ORTHODONTIC TOOTH MOVEMENT AND NEUROTROPHINS IN THE RAT DENTO-ALVEOLAR COMPLEX

Doctor of Clinical Dentistry (Orthodontics)

Thesis

October 2009

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AUSTRALIA

2009

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Figures and tables

Glossary of Abbreviated Terms

Ab	Antibody
Anti-NGF	Anti-Nerve growth factor
AP	Activator protein
ABC	Avidin-biotin complex
Ag	Antigen
cAMP	Cyclic adenosine monophosphate
CGRP	Calcitonin gene related peptide
CSF	Colony stimulating factor
DAB	3'-diaminobenzidine tetrahydrochloride
DNA	Deoxyribonucleic acid
DPM2	Distopalatal root of second molar
ECM	Extracellular matrix
EDTA	Ethylenediaminetetra-acetic acid
H	Hydrogen
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin like growth factor
IL	Interleukin
IFN-γ	Interferon gamma
IMVS	Institute of Medical and Veterinary Science
IP3	Inositol triphosphate
IR	Immunoreactive
IU	International units
K	Potassium
M	Molar (molarity)
M1	Maxillary first molar
M2	Maxillary second molar
*m*RNA	Messenger ribonucleic acid
NGF	Nerve growth factor
NGFR	Nerve growth factor receptor
NGS	Normal goat serum

NHS	Normal horse serum
NO	Nitric oxide
NT	Neurotrophin
O.C.T.	Optimal cutting temperature
PBS	Phosphate buffered solution
PDL	Periodontal ligament
RNA	Ribonucleic acid
TBS	Tris Buffered Solution
TGF-β	Transforming growth factor – beta
TNF	Tumour necrosis factor
Trk	Tyrosine receptor kinase

Abbreviations of length

m	Metre
mm	Millimetre
μm	Micron
nm	Nanometre

Abbreviations of time

d	Day
h	Hour
min	Minute
s	Second
wk	Week
y	Year

Abbreviations of volume

L	Litre
ml	Millilitre
µl	Microlitre

Abbreviations of weight

g	Gram
kg	Kilogram
mg	Milligram
ng	Nanogram
Da	Dalton
kDa	KiloDalton

Declaration

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Acknowledgements

I express my appreciation and gratitude to the following people for their invaluable assistance in the completion of this thesis.

Professor Wayne J. Sampson, P.R. Begg Chair in Orthodontics, University of Adelaide.

Associate Professor Mounir Ghabriel, Anatomical Sciences, School of Medicine, University of Adelaide.

Dr. Ian Ferguson, National Centre for Adult Stem Cell Research, Griffith University, Queensland.

Dr. Henry S.H. Ho, Specialist Orthodontist, Sydney, New South Wales.

Mr. Chris Leigh and Ms. Gail Hermanis, Technical Officers, Anatomical Sciences, School of Medicine, University of Adelaide.

Dr. Ian Parkinson, Senior Medical Scientist, Directorate of Surgical Pathology, South Australian Pathology and Hanson Institute, Adelaide; Lecturer, Discipline of Pathology, University of Adelaide.

Dr. Stephen Helps, Postdoctoral Fellow, Discipline of Pathology, School of Medical Sciences, University of Adelaide.

Ms. Sandie Hughes, Laboratory Officer, Oral Pathology, University of Adelaide.

Dr. Craig W. Dreyer, Senior Lecturer in Orthodontics, University of Adelaide.

Dr. Angela Pierce, Orthodontic Unit, School of Dentistry, University of Adelaide.

Professor P. Mark Bartold, Director, Colgate Dental Research Centre, Adelaide Dental Hospital, Adelaide.

Australian Society of Orthodontists Foundation for Research and Education for their funding.

My parents, Esther and James, brothers Hector and David, and pet dog Scooby for their support.

SECTION 1

Introduction

During orthodontic tooth movement, stress is applied on the dento-alveolar complex, initiating a biological response. This response results in the remodelling of the periodontal ligament and the alveolar bone. When a force is placed on a tooth, the periodontal ligament is stretched and compressed depending on the direction of force. On the side where the ligament is stretched, a response resulting in bone deposition is initiated. On the opposite side, where the periodontal ligament is compressed, a response resulting in the resorption of bone begins[1].

These responses are believed to be modulated by factors that are derived from the immune or nervous systems. When stress is placed on the periodontal ligament, it is believed that nerve fibres and neuroreceptors within the tissue are distorted, leading to the release of neurotrophins and a common concomitant clinical response of pain and pressure. These neurotrophins may interact with cells within the dentoalveolar complex, including fibroblasts, endothelial and alveolar bone cells, resulting in the initiation of bone resorption via the activation of intracellular secondary messengers, which leads to cellular proliferation and differentiation[2].

Neurotrophin levels may play a role in the modulation of cellular activity in the periodontal ligament during orthodontic movement[3]. They are a family of protein polypeptides which are important in neural cell differentiation and survival[3]. One relatively well studied member of the family, Nerve Growth Factor (NGF), is a polypeptide essential for supporting cholinergic innervation in the brain and sympathetic and sensory innervation in the peripheral tissues[4]. Within the dento-alveolar complex, the function and localization of neurotrophins and their receptors are yet to be determined.

Previous studies have shown that there is an increase in NGF expression in tissues in response to injury, suggesting that NGF expression may increase in regions within the dento-alveolar complex where inflammation and bone remodelling are occurring[5]. A study showed elevated levels of NGF mRNA in human periodontal ligament cells *in vitro* during increased transcription and translation of the bone-related proteins

alkaline phosphatase and osteopontin, suggesting NGF facilitates bone formation[3]. The NGF may be modulating cellular activity within the periodontal ligament.

O'Hara et al. used immunohistochemical staining in the rat dento-alveolar complex to show that there was evidence of an increase in NGF synthesis and release by certain cells or tissues within the region of alveolar bone remodelling during the initial injury response period to orthodontic tooth movement[5, 6].

Ho used a similar model to test the hypothesis that there may be a positive relationship between the presence of osteoclasts and pre-osteoclasts with areas of NGF localisation. He found no relationship between osteoclasts and areas of NGF; however, his findings showed areas of unknown tissue within the periodontal ligament that were associated with NGF[7].

To date, the relationship between NGF and the cellular process of orthodontic tooth movement remains unknown. Further studies are required to describe the distribution of neurotrophins and neurotrophic receptors and cellular interactions within the rat dento-alveolar complex.

LITERATURE REVIEW

1. Embryology of the Periodontium

The periodontium attaches the tooth to the alveolar bone, and maintains the integrity of the masticatory mucosa of the oral cavity. It forms the “attachment apparatus” and the “supporting tissues of the teeth”, establishing a developmental, biological and functional unit. This undergoes change according to changes in oral environment, due to morphological or functional causes.

The periodontium comprises (i) the gingiva, (ii) the periodontal ligament, (iii) the root cementum and (iv) the alveolar bone. The alveolar bone itself comprises two continuous components: (i) the alveolar bone proper and (ii) the alveolar process, forming the thin bone plate immediately lateral to the periodontal ligament[8].

1.1. Neural crest formation

The periodontium forms simultaneously with the development and formation of teeth. This process begins in the embryonic phase when cells are migrating from pleuripotent neural crest of the embryo into the first branchial arch. These cells form a band of ectomesenchyme beneath the epithelium of the stomatodeum, or primitive oral cavity[9].

1.2. Formation of the enamel organ

The neural crest cells migrate until they reach their desired locations within the jaw space. Epithelial cells within the stomatodeum release factors which initiate interactions between these two cell groups, causing the ectomesenchyme group to develop further into the dental epithelium or the enamel organ, which comprises the dental papilla and the dental follicle proper. Further processes are initiated which form the tooth and surrounding structures. Stages of this process have been characterised via the shape of the dental epithelium, into the: (i) bud, (ii) cap and (iii) bell stages of development. The enamel organ plays a key role in forming the dental papilla, which determines the shape and form of the tooth[9].

Each layer of the tooth germ forms an integral part of the tooth and attachment apparatus. The enamel organ forms the enamel component, the dental papilla forms the dentine-pulp complex and the dental follicle forms the attachment apparatus (the periodontium).

1.3. Root formation

The root and periodontium tissues develop in a similar pattern to crown formation. The root dentine forms originally from mantle dentine that projects from the coronal dentine. The unmineralised mantle dentine forms in an apical direction and is the framework on which the root is established. Acellular cementum then begins to form in a series of events which remain unclear, possibly involving Hertwig's epithelial root sheath. After root dentine formation has begun, these cells synthesise and secrete enamel-related proteins, from the amelogenin family. The root sheath becomes fenestrated towards the end of the period of protein synthesis, allowing ectomesenchymal cells from the dental follicle to penetrate and contact the root surface. When these cells contact the enamel-related proteins they differentiate into cementoblasts and secrete cementoid. Cementoid consists of an organic matrix of cementum, ground substance and collagen fibres. The cementum becomes firmly attached to the outer layer of the mineralised dentin via embedded collagen fibres. Some of the ectomesenchymal cells remain in the cementum on the apical third of the root[8].

1.4. Periodontal ligament and alveolar bone proper formation

The periodontal ligament and alveolar bone proper are formed by remaining parts of the dental follicle adjacent to the cementum. The follicle forms periodontal fibroblasts, fibres of the periodontal ligament and osteoblasts. Some ectomesenchymal cells may remain in the mature periodontium, as Rest cells of Mallasez, and are proposed to be involved in local tissue turnover[8].

2. Histology of the Periodontium

The periodontal ligament is a richly vascularised and cellular connective tissue which surrounds the roots of teeth. It joins the root cementum to the alveolar bone proper. Coronally, it is continuous with the lamina propria of the gingiva, to which collagen fibre bundles connect the alveolar bone crest to the root. Through the alveolar bone proper run vascular channels, known as Volkmann's canals. These canals allow for communication between the periodontal ligament with the marrow spaces of the alveolar bone[8].

The periodontal ligament space is hour-glass in shape, narrowing at the mid-root level and ranging in width from 0.15 to 0.40 mm. It is highly dynamic, having great capacity for remodelling allowing for orthodontic tooth movement.

2.1. Function

The main function of the ligament is to distribute and absorb forces, such as those caused by mastication, tooth contact or orthodontic force application, into the alveolar process via the alveolar bone proper. The ligament links the cementum of the tooth to the alveolar bone and provides anchorage through its orientated collagenous fibre bundles[8].

Apart from this primary function, the ligament also provides for the transmission of neural input to the masticatory apparatus to provide nutrition to the surrounding tissue, and to maintain alveolar bone height. Tissues are maintained continuously by the ligament, from the initial development of the tooth to its erupted functional state. A vast network of blood, lymphatic and neural tissues transports nutrients to and waste metabolites from the tooth[10].

2.2. Structure

Above the alveolar crest, the ligament merges with the gingival connective tissue, whilst at the apex the ligament is continuous with the pulpal tissue. The ligament links the root to the alveolar bone via an orientated network of collagenous fibre bundles.

These fibres form the bulk of the non-cellular component of the ligament. The cellular component of the ligament secretes, maintains and repairs the ligament and the adjacent structures of the cementum and alveolar bone. These cells play a role in bone remodelling during orthodontic tooth movement. This suggests the response to orthodontic force is mediated by the constituents of the periodontal ligament[11].

2.3. Cellular components

The cellular components of the ligament include fibroblasts (around 45 percent of periodontal ligament connective tissue), vascular-related cells, nerve-related cells, inflammatory cells, undifferentiated mesenchyme cells, and the epithelial rests of Malassez[10]. Near the periphery of the periodontal ligament, cementoblasts and osteoblasts line hard tissue surfaces, while in areas of resorptive activity osteoclasts and odontoclasts may be evident[12].

1. Fibroblasts are common and form the bulk of the cellular component of the ligament, contributing the structural proteins that allow the ligament to fulfil its main role. In a healthy ligament, fibroblasts regulate ligament tissue integrity and maintain homeostasis. They secrete structural proteins, including collagen and elastin, and ground substance, in the form of glycosaminoglycans and glycoproteins. They also secrete matrix-degrading enzymes including collagenase and other matrix metalloproteinases[13].

2. Osteoblasts line the bone surface of the periodontal ligament and may be found in active or resting states. They first appear as mononuclear cells that arise from the condensing mesenchyme in the embryonic facial region, and are generally cuboidal or slightly elongated in shape. Their main function is to synthesise organic bone matrix. Fully differentiated osteoblasts contain secretory organelles responsible for the production of type I collagen and other non-collagenous bone proteins. Mature osteoblasts undergo apoptosis, remain on the bone surface as bone-lining cells or become entrapped in the matrix to become osteocytes.

3. Osteocytes are entrapped in lacunae within the calcified bone. These small cells have reduced quantity of synthetic and secretory organelles, and are believed to be involved in slow turnover of the bone matrix. They are characterised by extensive cellular processes which communicate with adjacent osteocytes, osteoblasts and bone-lining cells throughout the bone canaliculi[13].

4. Multinucleated osteoclasts are derived from the blood-borne macrophage / monocyte lineage and are usually observed within Howship's lacunae in areas undergoing bone resorption. They are characterised by the presence of membrane infoldings or ruffled borders into which acid secretes, allowing for the solubilisation of bone minerals. Their formation is not well understood, and it is suggested that they originate within the haematopoietic tissues. They then migrate to sites of bone resorption, and differentiate into committed osteoclast precursors by fusing into the multinuclear form. This activation may be orchestrated by signalling molecules produced by osteoblasts. These cells contain receptors which recognise a resorptive signal, which is relayed to the osteoclasts via a largely unknown mechanism. It has been demonstrated that cell-to-cell contact between osteoblasts and osteoclast progenitors is required for osteoclast formation. There has been some suggestion that factors within the periodontal ligament are related to osteoclast formation and alveolar bone remodelling. These factors include osteoprotegerin (OPG) and the receptor activator of NF- κB ligand (RANKL)[14, 15].

5. Cementoblasts secrete the mainly collagenous matrix of cementum. They are found adjacent to the cementum at the periodontal ligament surface, particularly during times of high activity. Their appearance is characterised by an accumulation of numerous glycogen granules within their cytoplasm; however, they are difficult to distinguish histologically from the fibroblasts within the ligament[16]. During development, cementoblasts become trapped within the mineralised cementum and become cementocytes, responsible for collagen turnover and ground substance of the cementum. Cementum resorption is undertaken by cementoclasts and odontoclasts, both of the monocyte / macrophage cell lineage. This resorption only occurs under a

functional stimulus, when the force is greater than that which is required for bone resorption[11].

6. Inflammatory cells are commonly observed near blood vessels of the ligament, or near the junctional epithelium boundary, to regulate the host immune response. These cells include lymphocytes, plasma cells, mast cells and macrophages[13].

7. Epithelial rest cells of Malassez are remnants of the epithelial root sheath that lie near the cementum surface during root development. They are seen as closely packed strands of cuboidal cells near the cementum surface and secrete enamel-like proteins[11]. Calcitonin gene-related peptide (CGRP) and vasoactive intestinal peptide (VIP) have also been detected in these rest cell clusters suggesting an endocrine function[17]. The clusters are contained within an almost complete basal lamina and are characterised by a high nuclear to cytoplasmic ratio. They exhibit a prominent nucleus that contains condensed heterochromatin and often show invaginations in the cytoplasmic surface. With age the number of epithelial cells decreases[18]. Their function has yet to be determined, although it has been suggested that they may play roles in (i) maintaining the homeostasis of the periodontium, (ii) in repair cementogenesis, and in (iii) maintaining the periodontal ligament space[17].

8. Pluripotent Stem Cells are undifferentiated mesenchymal cells found within 5µm of blood vessels in the ligament, and are believed to provide new cells for the ligament when signals are released. The timing and source of these signals are yet to be determined[11].

2.4. Non-cellular components

These components form most of the extracellular matrix of the periodontal ligament, consisting predominantly of arranged collagen fibres, and lesser amounts of oxytalan and elastin fibres[10]. Proteoglycans and other non-collagenous proteins form a great part of the extracellular matrix.

1. Collagen is aggregated into groups histologically according to orientation, direction and localisation. These fibres extend across the width of the periodontal ligament space, from the cementum of the root surface to the cribiform plate of the alveolar bone proper. The fibres that extend across the ligament that are embedded in bone and cementum are known as Sharpey's fibres[10].

2. Oxytalan fibres are found essentially in the alveolar bone third of the ligament to which they are embedded in cementum and connect to the neurovascular supply. These fibres are usually associated with neural elements, while elastin is generally found associated with blood vessels. These fibres are thought to regulate vascular flow in relation to tooth function[11].

3. Vasculature of the Periodontium

The periodontal ligament undergoes high turnover and so is well vascularised for a connective tissue. The vasculature plays an important role in periodontal ligament function and has adapted to withstanding the high intermittent pressures and continuous pressures in the oral environment. Such pressures are exerted during normal function, during mastication, swallowing and speech, or iatrogenically during orthodontic tooth movement[11].

3.1. Blood Supply

The blood supplied to the ligament allows for substrate and metabolite exchange. The blood supplied to the teeth and periodontal ligament is via the maxillary artery, which branches to form the superior and inferior alveolar arteries. This supplies the

tooth root socket via the intraseptal artery, in which terminal branches penetrate the cribiform plate of the alveolus via rami perforantes[11]. These anastomose in the periodontal ligament space and supply the periodontal space. Branches of alveolar arteries directly enter the root canals of teeth after sending branches which anastomose with rami perforantes branches. These branches supply the apical portion of the periodontal ligament[11].

The various arteries which supply the gingiva and periodontal ligament form an entire system of blood vessels, each forming numerous anastomoses and perfusion networks which overlap to ensure adequate supply for the capillary network. The main blood supply of the free gingiva is derived from the supraperiosteal blood vessels, which anastomose with blood vessels from the alveolar bone and periodontal ligament within the gingiva. After entering the periodontal ligament, the blood vessels anastomose and form a polyhedral network that surrounds the root like a stocking[8].

It has been suggested that blood flow to the periodontal ligament is lower than in alveolar bone due to the smaller diameter of capillaries within the periodontal ligament. Blood flow through the periodontal ligament of rats has been shown to increase during orthodontic tooth movement, suggesting an increase in cellular activity during bone remodelling[17].

3.2. Lymphatic Supply

The lymphatic supply of the ligament is formed by an extensive network of lymph capillaries which drain in an apical direction toward larger diameter capillaries. The smallest of the lymph vessels are formed by a single layer of endothelial cells. Lymph from the periodontium drains into the various nodes of the head and neck[8].

4. Nerve Supply of the Periodontium

Cranial Nerve V provides the nerve supply to the masticatory apparatus, leaving the brain stem at the level of the pons-medullary interface. This nerve supplies the ligament via a maxillary branch (in the maxilla) and via the inferior alveolar branch (in

the mandible). The neural branching follows a similar path as the vascular branching that supplies the periodontal ligament. Branches of nerve fibres enter the ligament space through rami perforantes close to the root apices and run coronally. Some fibres enter through the lateral aspect of the ligament and send branches apically and coronally, forming plexuses within the ligament. Nerve fibres and the blood capillary network are found mainly in the ligament space adjacent to the alveolar bone[19].

4.1. Classification

Most of this nerve supply to the ligament is sensory, supplying pressure, touch, pain and proprioception. Classification of these neurons is according to axonal conduction velocity and their activating stimuli. Aβ fibres are sensory neurons with thick myelinated axons and are responsible for responding to very low threshold stimuli. Aδ fibres with thin myelinated axons are responsible for responding to high threshold noxious stimuli. Unmyelinated C fibres are the majority of sensory neurons in the ligament, and respond to touch, temperature and noxious stimuli[20].

These somatic mechanoreceptors record pain, touch and pressure, while proprioceptors within the ligament provide information on tooth movement and position. These proprioceptive receptors are very sensitive to even very small forces applied to teeth, from masticatory forces, food, tooth contact, and play an important role in chewing movements and forces. These properties are unique to the periodontal ligament[20].

4.2. Function

Many different types of neuro-receptors have been found within the ligament, most of which have unknown function. They have been described as Ruffini-like corpuscles, coiled endings and spindle-like endings according to their histological appearance. Their most common configuration is tree-like nerve endings that are found throughout the length of the root extending into the cementoblast layer. Most neuroreceptors originate from unmyelinated root axons, with a terminal end Schwann cell envelope. These neuroreceptors are believed to function as nociceptors and mechanoreceptors, responding to temperature and touch[11].

One such mechanoreceptor that has been well studied is the Ruffini-like receptor, which is found mainly in the apical segment of the periodontal ligament. These receptors appear in two forms: (i) a single neurite, or (ii) a compound neurite with several terminations that have finger-like processes that extend toward adjacent collagen fibre bundles[21]. It is believed that these processes serve to increase the receptive field of the receptor, by monitoring deformation of these fibre bundles. Oxytalan fibres lie between the venules and nerve fibres, suggesting a role in mechanoreception in the ligament[22].

The myelinated and unmyelinated nerve fibres of the ligament are often associated with blood vessels, suggesting a complex interaction between the two systems may exist. Nerve components of the autonomic nervous system innervate blood vessels of the periodontium. The postganglionic fibres of the sympathetic autonomic system that carry pain, touch and pressure arise from the superior cervical ganglion[23]. The cell bodies of these mechanoreceptors are located in either the trigeminal ganglion or in the mesencephalic trigeminal nucleus. This autonomic innervation may be related to venule diameter control, regulating supply of a region of the ligament through vasodilation and vasoconstriction[20].

5. Periodontal Response to Orthodontic Force

During orthodontic tooth movement, applied forces are transmitted through the tooth, to the periodontal ligament causing the ligament to become compressed in some areas, and in tension in others. Areas of compression result in the generation and influx of osteoclasts resulting in bone resorption. Areas of tension result in osteoblast differentiation and bone deposition. The direction of the applied force results in the creation of pressure and tension zones along the root surfaces resulting in movement through the alveolar bone. Histological cellular changes in the ligament occur soon after the force is applied, eliciting a biological response[24].

Several studies have attempted to describe this biological response to orthodontic force causing tooth movement.

Reitan applied light and excessive forces to a tooth in order to describe several phases associated with tooth movement. When light forces were placed, the tooth ceased to move after five days when the ligament was compressed. At excessive loads, this period of movement was shorter, followed by a 2 – 3 week period where only minor tooth movement was seen, coinciding with the undermining resorption of bone[25].

Oppenheim described the pressure – tension theory which related compression and tension of the ligament and the alteration in localised blood flow, to cellular changes and the release of a series of chemical signals. These signals mediated bone remodelling, such that resorption by osteoclasts occurred on the pressure side, while the deposition of bone occurred in the areas of tension[26].

A previous study by Waldo and Rothblatt[27] illustrated this using a rat dento-alveolar model. They devised a simple model of orthodontic force generation by using a specifically designed instrument to place an orthodontic elastic between the first and second maxillary molars to induce tooth movement. They studied the ligament histologically over specified periods of time from the movement's induction.

After 24 hours, separation of the molars had been achieved largely via tipping. The periodontal ligament appeared narrower in the pressure zones and wider in the tension zones, while an increase in inflammatory cells in the alveolar bone and vascularity of the periodontal ligament was noted.

After three days, a number of changes were evident in the region of the first and second molars: (i) haemorrhage, (ii) ligament compression, (iii) undermining bone resorption and (iv) large numbers of multinucleated osteoclasts. In comparision, the intensity of the response was reduced in the region of the third molar.

