Expression of Osteoprotegerin (OPG) and Receptor Activator of Nuclear Factor-κB Ligand (RANKL) on root surface after different extra-oral dry times

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ABSTRACT

Background: The current International and National Guidelines recommend that avulsed teeth be replanted as soon as possible to prevent desiccation and subsequent necrosis of the periodontal membrane. The extra-alveolar time prior to replantation has been found to be the most significant factor in determining the type of periodontal healing following this injury. Similar to orthodontic tooth movement and primary root resorption, it is assumed that periodontal healing is regulated by the molecular signalling system of osteoprotegerin (OPG) and receptor activator of nuclear factor-κB ligand (RANKL). The aim of this study was to investigate the expression and distribution of OPG and RANKL on root surfaces of teeth after exposure to different extra-oral dry times. **Methods:** Human teeth were extracted, left to air-dry for <5, 30 and 60 minutes prior to fixation. Immunohistochemistry was then performed on decalcified, paraffin-embedded longitudinal sections using antibodies raised against human OPG and RANKL. Secondary antibody horseradish-peroxidase labelling with haematoxylin as counterstain was used to visualise the expression of these antigens at the cervical, middle and apical regions of the root under light microscopy. **Results:** OPG was detected in the cementoblasts and periodontal ligament fibres with no statistical significance across the different dry times and regions (p>0.05). Five of the 8 specimens in the 60 minutes group had reduced or no RANKL immunoreactivity compared to the <5 or 30 minutes groups although this was not significant (p>0.05). Median RANKL scores were lower than that for OPG, with statistical significance noted at the apical region in the 60 minutes group (p<0.05). **Conclusion:** The expression for RANKL was consistently weaker than that for OPG at all dry times and locations. While OPG immunoreactivity did not differ significantly in all dry time groups and regions, RANKL expression decreased after 60 minutes of extra-oral dry time at the apical region.

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ABBREVIATIONS

APTES	3-aminopropyltriethoxysilane
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
DAB	3,3-diaminobenzidine
DNA	deoxyribonucleic acid
DPX	di-n-butylphthalate in xylene
EDTA	ethylenediaminetetraacetic acid
FFPE	formalin-fixed paraffin-embedded
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
H&E	haematoxylin and eosin
HBSS	Hank's balanced salt solution
HRP	horseradish peroxidase
IADT	International Association of Dental Traumatology
IGF	insulin-like growth factor
IL-1β	interleukin-1 beta
MMP-9	matrix metalloproteinase-9
mRNA	messenger ribonucleic acid
OPG	osteoprotegerin
PBS	phosphate-buffered saline
PDL	periodontal ligament
PDGF	platelet-derived growth factor
RANK	receptor activator of nuclear factor-κB
RANKL	receptor activator of nuclear factor-κB ligand

RNA   ribonucleic acid

RT-PCR   reverse transcriptase polymerase chain reaction

SD   standard deviation

sRANKL   soluble receptor activator of nuclear factor-κB ligand

TGF-β   transforming growth factor-beta

TNF   tumour necrosis factor

TNF-α   tumour necrosis factor-alpha

TRITC   tetramethylrhodamine isothiocyanate

1. INTRODUCTION

1.1. Anatomy of the tooth and the periodontium

The human tooth consists of a crown and a root which are demarcated by the cemento-enamel junction (Figure 1). The crown of the tooth, located above the cemento-enamel junction, comprises of an outermost enamel layer whereas the root is covered by a layer of cementum with dentine making up the bulk of the root. A tooth can have a single or multiple root(s) which attaches itself to the alveolar bone via the periodontal ligament. Collectively, the periodontal tissues form the supporting structures of the tooth (Cho and Garant 2000). The main components of the periodontium are:

- Gingiva

- Periodontal ligament

- Cementum

- Alveolar bone

The gingiva forms the soft tissue surrounding the tooth. It includes the oral epithelium, oral sulcular epithelium, junctional epithelium and connective tissue. The periodontal ligament constitutes primarily of vascular connective tissue and forms the attachment between the cementum and the alveolar bone. The cementum is a mineralised tissue layer covering the root surface which is laid down continuously throughout life. The alveolar bone makes up the walls of the tooth socket. The periodontal ligament fibres attach the cementum to the surrounding alveolar bone.
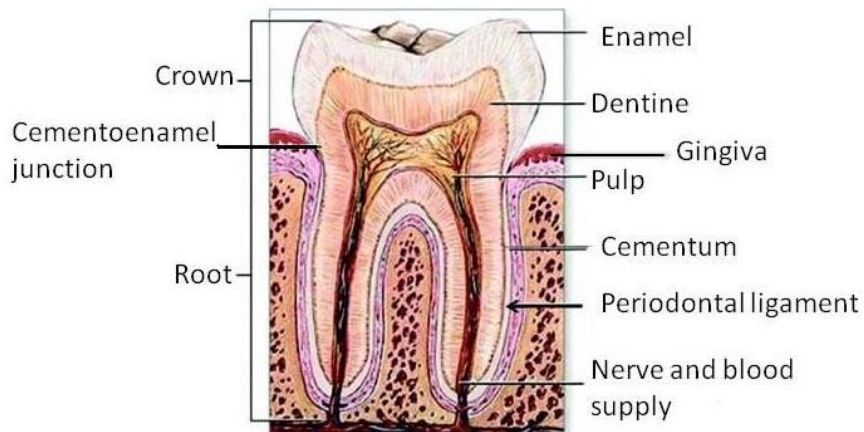
Figure 1.1 Anatomy of the tooth and the periodontium (A.D.A.M. Inc 2012).

1.2. Histology of the periodontium

1.2.1. Periodontal ligament

The periodontal ligament is a connective tissue made up of cells and an extracellular matrix. Healthy mature periodontal ligament (Figure 1.2) contains various cell populations such as fibroblasts, endothelial cells, epithelial cell rests of Malassez, sensory cells, osteoblasts, osteoclasts, undifferentiated mesenchymal cells and cementoblasts (Beertsen, McCulloch and Sodek 1997). The extracellular compartment consists of well-defined collagen fibre bundles, mainly types I and III, embedded within proteins such as glycosaminoglycans, glycoproteins and glycolipids. The predominant cell type in the periodontal ligament is the fibroblast. The migration and contraction of fibroblasts play a role in tooth eruption and wound healing (Gould, Melcher and Brunette 1980; Beertsen and Hoeben 1987). Also, the periodontal ligament fibroblasts close to areas of cementum and alveolar bone in a rat molar study were shown to express high levels of alkaline phosphatase activity (Groeneveld, Everts and Beertsen 1995), which in turn demonstrated an essential role of fibroblasts in the process of mineralisation (Van den Bos *et al.* 1995). Other cells such as the epithelial rests of Malassez could persist within the periodontal ligament indefinitely. These epithelial cell islands are remnants of Hertwig's epithelial root sheath. The undifferentiated

mesenchymal cells in the periodontal ligament can be induced to form osteoblast and cementoblast cell types (Cho and Garant 2000) although it is not certain which factors or conditions would influence differentiation to either cell type (Bosshardt 2005). Several molecules have been found to be involved in controlling the development, maintenance and regeneration of periodontal tissues. These include transforming growth factor-β (TGF-β) superfamily (including bone morphogenetic proteins), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), fibroblast growth factors (FGF) and others as summarised in Table 1.1 (Nanci 2008). The regulation of osteoblasts and cementoblasts in the periodontal ligament by the OPG/RANK/RANKL system will be discussed further in section 1.6.

Table 1.1 Molecular factors in the periodontal ligament.

Molecules	Suggested function(s)
Growth factors • TGF-β • PDGF, IGF • FGF	 Reported to promote cell differentiation and subsequent cementum formation during development and regeneration. These growth factors promote cementum formation by altering cell cycle activities. Fibroblast growth factors promote cell proliferation and migration as well as vessel formation – all key events for formation and regeneration of periodontal tissues.
Adhesion molecules • Bone sialoprotein • Osteopontin	 These molecules may promote adhesion of selected cells to the newly forming root. Bone sialoprotein may be involved in promoting mineralisation, whereas osteopontin may regulate the extent of crystal growth.
Epithelial/enamel proteins	Epithelial-mesenchymal interactions may be involved in promoting follicle cells along a cementoblast pathway. Some epithelial molecules may promote periodontal repair directly or indirectly.
Collagens	Collagens, especially types I and III, play key roles in regulating periodontal tissues during development and regeneration. In addition, type XII may assist in maintaining the periodontal ligament space against continuous formation of cementum.
Transcription factors • Runt-related transcription factor 2 (RUNx-2) • Osterix	 As for osteoclasts, these may be involved in cementoblast differentiation.
Signalling molecules • OPG • RANK • RANKL	 These molecules mediate bone and root resorption by osteoclasts.

1.2.2. Cementum

The composition of cementum resembles that of bone, consisting of approximately 50% inorganic material such as hydroxyapatite while the remaining being organic material such as collagen. The two main types of cementum that have different structural and functional features are acellular cementum and cellular cementum (Cho and Garant 2000). Acellular cementum serves to provide attachment for the tooth. The acellular layer is made up primarily of collagen fibres and is considered acellular because the cells that form it remain on its surface rather than within the cemental matrix. Cellular cementum functions to respond to tooth wear and movement as well as repair of the periodontal tissues. This layer is usually formed on unmineralised dentine surface near the advancing root edge. As the cementum thickens, the cementoblasts get entrapped within the extracellular matrix that they secret, thus forming cementocytes which reside in a lacuna each (Nanci 2008).
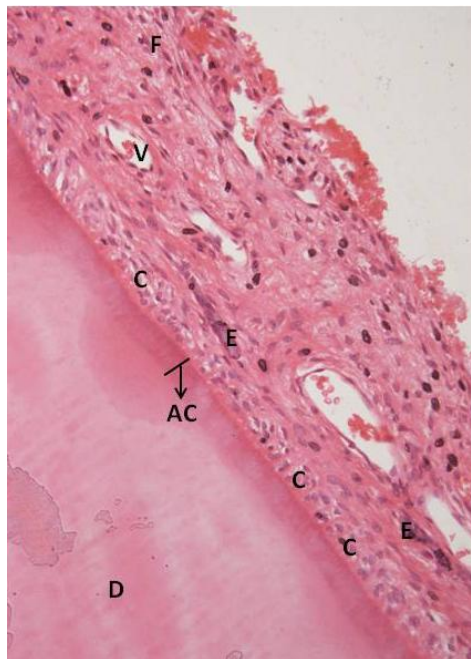
*Figure 1.2 Cells on the root surface. Cementoblasts (**C**) line the surface of acellular cementum (**AC**). Islands of epithelial cell aggregates which are the epithelial cell rests of Malassez (**E**) can be found within the periodontal ligament. (**D** = dentine, **V** = blood vessel, **F** = fibroblasts).*

1.2.3. Alveolar bone

The alveolar bone (or process) comprises of an outer cortical plate, a central trabecular space and bone lining the alveolus. The cortical plate is made up of layers of lamellar bone supported by compact Haversian system bone of different thickness. The trabecular space is filled with lamellar bone interspersed among red and yellow marrow (Nanci 2008). The bone lining the socket of the tooth is referred to as bundle bone due to the insertion of extrinsic collagen fibre bundles of the periodontal ligament within this bone, thus anchoring the tooth to the alveolar process (Cho and Garant 2000).
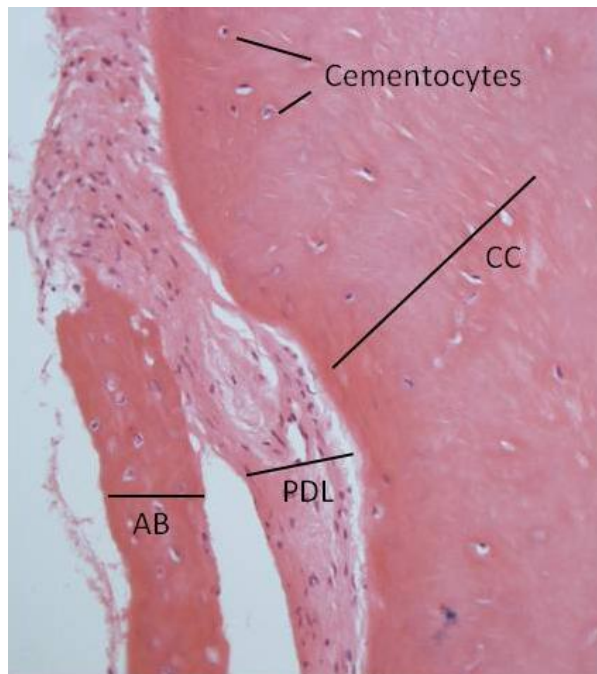
*Figure 1.3 Longitudinal section of periodontium at apical region of root. Cellular cementum (**CC**) containing cementocytes thickens towards the apex of the tooth (**PDL** = periodontal ligament, **AB** = alveolar bone).*

1.3. Tooth avulsion

Trauma to the tooth can result in injury to the periodontium and pulp. In the permanent dentition, traumatic injuries to the teeth may pose a long-term burden to the individual. A survey was carried out in Canada in an attempt to quantify treatment burden for

families following replantation of avulsed permanent incisors. They reported 90% of patients and 86% of parents had time off school and work in order to attend appointments for treatment and follow-up care (Nguyen, Kenny and Barrett 2004). One of the most complex injuries to the tooth is avulsion. Tooth avulsion is the complete displacement of the tooth from its socket. Incidence of avulsion varies, reportedly ranging up to 16% of all traumatic injuries to the permanent teeth (Andreasen and Andreasen 2007). Children aged 8 to 12 years old are most susceptible to avulsion, in which the maxillary permanent incisors are most frequently affected (McIntyre *et al.* 2009). This is postulated to be due to the softer bone surrounding these erupting incisors, thus making the teeth less resistant to extrusive forces upon impact (Andreasen and Andreasen 2007).

There are multiple aetiologies for avulsion injuries. These include sporting activities, cycling accidents, and assaults (Wright *et al.* 2007; Gulinelli *et al.* 2008). The mechanism of avulsion involves a large force hitting the labial portion of the tooth resulting in the tilting of the root apex labially and the crown palatally, thus rotating the tooth out of its socket. The nature of this impact results in the periodontal ligament being usually torn at the palatal surface and crushed at the apical aspect of the labial surface. Haas *et al.* (2008) investigated the amount and site of periodontal ligament remaining following avulsion injuries in a convenient human sample. They found 58% of root surface periodontal ligament remaining on avulsed incisors, with more remnant on the palatal than the labial surface of the root (Haas *et al.* 2008).

1.4. Healing of the periodontal ligament following replantation

Immediate replantation of the avulsed tooth is the optimal treatment for this injury. In reality, however, this rarely happens (Andreasen *et al.* 1995). The aim of replantation is

to ensure the survival of the cementum and periodontal ligament cells, thus increasing the chance of desirable wound healing. Following replantation of the tooth, the acute inflammatory cascade occurs in 2 phases, namely the destructive and the healing phases. In the destructive phase, there is active resorption of necrotic cells, bacteria, foreign material and toxic by-products. This process continues until the presence of inflammatory stimuli has been removed from the injured site. The subsequent healing phase is characterised by either cemental healing (with surface resorption) or by replacement resorption (Trope 2002b). There is ongoing competition between the cementoblasts and osteoblasts to repopulate the damaged root surface. Two outcomes are possible: 1) surface resorption, or 2) ankylosis (leading to replacement resorption). The outcome depends on the extent of root surface area damage sustained, with reportedly osseous replacement occurring if more than 20% of the root surface is damaged (Trope 2002b, 2002a). An early animal study by Andreasen and co-worker found that following the removal or drying of the periodontal ligament on the root surface, the healing capacity by proliferation of cells from adjacent intact cementum was approximately 1 - 1.5mm from the margins of the injury site, provided the surface area of periodontal ligament damage was less than 9mm^2 (Andreasen and Kristerson 1981). Other animal studies have also confirmed that beyond this critical size, ankylosis will occur (Lindskog, Blomlof and Hammarstrom 1983; Springer *et al.* 2005). Therefore, the main aim of treatment for tooth avulsion is to prevent any further damage and inflammatory response to the periodontal membrane.

