing nerves.[74] While nerves immunoreactive to VIP and neuropeptide Y (NPY) were seen in relation to blood vessels in the pulp and PDL, there were no significant neural changes during orthodontic tooth movement. This indicated that VIP and NPY might not be involved in tissue remodelling associated with tooth movement.[75] Nerves distributed with blood vessels in rat PDL have also been shown to express 5-hydroxytryptamine.[76]

Vandevska-Radunovic et al. found epithelial rests of Malassez in rats to be occasionally immunoreactive to protein gene product 9.5.[70] No nerves

were observed in root resorption areas after 7 days of tooth movement, but after 21 days calcitonin gene-related peptide immunoreactive nerves were found in close association with cementoclasts in root resorption lacunae.

Vandevska-Radunovic et al observed that axotomy delayed the expected increase in PDL blood flow associated with orthodontic tooth movement until sensory nerve re-innervation could be established. These authors proposed that neurogenic mechanisms are involved in the orthodontically induced inflammatory process.[77]

Yamashiro et al[78] evaluated the role of sensory nerve innervation in alveolar bone remodelling during tooth movement, by observing CGRP immunoreactivity (CGRP-IR). Tooth movement was induced in rats 7 days after the unilateral transection of inferior alveolar nerves. Denervation resulted in a decrease in CGRP-IR sensory nerve fibres compared to sham operated rat PDL and pulp, with no difference in the number of osteoclasts. Accompanying experimental tooth movement was a five-fold increase in CGRP-IR and in the number of osteoclasts. With nerve transection there were decreased CGRP-IR nerve fibres and osteoclasts. No significant change was seen in osteoblasts. Bone resorption was activated 24 hours after the induction of tooth movement and was associated with increases in CGRP-IR. This, however, did not indicate which neuropeptides acted directly or indirectly on osteoclasts although the results suggested that sensory nerves have an important role in regulation of bone resorption during tooth movement.[78]

Vandevska-Radunovic et al also observed that axotomy of the inferior alveolar nerve delayed recruitment of macrophage-like cells in the PDL of rat molars subjected to orthodontic forces. This would suggest that neuropeptide expressing nerve fibres interact with immunocompetent cells and are involved in the events of inflammation associated with tooth movement.[79]

3.5 Neurotrophins

3.5.1 Nerve Growth Factor (NGF)

Nerve growth factor (NGF) is a homologous, noncovalent homodimeric growth factor (12500 kDa/monomer), which elicits a wide variety of responses in selected neurons as well as some non-neuronal tissues.[80] NGF plays an essential role in the development, maintenance and survival of sensory and sympathetic nerves.[4]

It has been established that both neuronal and non-neuronal target cells have an important influence over the development and maintenance of innervating neurons.[81] NGF is synthesized and released from target tissues of parasympathetic neurons, a subpopulation of sensory neurons and cholinergic forebrain neurons in the central nervous system.[82] In peripheral sympathetic and neural crest-derived spinal sensory neurons, NGF is the retrograde messenger that provides a trophic effect. A number of cells produce NGF, including epithelial cells, smooth muscle cells, fibroblasts, and Schwann cells.[81] NGF is produced in target organs, reacts with specific receptors associated with the neuron and is transported to the cell body to maintain cell viability.[83] The majority of NGF responsive cells express both p75 and trk A receptors.[84]

Verge et al have demonstrated suppression of neuropeptide expression in transected rat sciatic nerves that have NGF receptors. They also suggested that NGF may be responsible for the reversal of injury-associated inflammatory responses.[85]

Wheeler et al studied the contributions of neurotrophins and their receptors to the injury response in rat molar teeth, over a 52 hour period.[86] The injury resulted from pulp exposure, acid etching, rinsing, and sealing with resin. Gelfoam implants were placed prior to sealing in some of the sample to determine retrograde transport of NGF. After 4-6 hours, an increase in NGF was noted in the dental pulp. Radiolabelled NGF was transported from the injury site to the trigeminal ganglion within 15 hours. Trk A expression was

elevated in the trigeminal ganglion by four hours, and reached a maximum by 52 hours. Trk A mRNA increase in the ganglion preceded the time at which conventional retrograde transport would deliver activated trk A to the cell body. It was proposed that trk A mRNA expression may be mediated by a rapid signalling mechanism.