From day three to day five, the separation of molars had appeared to be due to a combination of tipping and bodily movements. After day five, the differing ligament widths from the pressure and tension sides became even more apparent. In the areas of compression, root and bone resorption associated with clastic cell activity

was noted, while small Howship's lacunae with evidence of new bone deposition were seen on the tension side.

5.1. Histology of the Tension Zone

In this zone, the ligament becomes wider as the tooth is moved away from alveolar bone, causing collagen fibres to become stretched[28]. This transmits forces to alveolar bone, pulling it in direction of the force. Cell proliferation along the socket walls occurs within the first 30 – 40 hours, where osteoid tissue is laid[25]. The vascular activity and volume increases in this region, along with an increase in infiltration of macrophages and other leukocytes. The volume of collagen fibres in the zone decreases[29]. It has been suggested that macrophages may play a role in the remodelling process, via direct or indirect means. This is accompanied with an increase in fibroblast-like cells[29].

Within three to five days after force application, bone deposition becomes evident, via osteoblasts which are recruited from the ligament cell pool. The osteoblast cells have been seen aligned along the alveolar bone surface. Over days 2 – 14, an increase in non-oriented fibrils and elastic fibres occur. A high level of cellular activity is evident as indicated by increased levels of endoplasmic reticulum activity[30].

There are suggestions that the breakdown of collagen is largely extra-cellular, due to collagenases produced through an interaction between macrophages and fibroblasts[31].

In these areas of tension due to 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[31].

During early tooth movement, leukocytes, proteins and fluids, transport and migrate into the periodontal ligament from the blood vessels[30]. It has been suggested that these are involved in a phagocytic role and in producing a cascade of signalling pathways involved in the inflammatory processes of bone remodelling[1].

5.2. Histology of the Pressure Zone

Within this zone, the ligament becomes compressed causing vascularity to decrease. The initial effect may be due to interstitial fluid movement and tightening of randomly orientated fibres that lie in contact with interlacing blood vessels[32]. Osteoclasts are seen along the resorptive alveolar bone surface, along with collagen breakdown and the presence of macrophages. The bone resorption is usually observed within three and five days after force application[33].

Two patterns of resorption have been described, according to the magnitude of orthodontic force.

Light forces allow for the blood vessels to remain patent under compression, allowing for osteoclasts present within the ligament to commence resorption of the alveolus; this is termed frontal resorption[25].

When force application is large, the blood vessels are occluded and cellular mobilisation is inhibited by lack of supply of nutrients. A process of hyalinisation ensues, as the ligament becomes acellular and homogenised due to necrosis of ligament cells[25]. Undermining resorption occurs in these areas adjacent to the compressed zone where nutrient supply remains viable. Tooth movement stops until the adjacent alveolar bone is resorbed and the hyalinized tissue is removed[34].

6. Local Inflammatory Response to Orthodontic Force

An inflammatory process precedes the remodelling of the periodontal ligament and alveolar bone during orthodontic tooth movement[32]. Inflammatory vascular changes around the periodontal tissues during orthodontic treatment were described during histological examination of the periodontal ligament of mechanically stressed rabbit maxillary incisors[28]. Since then, many researchers have reported an increase in vascularity in paradental tissues around orthodontically-moved teeth[17, 35]. Recent studies have shown that the regulation of the inflammatory response involves a number of biological factors that have been demonstrated to increase in amount around the periodontium of mechanically stressed teeth[35].

Inflammation may be characterized macroscopically by redness, heat, swelling, pain and loss of function. Histologically, there is vasodilation and increased permeability of blood vessels, exudation of fluids and migration of leukocytes into extravascular space. These changes are mediated by endogenous biochemical substances such as histamine, leukotrienes, prostaglandins (PG) and cytokines[2]. Pro-inflammatory cytokines, such as IL-1 and TNF-α, are known to stimulate the synthesis of proteins that elicit acute or chronic inflammation and are also involved in bone remodelling processes[2]. PGs and leukotrienes induce blood vessel permeability, vasodilation and chemotaxis, and are involved in the process of bone remodelling. Substances that inhibit PG and leukotriene synthesis also inhibit tooth movement[33].

More recently, peripheral nerve fibres and neurotransmitters have been implicated in the process of inflammation and bone remodelling. Inflammatory processes such as vasodilation, increased vascular permeability, plasma extravasation, proliferation of endothelial cells and fibroblasts have been attributed to peripheral neurogenic mediation[35]. CGRP and substance P (SP) have also been reported to have direct influences on inflammatory cells. Monocytes, lymphocytes and mast cells have been shown to express neuropeptide receptors[36]. The peripheral nervous system was hypothesised to act as a link between physical stimulus and the biological responses[2]. CGRP and SP immunoreactive nerve fibres in the periodontal ligament of mechanically-stressed teeth had been shown to stain intensely [2, 37, 38], indicating increased sprouting and activity. Furthermore, the severance of the inferior alveolar nerve has been shown to reduce periodontal and pulpal blood flow and impede bone formation[39].

Davidovitch et al. hypothesised that mechanical stress distorts the cells and matrix of the periodontal ligament, which then strains the nerve fibres leading to the release of vasoactive neuropeptides[2]. The ensuing vasodilation leads to plasma extravasation and diapedesis of leukocytes. The leukocytes occupying the extravascular space release cytokines and growth factors that stimulate paradental cells to begin the process of periodontal ligament and alveolar bone remodelling.

Sensory neurotransmitters, such as CGRP, SP, vasoactive intestinal peptide (VIP) and nitric oxide (NO) have been shown to be involved in the regulation of osteoblasts

and osteoclasts, either directly or indirectly. These neurotransmitters are synthesized in the ganglion sensory cells and are distributed in the central and peripheral nervous system[2]. Stimulation of the mechanoreceptor and nociceptors trigger the release of the neurotransmitters. Receptor activation by these molecules leads to the generation of intra-cellular second messengers, namely cyclic adenosine 3', 5' monophosphate (cAMP) and inositol triphosphate (IP3) in the cells[40]. The second messenger molecules then transmit the signal to the nucleus via a series of kinases. The nucleus, on receiving the signals, synthesise immediate early genes (IEG), namely c-fos, c-jun and egr-1, depending on the type of signal received. IEGs levels have been shown to increase after exposure of cells to cytokines and mechanical stimulation[41]. The IEGs are then translated into activator protein-1 (AP-1), a transcription factor that modulates the activity of the gene it binds to and may produce proliferation or differentiation of the cells[24].

7. Molecular Biological Response to Orthodontic Force

When orthodontic force is applied to a tooth, the initial movement is largely physical. These physical changes associated with tooth movement consist of tissue fluid movement, distortion of the extracellular matrix and cellular distortion[42]. These cause a rapid cellular response followed by complex biological reactions which are not fully known and are yet to be clearly defined[43].

Several studies have been conducted to describe the periodontal ligament tissue responses to mechanical stress. Storey described the periodontium to display bioelastic and bioplastic properties related to the functioning of the dentition. Orthodontic forces applied to a tooth need to exceed the bioelastic limit to allow for translation of the tooth. This leads 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[28].

The metabolic and anabolic activities in bone are regulated by various systemic and local factors, including growth factors, cytokines, hormones and neurotrophins[42].

Neurotrophins have been implicated in the molecular reactions associated with cell-mediated remodeling of the periodontal ligament when subjected to mechanically-induced stress. This has suggested an involvement of the nervous system in bone metabolism within the periodontal ligament[33].

Many researchers proposed theories related to how a stressful event, such as orthodontic tooth movement, causes a biological response via signal transduction. Signal transduction may occur via an incompletely understood series of events involving signaling molecules[24].

7.1. Cell Matrix Interactions

The cell surface has many embedded proteins which form an extracellular matrix. The matrix is linked through the cytoplasmic membrane with the cytoskeleton, or inner framework, of the cell. During orthodontic force application, the extracellular matrix is distorted, causing activation of ion channels and enzyme release. Studies have demonstrated that during orthodontic strain, osteoblast-like cells within the ligament show an increase in Ca^{2+} intake. After initiation of force, phospholipase A becomes activated, which in turn cleaves arachidonic acid into its active form PGE_2. The elevated levels of PGE_2 cause an increase in levels of cAMP and cGMP, which are known second messengers that elicit a cell response[24, 44].

7.2. Cytoskeleton Force Transduction

Many receptors have been studied which are involved in mechanotransduction. These receptors, which are anchored in the plasma membrane, are connected to the extracellular matrix and transmit change at the cell surface into a biochemical signal. The signal transmits to the nucleus of the cell, resulting in alterations in the gene expression of the cell, causing a phenotypic or functional change[24]. Well-studied receptors come from the Integrin family of adhesion receptors, many of which are known to be anchored in the plasma membrane and connected to the extracellular matrix and cytoplasmic microfilaments[33].

This suggests that any factors that activate cellular receptors may be important regulators of cell and tissue growth[24].

7.3. Stretch-sensitive Ion Channels

Cells may have ion channels within their cytoplasmic membrane, which allow for the passage of calcium and potassium cations[24]. Application of mechanical stress to the cell may activate these ion channels and raise intracellular levels of calcium. Such activation is via unknown means. It may be via direct stimulation or due to secondary messengers. Osteoclasts may be controlled via this mechanism[24, 44].

7.4. Piezoelectric Potentials

Cells respond to mechanical stress through bioelectric signaling. This theory suggests that bone is surrounded by electrical layers through which electric charges flow when subjected to stress. When bending forces are applied to bone, the compression and tension on opposite sides cause a flow of electrically charged fluid through the bony canaliculi. This produces a stream of potentials as fluid is forced over cell membranes, causing a potential across the membranes which may directly stimulate cells. Some studies have shown areas of electronegativity associated with elevated osteoblastic activity and electropositive areas associated with high osteoclastic activity[1, 24].

7.5. Signalling molecules

These molecules play a role in the control and modulation of cellular activities. They are often markers for cellular activity and many have been implicated during bone remodeling following periodontal stress, including during orthodontic tooth movement; such molecules include neurotransmitters, cytokines and growth factors[2].

When the periodontal ligament is mechanically stressed, sensory nerve endings release their contents. Vasoactive intestinal polypeptide (VIP), Substance P, Calcitonin gene-related peptide (CGRP), and nitric oxide (NO) have all been demonstrated in the ligament during mechanical stress, via immunohistochemistry[2,

[43]. These signaling molecules affect bone cells and the vascular system via direct or indirect means[2].

Leukocytes in the ligament produce many signal molecules, and their products may be classified into cytokines, growth factors, colony stimulating factors and cell adhesion molecules. Osteoblasts, fibroblasts, epithelial cells, endothelial cells and platelets have been shown to synthesise many of these factors[1, 45].

The cytokines that are known to affect bone are from the interleukin family and include tumour necrosis factor-alpha (TNF-α), and gamma interferon (γ-IFN). These have been shown to play various roles in bone resorption via chemo-attracting leukocytes, stimulating fibroblasts and endothelial cells, and enhancing bone resorption[1, 45].

Growth factors have been found to act as regulatory molecules in the tissue changes caused by orthodontic force on the ligament. Such factors include transforming growth factor-β (TGF-β), platelet derived growth factor (PDGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), colony stimulating factors, such as granulocyte-colony stimulating factors (G-CSF) and macrophage-colony stimulating factors (M-CSF), nitric oxide (NO), parathyroid hormone related peptide (PTHrP) and the prostaglandins including PGE_2.[43]

7.6. Neurotransmitters

These are generally protein peptides that are found throughout the central and peripheral nervous system. Very few have known function, and two relatively well known transmitters are Protein Gene Product (PGP) 9.5 and Calcitonin gene-related peptide (CGRP).

1. PGP 9.5 is a general cytoplasmic marker of neurons and neuroendocrine cells, and may be used as a marker to visualise the entire innervation of a tissue. CGRP is a 37 amino acid neurotransmitter which is derived from precursor proteins in nerve cell bodies[46]. It has been localised in the hypothalamus, thalamus and hippocampus of the central nervous system, as well as within

the sensory, motor and autonomic fibres of the peripheral nervous system. It serves a prominent role in the proprioception of the periodontal ligament, as it is found in the small diameter neurons which give rise to unmyelinated C fibres, thinly myelinated Aδ fibres, and primary afferent fibres subserving the sensation of pain, temperature, and noxious stimuli[47].

2. CGRP's role in inflammation and the periodontium has been investigated in recent studies[48]. CGRP is involved in the inflammatory process and has been shown to (i) have a vasodilatory effect, (ii) regulate immune cells, (iii) proliferate endothelial cells, (iv) have an inhibitory action on bone resorption and (v) have a stimulatory effect on bone formation. CGRP nerve fibres have been demonstrated in concentrated numbers in the PDL adjacent to the tooth root apex, closely associated with blood vessels. Some of the fibres lie adjacent to the root surface and cellular cementum. Changes in the neural and blood vessel density, morphology, and distribution correspond to the connective tissue changes that occur with tooth movement[35].

A study by Vandevska-Radunovic in 1999 showed the effects of tooth movement on CGRP and PGP 9.5 labelled nerve fibres within the periodontium. After 7 days of orthodontic force, a significant increase in CGRP and PGP 9.5 labelled nerve fibres adjacent to the alveolar bone in the PDL and the dental pulp were demonstrated. This was associated with a peak in periodontal and pulpal blood flow. At this time, 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. The labelled nerve fibres were not associated with resorption areas until after 21 days[35, 49].

Kvinnsland et al found an increase in the number of immunoreactive nerve fibres in the coronal pulp and the periapical tissues after 5 days of molar movement. This suggested that CGRP immunoreactive (CGRP-IR) nerve fibres might be involved in tissue responses during tooth movement[37].

Saito et al demonstrated an association between CGRP-IR nerves and blood vessels in the PDL. They found that the nerves reached their maximum density and intensity

at three days, and then returned to control levels by 7 days. This suggested that CGRP may not only function as a neurotransmitter, but was also directly involved with the remodelling process associated with tooth movement[50].

7.7. CGRP and Increased Vascularity

There is a significant increase in the number of pulpal microvessels during orthodontic tooth movement, suggesting a related increase in diffusible angiogenic factors[51]. This suggests that there are pulpal substance P-expressing pain fibres and CGRP expressing fibres which provide a vasodilatory effect. No nerves have been identified thus far that provide a vasoconstrictor action[52].

CGRP-IR and substance-P IR 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[53].

7.8. CGRP and its Role in Bone Remodelling

Vandevska-Radunovic et al. demonstrated that epithelial rests of Malassez in rats were occasionally immuno-reactive to protein gene product 9.5. No nerves were observed in root resorption areas after 7 days of tooth movement. However, after 21 days, the CGRP-IR nerves were found in close association with cementoclasts in root resorption lacunae suggesting CGRP is involved with inhibiting cementoclasts / odontoclasts [49].

Vandevska-Radunovic et al. demonstrated that axotomy had delayed the normal increase in PDL blood flow associated with orthodontic tooth movement. This occurred until sensory nerve re-innervation could be established. This indicated that neurogenic mechanisms may be involved in the orthodontically induced inflammatory process[54].

Yamashiro et al. analysed the role of sensory nerve innervation, as indicated by CGRP-IR levels in alveolar bone remodelling after tooth movement was induced in

rats 7 days after the unilateral transection of inferior alveolar nerves. It was found that denervation resulted in a decrease in CGRP-IR sensory nerve fibres within the PDL and pulpal tissue when compared to control PDL and pulp with normal innervation. There was no difference in osteoclast numbers. After application of orthodontic force, there was a five-fold increase in CGRP-IR and in the number of osteoclasts. In comparison, in the region affected by nerve transection, there was a decrease in CGRP-IR nerve fibres and osteoclasts. No significant change was seen in osteoclast number. Bone resorption was activated 24 hours after the induction of tooth movement and was associated with increases in CGRP-IR. These results suggest that sensory nerves have an important role in regulating bone resorption during tooth movement[55].

Vandevska-Radunovic et al. also demonstrated that axotomy of the inferior alveolar nerve resulted in the delayed recruitment of macrophage-like cells in the PDL of rat molars under orthodontic force application. This indicated that neuropeptide expressing nerve fibers may interact with immunocompetent cells and are involved in the events of inflammation associated with tooth movement[39].

To date, no studies have quantitatively analysed a relationship between CGRP positive neurons and osteoclast activity in the periodontal ligament. This relationship if proven, may suggest the rate of bone resorption (indicated by the number of TRAP positive osteoclasts) may be selectively modulated by alteration of the rate of reinnervation by CGRP positive nociceptive nerve fibres. This may have a clinical implication in the use of analgesics which affect release of neuropeptides such as CGRP.

7.9. Growth Factors

Growth factors are believed to participate in the regulation of periodontal ligament cells. Growth factors have been found to affect bone and endothelial cells, as well as fibroblasts. Transforming growth factor β (TGF-β) is a small protein molecule produced by osteoblasts and fibroblasts. It is most abundant in bone and platelets[56]. It stimulates angiogenesis *in vitro* and is a chemoattractant to monocytes and

fibroblasts[57]. Enhanced TGF-β immunoreactivity was demonstrated in the periodontal ligament and alveolar bone during orthodontic tooth movement in cats[33]. Platelet-derived growth factor (PDGF) is derived from various cells, including platelets, and is believed to induce production of collagenase by osteoblasts. PDGF was reported to enhance the release of calcium from bone, *in vitro*[58]. Insulin-like growth factors (IGF) are proteins produced by the liver that have insulin-like metabolic effects. IGF promotes cell proliferation and differentiation and has been shown to cause an increase in DNA synthesis in periodontal ligament cells *in vitro*[59].

Nerve Growth Factor (NGF) was discovered in the 1940s in the male mouse submaxillary gland and was purified in large amounts and studied in the 1960s[60]. It is homologous, noncovalent and homodimeric (12500 kDa/monomer), and elicits a wide variety of responses in selected neuronal and non-neuronal tissues[60]. It plays an essential role in the development, maintenance and survival of sensory and sympathetic nerves[3].

NGF's neuronal and non-neuronal target cells influence the development and maintenance of the innervating neurons[3], and it is synthesized and released from the target tissues of parasympathetic neurons[61]. These neurons are a subpopulation of sensory neurons and cholinergic forebrain neurons in the central nervous system[62]. Within the sympathetic and neural crest derived spinal sensory neurons, NGF has been identified peripherally as a retrograde messenger that provides a trophic effect and has been shown to be produced by epithelial cells, smooth muscle cells, fibroblasts, and Schwann cells[63]. NGF reacts with specific receptors associated with its target cell's neurons, including p75 and trk A, and is transported to the cell body to maintain cell viability[64, 65].

Table 1: Molecules associated with orthodontic tooth movement associated with neural tissues and growth factors (from Biological Mechanisms of Tooth Movement, Krishnan & Davidovitch).

GENE	CELL EXPRESSION	FUNCTION	EFFECT ON TOOTH MOVEMENT
Calcitonin gene-related peptide (CGRP)	Neurons, pulp, periodontal ligament (PDL) and marginal gingiva	Neurotransmitter, vasodilation, increases osteoblast differentiation, inhibits osteoclast formation	Bone remodelling
Vasoactive intestinal peptide (VIP)	PDL, pulp	Neurotransmitter, vasodilation, increases osteoblast differentiation	Bone remodelling
NYP	Neurons	Neurotransmitter, vasodilation, increases osteoblast differentiation	Bone remodelling
Substance P (SP)	Pulp, periodontal ligament (PDL) and marginal gingival	Neurotransmitter, vasodilation, increases osteoblast differentiation	Bone remodelling
Preproenkephalin	Neurons	Neurotransmitter, vasodilation, increases osteoblast differentiation	Bone remodelling
Dynorphin	Neurons	Neurotransmitter, vasodilation, increases osteoblast differentiation	Bone remodelling
Protein gene product (PGP)-5	Neurons	Neurotransmitter, vasodilation, increases osteoblast differentiation	Bone remodelling
Connective tissue growth factor (CTGF)	ECM, osteoblasts and osteocytes	Proliferation and differentiation of osteoblasts / bone formation	Stimulates bone formation
Transforming growth factor (TGF) b1	PDL, Osteoblasts, pulp	Induces osteoclast apoptosis	Stimulates bone formation
Epithelial growth factor (EGF)	Fibroblasts, pulp	Remodeling	ECM and bone remodelling
Vascular endothelial growth factor (VEGF)	Pulp, osteoblasts	Neovascularisation, osteoclast recruitment	Stimulates bone resorption, accelerates movement
Bone morphogenetic proteins (BMPs)	Fibroblasts, osteoblasts	Osteoblast formation	Stimulates bone formation
Macrophage-colony stimulating factor (MCSF)	Fibroblasts	Osteoclast differentiation	Stimulates bone resorption
Fibroblast growth factor (FGF)	Osteoblasts, pulp	Osteoblast differentiation	Stimulates bone formation
Insulin-like growth factors (IGFs)	Fibroblasts, osteoblasts, osteocytes	Stimulates osteoblast and osteoclast function / bone remodelling	Bone remodelling
Platelet-derived growth factor (PDGF)	Pulp, fibroblasts	Osteoblast proliferation mediates through PGE	Stimulates bone formation

Several recent studies have aimed to clarify the function and localisation of NGF and its receptors. Verge et al. demonstrated the suppression of neuropeptide expression in transected rat sciatic NGF-IR nerves. They suggested that NGF may be responsible for the reversal of injury-associated inflammatory responses[66].

Streppel et al. demonstrated a decline in NGF expression following transection of the buccal branch of the facial nerve in a sample of rats. At 3 days NGF was first detected and then peaked in concentration at 5 days and declined at 6 days post axotomy within 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[67].

Neurotrophic receptors may be differentiated by their pharmacological properties, and are in two different classes[62] to which a ligand interacts to influence neuronal plasticity and survival[68]. NGF receptors may be categorized into high- and low-affinity, with the low-affinity receptors appearing 5-10 times more abundant[69]. When ligand binding occurs, the ligand-receptor complex may produce effects lasting from milliseconds to days[68]. Research has been aimed at clarifying the tissue distribution of nerve growth factor receptors, which thus far have been shown to alter during trauma, nerve transection, and orthodontic tooth movement[70].

Byers investigated the distribution of Nerve Growth Factor (NGF) receptors in teeth and the PDL. NGF receptor immunoreactivity (NGFR-IR) was observed in the perineurial sheath for a short distance after entering the tooth root pulp and within the predentine. Ruffini endings, their associated terminal Schwann cell membrane in the PDL, Schwann cells along unmyelinated axons, odontoblasts in contact with other NGFR-IR pulpal or neural cells, and pulp fibroblasts were also NGFR-IR positive[71].

An investigation by Saito et al. found NGF receptor immunoreactivity in rat PDL during orthodontic tooth movement. Their study involved time course data points at 1, 2, 6, and 12 hours and 1, 3, 5, and 7 days after the insertion of an elastomeric separator between maxillary first and second molars. The upper second molar's PDL was examined for changes in NGFR immunoreactivity, which was observed in the

control side, mainly in the apical and intermediate regions of the distal PDL and adjacent to blood vessels[70].

After the first hour of tooth movement, NGFR immunoreactivity was slightly reduced in the coronal and intermediate regions of the distal side. After 12 hours, an increase in immunoreactivity was seen in the coronal and intermediate portions compared to the controls. At 3 days, a further increase in immunoreactivity was noted on the distal side adjacent to the blood vessels and to resorptive lacunae on bone and root surfaces. At 5 days there was a decrease in reactive nerve fibre distribution on the distal side to approximately the same levels as the control, while on the mesial side there was an increase in nerve fibre distribution adjacent to blood vessels. After 7 days staining intensity for NGFR decreased[70].