1.4.1. Surface resorption (healing by cementum)

Cemental healing of the periodontal membrane has been termed as a "favourable" outcome (Wong and Sae-Lim 2002; Chen *et al.* 2008). In this type of healing, the cementoblasts repopulate the root surface, thus preventing osteoblasts/osteoclasts from

initiating replacement root resorption (Chen *et al.* 2008). Histology examination reveals that the superficial resorption lacunae are slowly repaired by formation of new cementum and reattachment of periodontal ligament fibres. This resorptive process is not progressive and the healing is initiated by the periodontal ligament-derived cells (Andreasen 1987).

1.4.2. Ankylosis (leading to replacement resorption)

An "unfavourable" outcome of periodontal ligament healing is replacement resorption. In situations of extensive damage to the cementum, osteoclast-like cells begin to resorb the root of the replanted tooth. However, instead of dentine deposition, the resorbed area is subsequently replaced with bone laid down by osteoblasts. Histologically, 2 forms of ankylosis have been demonstrated in animal studies: ankylosis with intact cementum and ankylosis with resorption of the cementum (Lindskog *et al.* 1985; Hammarstrom *et al.* 1986; Hammarstrom, Blomlof and Lindskog 1989). Hammarstrom and co-workers, using monkey incisors as an ankylosis model, demonstrated that administration of systemic antibiotics with endodontic treatment healed with bony deposition on cementum whereas the non-antibiotic with endodontic treatment group had bone deposited in resorptive cavities for teeth exposed to 60 minutes extra-alveolar dry time (Hammarstrom *et al.* 1986). This suggests that removal of inflammatory stimulus from the necrotic periodontal membrane along with endodontic treatment could reduce the occurrence of resorption before ankylotic fusion occurs.

1.4.3. Infection-related resorption (necrosis of the pulp)

The healing of periodontal ligament shares a symbiotic relationship with that of the pulp. Toxins, bacteria and other necrotic pulp contents can undesirably result in inflammatory root resorption. This phenomenon occurs when inflammatory stimulus

from necrotic pulp travels through the dentinal tubules and escapes to the periradicular region via areas of resorbed or damaged cementum on the root surface. This will give rise to external inflammatory resorption of the root (Andreasen 1981c). A recent recommendation by the International Association of Dental Traumatology (IADT) suggested the use of the term "infection-related resorption" to describe external inflammatory resorption stemming from necrotic pulp since all types of root resorption (both external and internal) are processes of inflammation. The process of periodontal healing in infection-related resorption is determined by the degree of cementum damage from the inflammatory process; progressing to either favourable periodontal ligament healing or ankylosis. Infection-related resorption can be minimised if the inflammatory stimulus is removed by timely pulp extirpation (Hinckfuss and Messer 2009).

1.5. Prognostic factors affecting periodontal ligament healing

The prognosis of replanted teeth depends on many different factors. Unless immediately replanted, most avulsed teeth will demonstrate progressive root resorption and inevitably end up being extracted or lost. The speed of root resorption is related to bony turnover. Thus in a pre-adolescent with immature root apices, this may occur within a few years but for an adult with complete root formation, it may take a decade or more (Barrett and Kenny 1997).

The healing of the periodontal ligament following replantation has been shown to be markedly influenced by the following factors (Andreasen *et al.* 1995):

- Stage of root development

- Immediate replantation

- Extra-alveolar dry time

- Duration and medium for wet storage

1.5.1. Stage of root development

Necrosis of the periodontal ligament has been found to be more likely in teeth with mature apices compared to those with incomplete root formation (Andreasen *et al.* 1995). This could be due to the presence of a thicker periodontal ligament in younger teeth and hence its ability to withstand longer dry periods and better healing potential compared to matured teeth which have a thinner periodontal ligament layer. Furthermore with increasing age and complete root formation, the bony alveolar socket becomes more rigid and therefore contributes to greater periodontal ligament tissue damage when a trauma occurs (Andreasen, Vinding and Christensen 2006).

1.5.2. Immediate replantation

Immediate replantation has been shown to be a major factor influencing periodontal ligament healing (Andreasen *et al.* 1995). The prognosis of healing is optimal when the replantation is carried out with minimal damage to the periodontal ligament. Unfortunately, this is usually difficult to achieve in real life. Often, there is a degree of contamination to the tooth during the avulsion which may affect the healing of the periodontal ligament and subsequent resorption of the tooth (Kinirons *et al.* 2000). Despite immediate replantation within 5 minutes, normal periodontal ligament healing was only noted in 73 percent of the cases (Andreasen *et al.* 1995). Rinsing the tooth for 10 seconds prior to replantation with low-chlorine tap water in an animal study has shown better healing than the use of saliva (Weinstein, Worsaae and Andreasen 1981), presumably due to the higher bacterial content in saliva and hence, increased risk of inflammation. However, the evidence on rinsing avulsed teeth with minimal contamination before replantation has not been conclusive.

1.5.3. Extra-alveolar dry time

Extra-alveolar dry time of the avulsed tooth is the most important factor which contributes to decreased chance for survival of the periodontal ligament cells. The longer the extra-alveolar dry time prior to replantation, the poorer the periodontal ligament healing (Andreasen *et al.* 1995). Desiccation of the root appears to render the periodontal membrane necrotic, with greater likelihood of ankylosis and replacement resorption occurring (Blomlof *et al.* 1983b; Andersson, Bodin and Sorensen 1989).

The following retrospective and animal studies have demonstrated a rapid decline of periodontal membrane healing with increasing extra-alveolar dry time. In a separate study of avulsed teeth replanted within 15 minutes, 15 out of 21 teeth followed up over an average of 5 years showed favourable healing (Andersson and Bodin 1990). When extracted monkey teeth were stored dry for 30 minutes before saline storage prior to replantation, a higher amount of ankylosis was observed than those which only had 15 minutes of dry time before saline storage (Andreasen and Schwartz 1986). This emphasises the detrimental effects of an additional 15 minutes dry time to periodontal membrane healing. Within 30 to 60 minutes of extra-alveolar dry time, necrosis of periodontal ligament is expected (Blomlof *et al.* 1983b; Kenny and Barrett 2001). Beyond 60 minutes of dry storage, replacement resorption is seen in almost all replanted teeth overtime (Andersson, Bodin and Sorensen 1989).

1.5.4. Wet storage

Pre-replantation storage in wet medium is preferred over dry storage if immediate replantation is not possible (Oswald, Harrington and Hassel 1980). It is important that the osmolality of the medium be similar to that of the periodontal ligament cells to maintain cell viability (Lindskog and Blomlof 1982). Among the commonly reported

mediums used are milk, saliva and tap water (Lindskog and Blomlof 1982; Andreasen *et al.* 1995; Kenny and Barrett 2001). Milk has the most suitable osmolality as a storage medium. It has been reportedly been able to maintain cell viability slightly better at a lower temperature of 4ºC, likely due to less desiccation through evaporation from the periodontal ligament cells (Blomlof and Otteskog 1980). Saliva and tap water are hypotonic solutions and therefore are poor in maintaining cell viability (Lindskog and Blomlof 1982). Commercial storage solutions such as Hank's Balanced Salt Solution (HBSS) have been tested as alternative storage media for avulsed teeth. HBSS has shown to be equally as good as milk in maintaining viability of progenitor cells (Lekic, Kenny and Barrett 1998; Ashkenazi, Sarnat and Keila 1999). Ashkenazi and co-workers did an *in vitro* study which concluded that despite milk and HBSS performing better than other tested media, the viability, mitogenicity and clonogenic capacity of the periodontal ligament cells were significantly reduced after 24 hours of incubation in these media (Ashkenazi, Sarnat and Keila 1999).

1.5.5. Total time tooth out of mouth prior to replantation

It was found that the total extra-alveolar time for a combination of dry and wet storage showed significantly reduced periodontal ligament healing if the teeth were out of the mouth for more than 20 minutes (Andreasen *et al.* 1995). This same clinical study also found that teeth stored dry for up to 9 minutes and later placed in saline prior to replantation demonstrated a lower periodontal ligament healing rate than those replanted immediately after dry storage only (Andreasen *et al.* 1995). The idea of pre-soaking avulsed teeth in HBSS prior to replantation to enable reconstitution of periodontal ligament cells has been challenged with an *in vitro* study showing no improvement of cell viability with pre-soaking avulsed teeth for 30 or 60 minutes in milk or HBSS after these teeth had been stored dry (Doyle, Dumsha and Sydiskis 1998). This highlights the

lethal effects of desiccation on periodontal membrane cells and that attempts to revitalise these cells with HBSS or milk prior to replantation are futile. It appears that immediate replantation remains the most essential factor in predicting favourable PDL healing. Nevertheless in reality, immediate replantation or a quick response to find an appropriate wet medium for the avulsed tooth is overshadowed by accident-associated factors such as the lack of knowledge and the extent of other non-dental injuries (Hamilton, Hill and Mackie 1997). Most avulsed teeth would have had gone through a period of desiccation and mechanical insult before being placed into a wet medium prior to replantation.

1.5.6. Other prognostic factors

The degree of contamination to the PDL and any cleaning of the root surface prior to replantation may also affect the PDL healing (Day and Duggal 2003). A significantly increased favourable PDL healing was noted when the tooth was not grossly contaminated with debris (Andreasen *et al.* 1995). In another study, bony healing was observed in a majority of the teeth that were replanted either with contamination or with prior rubbing of the PDL to remove any contamination (Kinirons *et al.* 2000).

Other predictors affecting root resorption include the age, type of endodontics and the presence of bacteria in the periodontal ligament, pulpal space or dentinal tubules (Andreasen, Vinding and Christensen 2006).

1.6. Homeostasis of the periodontal ligament

The periodontal ligament functions as the "barrier" between the cementum of the tooth and the alveolar bone socket. Its main role is to protect the root against physiologic processes of resorption and deposition of the alveolar bone (Lindskog *et al.* 1985).

Other functions of the periodontal ligament include cementum deposition by cementoblasts on root surface, maintenance of the ligament fibres by fibroblasts for tooth support and proprioception and pressure by innervation. The physiologic equilibrium of the periodontal ligament is maintained by cells on the root surfaces offering protection against the osteogenic cells. This is achieved by the molecular signalling system of osteoprotegerin (OPG) and receptor activator of nuclear factor-κB ligand (RANKL) (Fukushima *et al.* 2003).

1.6.1. Receptor activator of nuclear factor-κB (RANK) and its ligand RANKL

RANK is expressed as a 616-amino-acid peptide on the cell surface of osteoclast precursors while its ligand, RANKL is a 317-amino-acid peptide of molecular weight 38kDa (Tyrovola *et al.* 2008). RANKL is formed from three subunits to make an active trimeric molecule (Kong, Boyle and Penninger 2000). RANKL is expressed by cells of osteoblast origin and activated T cells, where it is found as a surface-bound molecule on osteoblasts and as a soluble form (sRANKL) when expressed by T-cells (Schoppet, Preissner and Hofbauer 2002). RANKL promotes osteoclast formation, fusion, differentiation, activation and survival, thus giving rise to bone resorption. It is the binding of RANK to RANKL which activates this resorption process by the osteoclasts. Both soluble and cell-bound RANKL are inhibited by the binding of osteoprotegerin, a competitor of RANK (Udagawa *et al.* 1999).

1.6.2. Osteoprotegerin (OPG)

OPG, RANKL and its receptor RANK share similar characteristics with members of the tumour necrosis factor (TNF) receptor superfamily. They are essentially paracrine regulators of bone metabolism and immune functions. However, compared to other TNF cytokine superfamily members, OPG lacks transmembrane and cytoplasmic domains

and is secreted as a soluble protein (Tyrovola *et al.* 2008). OPG consists of 401 amino acids and can exist as a monomer (60kDa) or a disulphide-linked homodimer (120kDa) (Tsuda *et al.* 1997). OPG is produced by various tissues such as the heart, lungs, kidneys, intestines and bone, as well as by hematopoietic and immune cells (Schoppet, Preissner and Hofbauer 2002). The major functions of OPG are inhibition of osteoclast differentiation, inhibition of osteoclast resorptive function, and stimulation of osteoclast apoptosis (Oshiro *et al.* 2002). Osteoprotegerin functions as a soluble receptor antagonist which neutralises RANKL, producing an effect opposite to that of RANK to RANKL binding (Kwon *et al.* 1998).

1.6.3. The OPG/RANK/RANKL system

As discussed, numerous cells such as preosteoblasts and T-cells express signal molecules in two forms of RANKL – membrane-bound and soluble form. The RANK receptor is found attached to the osteoclast progenitor cells. Osteoclast differentiation occurs when RANKL, expressed on osteoblasts and mesenchymal cells, bind to the RANK receptor. This will result in the merging of the osteoclast progenitor cells to form active osteoclasts (Kanzaki *et al.* 2001; Fukushima *et al.* 2003). The RANK-RANKL signalling prompts the resorptive activity of osteoclasts at the final stages of differentiation. OPG inhibits osteoclastogenesis by covering RANKL, thus preventing the binding between RANK and RANKL (Kanzaki *et al.* 2001). The signalling pathway of OPG-RANK-RANKL has been shown to be regulated by factors such as interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) (Palmqvist *et al.* 2002; Wada *et al.* 2004) as well as hormones (Boabaid *et al.* 2004; Ogasawara *et al.* 2004). Overall, these regulators and receptor molecules play an important role in balancing the induction and inhibition of osteoclastic activity.
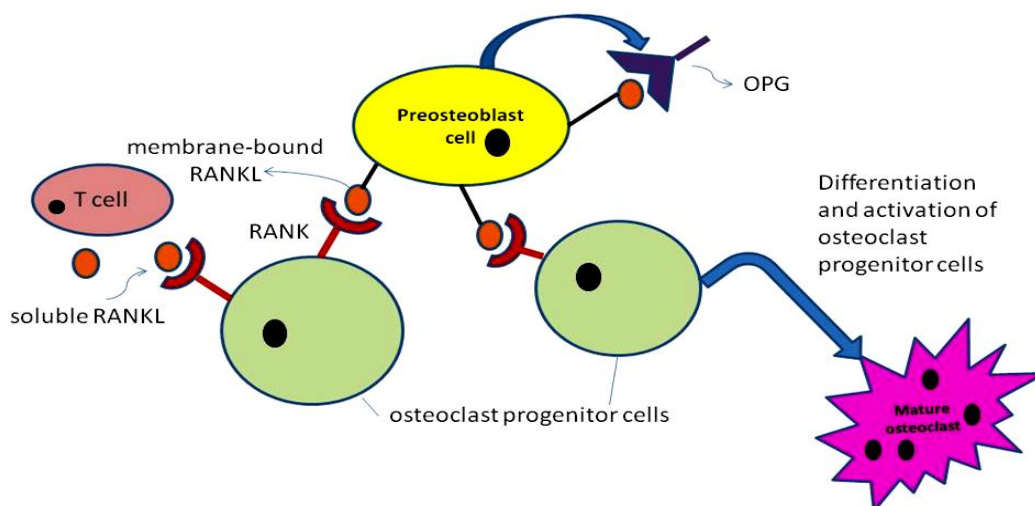
Figure 1.4 The interaction between OPG-RANK-RANKL.

In the medical field, the discovery of OPG-RANK-RANKL signalling system in the area of bone metabolism has expanded the fundamentals in understanding osteoclastogenesis in diseases such as osteopetrosis, Paget's disease and osteoporosis in post-menstrual women (Khosla 2001). Expression of these signalling molecules have also been detected in cancers associated with potential osseous destruction such as breast cancer (Thomas *et al.* 1999), prostate cancer (Krishnan and Davidovitch 2006) and myeloma (Uematsu, Mogi and Deguchi 1996). In dentistry, disruption in the balance of OPG, RANK and RANKL has been implicated in inflammatory bony destruction in periodontal disease (Jin *et al.* 2002; Wara-aswapati *et al.* 2007), detection of oral squamous cell carcinoma (Chuang *et al.* 2009) and cystic expansion of dentigerous and radicular cysts (Moraes *et al.* 2011). The alteration in expression levels of RANKL and OPG noted in the phenomenon of root resorption in deciduous teeth during exfoliation and in tooth movement for orthodontics (Fukushima *et al.* 2003; Yamaguchi *et al.* 2006) seemed to suggest that these signalling molecules could also affect mineralised dental tissues such as the cementum in a similar way to bone.