There was an increase in p75 in the trigeminal ganglion at 28 hours post injury, and reached a maximum at 52 hours. BDNF mRNA increased 12 hours after injury, reaching 3-fold levels after 52 hours. This would indicate that retrograde transport of NGF may be required for this response. Trk B increases were seen after 28 hours. Double labelling neurons with a retrograde tracer fluoro-gold and trk B or BDNF, showed that tooth-innervating trigeminal neurons express BDNF but not trk B.

Wheeler et al concluded that, after injury, there was an up-regulation of NGF with subsequent activation of trk A. Both trk A and p75 are up-regulated and promote nerve sprouting and signals involved in nociception and the process of healing. [86]

Streppel et al,[87] transected the buccal branch of the facial nerve in a sample of rats. They detected NGF at 3 days, which peaked in concentration at 5 days and declined at 6 days post axotomy in the proximal nerve stump. In the distal stump NGF was noted at 1 day, peaked at 4-5 days and had declined by 12 days. Axonal branching from the end-bulb of the proximal cut end was noted within hours.

3.6 Neurotrophic Receptors

There are two different classes of cell surface receptors, which can be differentiated by their pharmacological properties.[82] The ligand-receptor interactions influence neuronal plasticity and survival.[88] NGF receptors may be categorized into high- and low-affinity receptors with some tissues

expressing both high and low affinity NGF receptors. However, low affinity receptors are 5-10 times more abundant.[89] The ligand-receptor complexes may produce effects lasting from milliseconds to days.[88] The distribution of nerve growth factor receptor has been shown to alter during trauma, nerve transection, and orthodontic tooth movement.[90]

3.6.1 p-75 (NGFR)

The neurotrophic receptor p75 is a single peptide chain of approximately 400 amino acid residues. It has been historically categorised as a low affinity receptor for NGF, as well as BDNF, NT-3 and NT-4. According to Meakin et al, the binding of p75 to NGF does not necessarily induce biological activity; however, the ability of p75 to create a higher affinity binding component suggests that it may be involved in a signal transduction mechanism.[82] The low affinity p75 receptor demonstrates fast dissociation kinetics, does not internalise NGF, and the variety of cells in which it is expressed do not respond to NGF.[89]

The p75 receptor in many cells exhibits low affinity binding; however, it also exists in a high affinity binding form.[91]

It has been suggested that p75 may mediate cell growth and differentiation of non-neuronal tissue. Expression of p75 was observed to shift from mesenchymal cells of the dental papilla in the early cap stage to inner enamel epithelial cells at later stages. Expression of p75 associated with inner enamel epithelium decreased as cell proliferation ceased. It was proposed that p75 may have a role in differentiation and proliferation during odontogenesis.[92] Expression of neurotrophic receptors in developing rat teeth was investigated by Luukko et al, who found mRNA expression for p75, TrkB and TrkC but not Trk A was evident. The expression of these receptors was associated with innervation development. The expression of a truncated form of TrkB and p75 appeared to coincide with cellular differentiation. The expression of p75, Trk B and Trk C during morphogenesis in culture suggests

that these receptors are not dependent on innervation, but may be involved in the development of innervation as well as some non-neural function.[93]

The p75 receptor has a wide distribution in comparison to the Trk receptors, and is expressed on numerous cells, including Schwann cells, motor neurons, meningeal cells, dental pulp cells, hair follicle cells, and cerebellar Purkinje cells,[84] as well as non-neuronal cells such as basal keratinocytes, mammary epithelial cells and spleen cells.[89]   An increased affinity for binding and, therefore, a greater responsiveness to NGF occurs when p75 and Trk A receptors are co-expressed. Chao et al postulated that this could be due either to the rapid binding of NGF to p75 increasing the concentration of NGF for Trk A, or an interaction of p75 with Trk A leading to a conformational change, creating a high affinity binding site.[84]