Conclusion

These studies suggest a possible relationship between NGF / NGFR expression and orthodontic tooth movement. Presently, no quantitative studies have shown a relationship between CGRP immunoreactivity and NGF expression during orthodontic tooth movement. No studies have shown an association between orthodontic tooth movement measurements and CGRP immunoreactivity.

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Statement of Purpose

1. Aims

Paper 1 - The aim of the study was to examine changes that occur in the periodontal ligament of orthodontically traumatised teeth after localised injection of anti-NGF antibody.

Paper 2 - The aim of this study was to evaluate a potential association between CGRP-immunoreactive fibre density, and the amount of orthodontic tooth movement achieved over a given period of time..

2. Hypotheses

Paper 1 - There is no statistically significant association between CGRP immunoreactive nerve fibre intensity and localised injection of anti-NGF, in rat periodontal ligament.

Paper 2 - There is no statistically significant association between CGRP immunoreactive nerve fibre intensity and measured tooth movement, in the groups of localised injection of anti-NGF, injection of saline and in the non-injected sample.

SECTION 2

ARTICLE ONE

THE NEURAL RESPONSE TO ORTHODONTIC TOOTH MOVEMENT AND ANTI-NERVE GROWTH FACTOR IN THE RAT

FOR SUBMISSION TO THE ARCHIVES OF ORAL BIOLOGY

Abstract

Objectives: Nerve growth factor (NGF) and its receptors have been shown to increase expression following traumatic injury. The aim of the study was to examine changes that occur in the periodontal ligament of orthodontically traumatised teeth after localised injection of anti-NGF antibody.

Design: Orthodontic 100g NiTi pullcoil springs were ligated to the right maxillary first molar and maxillary incisors of 17, eight week old, male Sprague-Dawley rats which were divided into two groups. The first group were injected with anti-NGF palatal to the right maxillary first molar. The second group were injected with saline in the same location to serve as a control. The left maxillary molars from each animal served as internal controls. Animals from the two groups were sacrificed at 7 and 14 days. Six animals from a third matched group were neither injected nor had an orthodontic spring attached and were sacrificed to serve as an additional untreated control group. 25 μm horizontal frozen sections were obtained using a cryostat at -20°C and mounted on the slides to allow direct comparison between untreated and experimental sides of the mesiobuccal root periodontal ligament circumference. Immunohistochemical tissue labelling was performed, involving the avidin-biotin peroxidase technique and rabbit anti-calcitonin gene-related peptide (anti-rat-CGRP) as the primary antibody. Photomicrographs were analysed using Image J software with a colour deconvolution “plug-in” to compare and quantify the staining intensity of the immunolabelled tissue. Data were compared between each of the groups using the Neuman-Keul paired test on Graphpad Prism 5 software.

Results: A semi-quantitative comparison of the immunohistochemical CGRP staining showed a statistically significant ($p<0.05$) reduction in staining intensity in the anti-NGF injected group when compared with the saline injected group for both day 7 and day 14 groups. There was a statistically significant ($p<0.05$) reduction in staining intensity between the anti-NGF injected (group 1), the saline injected (group 2) and untreated control animals (group 3).

Conclusion: Data suggest that the injection of anti-NGF reduces NGF tissue levels and reduces the re-innervation by CGRP-positive fibres in the periodontal ligament of rat molars subjected to orthodontic tooth movement.

1. Introduction

Neurotrophin levels may play a role in the modulation of cellular activity in the periodontal ligament during orthodontic movement[1]. Neurotrophins have been implicated in the molecular reactions associated with cell-mediated remodeling of the periodontal ligament when subjected to mechanically-induced stress, suggesting involvement of the nervous system in bone metabolism within the periodontal ligament[2]. Neurotrophins are a family of protein polypeptides which are important in neural cell differentiation and survival[1]. One relatively well-studied neurotrophin is the beta subunit of 7S nerve growth factor complex, or Nerve Growth Factor (NGF). NGF is a polypeptide essential for supporting cholinergic innervation in the brain and sympathetic and sensory innervation in the peripheral tissues[3]. Within the dento-alveolar complex, the function and localization of neurotrophins and their receptors are unknown.

Previous studies have shown that there is an increase in tissue NGF expression in response to injury, suggesting that NGF expression may increase in regions within the dento-alveolar complex where inflammation and bone remodelling are occurring[4]. Elevated levels of NGF mRNA in human periodontal ligament cells were shown *in vitro* during increased transcription and translation of the bone-related proteins alkaline phosphatase and osteopontin, suggesting that NGF facilitates bone formation[1]. While NGF may modulate cellular activity within the periodontal ligament, its influence on orthodontic tooth movement is unknown. In addition, the effect of antiserum against NGF (anti-NGF) on orthodontic tooth movement has yet to be determined; however, previous studies suggest it may block sensory and sympathetic nerve regeneration and influence periodontal ligament and bone remodelling[5-8].

O'Hara et al. used immunohistochemical staining in a rat tooth movement model to show that there was evidence of an increase in NGF synthesis and release by certain cells within the region of alveolar bone remodelling during an initial injury response period[4]. Ho et al. used a similar model to test the hypothesis that there was a positive relationship between the presence of osteoclasts and pre-osteoclasts with areas of NGF localisation. He found no definitive relationship between osteoclasts and areas

of NGF; however, his findings showed NGF positive areas within the periodontal ligament but the cells responsible for NGF production could not be identified[9].

Calcitonin gene-related peptide (CGRP) is a neuropeptide believed to be a regulator of bone resorption [5-8]. It is a 37 amino acid neurotransmitter which is derived from precursor proteins in nerve cell bodies[10]. CGRP has been localised in the hypothalamus, thalamus and hippocampus of the central nervous system, as well as within the sensory, motor and autonomic fibres of the peripheral nervous system. It serves a prominent role in the proprioception capability of the periodontal ligament. It is produced in small diameter neurons which differentiate into unmyelinated C and thin myelinated Aδ fibres, for the conduction of pain, temperature and noxious stimuli[11]. In the cat periodontal ligament, CGRP immunoractive fibres were shown to extend close to the root cementum and have vasoactive, sensory and neuromodulating properties[12]. The wide distribution of CGRP-immunoreactive fibres in bone and the periodontal ligament suggest an important role in bone remodelling.

CGRP-immunoreactive nerve fibres have been demonstrated to stain intensely in periodontally stressed teeth [13, 14]. Saito et al [15] showed a denser region of CGRP-immunoreactive nerves in the periodontal ligament during tooth movement. Their study involved the use of time course data points at 1, 2, 6, and 12 hours and 1, 3, 5, and 7 days after the insertion of an elastomeric separator between rat maxillary first and second molars. The upper second molar PDL was examined for changes in NGFR immunoreactivity, which was observed in the control side, mainly in the apical and intermediate regions of the distal PDL and adjacent to blood vessels[16].

The stress placed on the periodontal ligament during orthodontic tooth movement is believed to cause a distortion in the nerve fibres and receptors within the tissue, as well as eliciting the sensation of pain and pressure. During such tooth movement, the degree of force exerted on the ligament may create a local region of inflammation and tissue degradation, with a temporary loss of the normal innervation pattern within the ligament. During the normal repair process following orthodontically induced injury, surviving nerve processes regrow into the injured area, largely in response to increased tissue levels of neurotrophic factors such as NGF [17]. NGF may play a role

in the proliferation and differentiation of periodontal ligament cells during orthodontic tooth movement.

Inflamed tissues have been shown to overexpress NGF [18], and it is known that sensory nerves play a role in inflammation and the immune response during wound healing. A study by Ro et al. [19] showed that anti-NGF applied locally to denervated adult rat skin prevents local sensory hypersensitivity and inhibits the sprouting of nociceptive fibres. This resumed after cessation of anti-NGF application [20]. It seems reasonable to propose that periodontal ligament remodelling may be influenced by anti-NGF, which may block neurochemical changes and inhibit cellular changes that cause bone resorption.

The present study aims to show the effect of anti-NGF injection on CGRP-immunoreactive fibre density and sprouting in the dento-alveolar complex in rats during orthodontic tooth movement. The null hypothesis to be investigated is that no relationship exists between CGRP positive nerve fibre density and the presence of anti-NGF during orthodontic tooth movement.

2. Materials and methods

The following study was performed with the approval of the University of Adelaide Animal Ethics Committee number M/023/2006. Twenty-three male, eight week old Sprague-Dawley rats were anaesthetised via a subcutaneuous injection containing a combination of Ketamine (100mg/ml) (Ketamil Injection, Troy Laboratories, Smithfield, Australia) and Xylazine (Xylazil, 20mg/ml) at a dosage of 2ml/kg. The two drugs were mixed at the ratio 2:1 (Ketamine:Xylazine) and diluted 1:1 with sterile water for injection. The rats weighed between 250 - 350g and were fed commercially manufactured standard pellets (Parastoc Feed, Ridley AgriProducts, Murray Bridge, Australia) and water, ad libitum. They were contained at the Animal House facility of the Medical School of the University of Adelaide.

2.1 Experimental groups

Research by Ho [9] provided seventeen animals, in which a 100g superelastic nickel titanium closing spring (Sentalloy, GAC, New York, USA), was bonded to the right maxillary first molar and to the maxillary incisors using 0.010" stainless steel ligatures and light cured bonding material (Z100 composite resin, 3M ESPE, St. Paul, USA)(see Appendix 1). This was to impart a mesially directed heavy force to the right maxillary first molar, using a protocol developed by Noxon et al [21]. Previous studies involved lighter forces ranging from 20 – 50 g. The test animals were injected with 1 µL (2 µg) of anti-NGF antibody (Courtesy of Dr. Ian Ferguson, Flinders University) into the gingival mucosa between the right maxillary first and second molars after application of the spring. A sham control group of animals were injected with the same volume of an isotonic saline solution into the gingival mucosa between the right maxillary first and second molar. Another six animals, that had no orthodontic movement and no injection performed, were used to establish age-matched, untreated controls.

Animals were provided with identification numbers and were sacrificed at day 0, 7 and 14 as shown in Table 1.

Table 1: Sample size and categories. Excepting Day 0, all had an orthodontic spring attached to the maxillary right first molar.

DAY	*CONTROL* *(i) no injection* *(ii) saline injected*	*ANTI-NGF* *INJECTED*	*TOTAL*
0	6 (i)	0	6
7	5 (ii)	6	11
14	3 (ii)	3	6
TOTAL	14	9	23

2.2 Harvesting of tissue

The animals were sacrificed via cardiac left ventricular perfusion with fixative solutions, after an intraperitoneal administration of chloral hydrate 5g/100ml H_2O (5mL). Of the seventeen animals that had spring placement and injection performed at day 0, eleven animals were perfused at 7 days and six animals were perfused at 14 days. The six untreated animals were also sacrificed, using the same perfusion protocol involving 200ml 0.1% sodium nitrite, and a 400ml solution of 50:50 para-formaldehyde and p-benzoquinone under positive pressure. Each animal had the maxilla and brainstem removed and stored in the 50:50 fixative solution for two hours, followed by 4% EDTA decalcifying solution pH 7.4 for 8 weeks. The decalcified tissue was then impregnated with an embedding medium (OCT, Sakura Finetek, Torance, CA, USA) for cryosectioning.

2.3 Section preparation

Horizontal sections 25µm thick were obtained using a Leitz 1720 cryostat at -20°C. The maxillae were orientated so that the crowns of teeth were uppermost, and sections were collected of the first and second molars below the furcation to the root apex (figure 1). Such sections allowed for direct comparison between untreated and experimental sides of the periodontal ligament circumference.

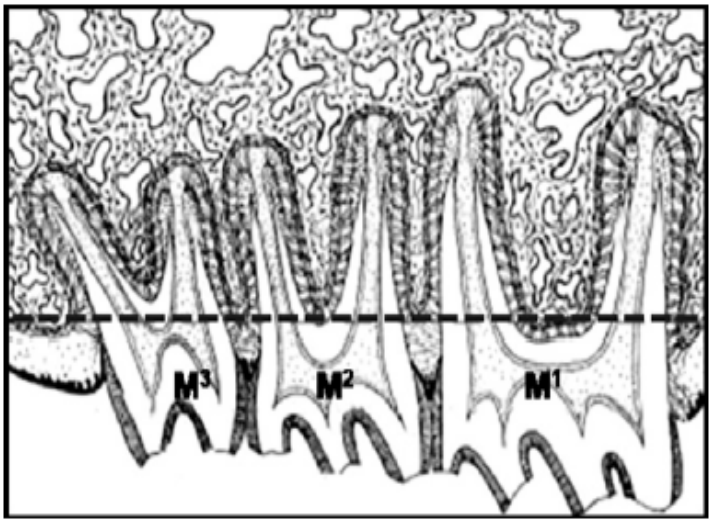

Figure 1: Schematic representation of the level at which the section was cut through maxillary first molar (M1) and maxillary second molar (M2) roots below the furcation

Serial sections were placed consecutively over 12 slides, so that each slide contained sections representing different levels of the periodontal ligament for comparison. Positive controls were provided by including a section of the trigeminal ganglion of the brainstem or the dorsal horns of the spinal cord, on each slide (figure 2).

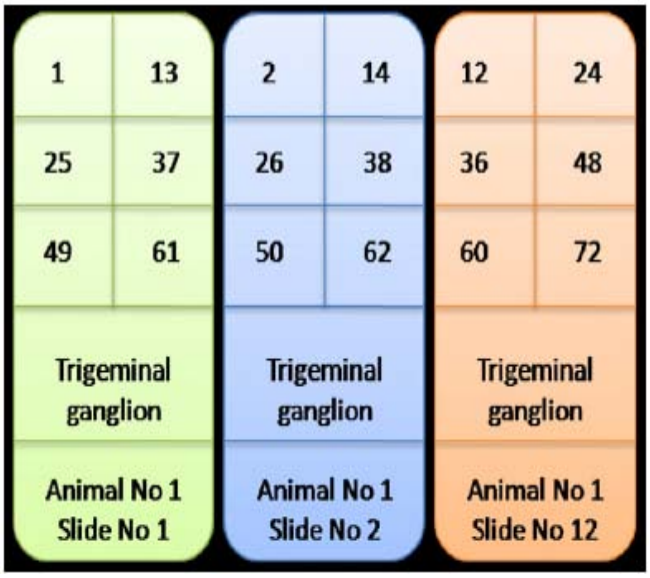

Figure 2: Arrangement of specimen sections on slides

2.4 Immunohistochemistry

The odd-numbered slides from each animal were used for immunohistochemical labelling for CGRP. Slides from the different groups were labeled in the same solutions simultaneously along with both positive and negative control tissue (rat brainstem) known to have CGRP-immunoreactive fibres. The avidin-biotin peroxidase complex technique was used involving rabbit anti-rat-CGRP serum (Peninsula Laboratories, ME, USA) as the primary antibody and biotinylated anti-rabbit IgG (Rockland, PA, USA) as the secondary antibody. The anti-rat-CGRP was diluted from lyophilised form (50µl) to 1:4000 in 1% normal goat serum (NGS in 0.1M PBS). The secondary antibody IgG (2mg/ml lyophilised) was diluted to 1:250 in 1% NGS.

The optimal primary antibody concentration used was found through a pilot study using similar tissue to the area studied. The immunocytochemistry protocol was previously developed (Appendices 2 & 3). Slides were incubated for 30 minutes in a blocking solution 0.3% H_2O_2 in 70% methanol:0.1M phosphate buffered saline (PBS), followed by three, five minute rinses in 0.1M PBS. Slides were then incubuted for one hour in 3% NGS (in 0.1M PBS). Between 800 – 1000 µL of diluted primary antibody was placed on the slides and incubated overnight (16-18 hours) at room temperature in a humidified chamber on a shaker.

Three, ten minute washes in 1% NGS were followed by a one hour incubation in diluted secondary antibody. The slides were rinsed with three, ten minute 0.1M PBS washes. A diluted streptavidin-peroxidase 1:1000 in 0.1M PBS was prepared into which slides were incubated for one hour. Three, ten minute 0.1M PBS washes were performed, followed by incubation in prepared DAB in 0.1M Tris buffer (pH 7.65) with 3% H_2O_2 added to initiate the reaction. The reaction was stopped after five to seven minutes with rinses in 0.1M Tris buffer, then 0.1M PBS. Following a haematoxylin counter stain, slides were mounted and cover slipped.

Photomicrographs were taken at a range of magnifications using an Olympus BH-2 photomicroscope (Altra20 camera and analySIS FIVE system) and stored as JPEG files of 300 – 350 kilobytes. The resulting digital photomicrographs were analysed using Image J with a colour deconvolution plug-in developed by Ruifrok & Johnston[22]

to compare staining intensity of the immunolabelled tissue (figure 3). The method converts the DAB staining intensity into a quantifiable form which allows for comparison between the groups. A region of interest involving the periodontal ligament around the mesiobuccal root was isolated digitally using Adobe Photoshop 9.0 software. Data from the mesiobuccal root of the first maxillary molar from both experimental and control sides were selected for comparison using the Neuman-Keul paired test on Graphpad Prism 5 software.

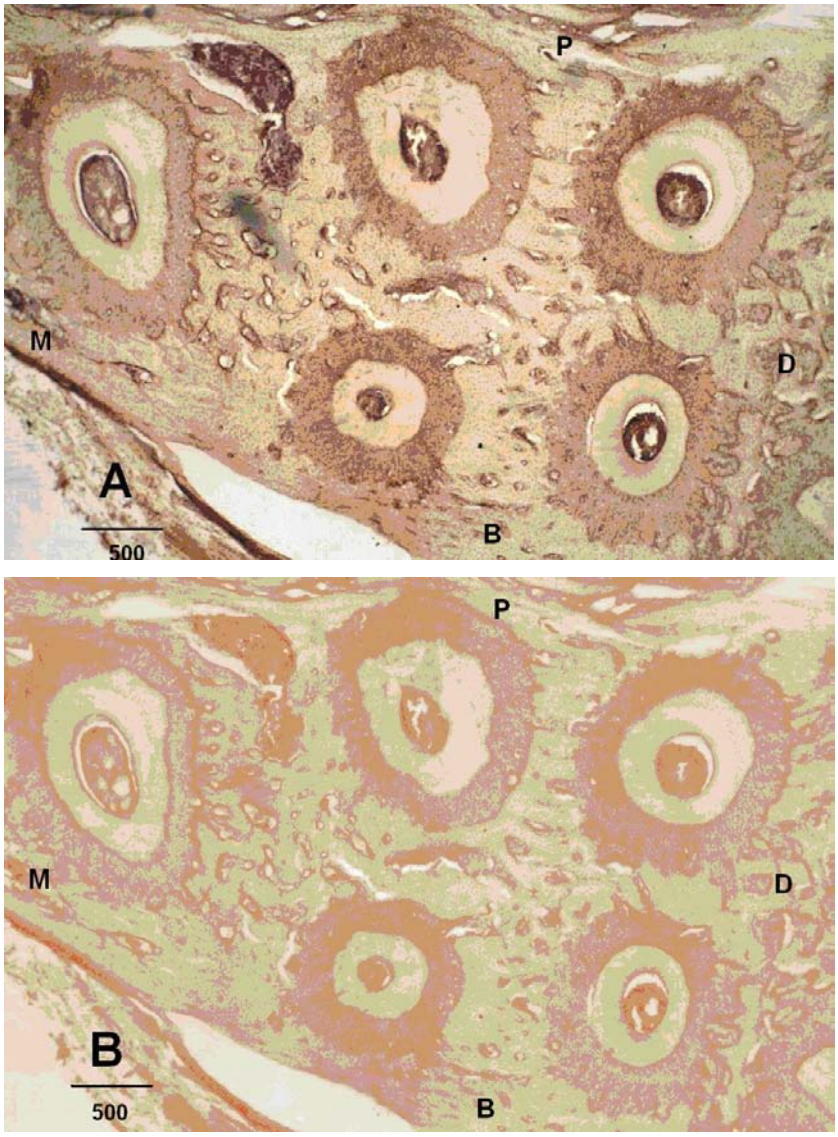

Figure 3: An example of the DAB semi-quantification protocol using the photomicrograph of the maxillary left first molar tissue from the spring attached, saline injected, 7 day group **(A)** with CGRP label (40x), **(B)** after Image J with DAB colour deconvolution (40x), **(C)** after the region of interest, the mesiobuccal root's periodontal ligament, has been isolated.

3. Results

3.1 General cellular and morphological changes

Tissue responses in the uninjected, non-orthodontically injured (non-experimental) day 0 sections were unremarkable for both the left and right sides. There was no evidence of inflammation or change in periodontal morphology. In the experimental saline-injected day 7 sections, a mild inflammatory response was observed, the intensity of which appeared to resolve in the day 14 sections of this group.

3.2 Immunohistochemistry

Observations are summarised in Table 2 and Figure 4. The CGRP label showed elevated staining intensity for day 7 sections in all groups, with a reduction of intensity at day 14. The injection of anti-NGF was seen to have the effect of reducing the intensity of CGRP staining in both day 7 and day 14 sections. Anti-NGF injected animals showed reduced staining when compared to the no anti-NGF injected control groups. CGRP staining appeared to be more intense in the experimental side in comparison to the left side control.

Table 2: Results of Newman-Keuls Multiple Comparison test for CGRP immunolabel staining intensity in the periodontal ligament of the mesiobuccal root of the first molar. Test = Anti-NGF injected. Control = Saline injected. R = spring attached side. L = no spring attached side. Untreated = No spring was attached.

