Chapter 6: Discussion

6.1 The experimental animal

The advantages of using the laboratory rat as an experimental animal include the similarity of the PDL to human PDL, better control of genetic variability, the practicality of increased sample sizes and a more manageable size of the animal. These conditions may not exist for experiments using other animals.[36]

The experimental animals were aged 8 weeks when sacrificed and weighed between 250 and 350gms. The dentition of the rats is complete by 3-4 weeks and so the age of the animals used in this study ensured a fully developed dental pattern.[101]

6.2 Anaesthesia

The Hypnorm/Hypnovel® anaesthetic combination has been used in previous animal studies[5, 102] with both rapid onset and profound anaesthesia produced. However, supplies of Hypnorm® are no longer available and the results achieved with Nembutal were only useful for animal sacrifice, given the long time required for onset and the varying dosage requirements.

In this study, anaesthesia for the placement of the Sentalloy closing coil spring and injection of anti-NGF into the gingival tissues was achieved with a combination of Ketamine (100mg/ml) (Ketamil Injection, Troy Laboratories, Smithfield Australia) and muscle relaxant Xylazine (Xylazil, 20mg/ml).

The two drugs were mixed at the ratio of 2:1 (Ketamine : Xylazine) and then diluted 1:1 with sterile water for injection. This was then administered intraperitoneally at a dosage of 2ml/kg of body weight.

Six animals were lost to the experiment due to unexpected death during the Sentalloy spring placement or during recovery from anaesthetic agents. Most animals died during the initial stage of the experiment when the operator was unfamiliar with the dosage of drugs and Sentalloy spring placement. After adjustment of the anaesthetic dosage to 2ml/kg, it provided satisfactory level of anaesthesia along with sufficient muscle relaxation to

facilitate placement of the closed coil spring between the maxillary first molar and the incisor teeth.

6.3 Application of orthodontic force to rat molars

The application of an orthodontic force to the molars of rats is a challenging procedure. Many methodologies have been advocated, with varying degrees of success. The tooth movement model as described by Waldo and Rothblatt [36] was a simple technique to simulate orthodontic movement. However, in the recent study by O'Hara[5], only 66% (26 out of 42) of the animals were found to have the elastic modules retained at the time of sacrifice. It was noted that the duration of the module between the teeth increased the chances of it loosening and, therefore, dislodging during mastication. Moreover, this method did not provide consistency in the direction of tooth movement and level of response. As the force delivered to the tooth was not caused by stretching but by decompression, tooth movement may have been translatory, tipping or rotational depending upon the depth of elastic module insertion, tightness of interproximal contact, and duration of retention.

Tooth morphology may also be a factor to be considered as pressure exerted by the inserted module may be transferred in varying directions by cusp and crown morphology.

Brudvig and Rygh advocated the use of a closed coil spring ligated between an eyelet on an incisor band and the upper first molar.[19, 103-107] Noxon et al. bonded a closed coilspring between the molar and incisor, delivering a force of 0.4N, designed to tip the maxillary first molar to the mesial and was shown to predictably stimulate orthodontic tooth movement as assessed by the histological appearance of resorption revealed by osteoclast activity on the mesial surface of the first molar.[57]

In the current study, the tooth movement model used was a modified technique described by Ren et al.[108] This tooth movement appliance has been

proved to be stable and able to deliver a continuous and constant force on the molar.[108, 109]

In the current study, ten closed-coil springs were randomly selected and the superelastic properties of the material and the delivered force were tested. A reproducible force of 100 cN was produced over a range of 3 to 6 mm of activation, which was within the activation range used for the test animals. The retention of the closing coil spring was about 61% i.e. 17 out of 28 animals were found to have the coil spring retained at time of sacrifice. 6 animals died before the day of sacrifice (with coil spring in placed), and 5 animals were found with the springs detached from the teeth and were excluded from the study. This retention rate was higher than the retention rate of elastic modules in a previous study.[5, 102] All animals appeared healthy during the study and managed their soft diet. 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.