The survival of neurons is determined by the balance of target-derived survival versus apoptotic factors. These might be mediated by differential signalling via Trk or p75 neurotrophin receptor interactions with particular neurotrophins.[94]   Neurons may die in the absence of p75 receptor; however, this receptor is required for rapid and appropriate apoptosis of the neuron on withdrawal of NGF. NGF withdrawal and p75 signalling may converge to activate the same cell death signal.[94]

Majdan et al,[94] proposed that when a neuron reaches an inappropriate target, Trk A is insufficiently activated due to lack of NGF. In contradistinction, p75 may still be activated by neurotrophins such as BDNF leading to rapid apoptotic elimination of that neuron. Such a mechanism would ensure active apoptosis when a neuron is unsuccessful in obtaining an appropriate supply of neurotrophins. According to Majdan et al, it is also possible that, in neurons expressing Trk B, NGF is the p75 receptor ligand responsible for the apoptotic signalling.[94]

The distribution of Nerve Growth Factor (NGF) receptors in teeth and the PDL was investigated by Byers.[95]   In the pulp, NGF receptor

immunoreactivity (NGFR-IR) was observed in the perineural sheath for a short distance after entering the root. Dental free nerve endings in the predentine mostly displayed NGFR-IR. In the PDL, Ruffini endings and their associated terminal Schwann cell membrane were labelled. Other cells displaying NGFR-IR included Schwann cells along unmyelinated axons, odontoblasts or other NGFR-IR pulpal or neural cells, and pulp fibroblasts. Three distinct NGFR-IR patterns were observed.

1.   Densely stained membrane and cytoplasm of thin fibres appear to be nociceptive fibres.

2.   Large Ruffini mechanoreceptors with NGFR-IR vesiculated terminal Schwann cells but no NGFR-IR of the terminal fingers.

3.   Large myelinated dental axons with little or no NGFR-IR of their terminal unmyelinated fibres but with much NGFR-IR of vesiculated endoneurial cells.

Saito et al.[90] investigated the NGF receptor immunoreactivity in the PDL of rats during orthodontic tooth movement. The time course data points used were 1, 2, 6, and 12 hours and 1, 3, 5, and 7 days after the insertion of elastic between maxillary first and second molars. The PDL of the upper second molar was examined for changes in NGFR immunoreactivity. NGFR stained nerve bundles in the control side were observed mainly in the apical and intermediate regions of the distal periodontal ligament and tended to be positioned around blood vessels.

After one hour of tooth movement, NGFR immunoreactivity was slightly reduced in the coronal and intermediate regions of the distal side. After 12 hours, greater immunoreactivity was evident in the coronal and intermediate portions compared to the controls. After 3 days, further intensity of stained nerves was noted on the distal side mostly distributed around blood vessels. Several nerve fibres were adjacent to resorptive lacunae on bone and root surfaces. After 5 days there was a decrease in distribution of reactive nerve fibres on the distal side to approximately the same levels as the control. On the mesial side there was an increase in immunoreactive nerve fibres, which

were close to blood vessels. After 7 days staining intensity for NGFR decreased.[90] They suggested that an alteration in the distribution and the intensity of immunoreactive staining for NGFR in the periodontal ligament is associated with the bone remodelling induced by orthodontic tooth movement.