Groups compared	Mean difference in intensity	q	Significance P < 0.05
Test day 7 R v Control day 7 R	-24.66	10.81	Yes
Test day 7 R v Control day 7 L	-24.22	11.82	Yes
Test day 7 R v Test day 7 L	-24.05	12.99	Yes
Test day 7 R v Untreated uninjected R	-16.19	9.817	Yes
Test day 7 R v Untreated uninjected L	-23.4	14.24	Yes
Test day 7 R v Test day 14 R	-1.238	0.6233	No
Test day 7 R v Test day 14 L	-17.62	8.873	Yes
Test day 7 R v Control day 14 R	-23.66	12.23	Yes
Test day 7 R v Control day 14 L	-20.32	10.63	Yes
Test day 14 R v Control day 14 R	-22.42	11.05	Yes
Test day 14 R v Control day 14 L	-19.08	9.508	Yes
Test day 14 R v Test day 14 L	-16.39	7.882	Yes
Test day 14 R v Untreated uninjected R	-14.95	8.498	Yes
Test day 14 R v Untreated uninjected L	-22.16	12.64	Yes
Test day 14 R v Control day 7 R	-23.42	9.911	Yes
Test day 14 R v Control day 7 L	-22.98	10.74	Yes
Test day 14 v Test day 7 L	-22.81	11.70	Yes
Untreated uninjected R v Untreated uninjected L	-7.21	-	No
Untreated uninjected R v Control day 7 R	-8.47	4.059	No
Untreated uninjected R v Control day 7 L	-8.035	-	No
Untreated uninjected R v Control day 14 R	-7.469	-	No
Untreated uninjected R v Control day 14 L	-4.132	-	No
Untreated uninjected R v Test day 7 L	-7.861	-	No
Untreated uninjected R v Test day 14 L	-1.439	-	No
Untreated uninjected L v Control day 7 R	-1.26	-	No
Untreated uninjected L v Control day 7 L	-0.8251	-	No
Untreated uninjected L v Test day 7 L	-0.6513	-	No
Untreated uninjected L v Control day 14 R	-0.2592	-	No
Control day 7 L v Control day 7 R	-0.4353	-	No
Control day 14 R v Control day 7 R	-1.001	-	No
Control day 14 R v Control day 7 L	-0.566	-	No
Control day 14 R v Test day 7 L	-0.3921	-	No
Test day 7 L v Control day 7 R	-0.6091	-	No
Test day 7 L v Control day 7 L	-0.1738	-	No
Test day 14 L v Control day 7 R	-7.031	-	No
Test day 14 L v Control day 7 L	-6.596	-	No
Test day 14 L v Test day 7 L	-6.422	-	No
Test day 14 L v Control day 14 R	-6.03	-	No
Test day 14 L v Untreated uninjected L	-5.771	-	No
Test day 14 L v Control day 14 L	-2.693	-	No
Control day 14 L v Control day 7 R	-4.338	-	No
Control day 14 L v Control day 7 L	-3.903	-	No
Control day 14 L v Test day 7 L	-3.729	-	No
Control day 14 L v Control day 14 R	-3.337	-	No
Control day 14 L v Untreated uninjected L	-3.078	-	No

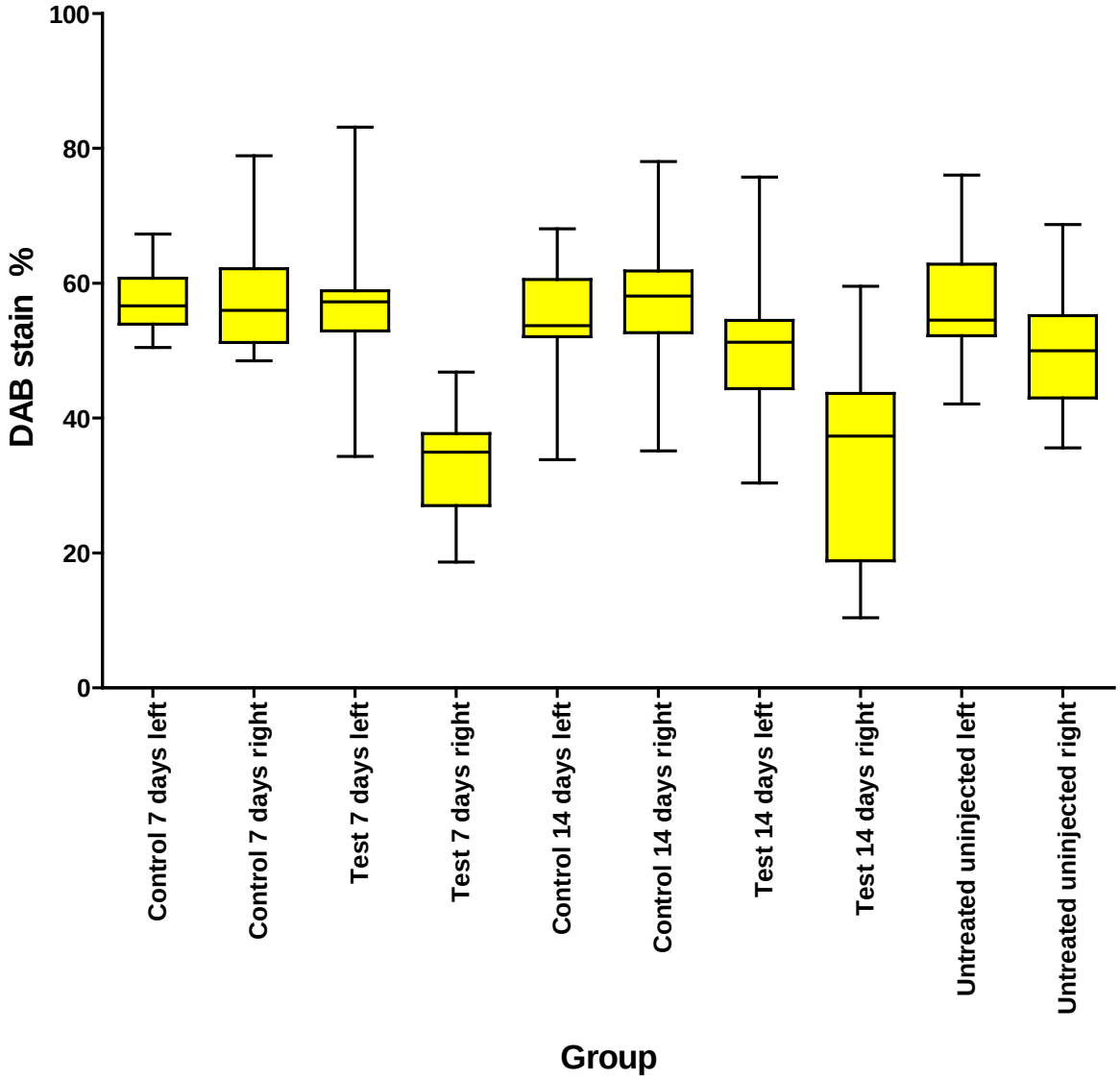

Figure 4: Box plot graphs representing each of the animal sample groups. Test = Anti-NGF injected. Control = Saline injected. R = spring attached side. L = no spring attached side. Untreated = No spring was attached.

In the comparison of different groups, the important findings relate to the Test group staining intensity and the control group staining intensity for similar periods of tooth movement. It was felt that these comparisons were semi-quantitative such that the Newman Keul test could be applied to paired data to detect levels of significant differences.

CGRP-immunoreactive tissue was seen as red-brown coloured areas in the periodontal ligament which were observable at high magnifications. Areas within the dental pulp were clearly CGRP-immunoreactive. The brain stem control tissue showed areas of staining around the dorsal horn, where CGRP-nerve fibres are known to exist. The intensity of CGRP staining was more evident once the photomicrographs of the sections of interest were filtered through imaging software involving a colour deconvolution programme with a specifically designed Haematoxylin-DAB plug-in. This software allowed the comparison of CGRP intensities between the relative groups in the study.

The main results are summarised below:

1. The anti-NGF injected (test) group sacrificed at day 7 showed a reduction in staining intensity when compared to the saline injected (control) group sacrificed at day 7. This difference was statistically significant ($p < 0.05$) when comparing the sides affected by tooth movement (the right maxillary first molar). There was no statistical significance in the difference when comparing the sides not effected by tooth movement (the left maxillary first molar).
2. The anti-NGF injected (test) group sacrificed at day 14 showed a reduction in staining intensity when compared to the saline injected (control) group sacrificed at day 14 (figure 4). The difference was statistically significant ($p < 0.05$) when comparing the sides affected by tooth movement, and there was no statistical significance in the difference when comparing the sides not effected by tooth movement.
3. When comparing tooth movement affected sides of the test groups of day 7 and day 14, whilst there was a slight increase in staining intensity evident at day 14, the difference was not statistically significant. The slight increase suggests resprouting of more CGRP-immunoreactive fibres as the effect of the initial anti-NGF injection at day 0 reduces.
4. When comparing the contralateral non-tooth movement affected sides of the test groups of day 7 and day 14 with the contralateral non-tooth movement affected sides of their respective control groups of day 7 and day 14, there was no statistically significant difference.
5. The comparison of untreated uninjected right and left sides showed no significant difference between the groups.

6. There was no significant difference between the controls for both day 7 and day 14 groups, with the untreated uninjected group for both the right and left sides, indicating there was no effect of the injection process with normal CGRP-immunoreactive tissue levels.

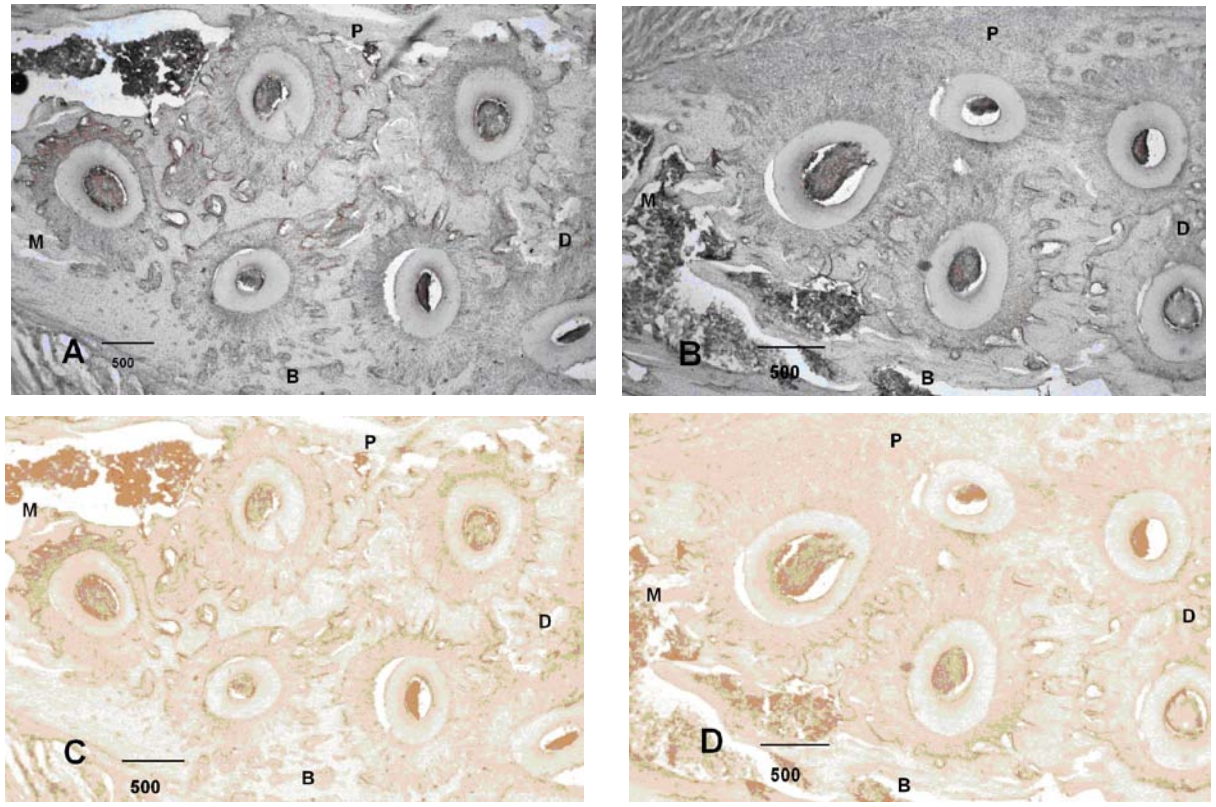

Figure 5: (A) Maxillary right first molar (spring attached), saline injected,14 day group with CGRP label (40x). **(B)** Maxillary right first molar (spring attached), anti-NGF injected, 14 day group with CGRP label (40x). **(C & D)** The same sections after Image J with H-DAB colour deconvolution plugin software applied. The sections show reduced DAB stain intensity in the anti-NGF injected section when compared to the saline injected control section. M = mesial D = distal B = buccal P = palatal. Scale shown is in micrometres.

4. Discussion

Laboratory rats were used in this investigation due to their biological and structural similarities to human periodontal ligament. The rat model also allows for better control of genetic variability, easier monitoring of animal number and conditions (diet and environment), increased sample size and more manageable size of the region to be studied (the maxillary molar).

All animals used in the study were male, eight-week-old Sprague-Dawley rats, weighing between 250 grams and 350 grams. The rat dentition is complete at age 3 – 4 weeks and the animals remained healthy during the study and were fed on a soft diet of pellets. Ho reported that, except for an initial temporary episode of weight loss in most animals for the first 3 days following appliance insertion, there was an overall gain in weight throughout the experimental periods [9]. Male rats were used to negate any hormonal-related variables [23].

The present study used tissue obtained from a previous study[9], after which the maxillary tissue of seventeen treated animals were stored in EDTA medium for up to 18 months in a dark store room at room temperature. Although every effort was made to preserve this tissue in its optimal state, the lengthy storage period may have precipitated some protein denaturation.

A weakness in the previous study[9] was remedied with the addition of six age-matched, male Sprague-Dawley rats which were not injected with anti-NGF or saline. These animals' maxillae were fixed, decalcified, and soaked in OCT following the same protocol as the seventeen animals provided by the previous study.

Application of orthodontic force to rat molars

Various methods have been described, with varying degrees of success. A previous study by O'Hara et al.[4] used the tooth movement model as described by Waldo and Rothblatt to simulate orthodontic movement[24]. However, only 66% (26 out of 42) of his animals were found to have the elastic modules retained at the time of sacrifice. O'Hara noted that the longer the module was left between the teeth, the greater the risk of loosening and module loss during mastication. It was also found that there was no consistency in the direction of tooth movement and level of response. The force delivered to the tooth was not a controlled force vector, but via decompression of an elastomeric, causing tooth movement to be translatory, tipping or rotational depending upon the depth of elastomeric module insertion, tightness of interproximal contact, and duration of application. Tooth morphology was found to be a factor that controlled the pressure exerted by the inserted module which varied according to the shape of the contact point, cusp and crown morphologies[4].

The method used in this experimental sample was based on a modified version of the Brudvig and Rygh protocol, which advocated the use of a closed coil spring ligated between an eyelet on an incisor band and the upper first molar[25-30]. Noxon et al. bonded a closed coil spring between the molar and incisor. This delivered a force of 0.4N to predictably tip the maxillary first molar mesially. Histologically, osteoclast activity was seen on the mesial root surfaces of the first molar[21]. The tooth movement obtained was stable and able to deliver a continuous and constant force[31, 32]. The present study used a continuous force level of 100 g generated by the NiTi coil spring. The retention of the closed coil spring from the modified Brudvig and Rygh protocol used in the seventeen animals obtained from the previous Ho study [9] was 82%.

Perfusion

The animals were perfused with a 1:1 solution of 0.2% para-benzoquinone and 2% para-formaldehyde solutions which has been shown to give good tissue preservation and retention of protein antigens[4].

CGRP-immunoreactivity

The results in this study indicate that the anti-NGF injection dampened the detection of CGRP-immunoreactivity, when compared to a saline-injected sample and a non-injected and non-orthodontically injured sample. The anti-NGF may have inhibited the sprouting of CGRP-immunoreactive nerve fibres.

The slight increase in CGRP-immunoreactive staining from day 7 to day 14 in the anti-NGF injected sample suggests that the effects of the initial anti-NGF injection may have reduced over this time period, and that more CGRP-immunoreactive fibre re-sprouting could occur. However, this slight increase was statistically insignificant and it is important to note that the sample number of animals in the day 14, anti-NGF, spring attached group was small (n=3), indicating a larger sample may find different results.

As noted by O'Hara et al.[33], the sprouting of CGRP-immunoreactive fibres was more pronounced in the pulp, periodontal ligament-bone interface and trabeculae surfaces, indicating the involvement of the fibres in bone remodelling. This finding is similar to those of previous studies by Kvinnsland et al. [13] and Hill & Elde [34]. CGRP-immunoreactive fibres were found to be related to areas of bone deposition and inhibition of bone resorption, and a study by Ballica et al found an increase in bone density in mice injected with CGRP[35]. CGRP fibres were also found near the root cementum suggesting a supporting role in the reparative process of the periodontal ligament during tooth movement [15, 36]. Imai and Matsusue suggested that CGRP has an effect on bone metabolism via neural and a local effect [7]. Their results of injection of animals with anti-NGF showed nil CGRP-immunoreactivity in the periodontal ligament or bone at any time periods for both experimental and control sides. A study by Christensen and Hulsebosch involving rat spinal cord reported that injection of anti-NGF inhibited sprouting of CGRP-immunoreactive fibres in the dorsal horn [37].

The observations of anti-NGF effect on CGRP-immunoreactive fibre staining intensity in this study correspond with the findings by O'Hara et al, which demonstrated highest intensity of NGF at day 7 [4]. Perhaps NGF has its greatest effect on CGRP-

immunoreactive fibre resprouting around this time period and, therefore, an anti-NGF injection would reflect this finding, reducing the amount of CGRP-immunoreactivity. NGF may play an important role in the reorganisation of the periodontal ligament and alveolar bone during orthodontic tooth movement.

The cells within the rat dentoalveolar complex which express NGF may signal for CGRP-immunoreactive fibre resprouting in orthodontically injured tissue. The nervous system may play an important regulatory role in the bone remodelling process. This suggests that NGF may be used to induce nerve sprouting and facilitate orthodontic tooth movement via the repair of tissue via upregulating the distribution of CGRP-immunoreactive fibres. Conversely, anti-NGF may be locally applied to a tooth which could inhibit the sprouting of CGRP-immunoreactive nerve fibres which, theoretically, could reduce the amount of tooth movement, reduce root resorption or inhibit pain sensation carried by these fibres.

Conclusion

The results of the present study indicate injection of anti-NGF reduces the NGF levels within the periodontal ligament and inhibits the innervation of the periodontal ligament by CGRP-immunoreactive fibres in an orthodontically-injured tooth, when compared to both the saline-injected, orthodontically-injured sample, and to the non-injected, non-orthodontically injured sample. The inhibiting effect of anti-NGF appears to have a greater effect at day 7 of tooth movement when compared to the day 14 group, although the difference is statistically insignificant, most likely due to a small sample number in the day 14 group.

Acknowledgments

Funding: The Australian Society of Orthodontists Foundation for Research and Education provided partial funding for the project.

Conflict of interest: There is no foreseeable conflict of interest.

Ethical approval: The ethics approval was obtained from the University of Adelaide Animal Ethics Committee (M/023/2006).

Statistician: Dr. Ian Parkinson, Senior Medical Scientist, Hanson Institute.

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ARTICLE TWO

ORTHODONTIC TOOTH MOVEMENT AND SLOW FIBRE DENSITY IN THE RAT

FOR SUBMISSION TO THE ARCHIVES OF ORAL BIOLOGY

Abstract

Objectives: Neural changes involving calcitonin gene-related peptide (CGRP) immunoreactive tissue have been shown to occur in the periodontal ligament of orthodontically traumatised teeth. These changes with tooth movement have been found to be associated with the injection of neurotrophin altering agents such as anti-nerve growth factor. The aim of this study was to examine whether neural changes involving CGRP have a linear association with measured orthodontic tooth movement in the rat.

Design: Orthodontic NiTi pullcoil springs (100g) were ligated to the right maxillary first molar and maxillary incisors of 17, eight week old, male Sprague-Dawley rats which were divided into two groups. The first group were injected with anti-NGF palatal to the right maxillary first molar. The second group were injected with saline in the same location to serve as a control. Animals from both groups were sacrificed at 7 and 14 days, perfused with 4% paraformaldehyde and the maxillae were decalcified in 4% EDTA. 25 μm horizontal frozen sections were obtained using a cryostat at -20°C. Immunohistochemical tissue labelling was performed, involving the avidin-biotin peroxidase technique and rabbit anti-calcitonin gene-related peptide (anti-CGRP) as the primary antibody. Photomicrographs were analysed using Image J software with a colour deconvolution plug-in to quantify the staining intensity of the immunolabelled maxillary first molar mesiobuccal root periodontal ligament circumference. The amount of tooth movement between the incisors and first molars was measured using direct and indirect means and compared with the CGRP staining intensity of each animal. A linear regression analysis was performed using the paired sets of data on Graphpad Prism 5 software.

Results: The linear regression analysis using the direct and indirect methods of measuring tooth distance showed there was no significant association between the CGRP staining intensity and the amount of tooth movement measured ($p>0.05$).

Conclusion: The results suggest that there was no significant association between CGRP staining intensity and amount of tooth movement achieved. Tooth movement and CGRP nerve fibre density are effected by multiple variables; therefore, a linear association between the neural response to tooth movement and the amount of tooth movement achieved may not be present.

1. Introduction

Orthodontic tooth movement applies stress on the dento-alveolar complex, causing a biological response resulting in the remodelling of the periodontium. The periodontal ligament is stretched and compressed depending on the direction of force[1]. Stress placed on the periodontal ligament may cause nerve fibres and neuroreceptors within the tissue to become distorted, causing the release of neurotrophins in areas of tissue damage and clinical responses of pain and pressure. These neurotrophins interact with cells including fibroblasts, endothelial and alveolar bone cells to initiate bone resorption, resulting in cellular proliferation and differentiation[2].

Calcitonin gene-related peptide (CGRP) is a neuropeptide involved in the regulation of bone resorption [2-5]. It has been localised in the hypothalamus, thalamus and hippocampus of the central nervous system, and in the sensory, motor and autonomic fibres of the peripheral nervous system. CGRP is produced in small diameter neurons that become unmyelinated C and thin myelinated Aδ fibres; they sense pain, temperature and noxious stimuli [6]. CGRP-immunoreactive fibres are widely distributed in bone and the periodontal ligament, suggesting an important role in bone remodelling [7].

Saito et al. [8] showed dense regions of CGRP-immunoreactive nerves in the periodontal ligament during tooth movement. Following orthodontically induced injury, surviving nerve processes regrew into the injured area, largely in response to increased tissue levels of neurotrophic factors such as NGF [9]. To date, no studies have shown an association between CGRP-immunoreactive nerve fibre intensity and the amount of orthodontic tooth movement achieved.

The present study aims to evaluate a potential association between CGRP-immunoreactive fibre density, and the amount of orthodontic tooth movement achieved over a given period of time. The null hypothesis to be investigated is there is no association between CGRP-immunoreactivity and the amount of orthodontic tooth movement.

2. Materials and methods

The following study was performed with approval of the University of Adelaide Animal Ethics Committee under number M/023/2006. The rats weighed between 250 - 350g and were fed commercially manufactured standard pellets (Parastoc Feed, Ridley AgriProducts, Murray Bridge, Australia) and water, ad libitum. They were contained at the Animal House facility of the Medical School of the University of Adelaide.

2.1 Experimental groups

A previous unpublished study by Ho [10] provided maxillae from seventeen male, eight-week-old Sprague-Dawley rats, in which a 100g superelastic nickel titanium closing spring (Sentalloy, GAC, New York, USA), was bonded to the right maxillary first molar and to the maxillary incisors using 0.010" stainless steel ligatures and light cured bonding material (Z100 composite resin, 3M ESPE, St. Paul, USA)(see Appendix 1). This was to impart a mesially directed force to the right maxillary first molar, using a protocol developed by Noxon et al [11]. The test animals were injected with 1 µL (2 µg) of anti-NGF into the gingival mucosa between the right maxillary first and second molars immediately after application of the spring. A sham control group of animals were injected with the same volume of an isotonic saline solution into the gingival mucosa between the right maxillary first and second molar.

Animals were provided with identification numbers and were sacrificed at day 7 and 14 as shown in Table 1.

Table 1: Sample size and categories. All had an orthodontic spring attached to the maxillary right first molar.

DAY	*CONTROL (saline injected)*	*ANTI-NGF INJECTED*	*TOTAL*
7	5	6	11
14	3	3	6
TOTAL	8	9	17

2.2 Measurement of tooth movement

The rats were anaesthetised via subcutaneuous injection of a combination of Ketamine (100mg/ml) (Ketamil Injection, Troy Laboratories, Smithfield, Australia) and Xylazine (Xylazil, 20mg/ml) at a dosage of 2ml/kg. The two drugs were mixed at the ratio 2:1 (Ketamine:Xylazine) and diluted 1:1 with sterile water for injection.

Ho [10] developed a protocol for molar-to-incisor distance measurement on the anaesthetised animal via direct and indirect means (see Appendix 2). The direct method involved the use of a digital calliper taking three sets of readings from the labial surface of the incisor to the mesial surface of the first molar. A mean value was calculated from the three readings. Readings were performed by the same operator, and recorded at Day 0, 3, 7, 10 and 14. In the present study, the measurements at Day 7 and 14 were used to form paired data, along with the quantified CGRP staining intensity data for each animal. This was because animals were sacrificed at either day 7 or day 14 for immunological staining. The indirect method involved a polyvinyl siloxane impression (Imprint 3, 3M ESPE) of the maxillary arch using a special tray on the anaesthetised animal. Images of the impressions were taken with a MZ16FA stereo microscope and were calibrated using Image analySIS software. Computer software was used to measure the distance between three constructed centroid points representing the incisors, the first molar and second molar on the digitised images (Appendix 2).