1.6.4. Regulation of OPG and RANKL

Various cytokines and growth factors have been found to affect bone metabolism (Kanzaki *et al.* 2004; Nishijima *et al.* 2006). These include sex hormones, parathyroid hormone, parathyroid hormone-related peptide and calcitriol. Deficiency in oestrogen has been associated with an increase in bone loss via reduced OPG expression by mature osteoblasts (Hofbauer *et al.* 1999b). An *in vitro* study using cultured human PDL cells noted that the expression of OPG was upregulated whereas that of RANKL was downregulated following treatment with oestrogen for 48 hours (Liang *et al.* 2008). In animal bone marrow cultures, parathyroid hormone was found to stimulate RANKL and inhibit OPG expression (Lee and Lorenzo 1999; Kanzawa *et al.* 2000). However, there is evidence that parathyroid hormone does not affect OPG levels in some osteoblastic cells (Suda *et al.* 1999) while another study found that high levels of parathyroid hormone suppressed the expression of both OPG and RANKL (Seck *et al.* 2001). Bone-resorbing promoters such as $1\alpha,25$-dihydroxyvitamin D_3 have also been found to influence the regulation of OPG/RANKL in human periodontal ligament cells (Zhang *et al.* 2004).

Other regulators which have also been found to affect the expression of OPG and RANKL include TNF-α, interleukin-1α, interleukin-18, transforming growth factor-β, bone morphogenetic protein, glucocorticoids, immunosuppressant, prostaglandin E2 and basic fibroblast growth factor (Brandstrom *et al.* 1998; Hofbauer *et al.* 1999a; Nakagawa *et al.* 1999; Hofbauer *et al.* 2001).

1.6.5. OPG/RANK/RANKL in the oral environment

Laboratory studies have shown that RANKL is expressed by odontoblasts, pulp and periodontal ligament fibroblasts and cementoblasts of human deciduous teeth

(Hasegawa *et al.* 2002; Fukushima *et al.* 2003) while RANK is expressed by multinucleated odontoclasts or by mononucleated precursors (Lossdorfer, Gotz and Jager 2002). OPG is expressed by odontoblasts, ameloblasts, periodontal ligament and dental pulp cells (Rani and MacDougall 2000; Zhang *et al.* 2004). The role of OPG/RANK/RANKL system in the periodontal tissue has been used to explain the biochemical processes underlying orthodontic tooth movement, primary tooth exfoliation and periodontal disease in dentistry.

The principle of tooth resorption has been suggested to be similar to that of bone and is regulated by cells known as odontoclasts (or dentinoclasts). Odontoclasts are large, multi-nucleated cells which originate from progenitor cells found in the dental pulp and periodontal ligament (Wang and McCauley 2011). Odontoclasts possess very similar properties to osteoclasts. Using histochemical analysis, Addison reported that the enzyme profiles and reaction products between osteoclasts and odontoclasts appeared to suggest similarity in their metabolic function (Addison 1979). It is believed that the only difference between these two cell kinds lies in the smaller size and nuclear numbers of odontoclasts. However, it can be difficult to differentiate between them within the periodontal membrane space (Pierce 1989). The enzyme H^+-K^+-ATPase along the ruffled borders of odontoclasts has been shown to demineralise the non-collagenous portion of the dentine in primary tooth resorption (Matsuda 1992). A further laboratory study found that multinucleated odontoclasts are able to resorb non-mineralised predentine matrix in a manner similar to odontoclasts resorbing demineralised dentine matrix (Sahara *et al.* 1994). In a mouse femur model, the enzyme cathepsin K was found to degrade bone type-I collagen following osteoclastic induction (Yamaza *et al.* 1998). Similarly, localisation of cathepsin K, matrix metalloproteinase-9 (MMP-9) and H^+-K^+-ATPase in osteoclasts and odontoclasts revealed lack of difference

in expressions of these enzymes between the two cell types. This suggested similar mechanisms could be involved in bone and teeth mineralisation (Sasaki 2003). Due to these morphological and functional similarities between odontoclasts and osteoclasts, it was thought that factors which regulate osteoclastic activity under physiological and pathological conditions could apply to the regulation of root resorption following traumatic injuries.

1.6.5.1. Role of OPG and RANKL in physiologic primary tooth root resorption

The discovery of OPG, RANK and RANKL has contributed to a better understanding of root resorption in primary tooth exfoliation. Fukushima and co-workers (2003) examined the expression of OPG and RANKL in primary teeth during physiological root resorption using immunocytochemistry and RT-PCR. They found increased root-resorbing activity through RANK-RANKL binding on odontoclasts in a manner similar to that of osteoclasts. In addition, regulation of OPG and RANKL levels has been demonstrated in dental follicles of rats, influencing osteoclastogenesis during tooth eruption (Wise *et al.* 2000; Boabaid *et al.* 2004). These studies suggest that OPG and RANKL are involved throughout the process of primary tooth exfoliation and subsequent permanent tooth eruption. Impairment in the nuclear factor-κB signalling pathway has been reported in ectodermal dysplasia patients (Doffinger *et al.* 2001) and thus led to postulations that tooth development could also be affected by RANK-RANKL signalling since patients with ectodermal dysplasia present with hypodontia. Using a mouse embryo explant culture model, RANK and RANKL could be detected in the developing tooth and surrounding alveolar bone during early tooth formation (Ohazama, Courtney and Sharpe 2004). This study also found that addition of exogenous OPG to the culture resulted in a delay in tooth development and mineralisation. Another animal study investigating the role of excessive RANK

expression suggested that tooth eruption was affected by RANK signalling pathway, in which RANK would induce early tooth eruption and accelerate root elongation (Castaneda *et al.* 2010).

In these physiological primary root resorption and tooth eruption studies, the activation of odontoclasts/osteoclasts was shown to be influenced by the balance of the OPG, RANK and RANKL. These studies strongly suggest the involvement of these signalling molecules in the mineralisation process, where regions with hard tissue resorption were associated with higher RANKL and lower OPG expressions.

1.6.5.2. Role of OPG and RANKL in orthodontic movement

The principle of tooth movement depends on alveolar bone resorption and apposition. In order for bone remodelling to occur, an inflammatory response is necessary. This is achieved by mechanical stimulus during orthodontics resulting in the release of inflammatory mediators which regulate biologic responses associated with bone remodelling (Krishnan and Davidovitch 2006). Levels of various inflammatory mediators such as interleukins and tumour necrosis factor were found to be increased in gingival crevicular fluid of patients undergoing orthodontic treatment (Uematsu, Mogi and Deguchi 1996). Nishijima and co-workers discovered an elevated level of RANKL and a decreased level of OPG in gingival crevicular fluid of orthodontic patients following application of retraction force (Nishijima *et al.* 2006)**.** Using a rat model, increased levels of OPG and RANKL mRNA were detected in bony tissues subjected to orthodontic forces (Low *et al.* 2005). Further immunohistochemical studies on rats by Kanzaki and co-workers found that the transfer of the RANKL gene to periodontal tissue promoted osteoclastogenesis whereas the transfer of the OPG gene inhibited osteoclastogenesis and subsequent tooth movement (Kanzaki *et al.* 2004; Kanzaki *et al.*

2006). Application of tensile stretch force to osteoblasts *in vivo* using rat teeth resulted in an increase of OPG mRNA in periodontal ligament cells (Kobayashi *et al.* 2000). An *in vitro* study found that compressive forces stimulated increased levels of s-RANKL (Yamaguchi *et al.* 2006). The same study also found that external apical root resorption could be induced with periodontal ligament cells expressing large amounts of RANKL and low OPG production. The cellular process governing root resorption has been thought to be similar to that of bone resorption (Pierce, Lindskog and Hammarstrom 1991). Therefore, the relative levels of OPG and RANKL are strongly believed to regulate bone resorption and deposition during orthodontic tooth movement.
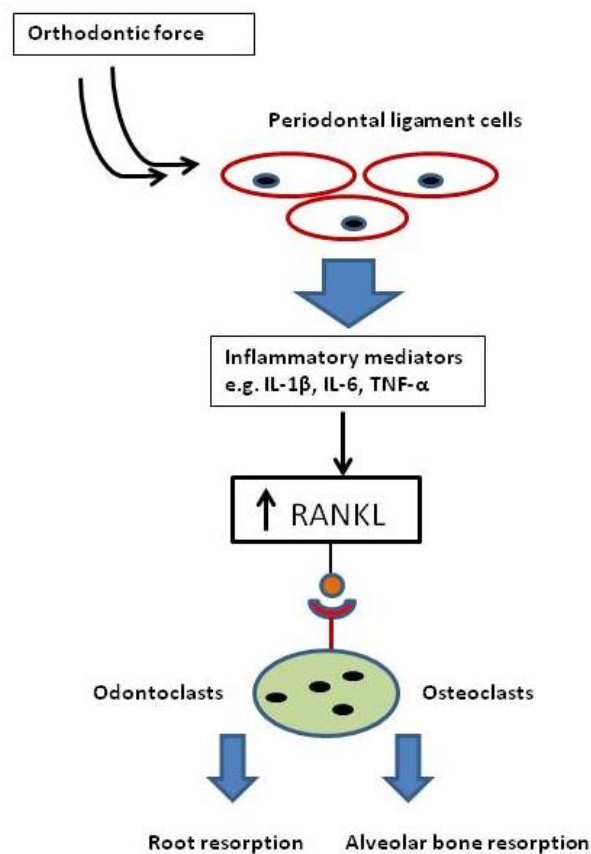
Figure 1.5 Summary of sequence of cellular events following orthodontic force application (Yamaguchi 2009).

1.6.5.3. Role of OPG and RANKL in periodontal disease

In addition to OPG/RANKL regulation in tooth root resorption, the role of these signalling molecules in periodontal inflammation has also been reported (Kanzaki *et al.* 2001; Wara-aswapati *et al.* 2007). Lipopolysaccharide, a cell component of gram-negative bacteria in periodontitis, has been thought to influence the expression of OPG/RANKL. One *in vitro* study using human periodontal ligament fibroblast cultures found that lipopolysaccharide stimulated both OPG and RANKL expression by upregulating other cytokines such as interleukin-1β and tumour necrosis factor-α (Wada *et al.* 2004). Using samples of exudate obtained from the gingival crevicular fluid in humans, Mogi and co-workers (1999) found significant increased concentrations of cytokines interleukin-1β and interleukin-6 in those with severe periodontitis. Further work by this same group found a higher ratio of RANKL to OPG concentration in gingival crevicular fluid of patients suffering from periodontal disease compared to controls (Mogi *et al.* 2004). Many other studies have also found an association between periodontal breakdown and cytokine response with OPG/RANK/RANKL signalling (Jin *et al.* 2002; Wada *et al.* 2004; Yamamoto *et al.* 2006b). Overall, the current available literature seems to suggest that RANKL and OPG contribute to osteoclastic bone destruction in periodontal disease.

1.6.5.4. Role of OPG and RANKL in dental traumatology

To date, there has been limited literature investigating the association between OPG/RANKL expression in the PDL and the prognostic factors affecting PDL healing following replantation of the avulsed tooth. The effect of extra-oral dry time on the expressions of OPG and RANKL on the tooth root surface has yet to be reported in the literature. A recent *in vitro* study published by Zhan and co-workers studied the effects of HBSS and milk on cultured PDL cells. They found that with increased time of

storage in these wet media, there was an increased number of osteoclast-like cells. Although there was statistically insignificant reduction of OPG expression with increasing time in the media, there was absence of RANKL expression in all the cell cultures, thus indicating osteoclastogenic induction in this case was probably RANKL-independent (Zhan *et al.* 2012).

1.7. Methods to detect expression of OPG and RANKL

One of the first studies to detect OPG and RANKL in dental tissues was by Rani and MacDougall (2000). Both protein and messenger ribonucleic acid (mRNA) level expressions were examined using mouse odontoblast/dental pulp cell lines. Reverse transcriptase polymerase chain reaction (RT-PCR) and Northern blotting techniques were used to detect OPG and RANKL at the mRNA level, whereas Western blotting and immunohistochemistry methods were used for detection at the protein level (Rani and MacDougall 2000). Various studies in the dental literature have also used the aforementioned methods in OPG and RANKL detection. RT-PCR appeared to be a common method for investigating the expression of OPG and/or RANKL in cultures of human gingival crevicular fluid and periodontal ligament cell samples obtained from surfaces of extracted teeth (Wada *et al.* 2004; Zhang *et al.* 2004; Yamamoto *et al.* 2006a; Wara-aswapati *et al.* 2007). Immunohistochemistry has also been widely used in studies localising OPG and RANKL in dental tissues (Wise *et al.* 2000; Lossdorfer, Gotz and Jager 2002; Crotti *et al.* 2004; Chuang *et al.* 2009; Moraes *et al.* 2011). Other less common techniques included the use of Western blot or Northern blot analysis to investigate RANKL expression (Udagawa *et al.* 1999; Yamaguchi *et al.* 2006) and *in situ* hybridisation for OPG detection (Kobayashi *et al.* 2000).

1.7.1. Detection methods at protein level

1.7.1.1. Immunohistochemistry

Immunohistochemistry involves the use of antibodies to identify an antigen within a tissue or within compartments of a cell. It is based on the concept of the strong and specific bond between an antibody and its antigen. There are the direct and indirect methods available. The direct method uses a labelled primary antibody whereas the indirect method uses a labelled secondary antibody which binds to the primary antibody, thus increasing the sensitivity of the reaction. Although antigen can be measured by other methods such as Western blots and radioimmunoassay, immunohistochemistry is the only technique which can identify an antigen in its tissue or cellular location *in situ* (Polak and Van Noorden 2003). However, evaluation of the antigen quantity through immunohistochemistry remains a challenge despite increasing efforts to improve it to a true quantitative immunoassay. Various factors such as fixation of the tissue, duration, antigen retrieval method, antibody specificity and antibody dilution affect the immunohistochemical method and outcome (Taylor and Levenson 2006; Walker 2006).

1.7.1.2. Western Blotting

Western blotting is the technique of transferring proteins from a gel to a membrane. It is used to identify and determine the relative quantity and molecular weight of a protein within a mixture of other proteins and molecules. Any tissue would first need to be homogenised to form a mixture and then treated with salts, buffers or proteases. The mixture is separated, usually by gel electrophoresis, such that the final positions of different proteins in the gel are in accordance to their molecular size. The separated proteins are transferred from the gel to a support membrane by electrophoresis such that the membrane becomes a replica of the separated macromolecules in the gel. The

position of the protein antigen on the membrane can then be detected by binding of an unlabelled primary antibody specific for that protein followed by a labelled second antibody which is usually either radioactive or fluorescent. This approach provides information about the relative antigen size and quantity. More commonly used in medical diagnostics, Western blotting can help detect if a particular protein is present. However, this technique is highly technique-sensitive, time-consuming and only gives the relative but not absolute quantity of the protein. As preparation of the sample involves mechanically breaking down the tissue, the precise location of the proteins cannot be detected with this method (Abbas, Lichtman and Pillai 2012).

1.7.2. Detection methods at mRNA level

1.7.2.1. Northern blotting

Northern blotting technique is considered the gold-standard for the detection of mRNA levels because it allows a direct comparison of the mRNA abundance between samples on a single membrane. The steps involved in Northern blotting technique are similar to those in Western blotting except that it involves transferring RNA instead of proteins from a gel to a membrane. As such, the labelled probe in this method would be either deoxyribonucleic acid (DNA) or RNA. This technique can be time-consuming as well as cumbersome compared to others and usually requires a certain minimum amount of RNA concentration to allow any detection. Even the slightest degradation of RNA can affect the quality and quantity of the expression.