3.6.2 Tyrosine Receptor Kinase (Trk) Family

The Trk receptors, are constituted with tyrosine kinase in their cytoplasmic domain, with a leucine rich and an Ig segment in the extracellular domain for binding of the different neurotrophins.[91]

1.6.2.1   Trk A

Tyrosine receptor kinase A (Trk A) is a heavily glycosylated peptide chain of approximately 790 amino acid residues. It displays high affinity binding and has slow dissociation kinetics. Trk A has been shown to mediate biological activities of NGF in the absence of p75.[82] Trk A binds to NGF with low affinity at most sites; however, due to studies revealing a small percentage of Trk A receptors having a high affinity binding, the receptor has been assigned as the high affinity binding site.[84]

Trk A is a high affinity membrane-bound protein that mediates responses to NGF. Yamashiro[96] found cell surfaces of epithelial rests of Malassez to be Trk A immunoreactive in the cervical and furcation regions of the rat molar. No immunoreactivity was observed in nerve fibres or other non-neuronal cells in the PDL. Denervation of the inferior alveolar nerve resulted in a decrease in the distribution area and size of clusters of immunoreactive cells after one week with a greater decrease still after three weeks. From this it was suggested that the epithelial rests may have a role in alveolar bone remodelling in association with the nerves in the PDL and that sensory nerve endings might regulate or maintain epithelial rests of Malassez expressing Trk A. The periphery of osteoclasts have also been reported to have weak immunoreactivity to Trk A in other studies.[97]

Woodnut and Byers[98] found that an antibody recognising the 41-kDa truncated form of Trk A showed an intense immunoreactivity in the epithelial rests of Malassez (ERM) in the rat and specifically around the periphery of clusters. These were usually found close to the cementum, but occasionally near alveolar bone or within alveolar lacunae. Tooth injury without PDL injury did not affect the immunoreactivity, form or amount of ERM. They suggested that the receptor may mediate some cellular or cluster function by binding NGF. Similar Trk A immunoreactivity was found between ERM and junctional epithelium. Nerves immunoreactive to p75 neurotrophin receptor were only rarely observed to be associated with ERM.

Chapter 4:   Materials and Methods

4.1   Materials

1. The initial part of the project consisted of examining previously prepared histological slides of posterior segments of rat maxillae, comprising three molar teeth.[5]

2. In the second stage of the project, the hypotheses were tested. 28, eight-week old male Sprague-Dawley rats, each weighting between 250 and 350g were used. The animals were fed a diet of commercially manufactured standard pellets (Parastoc Feed, Ridley AgriProducts, Murray Bridge, Australia) and water, ad libitum. They were housed in the Animal House facility of the Medical School of the University of Adelaide. The Ethics Committee of The University of Adelaide granted approval for the experimental procedure under ethics number M-023-2006.

   An initial pilot study with two animals was conducted to evaluate the appliance design and placement. The main experiment consisted of 28 animals divided into four groups. Two groups provided controls while the remaining two groups were test animals for the administration of anti-NGF. Each animal was provided with an identification number according to drug administration date and sequence. Animals were sacrificed at 7 and 14 days after the placement of closed-coil spring, according to Table 2.

Table 2. Sample size and categories

DAY	CONTROL	ANTI-NGF INJECTED	TOTAL
7	7	7	14
14	7	7	14
TOTAL	14	14	28

3. The final stage of the project involved histological examination using TRAP stain following the modified technique of Goldberg and Barka.[99] See Appendix 8.2

4.2 Methods

Part 1

4.2.1 Histological evaluations

Previously prepared histological slides from O'Hara[5] of posterior segments of rat maxillae, comprising three molar teeth were used. O'Hara[5] carried out a controlled animal intervention study on 42, eight-week old male Sprague-Dawley rats over 14 days period. Orthodontic forces were applied by placement of orthodontic alastics between the right maxillary first and second molars. In test animals, neutralising antibodies to NGF were injected into the molar region.

These histological slides were prepared to investigate the identity of the cells that exhibit changes in levels of NGF and NGF receptor expression in the first two weeks after application of orthodontic forces.

Part 2
4.2.2 Anaesthesia

The animals were weighed and anaesthetized to facilitate closed-coil spring placement, which provided forces for orthodontic tooth movement.