2.3 Harvesting of tissue

The animals were sacrificed via cardiac ventricular perfusion with fixative solutions, after an intraperitoneal administration of chloral hydrate 5g/100ml H_2O (5mL)(Appendix 3). Of the seventeen animals that had spring placement and injection performed, eleven animals were perfused at 7 days and six animals were perfused at 14 days. The perfusion protocol involved the use of 200ml 0.1% sodium nitrite, and 400ml of 50:50 para-formaldehyde and p-benzoquinone under positive pressure. Each animal had the maxilla and brainstem removed and stored in the 50:50 fixative solution for two hours, followed by 4% EDTA solution pH 7.4 for 8 weeks. The decalcified tissue was then impregnated with an embedding medium (OCT, Sakura Finetek, Torance, CA, USA) for cryosectioning.

2.4 Section preparation

Horizontal sections 25µm thick were obtained using a Leitz 1720 cryostat at -20°C. The maxillae were orientated so that the crowns of teeth were uppermost, and sections were collected of the first and second molars below the furcation to the apex of the first molar (figure 1).

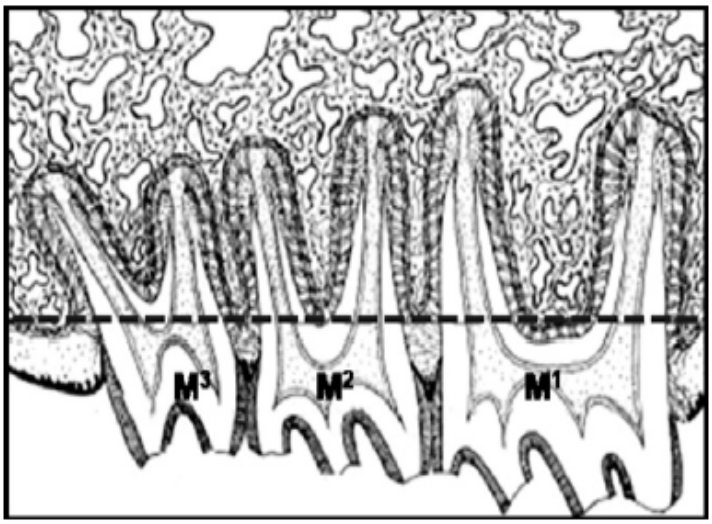

Figure 1: Schematic representation of section through maxillary first molar (M1) and maxillary second molar (M2) roots below the furcation

Serial sections were placed consecutively over 12 slides, so that each slide contained sections representing different levels of the periodontal ligament for comparison. Positive controls were provided by including a section of the trigeminal ganglion of the brainstem or the dorsal horns of the spinal cord, on each slide (figure 2).

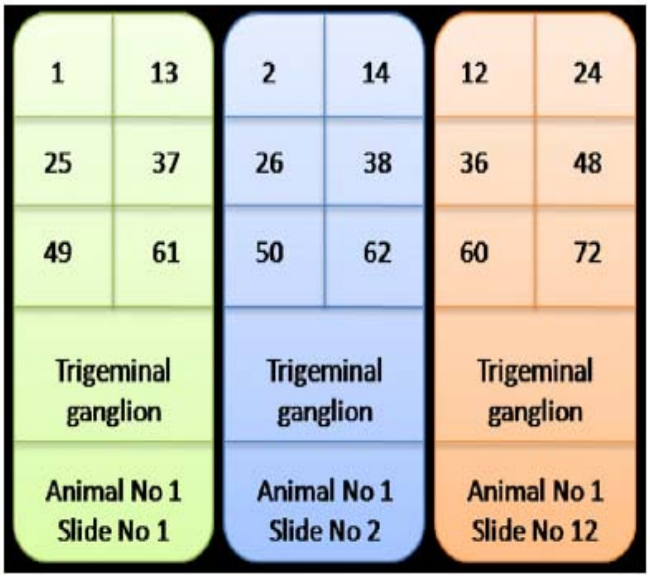

Figure 2: Arrangement of specimen sections on slides

2.5 Immunohistochemistry

The odd-numbered slides from each animal were used for immunohistochemical labelling for CGRP. Slides from the different groups were labeled in the same solutions simultaneously along with both positive and negative control tissue (rat brainstem) known to have CGRP-immunoreactive fibres. The avidin-biotin peroxidase complex technique was used involving rabbit anti-rat-CGRP (Peninsula Laboratories, ME, USA) as the primary antibody and biotinylated anti-rabbit IgG (Rockland, PA, USA) as the secondary antibody. The anti-CGRP was diluted from lyophilised form (50µl) to 1:4000 in 1% normal goat serum (NGS in 0.1M PBS). The secondary antibody IgG (2mg/ml lyophilised) was diluted to 1:250 in 1% NGS.

The optimal primary antibody concentration used was found through a pilot study using similar tissue to the area studied. The immunocytochemistry protocol was previously developed (Appendices 5 & 6). Slides were incubated for 30 minutes in a blocking solution of 0.3% H_2O_2 in 70% methanol:0.1M phosphate buffered saline (PBS), followed by three five minute rinses in 0.1M PBS. Slides were then incubuted for one hour in 3% NGS (in 0.1M PBS). Between 800 – 1000 µL of diluted primary antibody was placed on the slides and incubated overnight (16-18 hours) at room temperature in a humidified chamber on a shaker.

Three, ten minute washes in 1% NGS were followed by a one hour incubation in diluted secondary antibody. The slides were rinsed with three, ten minute 0.1M PBS washes. A diluted streptavidin-peroxidase 1:1000 in 0.1M PBS was prepared into which slides were incubated for one hour. Three, ten minute 0.1M PBS washes were performed, followed by incubation in prepared DAB in 0.1M Tris buffer (pH 7.65) with 3% H_2O_2 added to initiate the reaction. The reaction was stopped after five to seven minutes with rinses in 0.1M Tris buffer, then 0.1M PBS. A haematoxylin counter-stain was performed and slides were mounted and cover slipped.

Photomicrographs were taken at a range of magnifications using an Olympus BH-2 photomicroscope (Altra20 camera and analySIS FIVE system) and stored as JPEG files of 300 – 350 kilobytes. The resulting digital photomicrographs were analysed using Image J with a colour deconvolution plug-in developed by Ruifrok & Johnston [12] to compare staining intensity of the immunolabelled tissue (figure 3). The method converts the DAB staining intensity into a quantifiable form which allows for comparison between the groups. A region of interest involving the periodontal ligament around the mesiobuccal root was isolated digitally using Adobe Photoshop 9.0 software. Mean values were calculated for each animal, using the staining intensities for each section of that animal. Along with the animal's tooth movement measurements, the values were used to form the paired data for a linear regression analysis using Graphpad Prism 5 software.

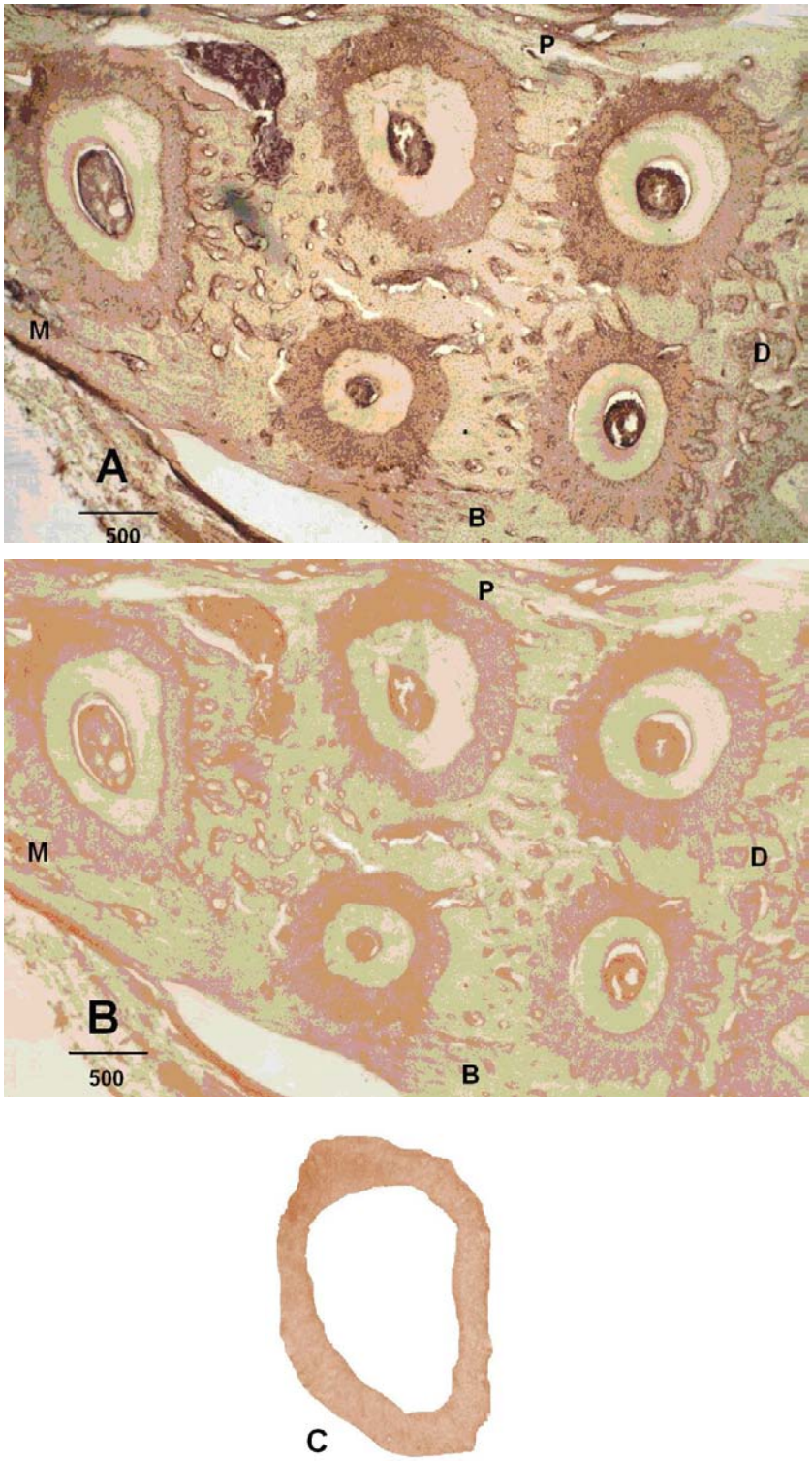

Figure 3: An example of the DAB semi-quantification protocol using the photomicrograph of the maxillary left first molar tissue from the spring attached, saline injected, 7 day group **(A)** with CGRP label (40x), **(B)** after Image J with DAB colour deconvolution (40x), **(C)** after the region of interest, the mesiobuccal root's periodontal ligament, has been isolated. (M) mesial, (D) distal, (B) buccal, (P) palatal. Scale bar 500 µm.

3. Results

3.1 Immunohistochemistry

CGRP-immunoreactive tissue was seen as red-brown coloured areas in the periodontal ligament which were observable at high magnifications and areas within the dental pulp were clearly CGRP-immunoreactive. The brain stem control tissue showed areas of staining around the dorsal horn, where CGRP-nerve fibres are known to exist. Once the photomicrographs of the sections of interest were filtered through imaging software, the intensity of CGRP staining was more evident. The software involves a colour deconvolution programme with a specifically designed Haematoxylin-DAB plug-in (figure 4), allowing for the semi-quantitative comparison of CGRP intensities between the relative groups in the study without the distraction of haematoxylin and background DAB colour variation.

The results of Article 1 indicate injection of anti-NGF reduces the NGF levels within the periodontal ligament and inhibits the innervation of the periodontal ligament by CGRP-immunoreactive fibres in an orthodontically-injured tooth, when compared to both the saline-injected, orthodontically-injured sample, and to the non-injected, non-orthodontically injured sample. The inhibiting effect of anti-NGF appears to have a greater effect at day 7 of tooth movement, when compared to the day 14 group, although the difference is statistically insignificant, most likely due to a small sample number in the day 14 group.

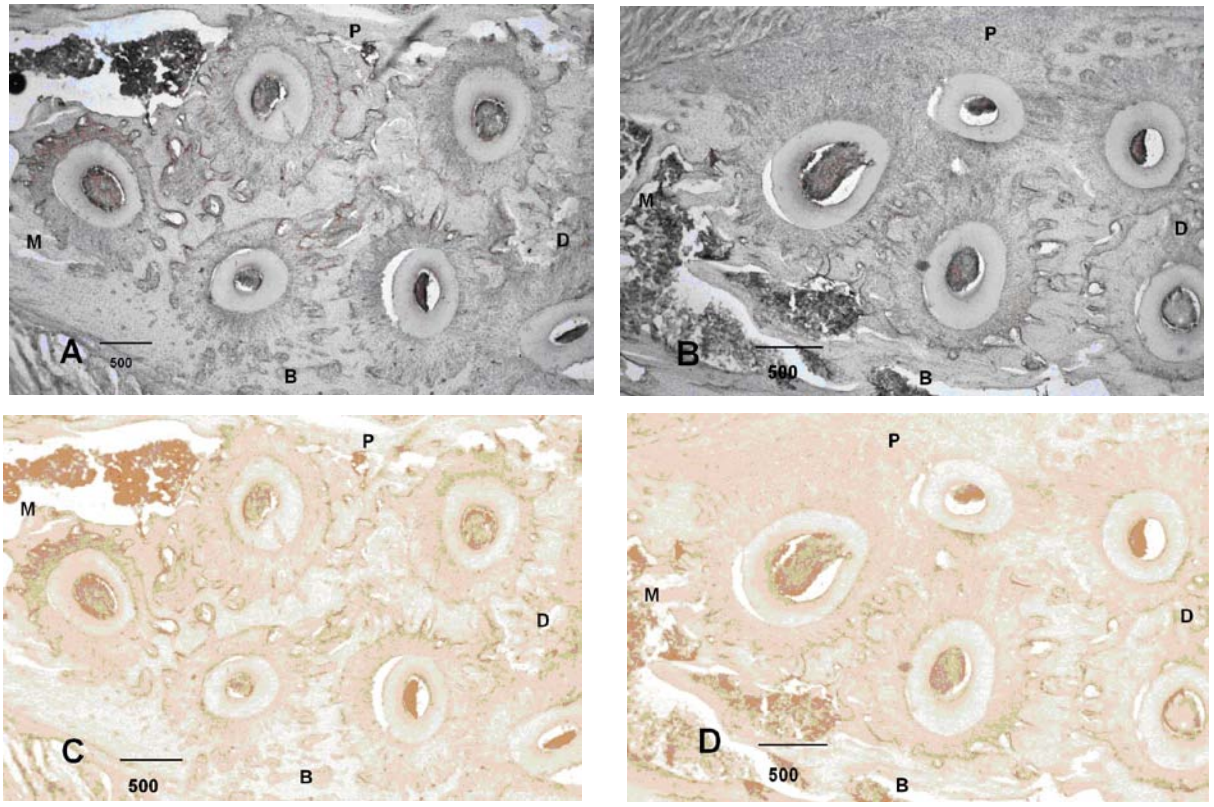

Figure 4: (A) Maxillary right first molar (spring attached), saline injected,14 day group with CGRP label (40x). **(B)** Maxillary right first molar (spring attached), anti-NGF injected, 14 day group with CGRP label (40x). **(C & D)** The same sections after Image J with H-DAB colour deconvolution plugin software applied. The sections show reduced DAB stain intensity in the anti-NGF injected section when compared to the saline injected control section. M = mesial D = distal B = buccal P = palatal. Scale shown is in micrometres.

3.2 Tooth measurement results

The results obtained in the previous study by Ho [10] are shown in Appendix 3. Measurement calculations were performed using SAS Version 9.1 software (SAS Institute Inc., Cary, NC, USA). The present study involved a linear regression analysis of scatter plot diagrams using the paired data shown in Table 2, figures 5 and 6.

Table 2: Tooth movement measured between left and right maxillary first molar and incisors over day 0 – day 7 via the direct method. CGRP stain intensity was for the left and right maxillary first molars. Animals were injected with saline (between right maxillary first and second molars).

	LEFT	LEFT	RIGHT	RIGHT
Animal	**CGRP stain intensity (%)**	**Distance day 0 – day 7 Direct (mm)**	**CGRP stain intensity (%)**	**Distance day 0 – day 7 Direct (mm)**
301	47.17	0.47	53.26	0.83
302	44.84	0.35	41.35	0.81
303	50.74	0.26	51.17	0.75
304	56.03	-0.14	55.59	-0.01

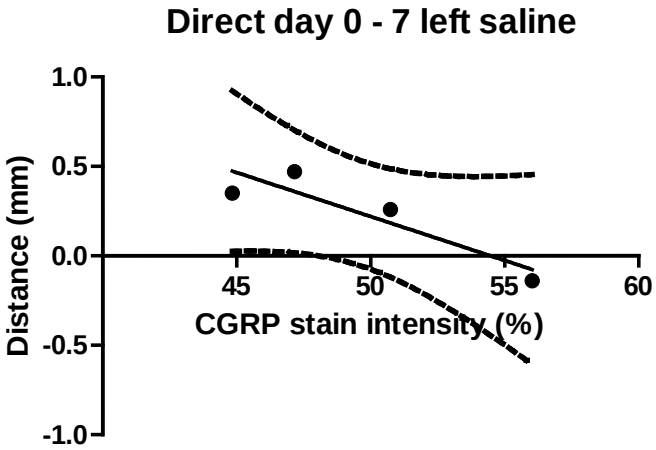

Figure 5: Scatter plot graph representing the paired data shown above. The solid line shows the line of best fit. Dotted lines show 95% confidence levels. R square value = 0.8219. S y.x = 0.1367. The slope is not significantly non-zero (P = 0.0934).

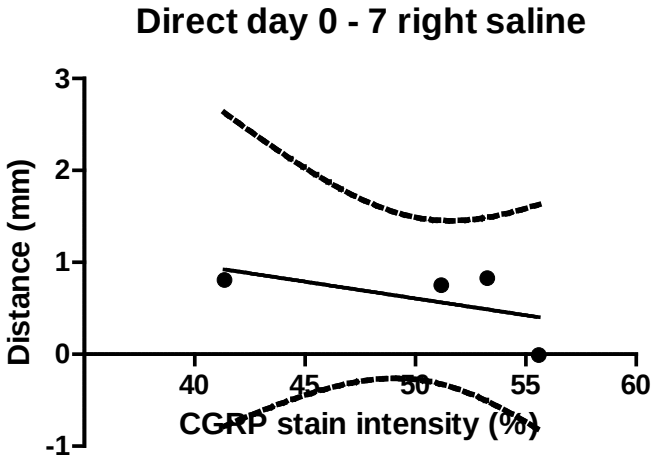

Figure 6: Scatter plot graph representing the paired data shown above. The solid line shows the line of best fit. Dotted lines show 95% confidence levels. R square value = 0.3289. S y.x = 0.4091. The slope is not significantly non-zero (P = 0.4353).

Table 3: Tooth movement measured between centroids of left and right maxillary first molar and incisors over day 0 – day 7 via the indirect method. CGRP stain intensity was for the left and right maxillary first molars. Animals were injected with saline (between right maxillary first and second molars).

	LEFT	LEFT	RIGHT	RIGHT
Animal	**CGRP stain intensity (%)**	**Distance day 0 – day 7 Indirect (mm)**	**CGRP stain intensity (%)**	**Distance day 0 – day 7 Indirect (mm)**
301	47.17	0.58	53.26	1.85
302	44.84	0.25	41.35	1.04
303	50.74	-0.04	51.17	0.81
304	56.03	0.38	55.59	1.24

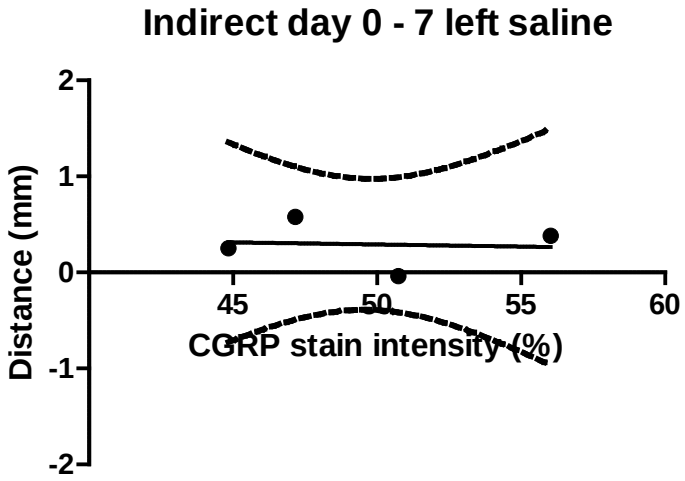

Figure 7: Scatter plot graph representing the paired data shown above. The solid line shows the line of best fit. Dotted lines show 95% confidence levels. R square value = 0.006814. S y.x = 0.3172. The slope is not significantly non-zero (P = 0.9175).

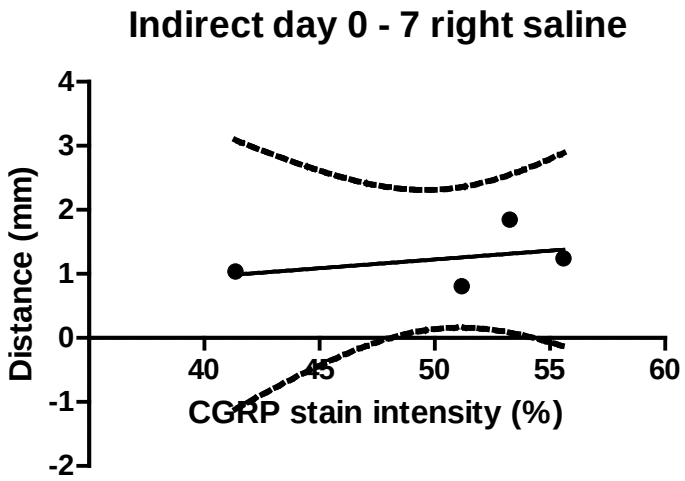

Figure 8: Scatter plot graph representing the paired data shown above. The solid line shows the line of best fit. Dotted lines show 95% confidence levels. R square value = 0.1478. S y.x = 0.5043. The slope is not significantly non-zero (P = 0.9175).

Table 4: Tooth movement measured between the centroids of right maxillary first and second molars over day 0 – day 7 via the indirect method. CGRP stain intensity was for the right maxillary first molar. Animals were injected with saline (between right maxillary first and second molars).