6.4 Tooth movement

In animals where the coil spring was retained, a clear separation between the first and second molars of the appliance side could be observed in both anti-NGF and control groups. No space was observed between the second and third molars, indicating lack of mesial movement of the second molar during the experiment. This finding is consistent with the previous study by Ong et al.[109]

6.5 Reliability/repeatability of measurements

The overall mean value of the intraclass correlation coefficient for all the direct measurements was 0.993. This shows that the reliability of direct measurement was within the acceptable limits, and the mean value of the 3 measurements was used for further statistical analysis. The repeatability of the indirect measurements was estimated using baseline data only. Data from subsequent time periods were not used to avoid the need to incorporate the

additional effects of treatment and time into the reliability calculations. Overall the differences between the first and second indirect measurements had a mean of -0.0089mm and a standard deviation of 0.0433mm. The levels of agreement between the first and second indirect measurement are illustrated in the Bland-Altman plot below. As the plot shows (Figure 30), there was a high degree of agreement between the two measures. The coefficient of repeatability[100] was calculated to be 0.0866, while the upper and lower limits of agreement were given by -0.0955mm and 0.0778mm respectively. Overall, these results indicated a high level of repeatability using the indirect measurement procedure.

Other studies found high reliability in indirect measurement methods. Fraser completed a comparison of measurements of lower arch length discrepancy from photocopies, photographs and directly from plaster models.[110] Ten subjects had repeated measurements taken utilizing the three different modes of analysis. The results indicated a better coefficient of reliability for the photographs and the photocopies compared to the direct measurement from the models.

6.6 Tooth measurement

In this study, a closing coil spring, which applied 100 g force, was used to provide mesial movement to the rat molar (M^1). This would provide histological reactions including widening of the PDL on the tension side, narrowing of the PDL on the compression side, areas of hyalinization on the compression side, frontal alveolar bone resorption on the compression side, and root resorption affecting the compression side root surface.

The injected administration of anti-NGF injection did not appear to affect the magnitude and rate of tooth movement. This result showed that there was no significant difference in the group mean magnitude of tooth movement between the anti-NGF and control groups in both direct and indirect measurement methods. (Figures 47) No tooth movement was evident

on the control side. The values recorded for tooth movement were similar to those obtained by Madan and Liu.[111]

In the statistical model, the effect of time on direct and indirect measurements was found to be highly significant ($p < 0.0001$). There was a significant change over the 14-day follow-up period across the two groups. In contrast, there was no evidence for a group effect or a group by time interaction effect. (Table 11)

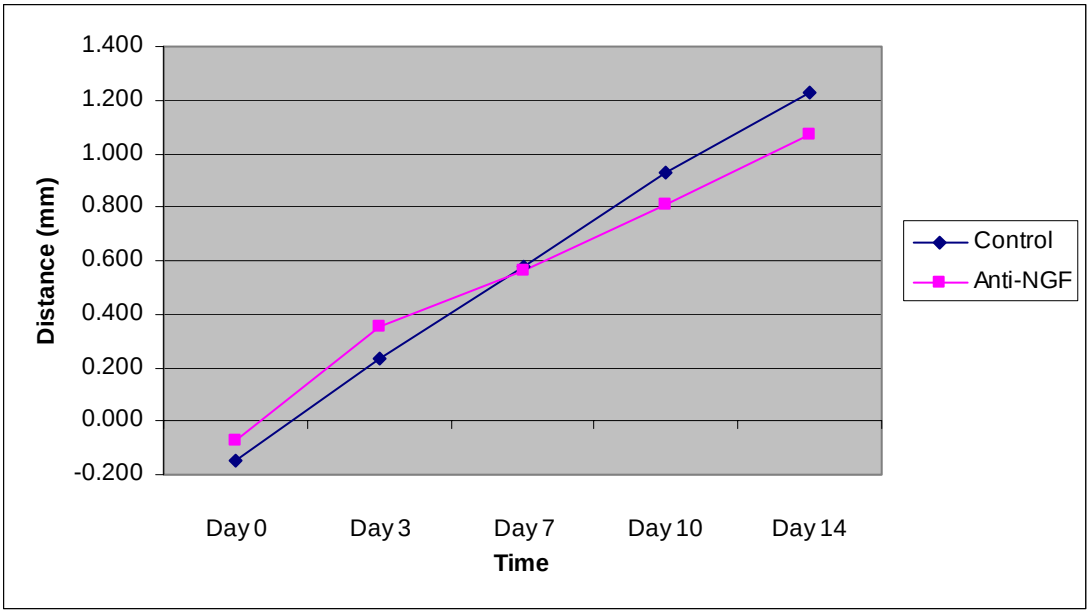

Figure 47: Indirect measurements from right maxillary first molar to second molar