1. For close coil spring placement and tooth movement measurements: Rats were anaesthetized by intraperitoneal injection of Ketamine (100mg/ml) (Ketamil Injection, Troy Laboratories, Smithfield Australia) and muscle relaxant Xylazine (Xylazil, 20mg/ml). The two drugs were mixed at the ratio of 2:1 (Ketamine: Xylazine) and then diluted 1:1 with sterile water for injection. This was then administered intraperitoneally at a dosage of 2ml/kg of body weight. This provided a short lasting

anaesthesia to allow placement of closed coil spring and record tooth movement measurements.

2. A second anaesthetic procedure was required immediately prior to sacrifice and was provided by chloral hydrate 5g/100ml H_2O. This was administered intraperitoneally at a dosage of 5ml solution per rat in order to provide anaesthesia for the intra-cardiac perfusion of fixative. Ketamine and Xylazine were used for the first anaesthetic procedure due to their known safety whilst chloral hydrate was chosen as the anaesthetic to facilitate sacrifice due to its ready availability.

General anaesthesia was tested via the plantar reflex of the anaesthetized animals and checking for chest movements.

4.2.3 Application of Orthodontic Force

The anaesthetized animal was placed onto a holding rack. The mouth was gently propped open by two rings attached with elastic bands to the rack (Figure 3). A split-mouth design was used, with the experimental side on the right and the control on the left side. A 100 gm superelastic sentalloy (NiTi) closing coil spring (GAC international Inc. Bohemia, NY, USA. Catalogue number 10-000-06) was used to impart a mesialising force to the right maxillary first molar tooth. The sentalloy springs were ligated with 0.010" stainless steel ligature wire anteriorly to the maxillary incisors and posteriorly to the cervical region of the left maxillary first molar. Light cured bonding material (3M ESPE Z100 Restorative, St. Paul, USA) was used for bonding the 0.010" stainless steel ligature end of the closed-coil spring to the specific rat's tooth. Care was taken not to injure the surrounding soft tissue (Figure 4).

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Figure 3: Animal was placed onto a purpose-built holding rack

Figure 4: Photograph of experimental spring setup with wire ligature

4.2.4 Calibration of force delivery

Ten closed-coil springs were randomly selected and the superelastic properties of the material and the delivered force were tested with a Hounsfield Universal Testing Machine (Figure 5, 6). The springs were fastened on the machine and the sensors were moved apart until activation of the spring was initiated. At the last half millimetre, the movements were set to

0.05mm per step until a force level of zero had been obtained. The distance between the sensors was then increased by steps of 0.5mm until a 100 per cent extension of the spring (6mm) had been reached.

Figure 5: Hounsfield Universal Testing Machine

Figure 6: Force delivered by the coil springs was calibrated

4.2.5 Tooth movement measurement

Measurement of tooth movement was done by two different methods. The first method employed a digital caliper at Day 0, 3, 7, 14 under general

anaesthesia (Ketamine/Xylazine). The distance between the most mesial point of the maxillary first molar and the most distal point of ipsilateral incisor (distance M-I) at the ginigival level was measured on the experimental and contralateral sides. All distances were measured 3 times.

The second method indirectly used VPS impressions (Imprint 3, 3M ESPE) taken intra-orally with a special tray, with the spring in place, at Day 0, 3, 7, 14. A plastic sleeve was placed around the closed-coil spring before the impression was taken to prevent the spring from becoming embedded in the impression. Photos of the impressions were taken under light microscopy (MZ16FA stereo microscope), and were calibrated by Image analysis software (analySIS® a SOFT IMAGING SYSTEM product).

Figure 7: Direct measurement with digital calliper

Figure 8: Impression taken with special tray

Figure 9: Photographic apparatus

The photographs were taken by the following method:

1. The impressions were securely placed on a glass table under the microscope;
2. The impressions were levelled with respect to the central incisors and the first molars;

All photographs were digitized on a PC computer utilizing Image analysis software (analySIS® a SOFT IMAGING SYSTEM product).