1.7.2.2. Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR is a variation of polymerase chain reaction used in molecular biology to study gene expression and allow analysis of mRNA in very minute amounts that cannot be picked up by Northern blotting (Kanzaki *et al.* 2006). RT-PCR can be performed on cell

cultures or tissue sample. In studies investigating OPG and/or RANKL expression in human periodontal ligament using the RT-PCR technique, the PDL tissue on the middle third of the root surface of extracted teeth were scraped and subsequently cultured prior to conducting RT-PCR (Zhang *et al.* 2004; Yamamoto *et al.* 2006a; Zhan *et al.* 2012). The first critical step for RT-PCR which will determine the yield and quality of the RNA involves total RNA isolation from cells or tissue with mechanical or chemical disruption. This step has now been made easy with the availability of commercial isolation kits. The mRNA is then reverse transcribed into its complement deoxyribonucleic acid (cDNA) using reverse transcriptase kits. Amplification of the cDNA is carried out in the PCR machine with heat-stable DNA polymerase and primers specific for the protein of interest. The final PCR product can be quantified conventionally by comparing the intensities of an ethidium bromide-stained standard and the target gene, or by using digital software to calculate the comparative cycle threshold values. Alternatively, the quantification can be performed real-time using a fluorescent probe. Despite widely used for gene expression studies, RT-PCR is a very sensitive technique requiring careful optimisation for accurate quantification which can be in turn time-consuming and costly. In conventional RT-PCR, there is also a risk of contamination from DNA during the amplification process (Bleve *et al.* 2003).

1.7.2.3. *In situ* hybridisation

In situ hybridisation is a technique involving the use of a labelled cDNA or RNA strand to localise a specific DNA or RNA sequence in a portion or section of tissue. This method allows visualisation of the tissue distribution of the RNA of interest without interference from the RNA of other cells within the same tissue sample. Therefore it is useful to detect RNAs that are present in only a small subset of cells within the sample (Taylor and Levenson 2006). Despite the advantages of *in situ* hybridisation, proper

controls are necessary to show that the labelling is due to hybridisation of the target rather than non-specific labelling. Samples usually require fairly prompt fixation to avoid rapid degradation of RNA. Immunocytochemistry can be carried out together with *in situ* hybridisation to help strengthen interpretations of the data (Kumar 2010). Just as with other mRNA detection techniques, an increased mRNA expression may not necessarily mean that increased protein is generated. The outcome from mRNA detection would show only a correlation to the cellular process but not causation. Ultimately, it is the protein interactions regulating the cellular process that most researchers would be interested in. The poor correlation between mRNA and protein expression levels could be due to the complex translational and transcriptional stages involved in turning the mRNA to the final protein such that it is not yet possible to calculate protein concentrations from mRNA. Also, different half-lives of the proteins *in vivo* and the limitations of the experiments leading to significant errors could all prevent the researcher from obtaining a clear idea of the correlation (Greenbaum *et al.* 2003).

1.8. Conclusions from the literature

One of the most important functions of the periodontal ligament is to protect the root against physiologic processes of resorption and deposition of the alveolar bone. Without the presence of the periodontal ligament, ankylosis results where the alveolar bone is directly deposited adjacent to the cementum of the root surface. The physiologic homeostasis of the periodontal ligament is maintained by cells on the root surfaces which prevent the root from being in direct contact with osteogenic cells.

Osteoprotegerin has been thought to protect the root from RANKL-induced activation of osteoclasts/odontoclasts (Fukushima *et al.* 2003). This is achieved by OPG binding to

RANKL and thus preventing RANK-RANKL binding. The balance between OPG and RANKL expression has been used to explain the phenomenon of root resorption in deciduous teeth during exfoliation and in tooth movement for orthodontics in various studies. The existing studies have implicated the involvement of OPG and RANKL in the resorptive process but the exact biological mechanism that regulates these proteins during resorption of mineralised tissue remain complicated.

1.9. Rationale for study

Most avulsed teeth that have been replanted demonstrate replacement resorption clinically overtime. To date, protein expression of OPG and RANKL on root surfaces of avulsed teeth has yet to be reported. The only study that attempted to look at the effects of storage media in relation to tooth avulsion performed the experiment on PDL cell cultures via the RT-PCR technique (Zhan *et al.* 2012). Since OPG and RANKL signalling system controls mineralisation activity, these molecules are expected to be present on the root surfaces of avulsed teeth in order for resorptive activity to take place. As the healing of the PDL depends on the extra-oral duration, the degree of root resorption would likely increase with greater extra-oral time. An insight into any observed change in the expressions of OPG and RANKL with extra-oral dry time would hopefully allow a better understanding into the molecular processes regulating replacement root resorption. This could then have relevance in terms of potential therapy for replantation of avulsed teeth.

1.10. Aim of study

The aim of this study was to investigate the expression and distribution of OPG and RANKL on root surface of extracted teeth with different extra-oral dry times.

1.11. Objectives

a) *Collection of extracted human permanent teeth.*

b) *To store dry freshly extracted human teeth according to the specified extra-oral dry time of <5 minutes, 30 minutes or 60 minutes immediately after extraction.*

c) *Histological preparation of teeth using paraffin-embedded sections.*

d) *Detection of OPG and RANKL on root surfaces using immunohistochemistry.*

e) *Investigate the staining intensity of OPG and RANKL expression and distribution the root surfaces under light microscopy.*

1.12. Hypothesis

The null hypothesis was that there is no difference in the expression of OPG and RANKL with different extra-oral dry times.

2. MATERIALS AND METHOD

2.1. Pilot work to validate the methodology

Before the location and quantification of OPG and RANKL could be evaluated, pilot work was required to validate the methodology. The purpose of this pilot study was to provide some basic information of protein expression and localisation of OPG and RANKL signalling molecules on extracted teeth samples. This initial pilot work was carried out to develop an optimal protocol for this study and is described below.

2.1.1. Tissue preparation

For the purpose of detecting the presence and distribution of proteins along the tooth root surface, formalin-fixed paraffin-embedded (FFPE) sections were chosen.

2.1.2. Antigen retrieval

A trial of 6 different antigen retrieval methods compared to no antigen retrieval was carried out. These are described below:

2.1.2.1. Heat-mediated antigen retrieval method at 100ºC in microwave

1) Vector H-3300 antigen unmasking solution diluted 1/100 (in distilled water) as per manufacturer's instructions (Figure 2.1).

2) Citrate buffer (prepared as a 0.1M citric acid solution – BDH 10081) at pH 6.0 (Figure 2.1).

3) 6M urea (BDH 44387) (Figure 2.2).

4) 1mM EDTA (Ethylenediaminetetra-acetic acid- BDH 10093) at pH 8.0 (Figure 2.2).
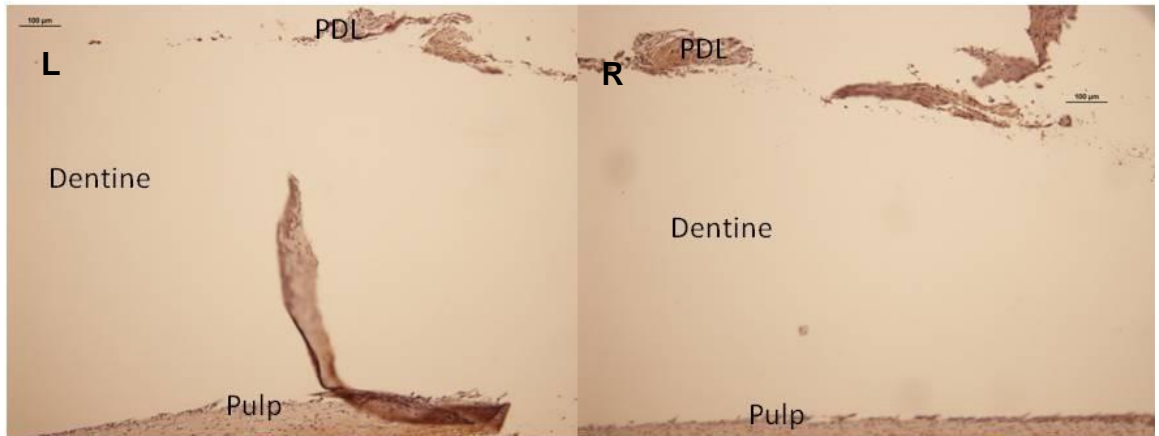
Figure 2.1 Photomicrographs representing specimen that had been treated with the antigen unmasking solution (L) and citrate buffer (R) in microwave. Note the complete loss of dentine and some of the periodontal ligament (PDL) tissue.
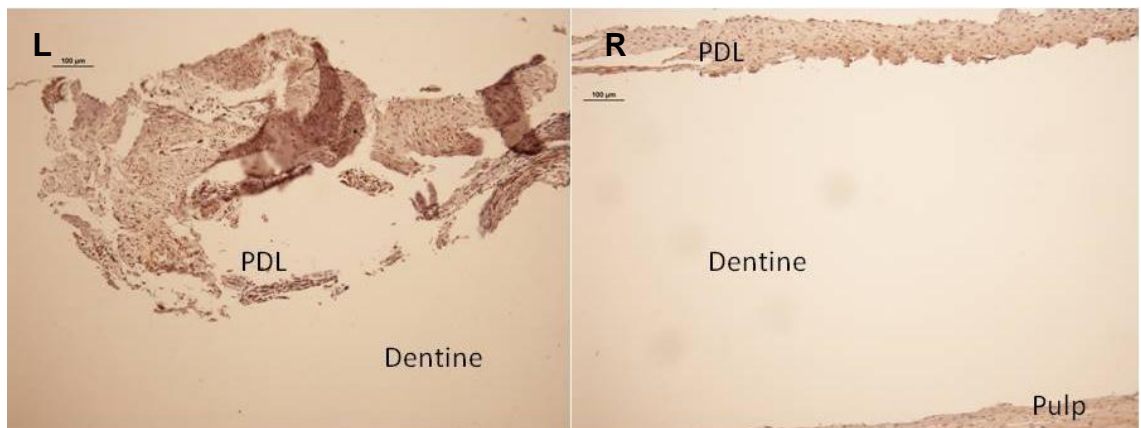
Figure 2.2 Photomicrographs representing specimen that had been treated with 6M urea (L) and 1mM EDTA (R) in microwave. Note again the complete loss of dentine tissue from both specimens.

2.1.2.2. Heat-mediated antigen retrieval method at 120ºC in pressure cooker

5) Vector H-3300 antigen unmasking solution diluted 1/100 (in distilled water) as per manufacturer's instructions (Figure 2.3).

2.1.2.3. Enzyme-mediated antigen retrieval method

6) 0.1% chymotrypsin (Sigma 4129 α-chymotrypsin from bovine pancreas) - prepared in 0.1% calcium chloride solution with pH 7.8 at 37ºC (Figure 2.3).
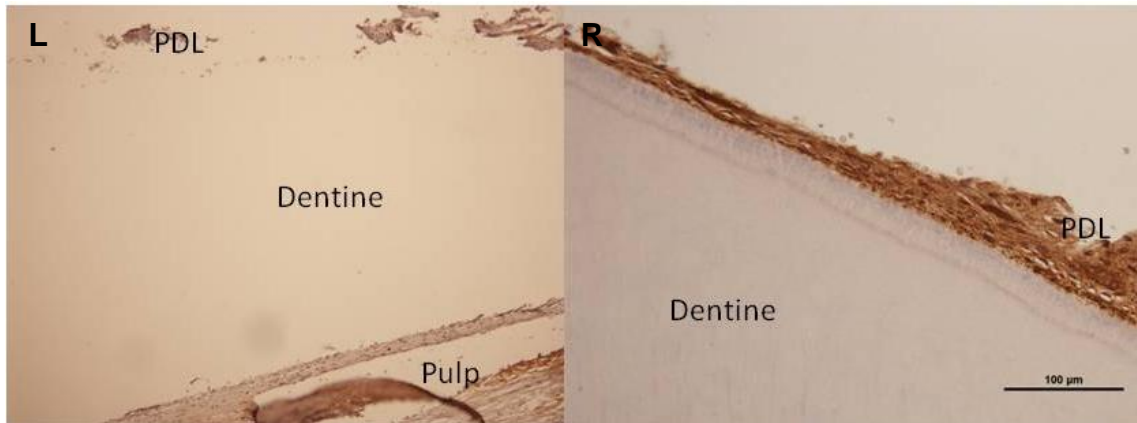
Figure 2.3 Photomicrographs representing specimen that had been treated with the antigen unmasking solution (L) in pressure cooker and chymotrypsin (R). Again, there was complete loss of dentine and some of the periodontal ligament (PDL) tissue in the specimen treated with antigen unmasking solution in the pressure cooker. For the specimen treated with chymotrypsin, there was excessive immunoperoxidase staining.

2.1.2.4. No treatment

7) No antigen retrieval method was employed (Figure 2.4).

2.1.2.5. Outcome of antigen retrieval trial

Following the antigen retrieval trial, no treatment was chosen as this retained the morphology of the structures with minimal tissue destruction (Figure 2.4). Furthermore, with no treatment to the tissue specimen, less artefacts were introduced to the final result of the immunostaining.
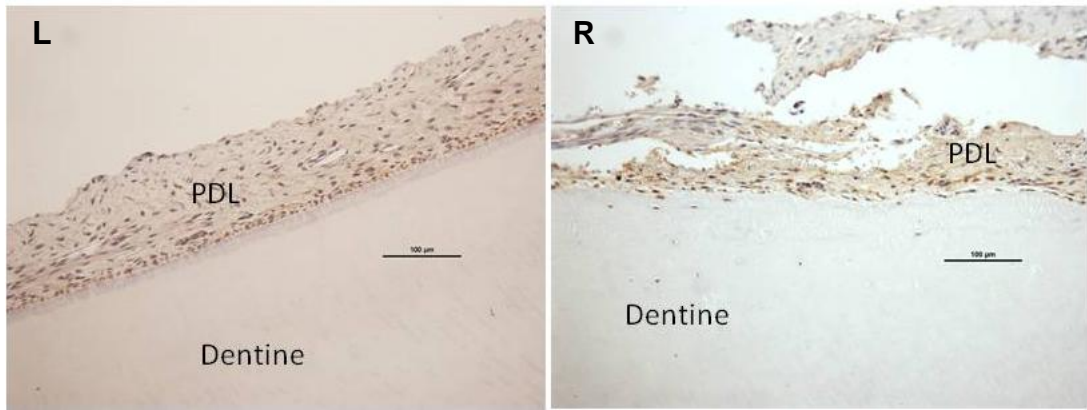
Figure 2.4 Photomicrographs representing specimens that had not undergone any antigen retrieval treatment; left specimen stained for OPG and right specimen stained for RANKL. Most of the tissue architecture was preserved and acceptable positive immunoperoxidase labelling could be observed in both specimens.

2.1.3. Direct and indirect immunostaining methods

Following careful consideration of the advantages and disadvantages, the indirect immunostaining method was used for this study. The primary antibodies chosen were raised in either rabbit or mouse according to the detection kit (Dako EnVision + Dual Link System – HRP K4065) to be used. The secondary antibody was labelled with horseradish peroxidase (HRP). When HRP was developed in a solution of 3,3-diaminobenzidine (DAB) chromogen and hydrogen peroxide (substrate), a mahogany brown colour was produced to visualise the location of the antigen. The product of this reaction was insoluble in organic solvents thereby allowing the sections to be dehydrated in alcohol and cleared in xylene prior to being mounted in di-n-butylphthalate in xylene (DPX) mounting medium.

2.1.4. Dilution trial

A primary antibody dilution trial was performed to discover the optimum dilution for use in this study. Dilution of the antibody helps to reduce background staining and is economical. The different dilutions tested for each antibody are shown in Table 2.1.

Table 2.1 Dilution trial for OPG and RANKL antibodies.