	RIGHT	RIGHT
Animal	**CGRP stain intensity (%)**	**Distance day 0 – day 7 Direct (mm)**
301	53.26	0.64
302	41.35	0.31
303	51.17	0.53
304	55.59	0.25

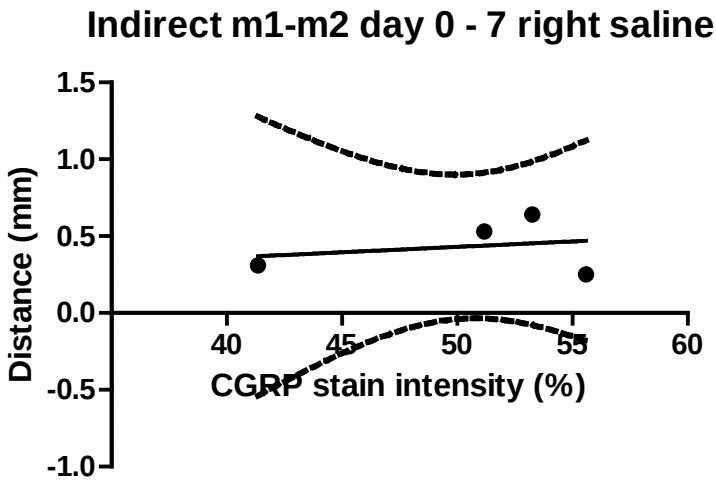

Figure 9: Scatter plot graph representing the paired data shown above. The solid line shows the line of best fit. Dotted lines show 95% confidence levels. R square value = 0.05815. S y.x = 0.2180. The slope is not significantly non-zero (P = 0.7589).

Table 5: Tooth movement measured between left and right maxillary first molar and incisors over day 0 – day 7 via the direct method. CGRP stain intensity was for the left and right maxillary first molar. Animals were injected with anti-NGF (between right maxillary first and second molars).

	LEFT	LEFT	RIGHT	RIGHT
Animal	**CGRP stain intensity (%)**	**Distance day 0 – day 7 Direct (mm)**	**CGRP stain intensity (%)**	**Distance day 0 – day 7 Indirect (mm)**
104	47.81	0.41	33.09	1.03
106	24.33	0.20	-	0.97
305	43.85	0.48	41.94	1.02
306	54.49	0.20	43.38	0.32
307	44.83	0.46	46.99	0.50
308	50.24	0.48	50.23	1.16

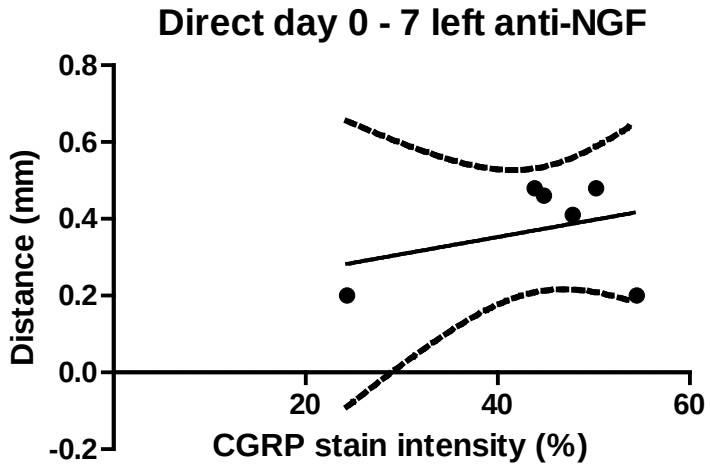

Figure 10: Scatter plot graph representing the paired data shown above. The solid line shows the line of best fit. Dotted lines show 95% confidence levels. R square value = 0.1194. S y.x = 0.1421. The slope is not significantly non-zero (P = 0.5023).

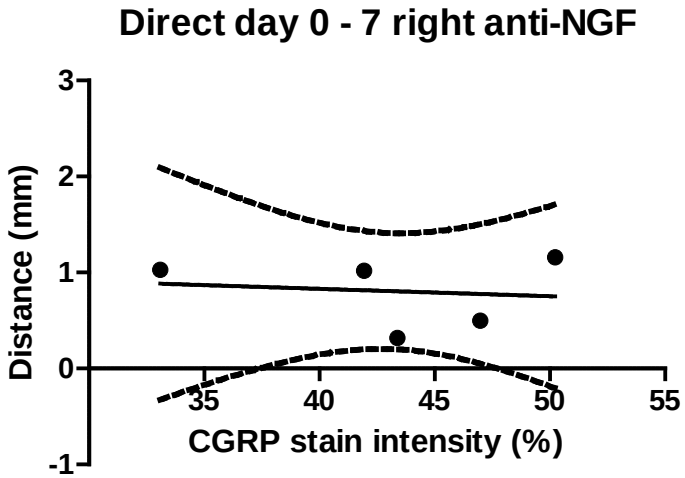

Figure 11: Scatter plot graph representing the paired data shown above. The solid line shows the line of best fit. Dotted lines show 95% confidence levels. R square value = 0.01815. S y.x = 0.4247. The slope is not significantly non-zero (P = 0.8290).

Table 6: Tooth movement measured between the centroid of left and right maxillary first molar and incisors over day 0 – day 7 via the indirect method. CGRP stain intensity was for the left and right maxillary first molars. Animals were injected with anti-NGF (between right maxillary first and second molars).

	LEFT	LEFT	RIGHT	RIGHT
Animal	**CGRP stain intensity (%)**	**Distance day 0 – day 7 Indirect (mm)**	**CGRP stain intensity (%)**	**Distance day 0 – day 7 Indirect (mm)**
104	47.81	-0.07	33.09	0.57
106	24.33	0.03	-	0.76
305	43.85	0.59	41.94	1.30
306	54.49	0.25	43.38	0.73
307	44.83	0.21	46.99	0.69
308	50.24	0.34	50.23	1.06

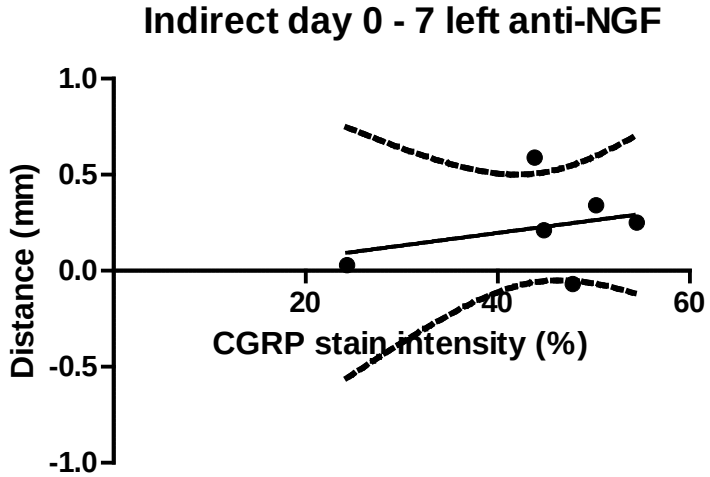

Figure 12: Scatter plot graph representing the paired data shown above. The solid line shows the line of best fit. Dotted lines show 95% confidence levels. R square value = 0.08749. S y.x = 0.2493. The slope is not significantly non-zero (P = 0.5693).

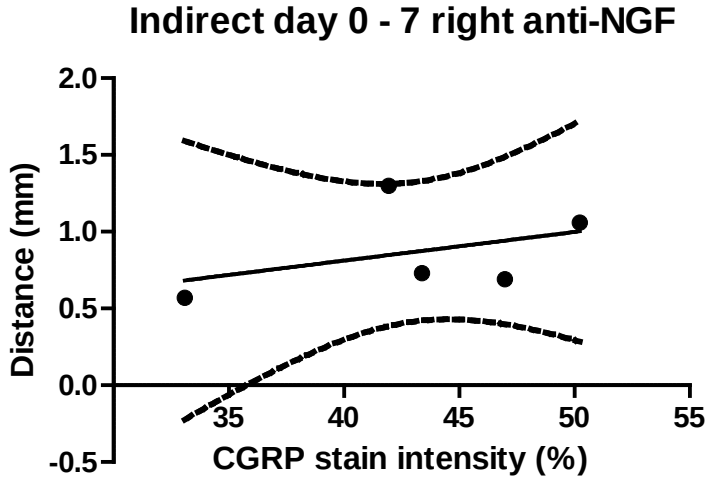

Figure 13: Scatter plot graph representing the paired data shown above. The solid line shows the line of best fit. Dotted lines show 95% confidence levels. R square value = 0.1601. S y.x = 0.3188. The slope is not significantly non-zero (P = 0.5044).

Table 7: Tooth movement measured between the centroids of right maxillary first and second molars over day 0 – day 7 via the indirect method. CGRP stain intensity was for the right maxillary first molar. Animals were injected with anti-NGF (between right maxillary first and second molars).

	RIGHT	RIGHT
Animal	**CGRP stain intensity (%)**	**Distance day 0 – day 7 Indirect (mm)**
104	33.09	0.38
106	-	0.58
305	41.94	0.49
306	43.38	0.42
307	46.99	0.40
308	50.23	0.18

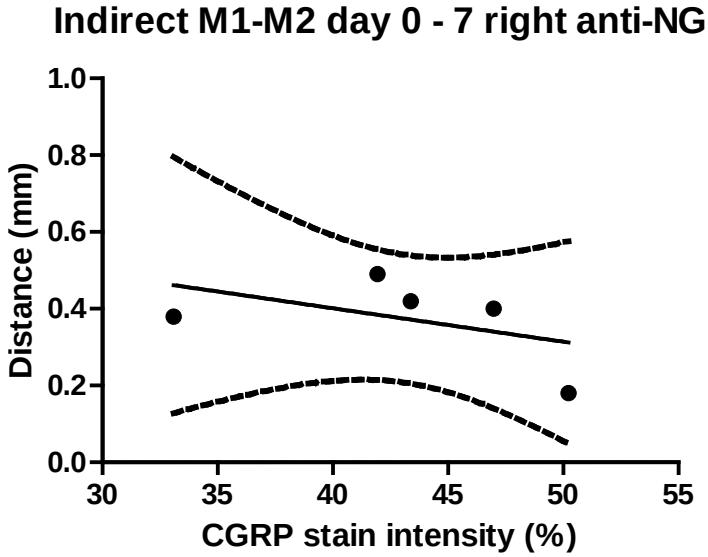

Figure 14: Scatter plot graph representing the paired data shown above. The solid line shows the line of best fit. Dotted lines show 95% confidence levels. R square value = 0.2368. S y.x = 0.1171. The slope is not significantly non-zero (P = 0.4058).

Table 8: Tooth movement measured between the left and right maxillary first molars and incisors over day 0 – day 14 via the direct method. CGRP stain intensity was for the left and right maxillary first molars. Animals were injected with saline (between right maxillary first and second molars).

	LEFT	LEFT	RIGHT	RIGHT
Animal	**CGRP stain intensity (%)**	**Distance day 0 – day 14 Direct (mm)**	**CGRP stain intensity (%)**	**Distance day 0 – day 14 Direct (mm)**
201	50.98	0.80	51.15	1.47
401	36.58	0.93	43.09	1.38
402	48.31	0.40	53.58	1.58

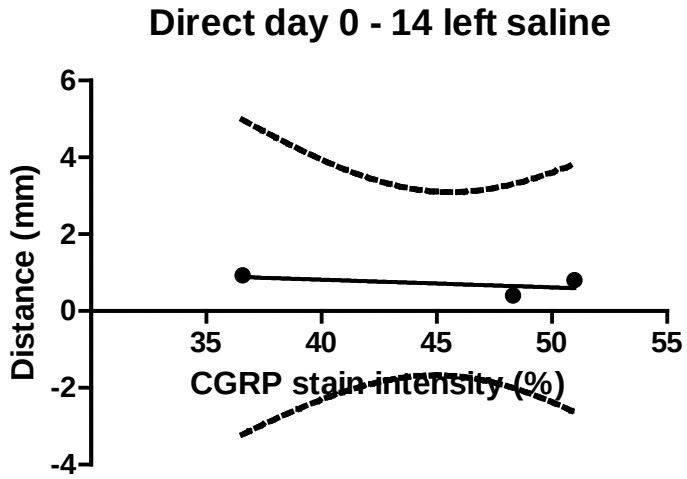

Figure 15: Scatter plot graph representing the paired data shown above. The solid line shows the line of best fit. Dotted lines show 95% confidence levels. R square value = 0.3057. S y.x = 0.3255. The slope is not significantly non-zero (P = 0.6270).

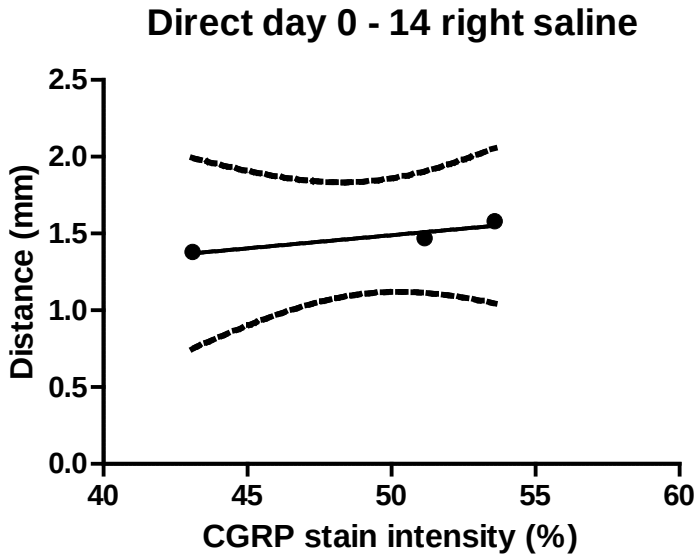

Figure 16: Scatter plot graph representing the paired data shown above. The solid line shows the line of best fit. Dotted lines show 95% confidence levels. R square value = 0.8769. S y.x = 0.04970. The slope is not significantly non-zero (P = 0.2282).

Table 9: Tooth movement measured between the centroids of the left and right maxillary first molar and incisors over day 0 – day 14 via the indirect method. CGRP stain intensity was for the left and right maxillary first molar. Animals were injected with saline (between right maxillary first and second molars).

	LEFT	LEFT	RIGHT	RIGHT
Animal	**CGRP stain intensity (%)**	**Distance day 0 – day 14 Indirect (mm)**	**CGRP stain intensity (%)**	**Distance day 0 – day 14 Indirect (mm)**
201	50.98	0.48	51.15	1.83
401	36.58	1.16	43.09	2.28
402	48.31	0.22	53.58	1.64

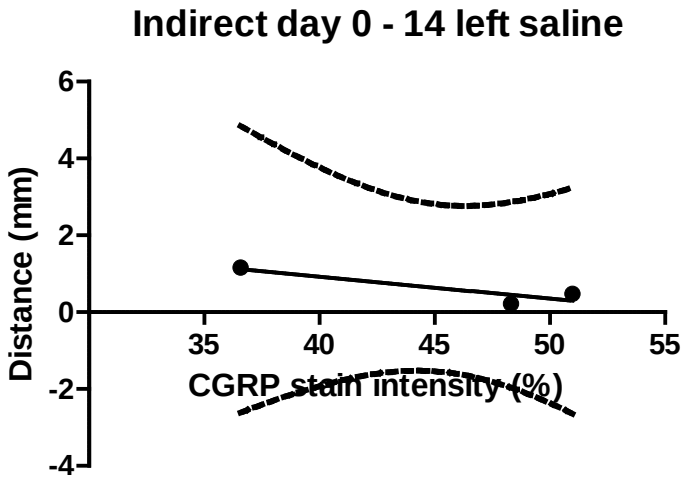

Figure 17: Scatter plot graph representing the paired data shown above. The solid line shows the line of best fit. Dotted lines show 95% confidence levels. R square value = 0.8136. S y.x = 0.2963. The slope is not significantly non-zero (P = 0.2842).

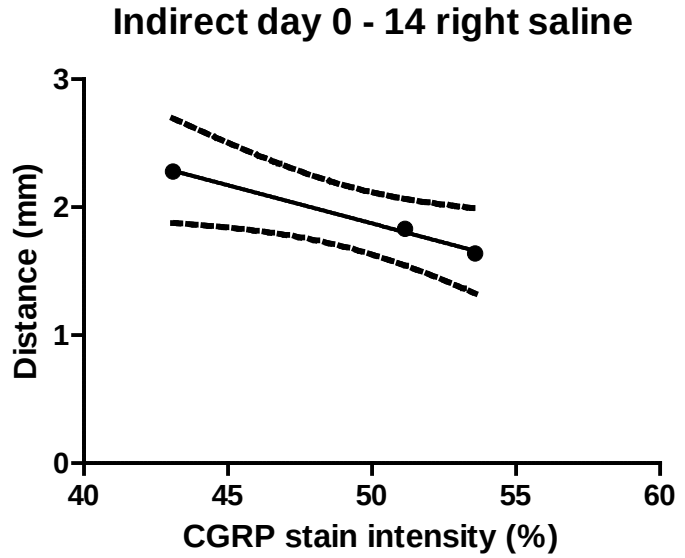

Figure 18: Scatter plot graph representing the paired data shown above. The solid line shows the line of best fit. Dotted lines show 95% confidence levels. R square value = 0.9951. S y.x = 0.03270. The slope is significantly non-zero (P = 0.0448).

Table 10: Tooth movement measured between the centroids of the right maxillary first and second molars over day 0 – day 14 via the indirect method. CGRP stain intensity was for the right maxillary first molar. Animals were injected with saline (between right maxillary first and second molars).

	RIGHT	RIGHT
Animal	**CGRP stain intensity (%)**	**Distance day 0 – day 14 Direct (mm)**
201	51.15	0.71
401	43.09	0.73
402	53.58	0.30

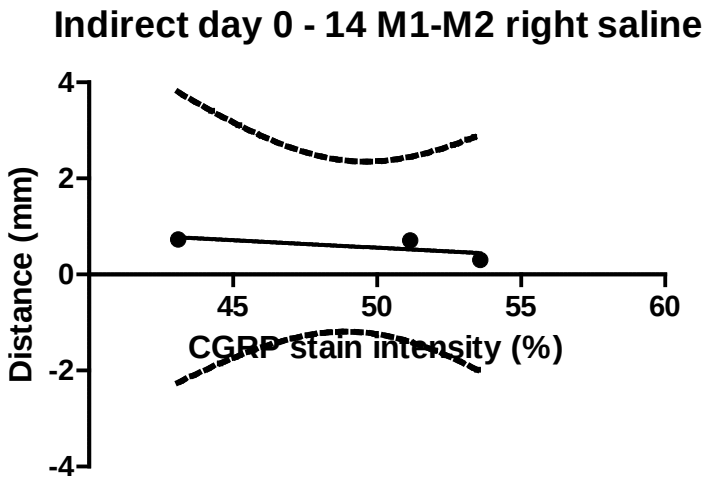

Figure 19: Scatter plot graph representing the paired data shown above. The solid line shows the line of best fit. Dotted lines show 95% confidence levels. R square value = 0.5022. S y.x = 0.2422. The slope is not significantly non-zero (P = 0.4986).

Table 11: Tooth movement measured between the left and right maxillary first molars and the incisors over day 0 – day 14 via the direct method. CGRP stain intensity was for the left and right maxillary first molars. Animals were injected with anti-NGF (between right maxillary first and second molars).

	LEFT	LEFT	RIGHT	RIGHT
Animal	**CGRP stain intensity (%)**	**Distance day 0 – day 14 Direct (mm)**	**CGRP stain intensity (%)**	**Distance day 0 – day 14 Direct (mm)**
405	45.02	0.48	49.62	1.01
407	48.32	0.56	53.68	1.84
408	29.35	0.70	26.50	1.43

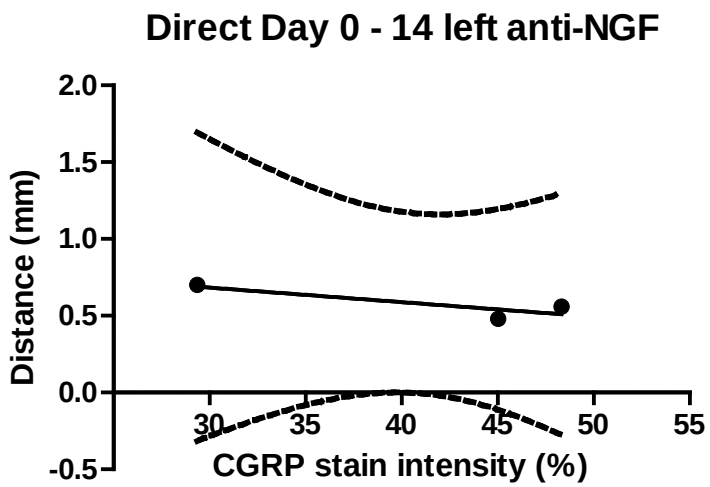

Figure 20: Scatter plot graph representing the paired data shown above. The solid line shows the line of best fit. Dotted lines show 95% confidence levels. R square value = 0.7436. S y.x = 0. 07974. The slope is not significantly non-zero (P = 0.3380).

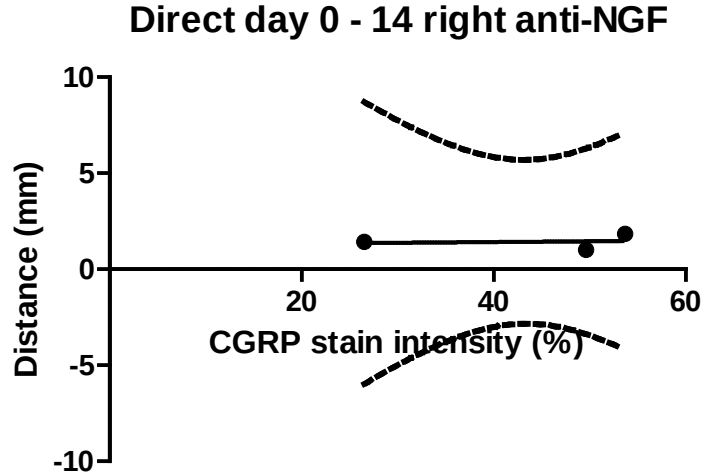

Figure 21: Scatter plot graph representing the paired data shown above. The solid line shows the line of best fit. Dotted lines show 95% confidence levels. R square value = 0.01727. S y.x = 0. 5818. The slope is not significantly non-zero (P = 0.9161).

Table 12: Tooth movement measured between the centroids of the left and right maxillary first molars and the incisors over day 0 – day 14 via the indirect method. CGRP stain intensity was for the left and right maxillary first molars. Animals were injected with anti-NGF (between right maxillary first and second molars).

	LEFT	LEFT	RIGHT	RIGHT
Animal	**CGRP stain intensity (%)**	**Distance day 0 – day 14 Indirect (mm)**	**CGRP stain intensity (%)**	**Distance day 0 – day 14 Indirect (mm)**
405	45.02	1.01	49.62	1.94
407	48.32	0.93	53.68	2.45
408	29.35	0.31	26.50	1.32

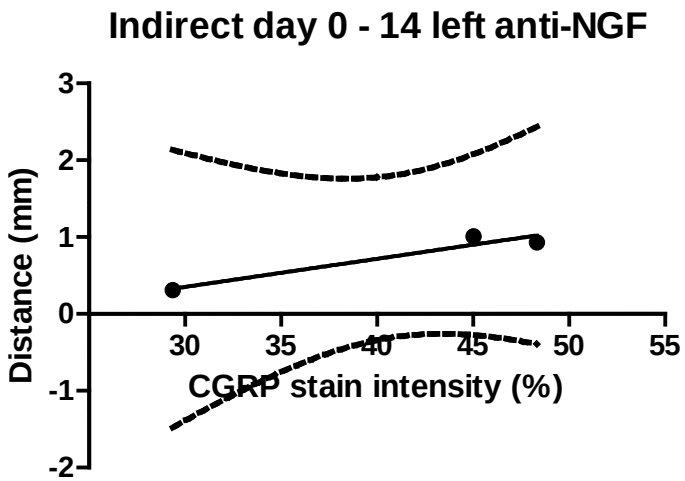

Figure 22: Scatter plot graph representing the paired data shown above. The solid line shows the line of best fit. Dotted lines show 95% confidence levels. R square value = 0.9298. S y.x = 0. 1435. The slope is not significantly non-zero (P = 0.1707).