The following digitizing procedure was employed:
1. The impression's details (animal number, date of impression) were recorded and entered into the computer programme;
2. Endpoints on the millimetre rule were digitized which accounted for magnification variation;
3. All landmarks were digitized in a specific order by aligning the cursor cross-hairs over the landmarks and gently pressing the button on the cursor which recorded the X and Y coordinates of the selected landmark. Anatomic points were digitized for M1 (molar) of both sides, and central incisors;
4. This procedure was repeated for each impression.
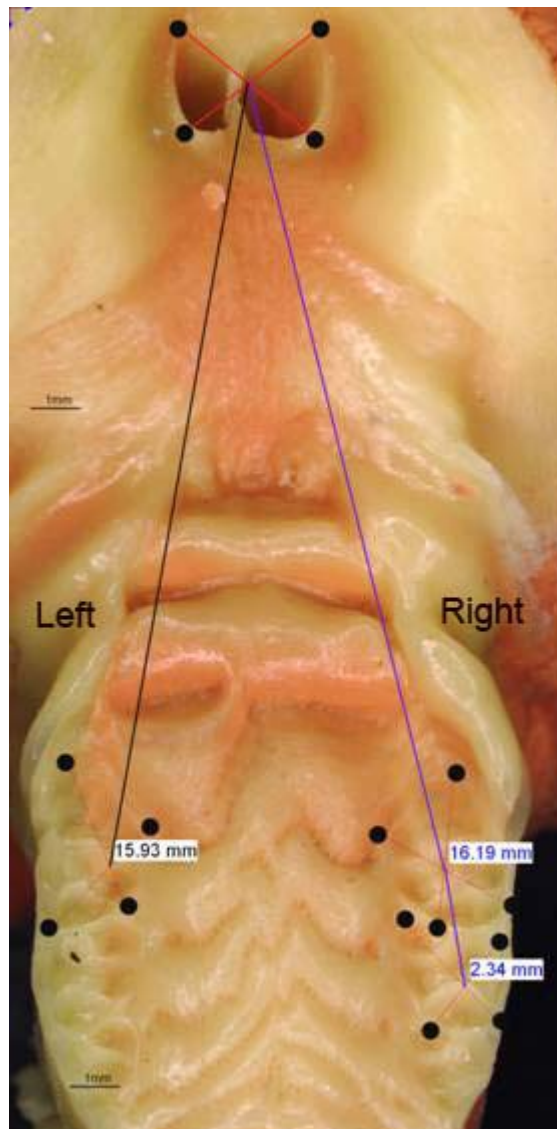
Figure 10: Impression landmarks

For M[1] and M[2], four points were recorded; most distobuccal point, most mesiobuccal point, most mesiopalatal point, most distopalatal point. For incisors, the most distobuccal points and most mesiobuccal points were recorded.

The centroid was determined by using these four points located on the circumference of the dental crown.
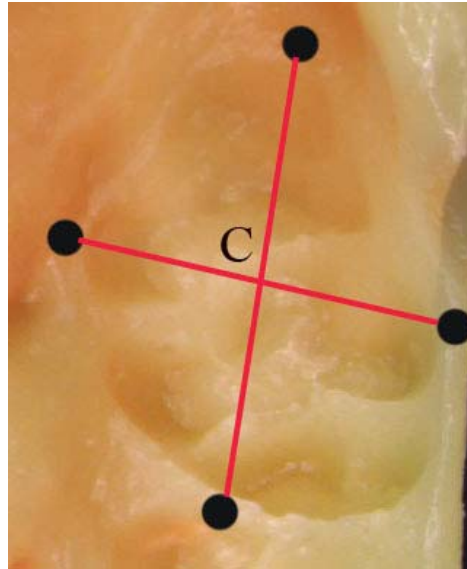
Figure 11: Illustration of centroid determination

4.2.6 Injection of Anti-NGF

In all animals of the test groups, 1.0 μl containing 2μg of anti-NGF (Appendix 8.3.7) was injected into the gingival mucosa between the first and second molars, which corresponded to the area of orthodontic force application. Same amount of saline was injected into the animals of the control groups. The injections were made after application of the closing coil spring and whilst the animals were still under anaesthesia and were repeated at Day 3 and 7. The animals were returned to cages in the animal house and allowed to recover.