Primary antibody	Host species	Type	Source	Dilution tried
RANKL	Mouse	Monoclonal (human)	*Abcam Ltd, Cambridge, UK ab 45039*	1:100 1:150 1:200
OPG	Rabbit	Polyclonal (human)	*Abcam Ltd, Cambridge, UK ab 73400*	1:400 1:500

A negative control slide for each tooth specimen was carried out. The negative control sections were subjected to the same immunostaining procedure as the test sections except that the primary antibody was omitted. The results of the dilution trial showed optimal dilution of RANKL to be 1:150 and OPG 1:500.

2.1.5. Counterstain trial

A counterstain trial was performed to obtain the best possible visualisation of the positive brown staining within the tissue. The following counterstain protocols were tested:

- Harris' Haematoxylin (Sugipath 01560BBE) for 30 seconds (Figure 2.5)
- Harris' Haematoxylin for 2 minutes (Figure 2.5)
- 1% aqueous Fast Green for 3 minutes (Figure 2.6)
- 1% aqueous Light Green for 3 minutes (Figure 2.6)
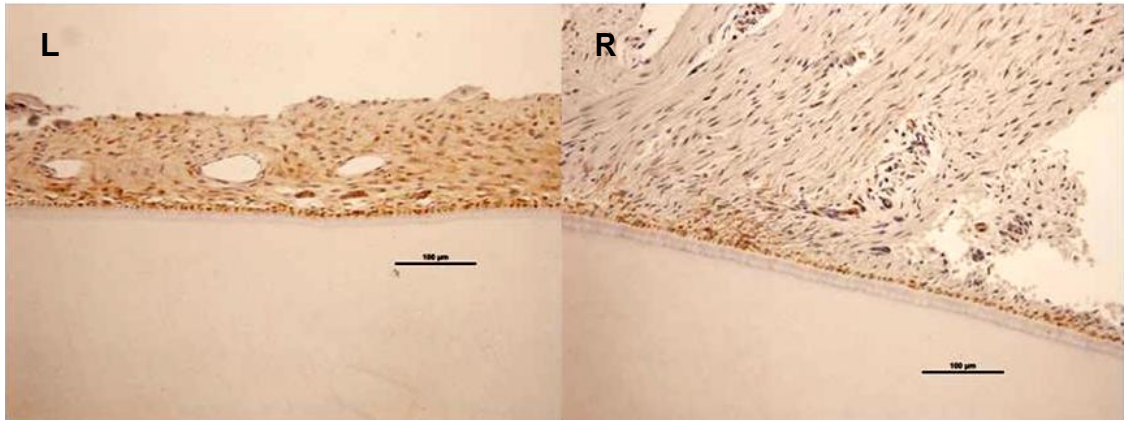
Figure 2.5 Photomicrographs showing the root surface region of specimens counterstained for 30 seconds (L) and 2 minutes (R) in Harris' haematoxylin. The brown precipitate and individual cell nuclei and fibres could be visualised distinctively.
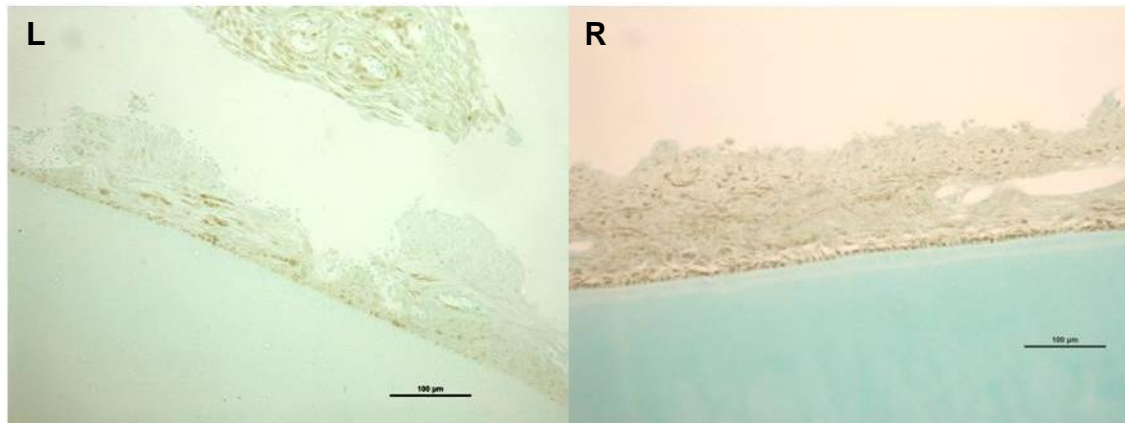
Figure 2.6 Photomicrographs showing the root surface region of specimens counterstained with light green (L) and fast green (R). Although the brown contrasted well against the green, the cell nuclei and fibre stains were less discernible.

The results indicated that the best image could be obtained with counterstaining using Harris' Haematoxylin for 30 seconds (RANKL) and 2 minutes (OPG) followed by tap water wash and subsequently mounted as appropriate.

2.2. Finalised protocol

2.2.1. Experimental material

2.2.1.1. Study population

Permanent teeth were obtained from children aged 9-16 years old who required routine dental extractions under general anaesthesia at either the Leeds Dental Institute (One Day Unit) or Leeds General Infirmary. Teeth were collected in the period between June 2011 and November 2011.

Ethical approval was obtained from the Leeds Dental Institute Research Ethics Committee Tissue Bank (Reference number: 261110/KT/49). Informed consent and assent were taken from parents or legal guardians and patients in accordance to the Tissue Bank protocol.

The sample consisted of 30 permanent teeth that had either minimal or no caries. Non-carious or minimally carious permanent teeth were extracted as part of an orthodontic treatment plan or as compensating extraction in the case for first permanent molars (Cobourne, Williams and McMullan 2009).

2.2.1.2. Inclusion criteria

- Healthy children with no relevant medical condition that might affect the periodontal ligament such as hypophosphatasia, Papillon Lefevre syndrome and other immunodeficiencies (Heasman and Waterhouse 2005).
- The extracted permanent teeth must be caries free or have minimal caries.
- The teeth should not have any clinical or radiographic signs of periodontal pathology.
- The teeth must be extracted intact with minimal damage to periodontal ligament.

2.2.1.3. Exclusion criteria

- Teeth with gross caries extending into the pulp.

- Teeth extracted from children with any significant medical history which might give rise to periodontitis or affect the health of the periodontal membrane (Heasman and Waterhouse 2005).

- Root(s) of teeth with evidence of caries, resorption or abnormal morphology.

- Traumatic extractions where root(s) had been fractured during extraction.

- Teeth that had less than half root formation (incomplete root formation).

- Teeth that were not collected immediately following extraction.

2.2.1.4. Tooth collection

After induction of the general anaesthesia and maintenance of the anaesthetic status, simple forceps extractions were performed by the staff and postgraduate students from the Department of Paediatric Dentistry, Leeds Dental Institute. Immediately following extraction, the teeth were collected in a clean, dry specimen pot. The teeth were left in the dry pot with no lid on according to the extra-oral dry time allocated (i.e. <5 minutes, 30 minutes and 60 minutes post-extraction) before being placed in a specimen pot containing 10% formal saline as a fixative agent.

Information regarding each tooth was recorded on a data collection sheet (see Appendix B). Each tooth was given a tooth bank number in accordance with the Tissue Bank for purpose of tracing and audit as necessary.

2.2.2. Formalin-fixed paraffin-embedded (FFPE) section preparation

The histological preparation steps that were carried out in this study to produce a thin section of the tooth mounted on a glass slide are summarised as follows in order:

- Fixation

- Decalcification

- Cassetting

- Dehydration

- Paraffin wax embedding

- Microtomy

- Staining procedure   – H & E stain for serial sections

                                          - Immunoperoxidase staining

2.2.2.1. Fixation

Following the extra-oral dry time allocated accordingly, the teeth were immersed in 10% formal saline for at least 24 hours at room temperature.

2.2.2.2. Decalcification

To facilitate easier sectioning, decalcification was carried out. The teeth were immersed in specimen pots containing 10% formic acid for the decalcification process. The 10% formic acid solution was changed every week. The end-point of decalcification was determined by radiographies (Figure 2.7). Following complete decalcification, the teeth were removed from the formic acid solution and immersed in a pH 7.4 phosphate buffer saline (PBS) solution (Sigma P3813).
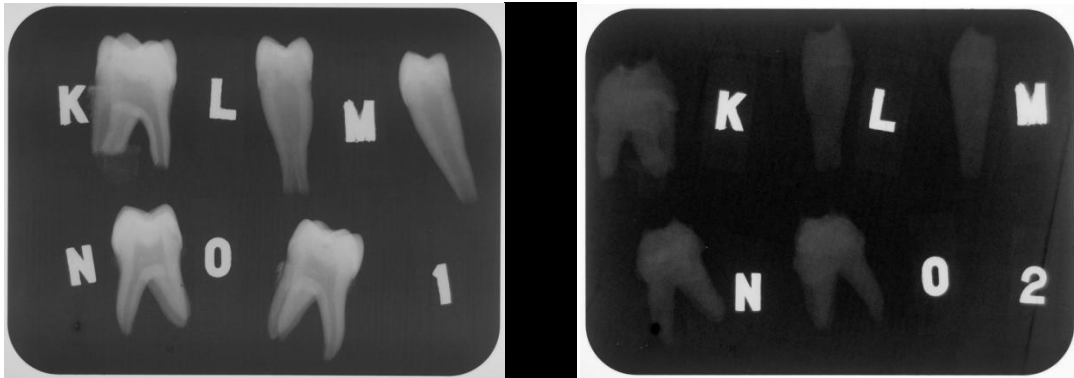
Figure 2.7 Series of radiographs of some teeth sample. Left: initial stage before decalcification; right: two weeks after decalcification.

2.2.2.3. Cassetting

Single-rooted teeth were cut into two halves with a sharp scalpel mesio-distally in a longitudinal fashion. The upper first permanent molar teeth were cut mesio-distally such that the palatal root was separated from the buccal roots. For purpose of uniformity, only the palatal roots of the upper molars were used in this study. In the case of lower first permanent molars, the teeth were separated mesio-distally to produce buccal and lingual halves. Each tooth segment was then placed in a small cassette secured with a metal lid and labelled appropriately.

2.2.2.4. Dehydration

The samples were dehydrated in an ascending series of alcohol concentrations (from 30% to 100% ethanol concentration) followed by xylene and finally wax to allow wax penetration into the tissues in preparation for paraffin-embedding. The cassettes were arranged in a basket kit and transferred to an automated tissue processor (Thermo Scientific Excelsior ES) (Figure 2.8) for dehydration. The tissue processor was programmed on an overnight cycle.

Figure 2.8 Automated tissue processor machine for dehydrating teeth samples, and basket kit in dehydration chamber of machine.

2.2.2.5. Paraffin wax embedding

Following the dehydration cycle, the holder containing all the cassettes was transferred to the embedding workstation (Figure 2.9). The teeth samples were carefully removed from the cassettes and placed into small metal trays. These trays were then filled with molten paraffin wax until the teeth samples were just immersed completely in the wax. The surface of the sample and wax was then covered with the appropriate corresponding labelled cassette. The mould (containing the tooth sample and molten wax) was placed on a cooling plate to allow solidification of the wax for at least half a day. Once the wax had completely set, the metal tray was removed to reveal a wax block (Figure 2.10). The wax blocks were stored in a cool, dry place until they were sectioned.
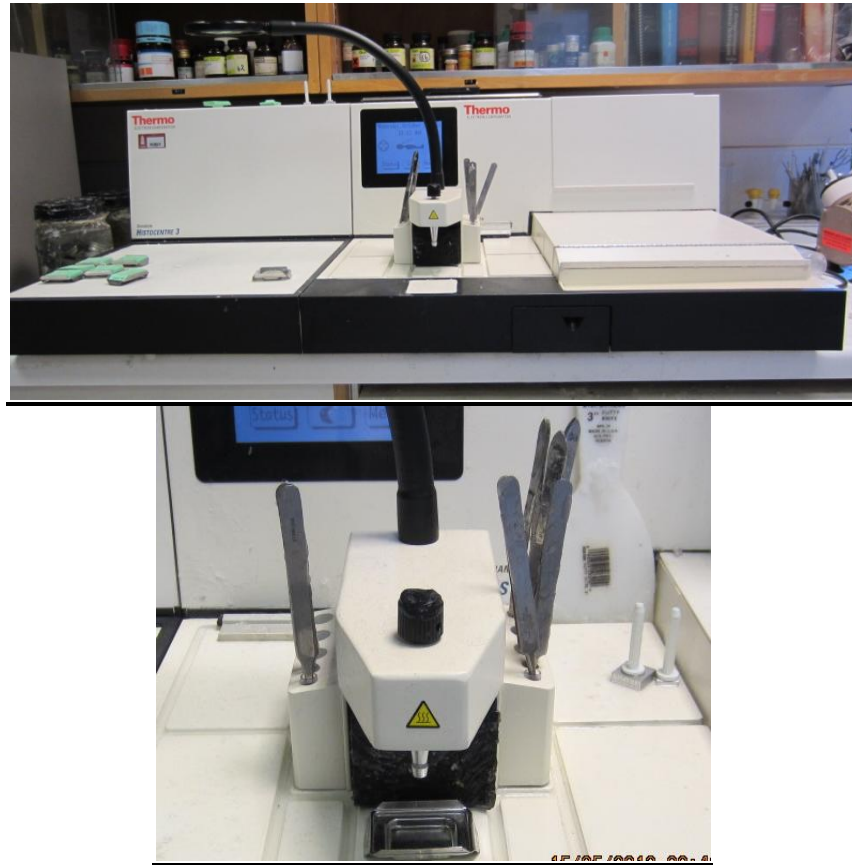
Figure 2.9 Paraffin wax embedding workstation.
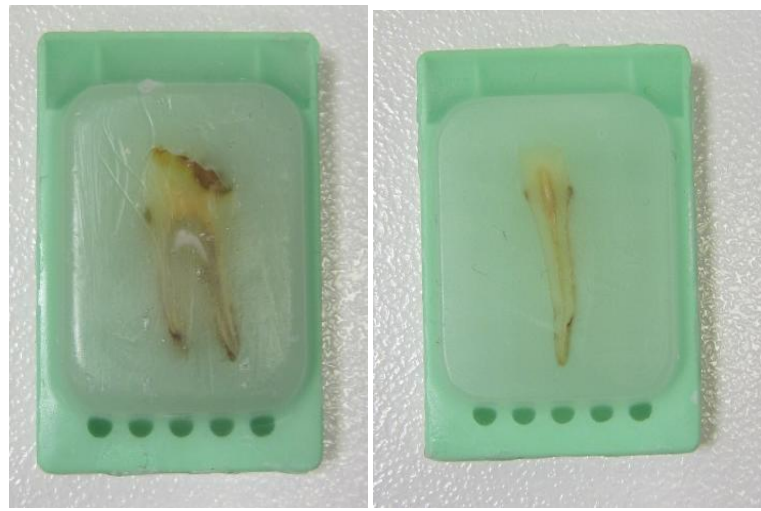
Figure 2.10 Paraffin wax blocks of samples.

2.2.2.6. Microtomy and incubation

The wax block was mounted on a microtome (Figure 2.11) and tooth sections of 5-7µm

thickness were sliced. The sections were then floated on the surface of 40ºC water bath

and "picked up" on glass slides which were labelled accordingly. The slides were left in

an upright position to drain excess water before being incubated at 37ºC.
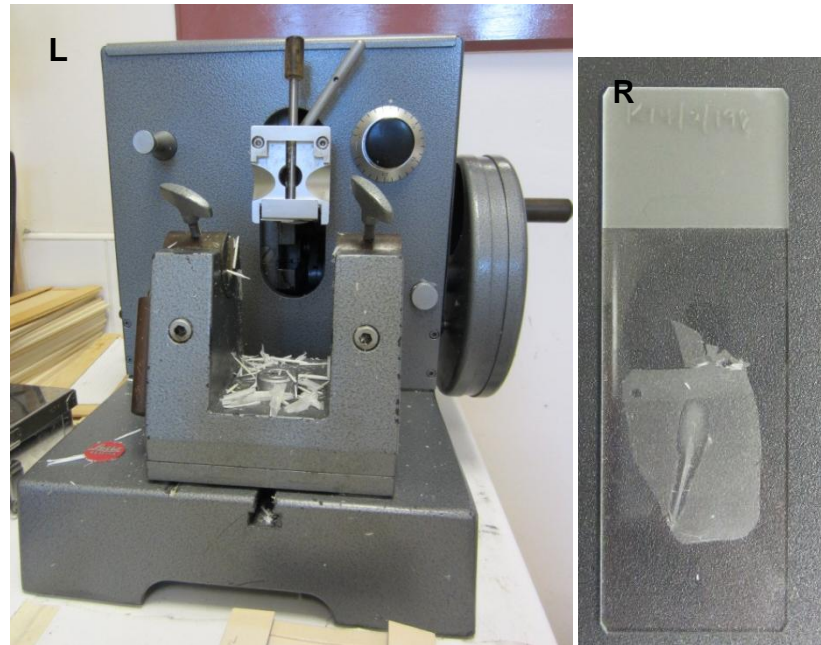
Figure 2.11 Rotary microtome (L) and mounted tooth section on glass slide (R).