6.7 TRAP Staining

Andersson and Marks considered TRAP staining to be a useful marker of osteoclast ontogeny and function.[112] Tartrate resistant acid phosphatase has been demonstrated to be associated with osteoclasts and extracellularly in the lining of resorption lacunae.[113] It has been further localised to bone surfaces facing the ruffled border of osteoclasts and more diffusely in adjacent bone areas, as well as cells that are considered osteoclast precursors.[114, 115]

TRAP staining of the experimental tissues showed intense red staining associated with osteoclasts and osteoclast precursor cells. One percent fast green solution was used to provide background staining and successfully improved contrast within the sections. Control sections for TRAP staining were produced via the omission of the substrate AS-BI phosphoric acid solution and increasing the sodium acetate solution to 52mls. This produced no red stained cells.

The original technique of Goldberg and Barka, proved troublesome as severe tissue damage occurred during the drying stage immediately prior to slide mounting.[99] Following trial runs, it was found that the stained sections presented better if this step was eliminated and the sections were not allowed to dry prior to mounting.

6.8 NGF intensity and TRAP activity

Kawamoto and Matsuda have shown that there is an increase in NGF expression in tissues in response to injury.[116]

The histological results of the material collected from O'Hara's study[5] indicated that no tissue response was observed in Day 0 as expected. Day 3 sections demonstrated increased intensity on both sides, with the experimental side showing higher intensity. Staining of the cells was especially distinct in pulp tissue and in the PDL near the bone-PDL interface. In bone, staining could be seen near trabecular surfaces particularly on the experimental side. These cells are believed to be involved in the removal of pathologically-affected tissue and production of signalling molecules.[117] Areas of tension showed increased vascular activity with more distended vessels seen near the PDL-bone interface. This is a common finding reported by most tooth movement studies.[118]

On the compression side of the injured PDL of Day 3 sections, there were fewer vessels noted. This could be attributed to the applied force occluding the vascular supply resulting in ischaemia and degenerative

changes.[117] Reitan observed resorption of the alveolar bone surface on the compression side at Day 3.[37]

In Day 7 sections, the width of the PDL on the tension and compression side was similar. There was increased inflammatory infiltrate within the PDL and bone suggesting continued reaction to the orthodontic force. The internal control side was not affected and showed no evidence of inflammation or change in PDL morphology.

TRAP-positive cells were detected on both experimental and control sides of test animals. The intensity of TRAP activity was greatest in areas associated with compression-induced resorption and repair. However, the TRAP-positive cells were not stained intensely with NGF immunolabelling. On the other hand, cells that were stained positively for NGF, did not demonstrate TRAP-positive reactions. The results in this investigation did not support the hypothesis that cells at the PDL-bone interface and in bone trabeculae that were positive for NGF were osteoclasts and pre-osteoclasts.[119, 120]

The findings in this study suggested that the rate of bone resorption and deposition was not significantly altered by the presence or absence of sympathetic and nociceptive innervation. However, a number of studies have shown that sympathetic and nociceptive nerves can affect bone metabolism. Hill et al. showed that the rate of bone resorption was increased in animals that were sympathetomised at birth and the rate of bone resorption was decreased in animals depleted of nociceptive innervation since birth[121]. It is suggested that NGF responsive sympathetic or nociceptive nerves can, independently, retard or promote bone resorption.

Roberts,[53] in an explanation of orthodontic tooth movement, divided the process into three phases; initial strain, lag phase and progressive tooth movement. During the lag phase there was no tooth movement. This phase coincided with the time taken for hyalinized tissue to be eliminated, and for the cortical bone to be resorbed from the bone marrow side. The time required for this was variable and may take from 2 to 10 weeks. (Figure 1)

The current experiment was carried over a 14-day period, which was in the lag phase of orthodontic tooth movement, NGF may not have expressed its full effect on the rate of tooth movement.