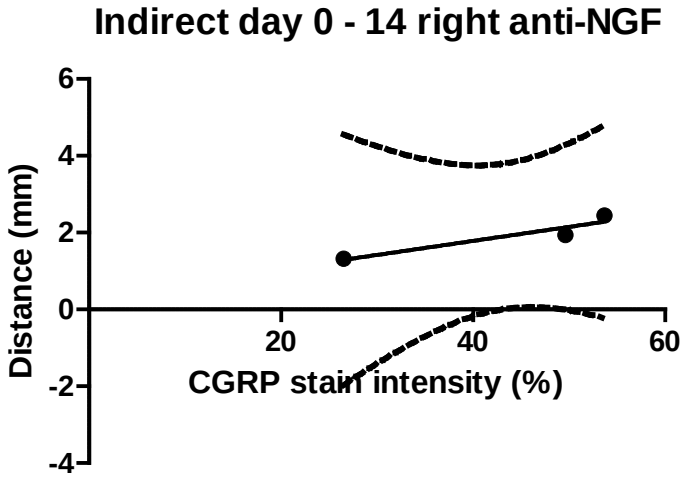

Figure 23: Scatter plot graph representing the paired data shown above. The solid line shows the line of best fit. Dotted lines show 95% confidence levels. R square value = 0.8958. S y.x = 0. 2583. The slope is not significantly non-zero (P = 0.2092).

Table 13: Tooth movement measured between the centroids of the right maxillary first and second molars over day 0 – day 14 via the indirect method. CGRP stain intensity was for the right maxillary first molar. Animals were injected with anti-NGF (between right maxillary first and second molars).

	RIGHT	RIGHT
Animal	**CGRP stain intensity (%)**	**Distance day 0 – day 14 Indirect (mm)**
405	49.62	0.49
407	53.68	1.16
408	26.50	0.39

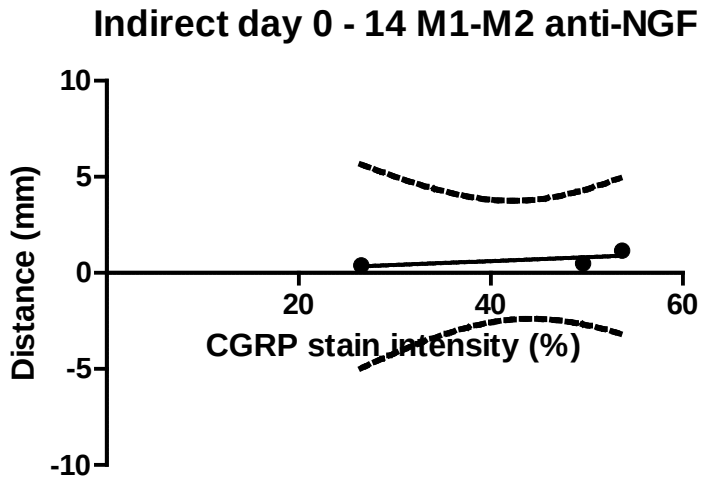

Figure 24: Scatter plot graph representing the paired data shown above. The solid line shows the line of best fit. Dotted lines show 95% confidence levels. R square value = 0.4967. S y.x = 0. 4201. The slope is not significantly non-zero (P = 0.5021).

The results of the linear regression analysis using the paired data of tooth movement versus the CGRP stain intensity for each group are summarised in the table below.

Table 14: Summary of results of the linear regression analysis. M1 = first molar, M2 = second molar, I = incisors.

Distance measured	Method used	Side	Period (days)	Injection (Right)	R square value	S y.x	P value	Significance (p ≤ 0.05)	Slope
M1 to I	Direct	Left	0 – 7	Saline	0.8219	0.1367	0.0934	No	-0.04921 ± 0.01620
M1 to I	Direct	Right	0 – 7	Saline	0.3289	0.4091	0.4353	No	-0.03652 ± 0.03774
M1 to I	Indirect	Left	0 – 7	Saline	0.006814	0.3172	0.9175	No	-0.004405 ± 0.03761
M1 to I	Indirect	Right	0 – 7	Saline	0.1478	0.5043	0.9175	No	0.02740 ± 0.04652
M1 to M2	Indirect	Right	0 – 7	Saline	0.05815	0.2180	0.7589	No	0.007065 ± 0.02011
M1 to I	Direct	Left	0 – 7	Anti-NGF	0.1194	0.1421	0.5023	No	0.00446 ± 0.00605
M1 to I	Direct	Right	0 – 7	Anti-NGF	0.01815	0.4247	0.8290	No	-0.007725 ± 0.03281
M1 to I	Indirect	Left	0 – 7	Anti-NGF	0.08749	0.2393	0.5693	No	0.006578 ± 0.01062
M1 to I	Indirect	Right	0 – 7	Anti-NGF	0.1601	0.3188	0.5044	No	0.01862 ± 0.02462
M1 to M2	Indirect	Right	0 – 7	Anti-NGF	0.2368	0.1171	0.4058	No	-0.00873 ± 0.00905
M1 to I	Direct	Left	0 – 14	Saline	0.3057	0.3255	0.6270	No	-0.01993 ± 0.03004
M1 to I	Direct	Right	0 – 14	Saline	0.8769	0.0497	0.2282	No	0.01708 ± 0.006400
M1 to I	Indirect	Left	0 – 14	Saline	0.8136	0.2963	0.2842	No	-0.05714 ± 0.02735
M1 to I	Indirect	Right	0 – 14	Saline	0.9951	0.0327	0.0448	Yes	-0.0597 ± 0.00421
M1 to M2	Indirect	Right	0 – 14	Saline	0.5022	0.2422	0.4986	No	-0.03132 ± 0.03118
M1 to I	Direct	Left	0 – 14	Anti-NGF	0.7436	0.0797	0.3380	No	-0.00948 ± 0.00556
M1 to I	Direct	Right	0 – 14	Anti-NGF	0.01727	0.5818	0.9161	No	0.003721 ± 0.02806
M1 to I	Indirect	Left	0 – 14	Anti-NGF	0.9298	0.1435	0.1707	No	0.03646 ± 0.01002
M1 to I	Indirect	Right	0 – 7	Anti-NGF	0.8958	0.2583	0.2092	No	0.03654 ± 0.01246
M1 to M2	Indirect	Right	0 – 7	Anti-NGF	0.4967	0.4201	0.5021	No	0.02013 ± 0.02026

The results are summarised below:

1. The linear regression analysis using the direct and indirect methods of measuring tooth distance showed only one significant association between CGRP staining intensity and the amount of tooth movement measured ($p < 0.05$). This was found for the right first maxillary molar to incisor measured by the direct method, day 0 – day 14, saline injected group. The group had a R square value of 0.9951, an S y.x value of 0.0327 and p value of 0.0448.
2. All other groups showed no significant association between the CGRP staining intensity and the amount of tooth movement measured ($p>0.05$). This may be due to the small sample sizes, variability in measurements and method errors.
3. The non-linearity of the results make it difficult to compare the slopes and Y-intercept for the different groups. Slopes of the line of best fit varied greatly in positive to negative values. The accuracy of the results due to the small sample size and method error remains questionable. In some cases movement was measured on the unaffected side (no orthodontic spring attached), which may be explained by measurement error or due to loss of incisor anchorage.
4. The results suggest there was no significant association between CGRP staining intensity and amount of tooth movement achieved. Tooth movement and CGRP nerve fibre density may be affected by multiple unrelated variables; therefore, a linear association between the neural response to tooth movement and the amount of tooth movement achieved may not be present.
5. The scatter plot diagrams involving paired data of the indirect measurement method to CGRP staining intensity showed a more linear relationship than the paired data involving the direct measurement method. This suggests the indirect measurement method may be more reproducible.

4. Discussion

Laboratory rats used in the study were male, eight-week-old Sprague-Dawley rats, and weighed between 250 grams and 350 grams. This age ensures a fully developed dentition was present, and male rats were used to negate any hormonal related variables [13].

The present study used tissue obtained from a previous study [10], in which the maxillae of the seventeen treated animals were stored in EDTA medium for up to 18 months, during which the tissue may have endured some protein denaturation. This number represents a small sample size when separated into subgroups whereas a sample size approaching 25 – 30 in each of the subgroups would allow greater statistical robustness.

4.1 Application of orthodontic force to rat molars

A previous study by O'Hara [14] used the tooth movement model as described by Waldo and Rothblatt to simulate orthodontic movement[15]. He noted that the longer the module was left between the teeth, the greater the risk of loosening and module loss during mastication. At the time of sacrifice, only 66% (26 out of 42) of his animals were found to have the elastic modules retained at time of sacrifice. There was no consistency in the direction of tooth movement and level of response. The force delivered to the tooth was not controlled and was translatory, tipping or rotational, dependent upon the depth of elastomeric module insertion, tightness of interproximal contact, and duration of retention [14].

The method used in this study involved the use of a closed coil spring ligated between an eyelet on an incisor band and the upper first molar[16-21]. Noxon et al. bonded a closed coil spring between the molar and incisor, delivering a force of 0.4N to tip the maxillary first molar mesially [11]. They found the tooth movement obtained was stable and the spring was able to deliver a continuous and constant force[22, 23]. The present study used a lower, continuous force level generated by the NiTi coil spring.

The present study involved the comparison of measured tooth movement to the semi-quantitative staining intensity of CGRP positive neurons. The idea was based on previous findings of the known positive association between CGRP positive neurons and the presence of orthodontic force causing tooth movement and inflammation in the periodontal ligament. Results showed that although CGRP positive neurons were present in areas undergoing orthodontically induced inflammation, there was no statistically significant linear association between the CGRP staining intensity and distance moved by the tooth. Perhaps there are other factors that may be confounding the amount of CGRP positivity in this study, such as the difficulty in obtaining reliable and predictable orthodontic tooth movement, and the effects of masticatory forces on the orthodontic appliance activity. A longer time period of orthodontic force application and a larger sample size may reduce these confounders.

4.2 CGRP-immunoreactivity

The sprouting of CGRP-immunoreactive fibres was more pronounced in the pulp, periodontal ligament-bone interface and trabeculae surfaces, indicating the probable involvement of the fibres in bone remodelling. This finding is similar to those of previous studies by Kvinnsland et al [24] and Hill & Elde [25] in which CGRP-immunoreactive fibres were found to be related to areas of bone deposition and inhibition of bone resorption. CGRP fibres were also found near the root cementum suggesting a supporting role in the reparative process of the periodontal ligament during tooth movement [8, 26].

The nervous system may play an important regulatory role in the bone remodelling process. This suggests that factors that regulate neural processes may be used to induce nerve sprouting and facilitate orthodontic tooth movement via upregulating the distribution of CGRP-immunoreactive fibres.

Conclusion

The results of the present study indicate there was no statistically significant association between the CGRP staining intensity and the amount of tooth movement measured ($p>0.05$). This suggests that there was no definite linear association between CGRP staining intensity and amount of tooth movement achieved. Tooth movement and CGRP nerve fibre density may be affected by multiple unrelated non-linear variables; therefore, a simple linear association between the neural response to tooth movement and the amount of tooth movement achieved may not be present.

Acknowledgements

Funding: The Australian Society of Orthodontists Foundation for Research and Education provided partial funding for the project.

Conflict of interest: There is no foreseeable conflict of interest.

Ethical approval: The ethics approval was obtained from the University of Adelaide Animal Ethics Committee (M/023/2006).

Statistician: Dr. Ian Parkinson, Senior Medical Scientist, Hanson Institute, Adelaide.

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SUMMARY

The literature review described the structure and function of the periodontium, showing it to be a complex and highly structured tissue of which much of its molecular biology remains unknown. There is a complex interplay of molecular factors which control its various functions, many of which are still under examination.

The role the periodontium plays in orthodontic tooth movement is the subject of many research projects, many of which have conflicting results and conclusions, indicating that further investigations are required before a substantive conclusion can be made.

In this study, the periodontium's neural response to orthodontic tooth movement was investigated using the dento-alveolar region of Sprague-Dawley rats. A known neural protein associated with slow-reacting neural fibres in the periodontal ligament called calcitonin-gene-related-peptide (CGRP) was studied previously and found to have an association with the control of the tissue responses during tooth movement. These tissue responses involve inflammatory mediation and bone remodelling. CGRP immunoreactive nerve fibres were found to increase in staining intensity during orthodontic tooth movement. However, a statistically significant, quantitative approach has not been performed. A growth factor that is believed to participate in the regulation and control of neurons is nerve growth factor (NGF). It has been shown to play an essential role in the development, maintenance and survival of sensory and sympathetic nerves. NGF has been shown to be distributed in teeth and the periodontal ligament and may play a role in the periodontal response to orthodontic tooth movement.

Following from previous studies undertaken by researchers at the University of Adelaide, our investigation was aimed at the relationship between CGRP immunoreactivity and NGF expression during orthodontic tooth movement, using a semi-quantitative approach to test the hypothesis that there was no statistically significant association between anti-NGF injection and CGRP fibre intensity during orthodontic tooth movement. A further study was undertaken to show whether there was an association between the amount of orthodontic tooth movement and CGRP fibre intensity, with anti-NGF injection, saline injection or no injection.

The previous studies provided a working protocol and tissues which had been previously prepared. Tooth movement had been performed on the maxillary right first molar via an orthodontic NiTi pull coil spring. Some of the animals had an injection of anti-NGF performed adjacent to the right maxillary first molar. In addition to the previous tissues, an extra sample of animals was added to which no orthodontic tooth movement or administration of anti-NGF was performed, in order to provide a control group sample. An immunohistochemical protocol was devised which involved the “Avidin-Biotin Complex” protocol, using CGRP as the primary antibody and diaminobenzidine (DAB) as the staining substrate. The resulting sections were photomicrographed and data was analysed using colour deconvolution software that quantified the intensity of the CGRP immunoreactive fibre staining in the region of interest, the periodontal ligament space surrounding the mesiobuccal roots of the maxillary first molar.

The data from the sections of different groups were compared statistically, and it was found that the injection of anti-NGF had reduced the amount of CGRP immunoreactive staining in the animals undergoing orthodontic tooth movement. This difference was statistically significant when tested with the Neuman-Keul paired test.

The second study involved an investigation of the amount of tooth movement obtained from the previous study samples, and its possible association with the amount of CGRP immunoreactive fibre staining intensity. The previous study provided tooth measurement data, which was used to compare to the semi-quantitative data found in this study for CGRP immunoreactive fibre staining intensity, using a regression analysis. No association was found in this comparison.

The overall conclusions drawn from these studies suggest that NGF plays a significant role in the resprouting of CGRP immunoreactive nerve fibres during orthodontic tooth movement. This may have long reaching effects on clinical analgesics which may regulate NGF and / or CGRP immunoreactive nerve fibre development. The study did not show there was a significant relationship between CGRP immunoreactive fibre intensity and the amount of measured tooth movement. More investigation is required, involving a large sample size and long term data for tooth movement.

Appendix 1.

The Application of Orthodontic Force on the Rat Molars

Seventeen animals were prepared in a previous thesis study. The animals were weighed and anaesthetized to allow for placement of the closed-coil spring, and also prior to perfusion fixation at the end of the study.

1. Placement of Closed-Coil Spring: The rats were anaesthetized with Ketamine (100mg/ml) (Ketamil Injection, Troy Laboratories, Smithfield Australia) and Xylazine (Xylazil, 20mg/ml) via intraperitoneal injection at a dosage of 2ml/kg of body weight. The two drugs were mixed at the ratio of 2:1 (Ketamine: Xylazine) and then diluted 1:1 with sterile water for injection. This allowed a short period of anaesthesia allow for the placement of closed coil springs. Anaesthesia was tested via observation of a plantar reflex and chest movements.

2. Application of Orthodontic Force: The anaesthetized animal was placed onto a holding rack (figure 1). The mouth was gently propped open by two rings attached with elastic bands to the rack. A split-mouth design was used with the experimental side on the right, and the contralateral as the control. A 100 g super-elastic NiTi closing coil spring (Sentalloy, GAC, New York, USA) was used to impart a mesially-directed force to the right maxillary first molar tooth. The springs were ligated with 0.010” stainless steel ligature wire anteriorly to the maxillary incisors and posteriorly to the cervical region of the right maxillary first molar. Springs were previously calibrated such that the force levels studied were within its superelastic range. Light curing 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 tooth. Care was taken not to injure the surrounding soft tissue (Figure 2).

Figure 1: Animal was placed onto a purpose-built holding rack

Figure 2: Photograph of experimental spring setup with wire ligature

3. Injection of Anti-NGF: In the animals of the test group, 1.0 μl (2μg) of anti-NGF was injected into the gingival mucosa between the first and second molars (figure 3). This was the area where the orthodontic force was applied. The injections were made after application of the closing coil spring and whilst the animals were still under

anaesthesia. The animals were returned to cages in the animal house and allowed to recover.

In animals of the sham control group, a saline solution was injected into the gingival mucosa between the first and second molars in a similar region to the test group. This was to provide a control group for comparison of the effect of injecting anti-NGF on CGRP positive neurons.

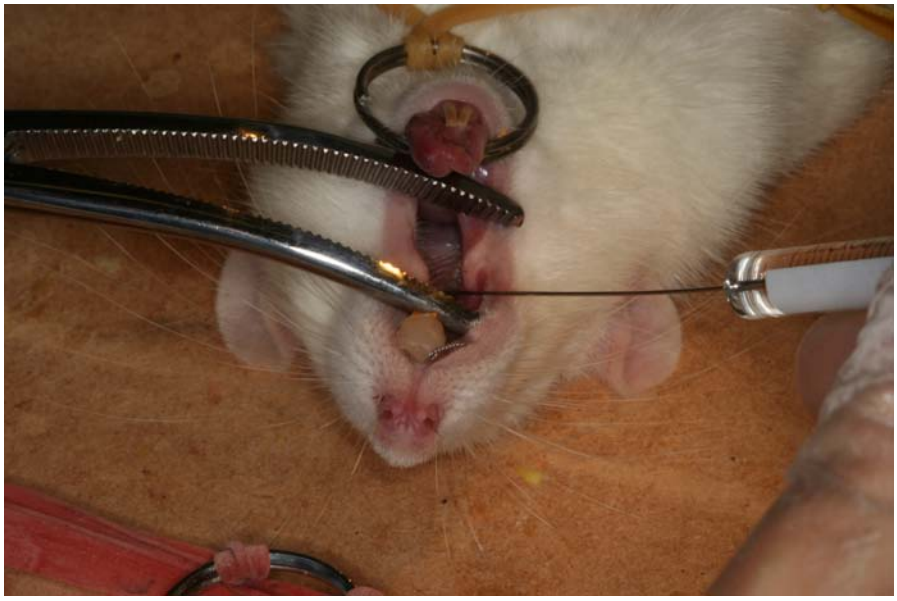

Figure 3: Injection of NGF into the gingival mucosa

Appendix 2.

Measurement of tooth movement

Measurement of tooth movement was done in two different methods.

1. The direct method used a digital calliper to measure the distance from the mesial surface of the first molar to the labial surface of the maxillary incisors of the same quadrant (Figure 1). Readings were taken at Days 0, 3, 7 and 14 under the same anaesthesia protocol outlined in Appendix 1. In the present study, only readings at Day 7 and 14 were used, with the CGRP staining intensity data for that animal, to form the paired data used in the regression analysis.

2. The indirect method used polyvinyl siloxane impressions (Imprint 3, 3M ESPE) which were taken intra-orally with a special customised acrylic tray at Days 0, 3, 7 and 14 (figure 2). Photos of the impressions were taken under light microscopy (MZ16FA stereo microscope) and were calibrated by Image analysis software.

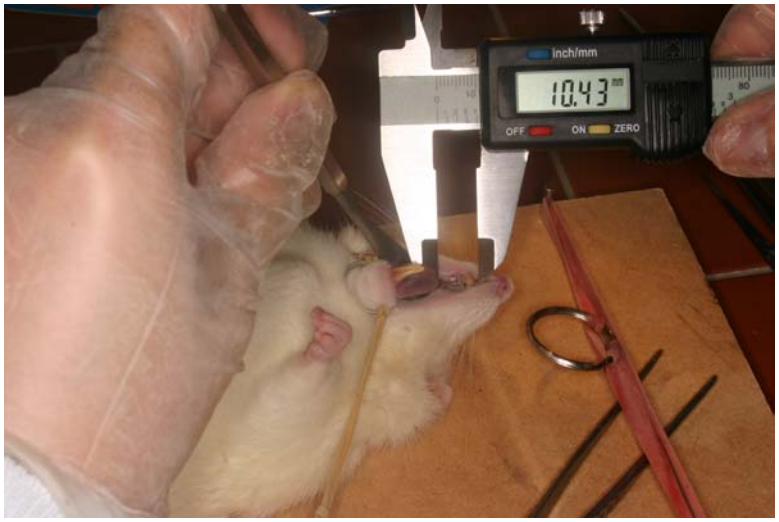

Figure 1: Direct measurement with digital calliper

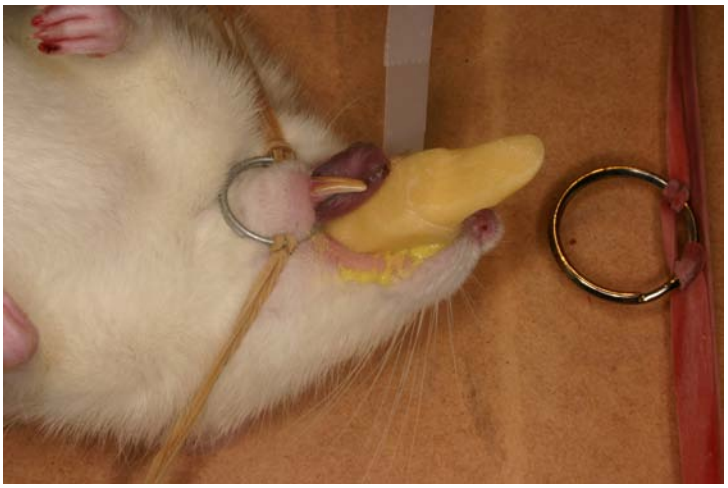

Figure 2: Impression taken with special tray

3. The image capturing procedure involved recording the animal number and date of impression. A millimetre rule was photographed adjacent to the set impression, and the image captured and digitised using Image analysis software.

4. Landmarks of the first molar of both sides and the central incisors were located and digitised in a specific order and their X and Y coordinates were collected (figure 3). Four anatomical landmarks along the circumference of the clinical crown were recorded for the incisors, the first and second molars. These were the distobuccal, mesiobuccal, mesiopalatal and distopalatal points, and a centroid was constructed by using these four points (figure 4).

5. Measurements were made between the centroids of the first and second molars, and between the centroids of the first molar and incisors, for both the left and right sides. These were recorded and tabulated at specified time points (Appendix 3).