Figure 12: Injection of NGF into the gingival mucosa

4.2.7 Perfusion of animal

The animals were perfused at 7 and 14 days after coil spring placement and anti-NGF administration at the Animal House. Animals were anaesthetised with the chloral hydrate solution (5gms/100mls) as above. The chest cavity was opened and rib cage removed to allow open access to the heart and aorta. A wide bore needle of the perfusion apparatus was placed into the left ventricle and the right atrium was cut to allow venous return to drain. Each animal was pre-perfused under positive pressure with 200-300ml of previously prepared 0.1% sodium nitrite solution for 5 minutes which caused vascular dilation to facilitate tissue perfusion. (Figure 13)

Figure 13: Perfusion apparatus

Figure 14: Animal at completion of perfusion, note the brown colour of all tissues and extremities

4.2.8 Fixation

200ml of 0.4% *p*-benzoquinone solution and 200ml of 4% para-formaldehyde were combined and perfused into the left ventricle under positive pressure for approximately 15 minutes. A satisfactory level of fixation was indicated when the extremities became brown in colour. (Figure 14)

4.2.9 Dissection of the maxilla

The maxilla and cranial vault were dissected out and superfluous soft tissues removed. The removal of the cranial vault allowed visual inspection of the brain and the degree of tissue perfusion. The qualitative measure of staining within the brain was the deep brown neural discoloration. The dissected maxilla and cranium were then placed into *p*-benzoquinone and 4% para-formaldehyde for approximately 2 hours.

4.2.10 Decalcification

After 2 hours of immersion fixation, the tissues were placed in 4% EDTA solution at ph 7.0. This solution was changed every second day for 14 days and then twice a week for a further 6 weeks, giving a total decalcification time of 8 weeks. At the completion of this time frame the tissues were examined radiographically to confirm decalcification. All the tissues were then stored in EDTA solution awaiting further analysis in future projects.

Part 3
4.2.11 Removing cover slips protocol

Previously prepared histological slides from O'Hara[5] of posterior segments of rat maxillae, comprising three molar teeth were used. All cover slips were removed from the slides according to the protocol as shown in Table 3, and prepared for TRAP staining. (see Appendix 8.2)

Table 3: Immersion protocol for coverslips removal

	Solution	Duration
1	Xylene	48 hours
2	100% Ethanol	2 min
3	50% Ethanol	2 min

4.2.12 Coverslipping protocol

Cover-slipping of the stained slides were undertaken with care to avoid dehydration of the sections, which could distort tissue morphology. Care was also taken not to introduce bubbles within the mounting media. Slides containing bubbles that were detected at the microscopy stage were later immersed for a few days in xylene and re-coverslipped.

4.2.13 Light microscopy

A light microscopic survey was done on all the slides to select a representative section for each slide. Photomicrographs of sections of interest were taken using a Multiphoton microscope (Bio-Rad Radiance 2000MP Visualising System) at Adelaide Microscopy; the University of Adelaide. Photomicrographs were taken of the roots of the maxillary first and second molars at 4x, 10x, 20x and 40x magnifications. The resulting digital photomicrographs were then collated for assessment.

4.2.14 Error Study

To determine method error of the tooth measurement, all measurements were repeated at the completion of the experimental time periods. The impressions were re-scanned and re-digitised for the anatomical landmarks. These were then re-measured for the tooth movement between maxillary first molar and the incisors.

All the measurements were recorded by one examiner, which eliminated inter-examiner error.

4.2.15 Statistical Analysis

All calculations were performed using SAS Version 9.1 (SAS Institute Inc., Cary, NC, USA). The Type 3 Tests of Fixed Effects were used to show the significance of anti-NGF on tooth movement.