An alternative explanation for the observation that the rate of tooth movement was not significantly affected in the presence or absence of sympathetic and nociceptive nerves was that the two normally function in counter balance to modulate bone resorption. This raises the possibility that the rate of orthodontic tooth movement may be modulated by using pharmaceutical agents that selectively alter sympathetic or nociceptive nerve function.

NGF expression was evident within the pulp, the root-PDL interface, within the PDL, and at the PDL-bone interface. This suggested that cells within the PDL, such as the osteoclasts, cementoblasts, fibroblasts and bone-lining cells, might be responsible for NGF expression in the dentoalveolar complex.

6.9 Suggestions for future research

The mechanisms involved in tooth movement and, in particular, the effect of NGF on tooth movement and regulation of osteoclasts in the dentoalveolar complex are still incompletely understood. The present study investigated the effect of anti-NGF on the rate and tooth movement over a 14-day period. The results indicated that the administration of anti-NGF did not affect tooth movement rate significantly.

6.9.1 Identification of cells expressing NGF

Cells at the PDL-bone interface and in bone trabeculae that were positive for NGF were thought to be osteoclasts and pre-osteoclasts.[119, 120] However, in this study, cells that were stained positive for NGF, did not stain positively with TRAP staining.

In this study, NGF expression was evident within the pulp, the root-PDL interface, within the PDL, and at the PDL-bone interface. This led to the suggestions that the predominant cells within the PDL, the fibroblasts, might be responsible for NGF expression in the dentoalveolar complex.

Identification of NGF expressing cells within the rat dento-alveolar complex will further add evidence to the suggestion that the nervous system plays an important regulatory role in the process of bone remodelling.

6.9.2 Tooth movement model

The current tooth movement model using closing coil spring was a reliable method of tooth movement which provided a more consistent result. The method of tooth movement measurement was also shown to be highly reproducible and reliable.[108, 111]

Future research involving tooth movement models should follow the protocol used in this study in order to standardize the response. Moreover, further research should be carried over a longer period of time, which surpasses the lag phase, and minimizes the effect that undermining resorption might have on tooth movement rate. Results of administrating NGF with increased dosage should also be tested.

Chapter 7: Conclusions

From the results of the study the following conclusions may be drawn:

1. The periodontal administration of anti-NGF at a dose of 1.0 μl (2μg) did not significantly affect tooth movement.
2. TRAP-positive cells were not stained intensely with NGF immunolabelling. On the other hand, cells that were stained positively for NGF, were shown to be TRAP-negative.
3. TRAP staining provided a useful and reliable labelling technique for the identification and localization of clastic cell activity within the dentoalveolar complex.
4. The modified tooth movement model of Ren et al.[108] provided reliability in the magnitude, direction and duration of force application.
5. The indirect method for tooth movement measurement was reliable and highly reproducible.

Chapter 8: Appendices

8.1 Rat perfusion protocol

1. Solutions:
    - Pre-perfusion solution: 10gms Na nitrite + 100ml 0.4M phosphate buffer + H_2O to make up 1000mls. Solution is 0.1% Na nitrite solution.
    - Perfusion solution: 0.800gms *p*-benzoquinone + 200ml H_20 dissolved on roller at 4^oC for approximately 30 minutes.
    - Make up 200ml 0.1M $NaPO_4$ buffer @ ph 7.4 by using 50ml of 0.4M $NaPO_4$ buffer @ ph7.4 + 150ml H_2O.

2. Pre-perfuse each rat through the left ventricle with 200-300ml of solution for 5 minutes under positive pressure.
3. Place the mixture of prepared 200ml *p*-benzoquinone solution + 200ml 4% para-formaldehyde into a separate container, then perfuse each rat under positive pressure through the left ventricle for 15 minutes until all extremities are brown in colour and fixed.
4. Dissect out maxilla and brain and place into a container with the mixture of *p*-benzoquinone + 4% para-formaldehyde for approximately 2 hours.
5. After 2 hours, complete final dissection with removal of the palate into prepared EDTA solution at ph 7.4.

8.2 TRAP staining

TRAP solutions comprised:

: 50mls of sodium acetate.3 H_2O , pre-warmed to 37^0C.

: 3ml of hexazotized basic fuchsin (see below).