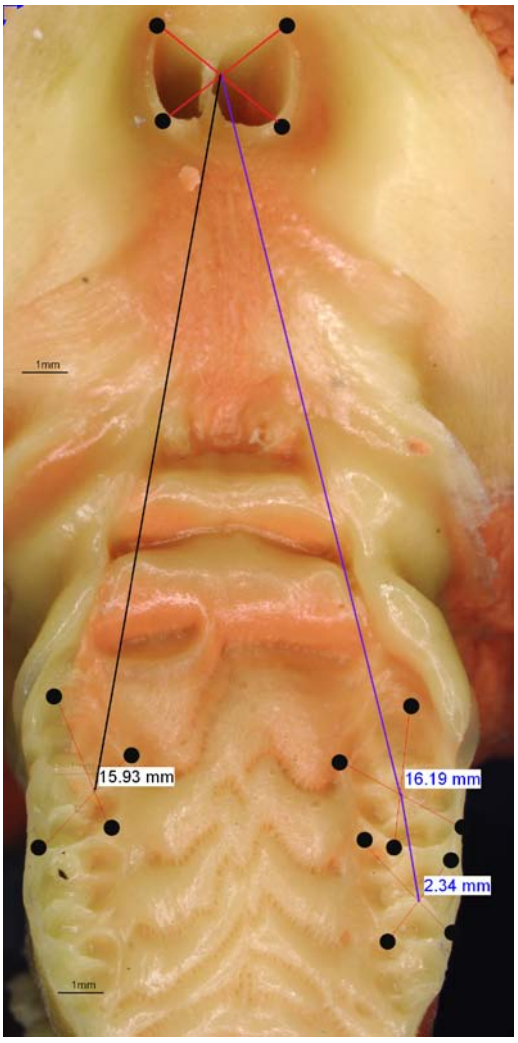

Figure 3: Impression landmarks

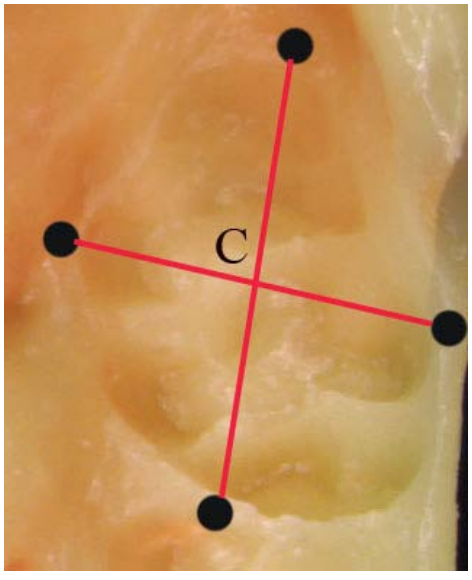

Figure 4: Illustration of centroid determination

Appendix 3.

Tooth measurement results

Table 1: Tooth movement measurements of Day 0 (* = animals which died during the experimental procedure, C = uninjected left side, T = saline (s) or anti-NGF (n) injected right side)

Tooth movement measurements of Day 0											
	Day 0-C (direct)				Day 0-T (direct)				Indirect		
rat no.	M1	M2	M3	Mean	M1	M2	M3	Mean	C	T	Molar 1-2
101s	13.74	13.78	13.78	13.77	13.70	13.48	13.61	13.60	*	*	*
102s	14.09	13.96	13.92	13.99	13.68	14.00	13.96	13.88	16.80	16.42	1.91
103s	13.12	13.17	13.19	13.16	13.38	13.32	13.45	13.38	16.17	16.27	2.09
104n	13.89	13.90	13.89	13.89	14.17	14.11	14.09	14.12	16.26	16.72	2.06
105n	*	*	*	*	*	*	*	*	*	*	*
106n	13.94	13.92	13.99	13.95	13.97	13.97	14.01	13.98	16.65	16.68	2.09
201s	12.34	12.40	12.44	12.39	12.49	12.44	12.56	12.50	14.79	15.22	2.30
202s	12.37	12.45	12.46	12.43	12.47	12.54	12.52	12.51	14.38	14.95	2.01
203s	12.54	12.54	12.54	12.54	12.49	12.57	12.58	12.55	15.22	15.23	2.32
204n	*	*	*	*	*	*	*	*	*	*	*
205n	12.48	12.49	12.53	12.50	12.52	12.56	12.55	12.54	14.84	15.08	2.16
206n	11.60	11.59	11.59	11.59	11.85	11.75	11.86	11.82	*	*	*
301s	12.90	12.93	12.92	12.92	12.60	12.62	12.68	12.63	15.93	16.19	2.34
302s	13.65	13.64	13.62	13.64	13.40	13.51	13.46	13.46	16.30	16.49	2.33
303s	13.33	13.33	13.27	13.31	13.26	13.26	13.24	13.25	15.86	15.96	2.25
304s	12.93	12.91	12.94	12.93	13.01	12.94	12.83	12.93	15.92	16.15	2.19
305n	13.35	13.29	13.28	13.31	13.34	13.50	13.37	13.40	16.00	16.11	2.05
306n	13.08	13.09	13.06	13.08	13.06	13.09	13.05	13.07	16.00	16.06	2.18
307n	13.30	13.26	13.30	13.29	13.26	13.38	13.31	13.32	16.19	16.26	2.36
308n	13.08	13.03	13.10	13.07	13.35	13.29	13.40	13.35	15.94	16.02	2.51
401s	12.83	12.90	12.80	12.84	12.28	12.33	12.22	12.28	15.84	15.82	2.19
402s	13.18	13.18	13.21	13.19	13.23	13.27	13.34	13.28	15.94	16.38	2.20
403s	12.66	12.79	12.69	12.71	12.63	12.73	12.74	12.70	15.71	15.58	2.32
404s	13.25	13.21	13.21	13.22	13.16	13.13	13.20	13.16	16.06	16.16	2.31
405n	12.68	12.66	12.69	12.68	12.71	12.76	12.74	12.74	15.41	15.73	2.05
406n	13.11	13.14	13.18	13.14	13.06	13.01	13.15	13.07	16.05	15.78	2.38
407n	12.41	12.41	12.44	12.42	12.35	12.35	12.28	12.33	15.25	15.05	2.20
408n	12.79	12.89	12.81	12.83	12.84	12.79	12.82	12.82	15.69	15.56	2.37

Table 2: Tooth movement measurements of Day 3 (* = animals which died during the experimental procedure, C = uninjected left side, T = saline or anti-NGF injected right side)

Tooth movement measurements of Day 3											
	Day 3-C (direct)				Day 3-T (direct)				Indirect		
rat no.	M1	M2	M3	Mean	M1	M2	M3	Mean	C	T	Molar 1-2
101s	13.46	13.44	13.41	13.44	13.19	13.04	13.38	13.20	*	*	*
102s	13.86	13.75	13.86	13.82	12.54	12.88	12.52	12.65	17.06	16.59	2.54
103s	13.07	13.17	13.09	13.11	12.21	12.10	12.14	12.15	15.88	15.47	2.36
104n	13.21	13.38	13.26	13.28	12.95	12.80	12.81	12.85	16.20	16.04	2.31
105n	*	*	*	*	*	*	*	*	*	*	*
106n	13.47	13.56	13.39	13.47	13.00	12.89	13.00	12.96	15.63	15.17	2.35
201s	12.17	12.18	12.01	12.12	11.35	11.34	11.47	11.39	14.23	14.14	2.32
202s	12.06	11.96	11.93	11.98	11.48	11.64	11.63	11.58	14.07	14.11	2.53
203s	12.08	12.07	12.12	12.09	11.46	11.49	11.55	11.50	15.20	14.79	2.63
204n	*	*	*	*	*	*	*	*	*	*	*
205n	11.91	11.90	11.91	11.91	11.75	11.83	11.86	11.81	14.17	14.13	2.21
206n	11.22	11.23	11.22	11.22	10.00	10.25	10.16	10.14	*	*	*
301s	12.58	12.39	12.52	12.50	11.86	11.98	11.96	11.93	15.22	14.80	2.56
302s	13.16	13.14	13.14	13.15	11.98	12.07	11.96	12.00	15.85	15.59	2.51
303s	13.29	13.34	13.28	13.30	12.80	12.73	12.86	12.80	16.36	16.00	2.42
304s	12.47	12.48	12.52	12.49	11.54	11.67	11.62	11.61	15.28	15.30	2.33
305n	12.78	12.77	12.78	12.78	12.03	12.04	11.83	11.97	15.78	15.56	2.47
306n	12.23	12.24	12.28	12.25	11.92	11.74	11.91	11.86	15.35	14.93	2.31
307n	12.71	12.76	12.79	12.75	11.79	11.77	11.83	11.80	15.76	15.43	2.51
308n	12.90	12.89	12.90	12.90	12.18	12.04	11.96	12.06	15.45	15.06	2.65
401s	12.53	12.52	12.59	12.55	12.07	11.96	11.80	11.94	15.60	15.31	2.45
402s	12.99	12.88	12.87	12.91	11.51	11.68	11.86	11.68	15.30	15.61	2.29
403s	12.00	12.05	12.12	12.06	11.65	11.56	11.81	11.67	15.11	14.80	2.68
404s	13.18	13.18	13.21	13.19	12.82	12.93	12.97	12.91	15.72	15.36	2.76
405n	12.30	12.35	12.38	12.34	11.50	11.55	11.67	11.57	15.11	15.15	2.10
406n	12.76	12.77	12.73	12.75	12.49	12.43	12.40	12.44	15.52	15.16	2.58
407n	12.10	12.18	12.17	12.15	11.65	11.68	11.71	11.68	14.83	13.91	2.54
408n	12.54	12.70	12.63	12.62	11.50	11.35	11.38	11.41	15.54	14.96	2.53

Table 3: Tooth movement measurements of Day 7 (* = animals which died during the experimental procedure, C = uninjected left side, T = saline or anti-NGF injected right side)

Tooth movement measurements of Day 7											
	Day 7-C (direct)				Day 7-T (direct)				Indirect		
rat no.	M1	M2	M3	Mean	M1	M2	M3	Mean	C	T	Molar 1-2
101s	*	*	*	*	*	*	*	*	*	*	*
102s	13.54	13.52	13.53	13.53	13.29	13.26	13.28	13.28	16.81	16.22	2.73
103s	*	*	*	*	*	*	*	*	*	*	*
104n	13.49	13.47	13.48	13.48	13.17	12.99	13.10	13.09	16.33	16.15	2.44
105n	*	*	*	*	*	*	*	*	*	*	*
106n	13.70	13.75	13.80	13.75	13.07	12.95	13.01	13.01	16.62	15.92	2.67
201s	12.04	12.03	11.86	11.98	11.21	11.38	11.23	11.27	14.41	14.24	2.59
202s	11.68	11.67	11.70	11.68	11.37	11.33	11.34	11.35	10.91	10.52	2.55
203s	12.02	11.96	12.05	12.01	11.17	11.07	11.14	11.13	14.69	14.16	2.71
204n	*	*	*	*	*	*	*	*	*	*	*
205n	12.30	12.32	12.35	12.32	11.55	11.64	11.60	11.60	15.02	14.51	2.56
206n	*	*	*	*	*	*	*	*	*	*	*
301s	12.43	12.47	12.45	12.45	11.79	11.82	11.80	11.80	15.35	14.34	2.98
302s	13.22	13.36	13.28	13.29	12.69	12.62	12.65	12.65	16.05	15.45	2.64
303s	13.00	13.05	13.11	13.05	12.46	12.55	12.48	12.50	15.90	15.15	2.78
304s	13.05	13.05	13.12	13.07	12.84	13.02	12.95	12.94	15.54	14.91	2.44
305n	12.89	12.77	12.82	12.83	12.40	12.36	12.39	12.38	15.41	14.81	2.54
306n	12.86	12.89	12.88	12.88	12.71	12.83	12.70	12.75	15.75	15.33	2.60
307n	12.93	12.92	12.65	12.83	12.87	12.89	12.70	12.82	15.98	15.57	2.76
308n	12.62	12.54	12.60	12.59	12.18	12.22	12.18	12.19	15.60	14.96	2.69
401s	12.47	12.48	12.50	12.48	11.90	11.96	12.00	11.95	15.49	14.82	2.50
402s	12.96	13.04	13.08	13.03	12.38	12.42	12.50	12.43	15.95	15.65	2.46
403s	12.22	12.25	12.37	12.28	11.24	11.23	11.12	11.20	15.46	14.96	2.70
404s	*	*	*	*	*	*	*	*	*	*	*
405n	12.28	12.27	12.28	12.28	12.17	12.07	12.18	12.14	15.03	14.72	2.25
406n	*	*	*	*	*	*	*	*	*	*	*
407n	12.17	12.07	12.09	12.11	11.21	11.25	11.20	11.22	14.77	13.62	2.53
408n	12.47	12.37	12.53	12.46	10.46	10.48	10.42	10.45	15.42	14.83	2.63

Table 4: Tooth movement measurements of Day 10 (* = animals which died during the experimental procedure, C = uninjected left side, T = saline or anti-NGF injected right side)

Tooth movement measurements of Day 10											
	Day 10-C				Day 10-T				Indirect		
rat no.	M1	M2	M3	Mean	M1	M2	M3	Mean	C	T	Molar 1-2
201s	11.67	11.62	11.71	11.67	11.24	11.15	11.21	11.20	14.28	13.96	2.71
202s	11.64	11.76	11.69	11.70	11.08	11.10	11.03	11.07	14.53	13.49	3.05
203s	11.95	11.96	11.89	11.93	10.65	10.73	10.98	10.79	14.86	13.76	2.95
204n	*	*	*	*	*	*	*	*	*	*	*
205n	12.26	12.28	12.28	12.27	11.56	11.76	11.79	11.70	15.20	14.48	2.67
206n	*	*	*	*	*	*	*	*	*	*	*
401s	12.28	12.35	12.32	12.32	11.90	11.79	11.93	11.87	15.22	14.45	2.78
402s	12.99	13.04	12.96	13.00	11.85	11.95	11.92	11.91	16.12	15.32	2.75
403s	*	*	*	*	*	*	*	*	*	*	*
404s	*	*	*	*	*	*	*	*	*	*	*
405n	12.24	12.25	12.26	12.25	11.46	11.44	11.55	11.48	15.10	14.55	2.38
406n	*	*	*	*	*	*	*	*	*	*	*
407n	11.95	11.94	11.99	11.96	11.03	10.97	11.00	11.00	14.88	13.65	2.69
408n	12.38	12.39	12.39	12.39	11.19	11.19	11.15	11.18	15.20	14.26	2.74

Table 5: Tooth movement measurements of Day 14 (* = animals which died during the experimental procedure, C = uninjected left side, T = saline or anti-NGF injected right side)

Tooth movement measurements of Day 14											
	Day 14-C (direct)				Day 14-T (direct)				Indirect		
rat no.	M1	M2	M3	Mean	M1	M2	M3	Mean	C	T	Molar 1-2
201s	11.58	11.60	11.59	11.59	11.06	11.00	11.02	11.03	14.31	13.39	3.01
202s	*	*	*	*	*	*	*	*	*	*	*
203s	*	*	*	*	*	*	*	*	*	*	*
204n	*	*	*	*	*	*	*	*	*	*	*
205n	*	*	*	*	*	*	*	*	*	*	*
206n	*	*	*	*	*	*	*	*	*	*	*
401s	11.89	11.89	11.94	11.91	10.87	10.94	10.90	10.90	14.68	13.54	2.92
402s	12.78	12.83	12.76	12.79	11.68	11.70	11.73	11.70	15.72	14.74	2.50
403s	*	*	*	*	*	*	*	*	*	*	*
404s	*	*	*	*	*	*	*	*	*	*	*
405n	12.19	12.19	12.22	12.20	11.73	11.73	11.74	11.73	14.40	13.79	2.54
406n	*	*	*	*	*	*	*	*	*	*	*
407n	11.73	12.07	11.77	11.86	10.47	10.51	10.50	10.49	14.32	12.60	3.36
408n	12.10	12.16	12.12	12.13	11.38	11.45	11.35	11.39	15.38	14.24	2.76

Appendix 4.

Harvesting of Tissue

1. Animal Sacrifice: A second anaesthetic procedure, involving administered intra-peritoneal administration of chloral hydrate 5g/100ml H_2O (5 mL per animal), was required immediately prior to sacrifice to provide anaesthesia for intra-cardiac perfusion of fixative.

2. Perfusion of Animal: The animals were perfused at 7 and 14 days after coil spring placement at the Animal House, Medical School. 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 blood returning to the heart to drain. Each animal was initially perfused with 200-300ml of previously prepared 0.1% sodium nitrite solution for 5 minutes to cause sufficient vasodilation to facilitate tissue perfusion with the fixative (figure 1).

Figure 1: Perfusion apparatus

3. Perfusion with Fixative: 200ml of *p*-benzoquinone solution and 200ml of 4% para-formaldehyde were combined and perfused into the left ventricle under positive pressure for approximately 15 minutes (figure 2). A satisfactory level of fixation was

indicated when the extremities became brown in colour. The removal of the cranial vault allowed visual inspection of the brain and the degree of tissue perfusion.

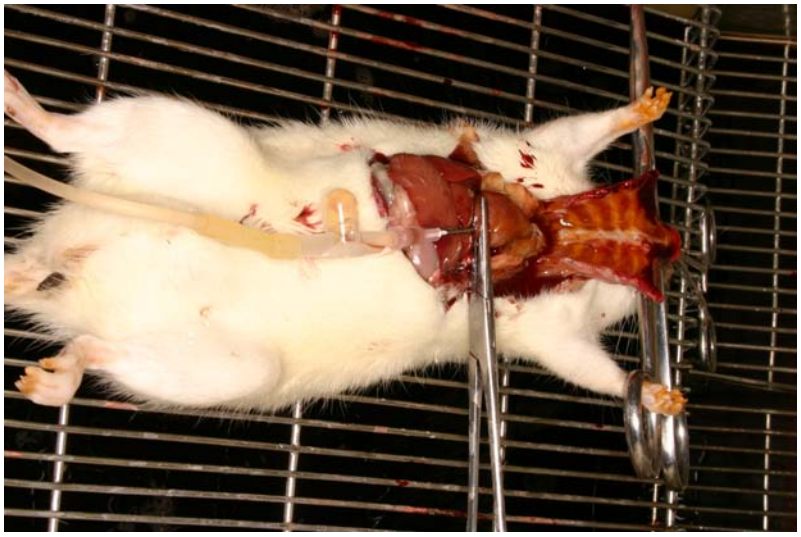

Figure 2: Animal at completion of perfusion, note the brown colour of all tissues and extremities

4. Dissection of the maxilla: The maxilla and cranial vault were dissected out and superfluous soft tissues removed. The dissected maxilla and cranium were then placed into 0.2% *p*-benzoquinone and 2% para-formaldehyde for approximately 2 hours.

5. Decalcification: After 2 hours of immersion in the fixative solution, the tissues were then 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 the minimum total decalcification time of 8 weeks. These tissues were then examined radiographically to confirm decalcification prior to usage. The maxillae from the seventeen animals were stored in the EDTA solution until use in the current study.

6. Cryo-protection: In preparation for cryo-protection, the maxilla and the brain stem were dissected further into two portions, separating the maxilla from the brainstem with the trigeminal ganglion attached. The tissues were impregnated with embedding medium (O.C.T. Sakura Finetek USA Inc Torance, CA 90504 USA), via a gradual increase in concentration and gentle agitation. Over an 8 day period, the

concentration of the embedding medium was steadily increased in a 30 % sucrose solution until 100% embedding medium was obtained by day 8.

7. Sectioning: Specimens were sectioned horizontally at 25 µm on a Leitz 1720 cryostat at -20°C with the blade set at 6°. Sections were collected directly onto previously prepared APT-coated slides. Each maxilla was orientated so that the crowns of the teeth were uppermost. The initial transverse sections through to the pulpal regions were removed and discarded. The slides were placed into a slide box, and left over night at room temperature to allow the sections to fully dry and adhere to the slide. The following day the slide box was sealed into a plastic bag, labelled and placed into a -20°C refrigerator until required.

8. Immunohistochemical Staining Protocol: After cryosectioning, the sample was incubated with blocking agents. 0.3% H_2O_2 (in 70% methanol in 0.1M PBS) was used to block endogenous peroxidase and then rinsed in 0.1M PBS. The sections were then incubated in 3% NGS (normal goat serum) in 0.02% Triton X-100 for one hour, to prevent non-specific binding. After blocking and rinsing, the sample was incubated with rabbit polyclonal CGRP antibodies and rinsed. The sample was then incubated with a biotin labelled secondary antibody (Biotin conjugated affinity purified anti-rabbit IgG (Goat)), which acts to bind to the primary antibody, to amplify the resultant amount of visual staining.

An enzyme-based immuno-staining method was used, involving the secondary antibody that is tagged with biotin. Steptavidin linked to horseradish peroxidase was then applied to the tissue sections and diaminobenzidine (DAB) was added, resulting in a brown precipitate forming where the antibody has bound. The enzyme labelled sections were then counterstained with haematoxylin to enhance visualization prior to viewing under a light microscope.

Positive immunohistochemistry controls were provided by sections of trigeminal ganglion / nerve which had been perfused and placed in fixative according to the same protocol as the maxillary tissue.

Appendix 5

Avidin-Biotin Peroxidase Complex immunolabelling protocol

1. Sections were collected of APT coated slides to increase adhesion
2. Endogenous peroxidase was blocked by rinsing slides using 0.3% H_2O_2 (in 70% methanol in 0.1 M PBS) for 30 minutes.
3. The slides were then rinsed in 0.1 M PBS three times for 5 minutes.
4. The slides were wiped and the sections were encircled with a liquid repellent slide marker pen to retain the staining solutions on the specimens
5. A solution of 3% Normal goat serum (NGS) was made up using 3 ml 100% NGS : 97ml 0.1M PBS, with Triton X-100. The solution was pipetted onto the sections and the slides were incubated for 60 minutes.
6. A solution of 1:4000 CGRP (mouse) primary antibodies in 1% NGS was made. The solution was pipetted onto the sections and then incubated overnight at room temperature on a shaker.
7. The slides were then rinsed with 1% NGS three times for 10 minutes.
8. A solution of 1:250 biotinylated secondary antibody (anti-rabbit goat immunoglobulin G) in 1% NGS was made. This solution was pipetted onto the sections and then incubated for 60 minutes.
9. The slides were rinsed thoroughly in 0.1M PBS three times for 10 minutes.
10. A solution of streptavidin-peroxidase conjugate 1:1000 in 0.1M PBS was made. This was pipetted into the sections and incubated for 60 minutes at room temperature on a shaker.
11. The slides were rinsed thoroughly in 0.1M PBS three times for 10 minutes.

Appendix 6

DAB and counter-staining protocol

1. A DAB solution in 0.1M Tris buffer (pH 7.65) was prepared using 5 mg in 10 ml of 0.1M Tris. Care was taken due to the carcinogenic properties of DAB. The solution was kept in the dark to ensure the reaction did not proceed turning the solution from pink (normal) to brown (unusable).
2. A solution of 3% H_2O_2 was made by diluting 1 ml 30% H_2O_2 in 9 ml double distilled water. Just prior to use of the DAB, 30 μl of the 3% H_2O_2 was added to 10 mL of DAB.
3. The H_2O_2 and DAB solution was pipetted onto the sections and incubated for no longer than 7 minutes and the colour development was observed.
4. Tris buffer pH 7.65 was pipetted onto the sections once to stop the reaction, followed by three washes with 0.1M PBS.
5. The sections were stained with haematoxylin for 1 minute then washed in running water to remove the excess stain. They were then dipped in acid alcohol, followed by a wash in running water, then placed in ammonia for 1 minute and washed again in running water.
6. The sections were dehydrated with 100% ethanol in three incubations for 10 minutes. The sections were then incubated in D-Limonene (Histoclear) twice for 5 minutes to prepare them for mounting.
7. The sections were mounted and covered with large coverslips.