2.2.3. Serial sectioning and H&E staining

To ensure some periodontal tissue was present on the root surface, serial sectioning was

carried out and every 20th section was stained with haematoxylin and eosin (H&E).

These were then viewed under light microscopy to examine the range of sections which

would be suitable for immunostaining. The procedure for H&E staining is summarised

in Figure 2.12.

Tooth section picked up on a poly-L-lysine coated slide and left to dry on a heated 70ºC hot plate

Slides transferred to an incubator for overnight incubation at 37ºC

Slides prepared for H&E staining as follow:

- Arrange slides in staining rack

- Immerse slides in xylene for 5 minutes, 2 changes of absolute alcohol for 5 minutes and tap water wash

- Stain with Harris' haematoxylin solution for 3 minutes then tap water wash

- 1% acid alcohol in 3 quick dips then tap water wash

- Scott's tap water up to 2 minutes then tap water wash

- Counterstain with 1% aqueous eosin for 3 minutes then tap water wash

- Dehydrate in 2 changes absolute alcohol for 5 minutes each

- Clear in xylene for 5 minutes

- Mount cover slip on specimen using DPX as mounting medium

Allow slides to lie flat and dry for at least 24 hours before viewing microscopically

Figure 2.12 Flowchart summarising the steps from microtomy to staining using H&E.

Figure 2.13 illustrates an example of the range of sections (80th to 120th) from a tooth specimen which was deemed suitable for immunohistochemistry following serial sectioning and H&E staining.

Proximal view of lower molar

Sections 80-120 from cut surface

Section 1 from cut surface

Tooth cut into half
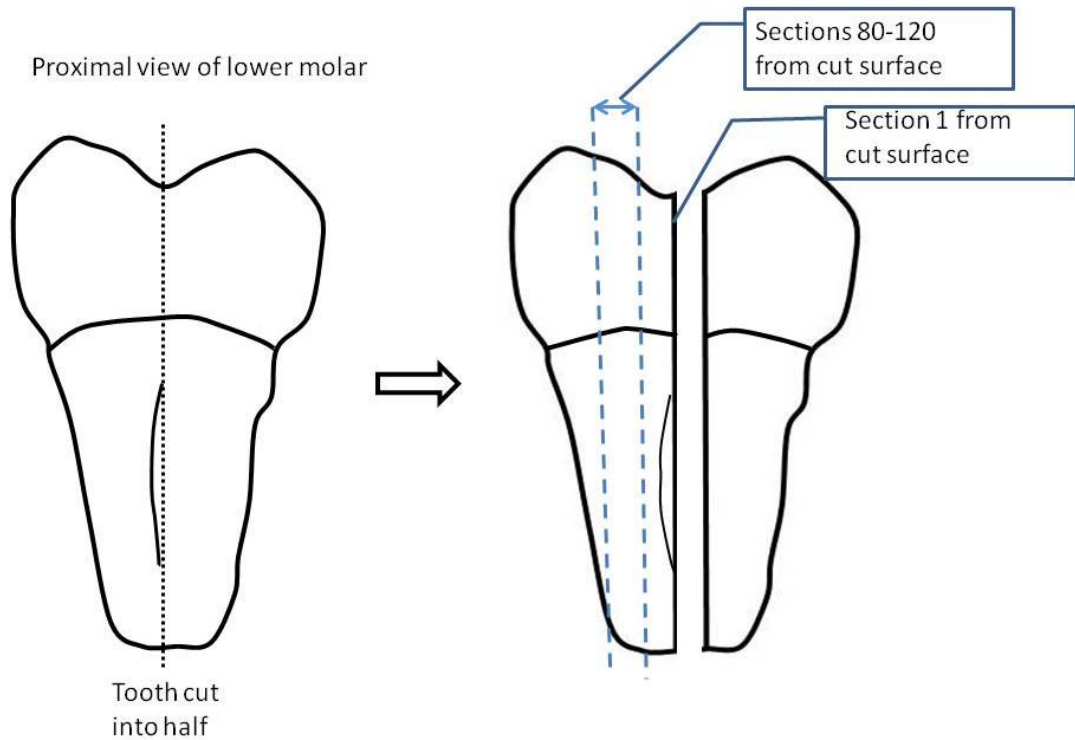
Figure 2.13 Illustration showing how the range of sections were determined for immunohistochemistry in a lower first permanent molar as viewed from the proximal surface. Every slice of section measured 5µm thick.

Once the range of sections to be obtained from each tooth wax block had been determined, preparation of slides for immunohistochemistry was carried out. For each tooth specimen, three consecutive sections mounted on individual slides were obtained to perform OPG staining, RANKL staining and a joint negative control. Small differences in sample slide preparation exist between those for immunohistochemistry and for H&E staining. The tooth sections for immunohistochemistry were mounted on 3-aminopropyltriethoxysilane (APTES) coated microscope slides and not on poly-L-lysine ones. The slides were placed upright to drain excess water at room temperature instead of on a hot plate to minimise protein degradation through heat. The slides were then incubated overnight at 37ºC. Following that, these slides were stored in a cold room until immunohistochemistry was performed to preserve the antigens.

2.2.4. Immunohistochemistry

2.2.4.1. Deparaffinisation and blocking of endogenous peroxidase

Slides were arranged in a staining rack and immersed in 2 changes of xylene for 20 minutes each. Subsequently, the slides were immersed in 2 changes of absolute alcohol for 5 minutes each and finally in tap water. These steps serve to remove the paraffin wax from the sections. The slide sections were then immersed in 2% hydrogen peroxide/methanol solution for 20 minutes to block any endogenous peroxidase.

2.2.4.2. Immunostaining workstation

The staining rack of slides was then transferred into a washbox filled with PBS pH 7.4 solution and placed on a magnetic stirrer for a minimum of 5 minutes. Coverplates were then attached to each slide carefully and loaded into the Shandon Sequenza Immunostaining Workstation (Figure 2.14). The coverplate wells were filled with PBS pH 7.4 solution and allowed to drain for 5 minutes. Normal goat serum diluted 1/5 was applied to each slide. The primary antibodies OPG and RANKL were applied to the appropriate slides as follows:

- Human RANKL raised in mouse (Abcam Ltd Ab45039) 1:150 dilution
- Human OPG raised in rabbit (Abcam Ltd Ab73400) 1:500 dilution

The slides were then left in the immunostaining workstation overnight at room temperature.

Figure 2.14 Shandon Sequenza immunostaining workstation.

2.2.4.3. Developing slides with HRP-labelled secondary antibody

Following the overnight treatment, the coverplate wells were filled with PBS pH 7.4 solution and left to drain for a minimum of 5 minutes. Two drops of horseradish peroxidase-labelled polymer solution (Dako Envision®+ Dual Link System-HRP (DAB+) kit K4065) were applied to each slide and left to drain for 30 minutes. The coverplate wells were filled with PBS pH 7.4 solution and left to drain for a minimum of 5 minutes. The slides were removed from the immunostaining workstation and placed on a flat bed (Figure 2.15). The tissues on each slide was covered with 3,3'-diaminobenzidine (DAB) substrate/chromogen solution (Dako Envision®+ Dual Link System-HRP (DAB+) kit K4065) for a maximum of 10 minutes to obtain the correct staining intensity. The slides were then placed into a staining rack immersed in PBS pH 7.4 solution to halt any further staining reaction.
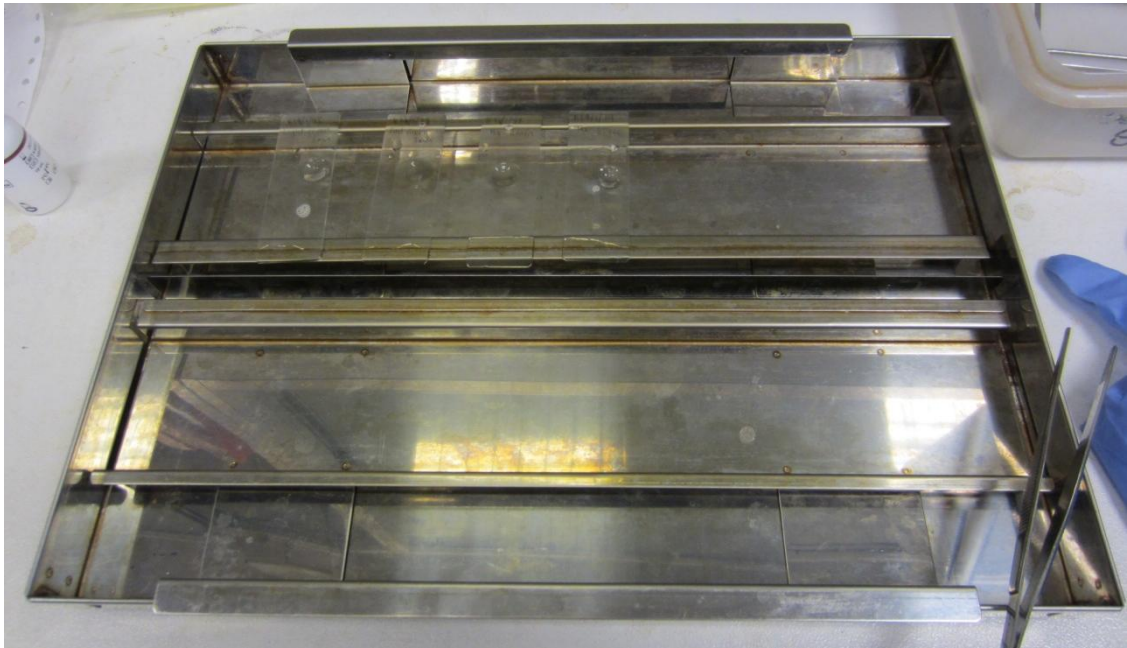
Figure 2.15 Slides placed flat in a humid chamber for secondary antibody development.

2.2.4.4. Controls

For each tooth sample, a joint negative control was carried out by subjecting the sample to the same immunostaining procedures except that the primary antibodies were omitted. In each immunostaining batch, a positive control section per primary antibody was used to ensure that the antibodies and solutions were working correctly during each immunostaining run. Positive control sections were obtained from a freshly extracted healthy premolar tooth which was tested initially during the pilot work and consistently showed positive immunostaining in the periodontal tissue for OPG and RANKL.

2.2.4.5. Counterstaining and mounting

Following secondary antibody labelling, the slides were then given a tap water wash. Counterstaining was carried out with Harris' Haematoxylin (Sugipath 01560BBE) for 30 seconds to 2 minutes depending on the intensity desired. Tap water wash was then carried out followed by immersion in Scott's tap water for 1 minute. The slides were then given a tap water wash until clear. Two changes of absolute alcohol for 2 minutes

each and then a further two changes of xylene for 2 minutes each were performed. The specimens were then covered with coverslips using DPX as the mounting medium and left to dry on a flat surface for 24 hours before viewing under light microscopy.

2.2.5. Microscopy and image field selection

The root length was divided into 3 equal regions to represent cervical, middle and apical portions of the root as illustrated in Figure 2.16.
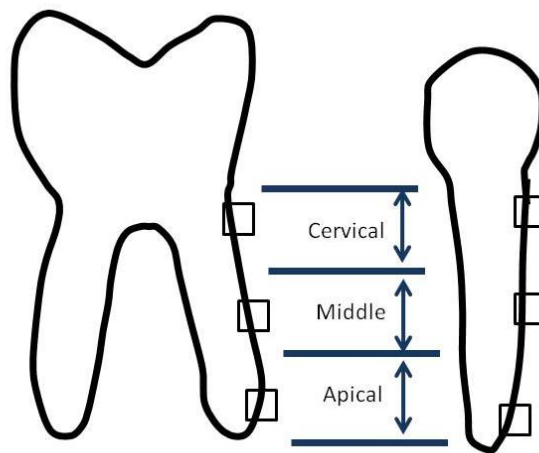
Figure 2.16 Illustration of the divided areas along the root surface. Square boxes represent the areas in which images were captured for analysis.

The distribution of the antigens OPG and RANKL was examined with light microscopy under objectives x10, x20 and x40. Images representative of each region from every tooth sample were obtained using a light microscopy (Olympus BX50) attached to a digital camera (Nikon DS-Fi1) (Figure 2.17). Images were obtained from either of the proximal surfaces in the case of palatal roots and single-rooted teeth sections. For the sections produced from lower molar halves, images were taken from the proximal regions excluding the furcation surfaces of the roots (i.e. most lateral surfaces of the tooth sections).
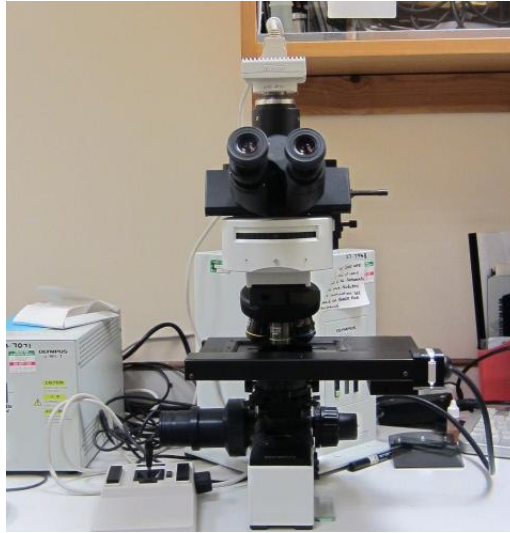
Figure 2.17 Light microscope attached to a digital camera used for capturing images.

2.2.6. Image analysis

2.2.6.1. Descriptive analysis

Each image was analysed for identification of any stained structures/cells within the periodontal membrane along the root surface. The general pattern of staining distribution and intensity observed was examined under low power objectives x10 and x20 light microscopy. Trends between OPG and RANKL across the different extra-oral dry times were also examined.

2.2.6.2. Semi-quantitative analysis

The images were analysed using NIS Elements BR 3.0 software programme. Scoring was done according to the intensity of staining (Chuang *et al.* 2009) as shown in Table 2.2. Due to the uneven presence of periodontal tissue on the root surface, only areas with a periodontal tissue thickness of 100µm from the surface of the cementum were scored. If one proximal surface of a section was unclear or could not be used for any other reason, the other proximal surface was then analysed instead. Training for scoring was undertaken with one of the supervisors (XY) and repeated until agreement was

reached with consistency. Scoring of all specimens was then performed by the investigator (KST).

Table 2.2 Semi-quantitative scale used to assess staining intensity.

Score	Description
0	No staining
1	Light yellow (weak staining)
2	Brown (moderate staining)
3	Dark brown (strong staining)

2.2.6.3. Intra-examiner reliability

Intra-examiner reliability for scoring the staining intensity was carried out using 20% of the sample (6 teeth). The repeated scores were carried out at 1 month after the first scores were recorded. The examiner was blinded to the first set of scores while recording the second set of scores. Following this, a Bland-Altman plot was constructed to assess the intra-examiner agreement.