: 2ml of 0.67mol/L L (+) tartrate solution (Sigma).

: 2ml of 12.5mg/ml naphthol AS-BI phosphoric acid (Sigma).

Hexazotized basic fuchsin solution was prepared by mixing equal volumes of basic fuchsin solution (Aldrich Chemical Company) and 4% sodium nitrate

solution ($NaNO_2$). Basic fuchsin solution was prepared by dissolving 800mg of basic fuchsin in 16ml of distilled water and 4ml of concentrated (36%) hydrochloric acid. This was mixed well on a heated plate until dissolved and subsequently filtered. An equal volume was mixed thoroughly with sodium nitrite solution immediately before use.

The final solution was poured into Coplin jars, each containing 10 sections. Control solutions were prepared by omitting the substrate naphthol AS-BI phosphoric acid solution and increasing sodium acetate solution to 52 ml. Only two sections were used in the control solutions in each run. Slides were incubated for 2 hours at 37^0C, after which solutions were decanted and slides washed for 10 mins in running water. Slides were counterstained with 1% Fast Green FCF (Sigma) for 5 minutes, washed in running water for 1 minute, and subsequently mounted with Histomount. Sections were studied under light microscopy for evidence of TRAP stained cells.

8.3 Solution Preparation:

8.3.1 0.1M $NaPO_4$

Prepared from original mixture:

- 1M NaH_2PO_4 – 39.0025gm NaH_2PO_4 + 250ml distilled water.
- 1M Na_2HPO_4 – 35.49gm NaH_2PO_4 + 250ml distilled water.
- 77.4ml NaH_2PO_4 + 22.6ml NaH_2PO_4 + 900ml distilled water.
- 0.1M $NaPO_4$ @ ph7.4

8.3.2 Pre-perfusion solution

Pre-perfusion solution was made up by dissolving 10gms of Na nitrite with 100mls 0.4M phosphate buffer with distilled water to make up 1000mls. This solution is in effect 0.1% Na nitrite solution.

8.3.3 Perfusion solution

These solutions were made by dissolving 0.800gms *p*-benzoquinone into 200ml of distilled water on a roller at 4^oC for approximately 30 minutes. At the time of perfusion 200ml of *p*-benzoquinone solution and 200ml of 4% para-

formaldehyde were combined in a container and perfused directly into the left ventricle under positive pressure for 15 minutes until all extremities are brown in colour.

8.3.4 4% paraformaldehyde/0.1M phosphate buffer pH7.4

Preparation:

160g Paraformaldehyde

H_20 1600mls heated

NaOH dropwise to clarify

800mls 0.4M sodium phosphate buffer pH7.4

Add H_20 to 4000mls

8.3.5 Para benzoquinone

Preparation

Dissolve 0.800gms p-benzoquinone

Into 200mls H_20

On a roller at 4°C for approximately 30min.

8.3.6 Ethylenediaminetetra-acetic acid (EDTA) solution (4% EDTA in 0.1M $NaPO_4$ pH7.4)

Preparation:

80g sodium ethylenediaminetetra-acetic acid (EDTA) into 1500mls H20 on Stimer mixer, with pH electrode in place.

Pellets of NaOH added one at a time until pH6 reached and EDTA in solution.

500mls of 0.4M NaPO4 pH7.4 added.

8.3.7 Anti-NGF

1. Antibodies to mouse NGF were raised in rabbits using standard procedures

2. Immune anti-NGF serum from two rabbits was pooled and IgG fraction precipitated by addition of 50% saturated ammonium sulphate
3. Precipitated protein was dialysed against three changes of PBS and passed through an immunoaffinity column containing 7mg NGF covalently bound to CNBr activated Sepharose (Pharmacia)
4. Column was washed with 10 column volumes of PBS followed by 2 column volumes of 0.1 Tris buffered saline pH 7.4 plus 0.2M NaCl to remove weak to moderate affinity antibodies
5. High affinity antibodies were eluted with 4M $MgCl_2$ in 200mM Acetate pH 5.0, dialysed against 50mM anmmonium acetate pH 6.5 (3 changes) before aliquoting at 20μg per vial and lyophilising.
6. Antibodies neutralize biological activity of NGF in chick DRG Bioassay.