2.2.7. Data processing

2.2.7.1. Data presentation

The staining intensity for each tooth sample at each region was recorded on a raw data collection sheet (Appendix B). The median score for each extra-oral dry time group at each distribution region were obtained in the form of tables produced using Microsoft Excel 2007 (Microsoft Corporation, USA).

2.2.7.2. Data screening

As the expressions of OPG and RANKL were assessed in the ordinal data form, non-parametric statistical tests were used.

2.2.7.3. Statistical analysis of main results

Statistical analysis was performed using a software package Statistical Package for Social Sciences 19 (IBM Corporation, USA). The Kruskal-Wallis test was used to test for statistical significant differences between different dry time groups according to each location followed by a post-hoc analysis to identify which specific group differed from another if any statistical significance was detected in the Kruskal-Wallis test. The Friedman test for dependent variables was used to compare across the different locations on the root surface and between OPG and RANKL expressions according to each dry time group. To compare between OPG and RANKL expressions within the same sample at a given dry time and location, the sign test was performed.

2.3. Preliminary work for confocal microscopy

Preliminary work was carried out to determine a protocol for future 3-dimensional reconstruction using confocal microscopy on formalin-fixed paraffin-embedded (FFPE) sections.

2.3.1. FFPE section preparation

For this preliminary work, two teeth from each <5 minutes and 60 minutes dry time group were used. The FFPE sections were previously prepared as described in section 2.2 except that the thickness of each section for this part of the study was 10μm. The sections were mounted on APTES slides and left to be incubated at 37ºC overnight.

2.3.2. Deparaffinisation and primary antibody application

Slides were arranged in a staining rack and immersed in 2 changes of xylene for 20 minutes each. Subsequently, the slides were immersed in 2 changes of absolute alcohol for 5 minutes each and finally in tap water. The staining rack of slides was then transferred into a washbox filled with PBS pH 7.4 solution. Coverplates were then attached to each slide carefully and loaded into the Shandon Sequenza Immunostaining Workstation (Figure 2.14). The coverplate wells were filled with PBS pH 7.4 solution and allowed to drain for 5 minutes.

Normal rabbit serum (X0902, Dako UK Ltd) diluted 1/5 was applied to each slide that would be stained for RANKL. Normal swine serum (X0901, Dako UK Ltd) diluted 1/5 was applied to each slide that would be stained for OPG. The primary antibodies OPG and RANKL were diluted with 1% bovine serum albumin (BSA) (A9647, Sigma-Aldrich UK) prepared by dissolving 0.1g of BSA crystals in 10ml of PBS. The primary antibodies were then applied to the appropriate slides as follows:

- Human RANKL raised in mouse (Abcam Ltd Ab45039) 1:10 dilution
- Human OPG raised in rabbit (Abcam Ltd Ab73400) 1:25 dilution

The slides were then left in the immunostaining workstation overnight at room temperature.

2.3.3. Developing slides with fluorescein-labelled secondary antibodies

Following the overnight treatment, the coverplate wells were filled with PBS pH 7.4 solution and left to drain for a minimum of 5 minutes. The secondary antibodies used were labelled with fluorescein isothiocyanate (FITC) and were applied to each slide accordingly and left to drain for 30 minutes. For RANKL-stained slides, the secondary

antibody used was FITC rabbit anti-mouse (F0232, Dako UK Ltd). For OPG-stained slides, the secondary antibody used was FITC swine anti-rabbit (F0205, Dako UK Ltd).

The coverplate wells were filled with PBS pH 7.4 solution and left to drain for a minimum of 5 minutes. This was repeated four times to ensure all the slides were adequately washed. The slides were then removed from the immunostaining workstation and arranged in a staining rack immersed in a washbox containing PBS pH 7.4 solution. The washbox containing the slides was placed on a magnetic stirrer and left to stir with agitation for 1.5 hours. After stirring was completed, the slides were removed from the washbox and placed in a humid chamber. Counter nuclei staining was applied using TO-PRO®-3 stain (T3605, Invitrogen Ltd Scotland) diluted 1/100 in PBS for 20 minutes and the humid chamber covered during this period. The slides were then placed into a staining rack immersed in PBS pH 7.4 solution for 10 minutes.

2.3.4. Mounting and confocal microscopy

The slides were mounted in a special mounting medium, which consisted of a mixture of PBS, glycerol and 1,4-diazabicyclo-octane (DABCO) solutions, and covered with glass slips as appropriate. These slides were placed on slide trays and wrapped in aluminium foil before being stored in a fridge at 4°C. Negative controls were carried out accordingly following the described protocol above except that the primary antibodies were omitted.

Viewing of the slides was carried out using a confocal microscope (Leica TCS SP2, Leica Microsystems Heidelberg) within three weeks upon completion of immunostaining. Images were captured with Leica Confocal Software (Leica Microsystems Heidelberg) programme.

3. RESULTS

3.1. Experimental material

3.1.1. Sample

A total of 30 teeth were collected following informed consent and ethical approval in accordance with the Leeds Dental Institute Tissue Bank (reference number: 261110/KT/49).

Two teeth were excluded due to poor tissue sectioning. The final sample for this study was 28 teeth. For semi-quantitative analysis, sections which did not have adequate periodontal tissue thickness (100µm) were excluded. For OPG analysis, three specimens were excluded whereas for RANKL analysis, one specimen was excluded. Therefore, a total of 25 teeth were analysed for OPG and 27 teeth for RANKL.

3.1.2. Tooth-related variables

The breakdown of the sample of 28 permanent teeth according to the tooth type is shown in figure 3.1. For this study, a total of 28 teeth were used, which consisted of 11 upper first permanent molars, 12 lower first permanent molars and 5 single-rooted premolars.
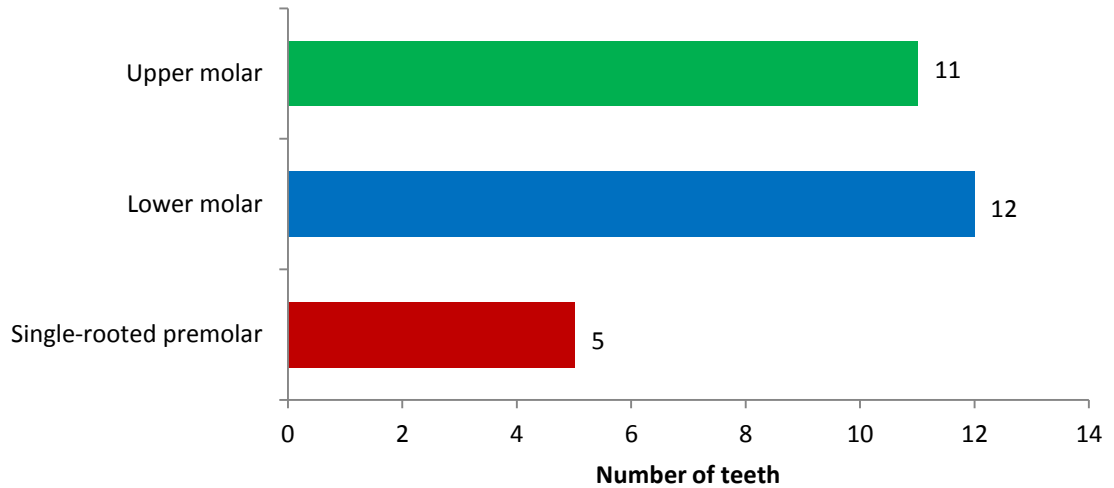
Figure 3.1 Distribution of sample according to tooth type.

The distribution of the sample according to the extra-alveolar dry times is shown in figure 3.2. Both the <5 and 30 minutes dry time groups had 10 teeth in each group while the 60 minutes group had 8 teeth.
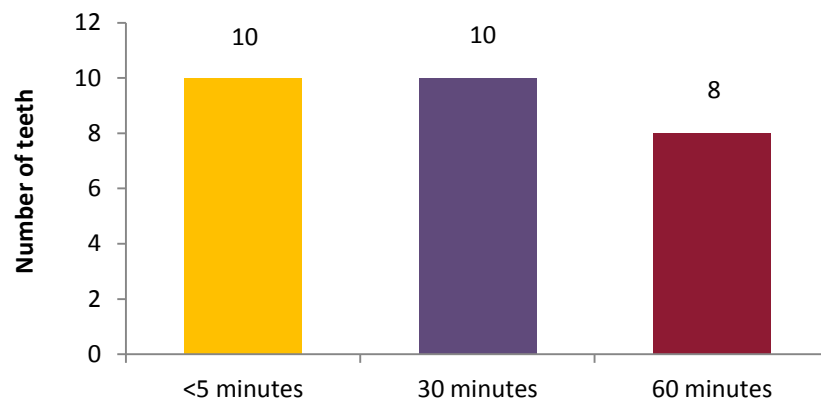
Figure 3.2 Number of teeth according to each dry time group used in this study.

3.2. Quality of immunostaining

The overall quality of staining was acceptable, with all controls confirming the specificity of the labels on the test sample sections. As mentioned in section 3.1.1, to ensure uniformity of immunostaining analysis, sections which had a lack of periodontal tissue thickness at any one or more regions of interest along the root surface were excluded completely from the analysis.

3.3. Assessment of intra-examiner reliability

Semi-quantitative scoring of the stains using a scale of 0 to 3 for both OPG and RANKL was repeated on 6 teeth by the same examiner (KST). These 6 teeth were chosen as a convenient sample. The Bland-Altman plot was based on the two sets of readings and presented in Figure 3.3. The mean difference between the measurements was 0.08 with a standard deviation (SD) of 0.50. The confidence interval range was from -0.90 to 1.06. Acceptable agreement was found, with a majority of the measurements falling within 95% limits of agreement. The complete first set of readings was used for the results in this study.
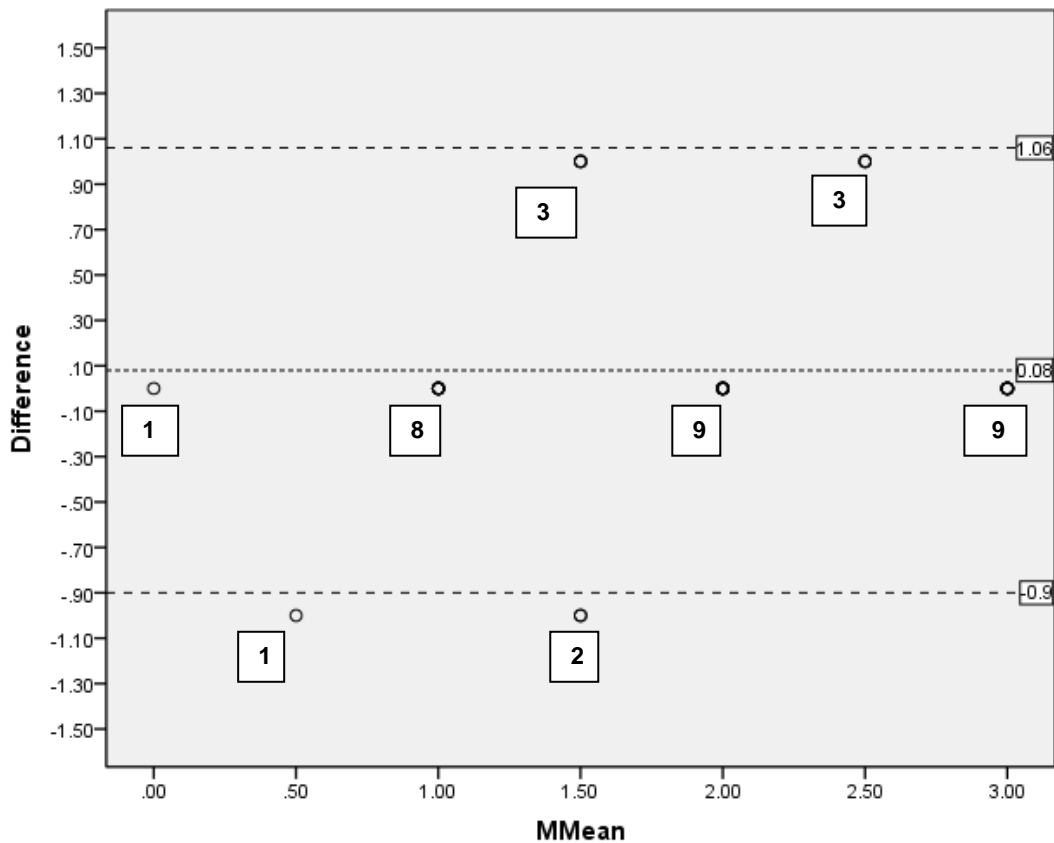
Figure 3.3 Bland-Altman plot representing the intra-examiner agreement for grading of staining intensity. The upper and lower dashed lines represent the 95% limits of agreement while the centre dotted line represents the mean of the difference. The bold numbers at each corresponding dot represent the number of points with the same coordinates.

3.4. Qualitative observations

3.4.1. Histology and H&E serial sections

Histological sections using H&E stain revealed the presence of continuous periodontal ligament tissue along the proximal root surfaces for all tooth specimens. It was found that there was sufficient amount of periodontal ligament present on the root surfaces in the 80th to 120th sections (i.e. 400-600µm depth from the cut surface of specimen).

Comparing teeth with minimal caries and non-carious teeth, there was no histological difference in the periodontal ligament. Normal periodontium with typical vascularisation, fibroblasts and cementoblasts lining the root surfaces were observed between carious and non-carious teeth (Figure 3.4). For the carious teeth, inflammatory cells such as lymphocytes were confined within the carious cavity in the coronal part of the tooth. The pulps of these teeth revealed almost normal pulp histology containing an odontoblastic layer adjacent to dentine, vessels and stromal cells (Figure 3.5)
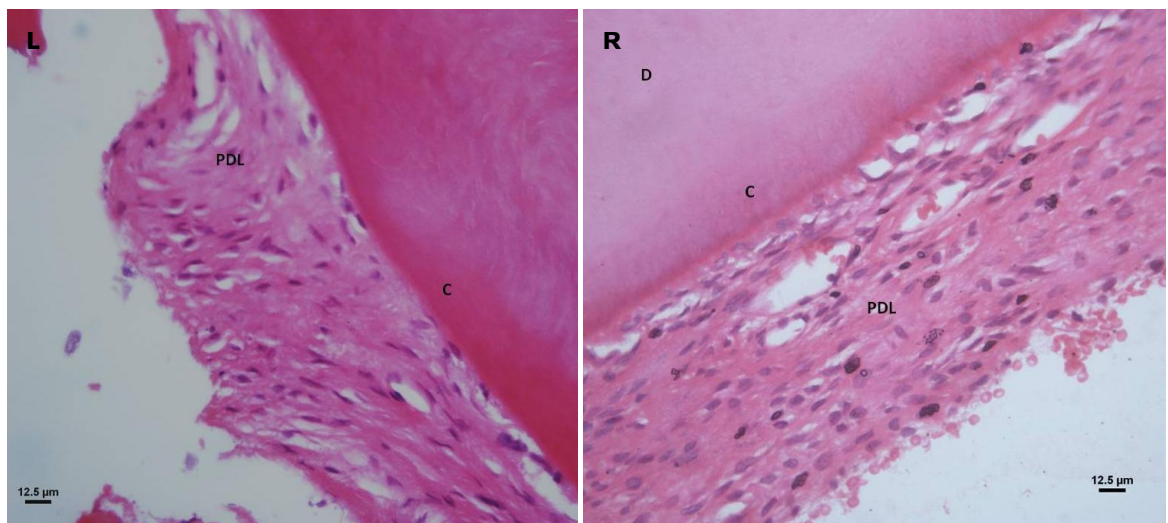
*Figure 3.4 Sections showing normal periodontium along the apical half of the root surface in minimally carious (left) and non-carious teeth (right) (**D** = dentine, **C** = cementum, **PDL** = periodontal ligament). The left picture was taken from a tooth sample in the 60 minutes dry time group while the right picture was taken from another tooth in the <5 minutes dry time group.*
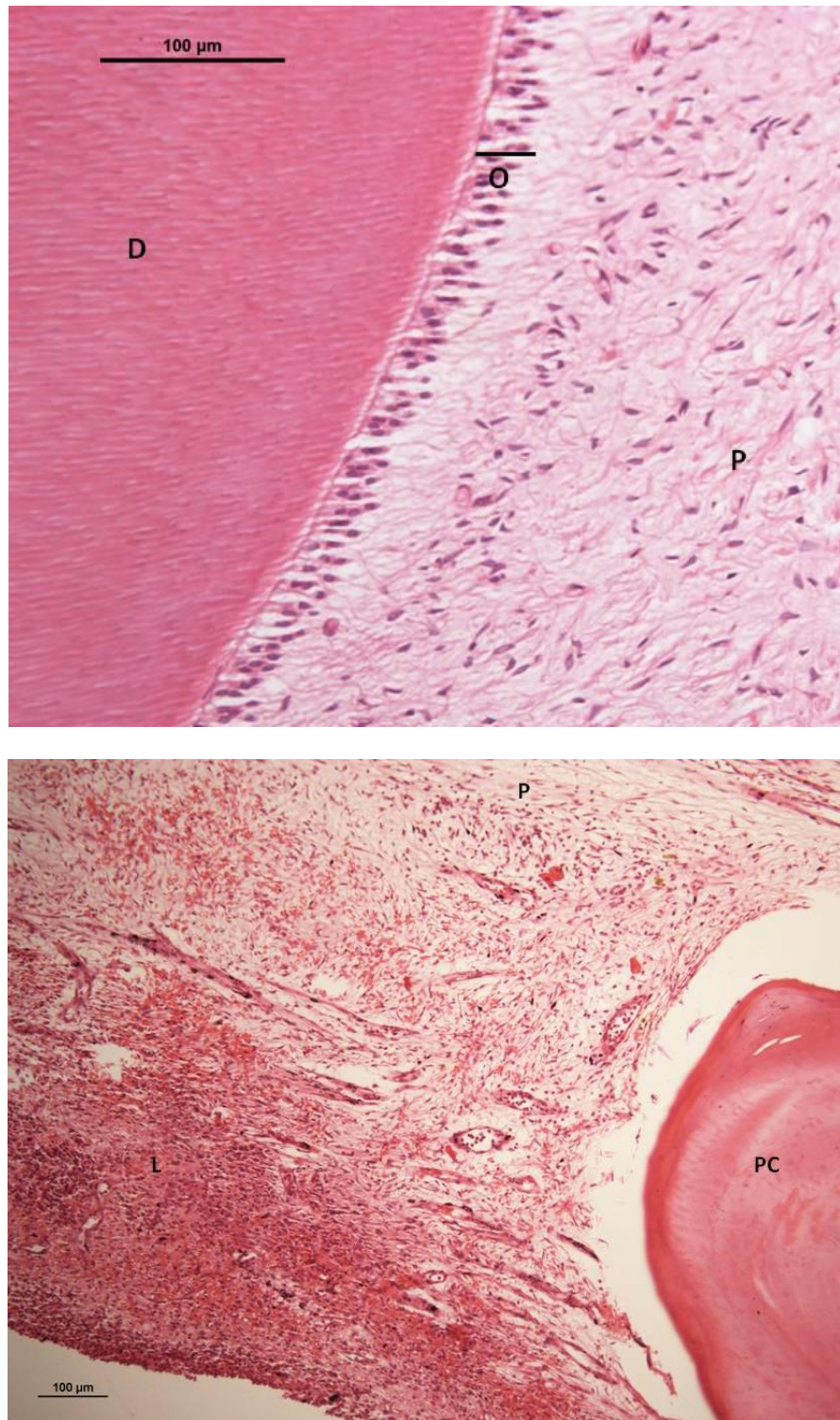
*Figure 3.5 Sections from coronal portion of pulp in teeth from <5 minutes dry time group. Top picture taken from a non-carious tooth shows a healthy pulp consisting of an odontoblast layer adjacent to dentine and pulp fibroblasts. The bottom picture shows lymphocytic infiltration in the pulp and part of a pulp calcification arising following insult to the pulp in a carious tooth (**D** = dentine, **O** = odontoblast later, **P** = pulp, **L** = lymphocytes, **PC** = pulp calcification).*

3.4.2. OPG immunohistochemistry

For all the specimens, there was consistently positive OPG staining of various intensities within the periodontal ligament. Immunoreactive cells such as the cementoblasts and PDL fibroblasts showed cytoplasmic staining. The extracellular matrix component of the periodontal ligament also exhibited positive immunostains in some of the samples. Moderate to heavy stains for OPG along the root surface were observed in cementoblasts, fibroblasts and epithelial cell rests as well as along the fibres of the periodontal ligament in majority of the samples at the cervical and middle regions in 22 of 25 specimens (Figure 3.6). Weak to moderate OPG stain was observed more commonly at the apical region in 18 of the 25 specimens, with most of the stains only evident within the cementoblast layer (Figure 3.7).

Within the pulp, the non-carious specimens had positive OPG immunoreactivity only within the odontoblastic layer (Figure 3.8). However, in the presence of inflammation within the coronal pulp, there was positive OPG immunoreactivity in the pulp chamber fibroblasts as well (Figure 3.9).
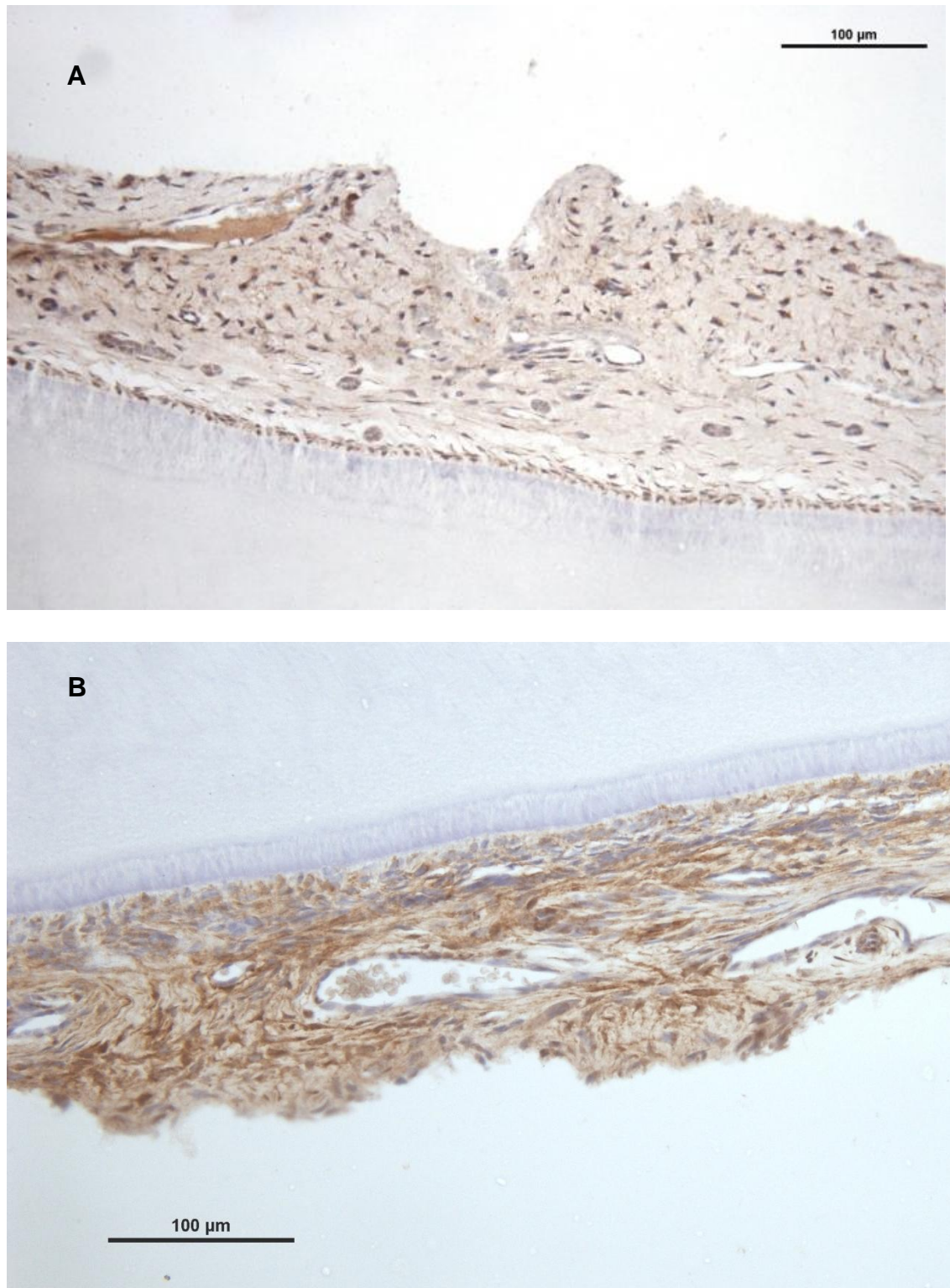
Figure 3.6 Moderate (top picture A) to heavy (bottom picture B) stains for osteoprotegerin along the root surface were observed. Positive osteoprotegerin stains were evident in cementoblasts, periodontal ligament fibroblasts and the epithelial cell rests of Malassez. The top picture was taken at the cervical third of the root surface of a 30-minutes dry time group sample whereas the bottom picture represents the middle third of the root region in <5 minutes dry time group sample.
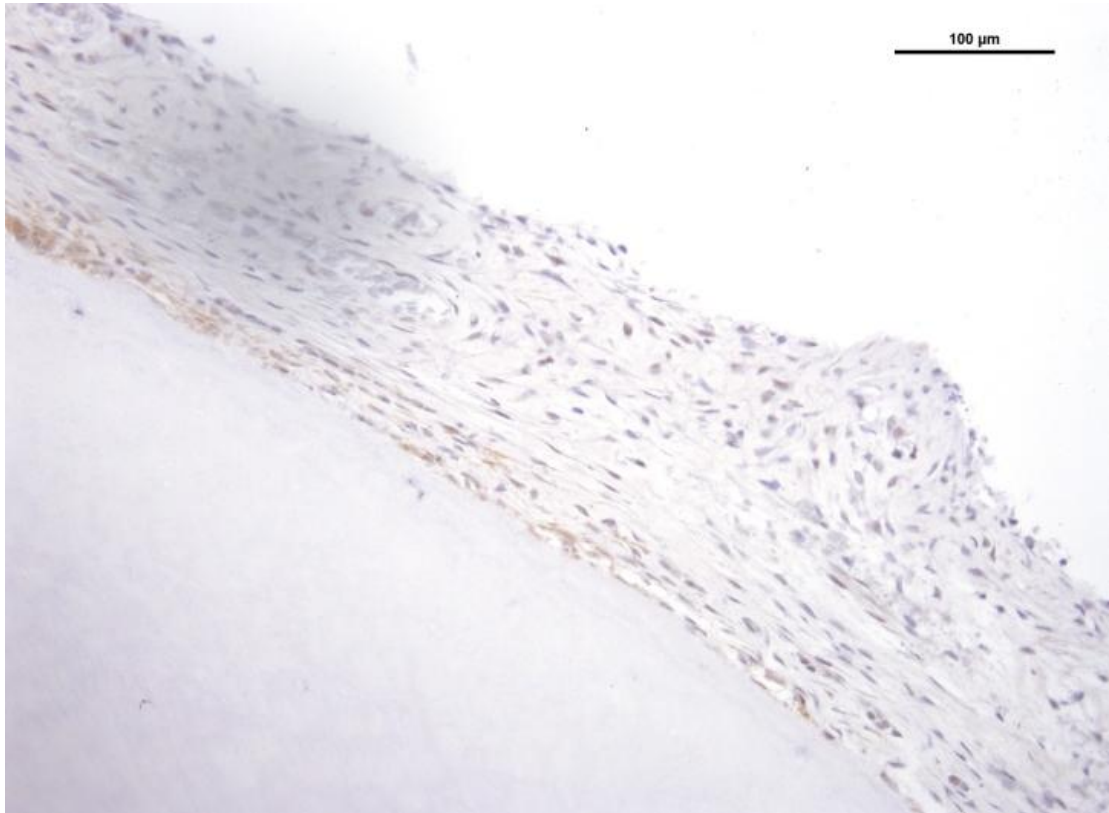
Figure 3.7 Positive OPG stains at the apical region located primarily within the cementoblast layer. Weaker immunoreactivity observed at the apical third of the root surface compared to the cervical or middle third regions (Figure 3.6A and 3.6B).

Figure 3.8 Positive osteoprotegerin noted within the odontoblast layer but not in the pulp fibroblasts region for non-carious specimens.
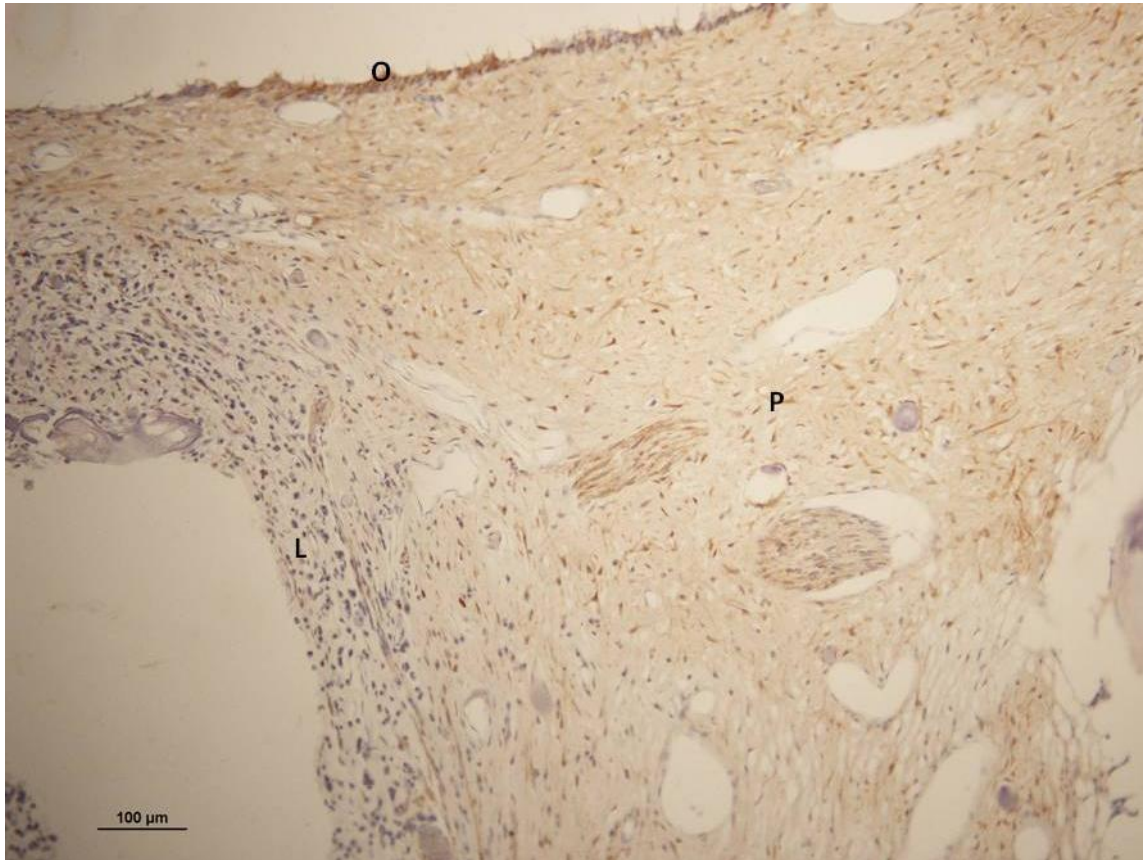
*Figure 3.9 In the presence of lymphocytic infiltration in the pulp, there was positive immunostaining for osteoprotegerin in the odontoblast layer as well as in pulp fibroblasts (**P**=pulp, **L**=lymphocytes, **O**=odontoblasts).*

Similar pattern of OPG staining intensity was observed in all three root surface regions for most of the specimens in all three different dry time groups (Figure 3.10 and 3.11).
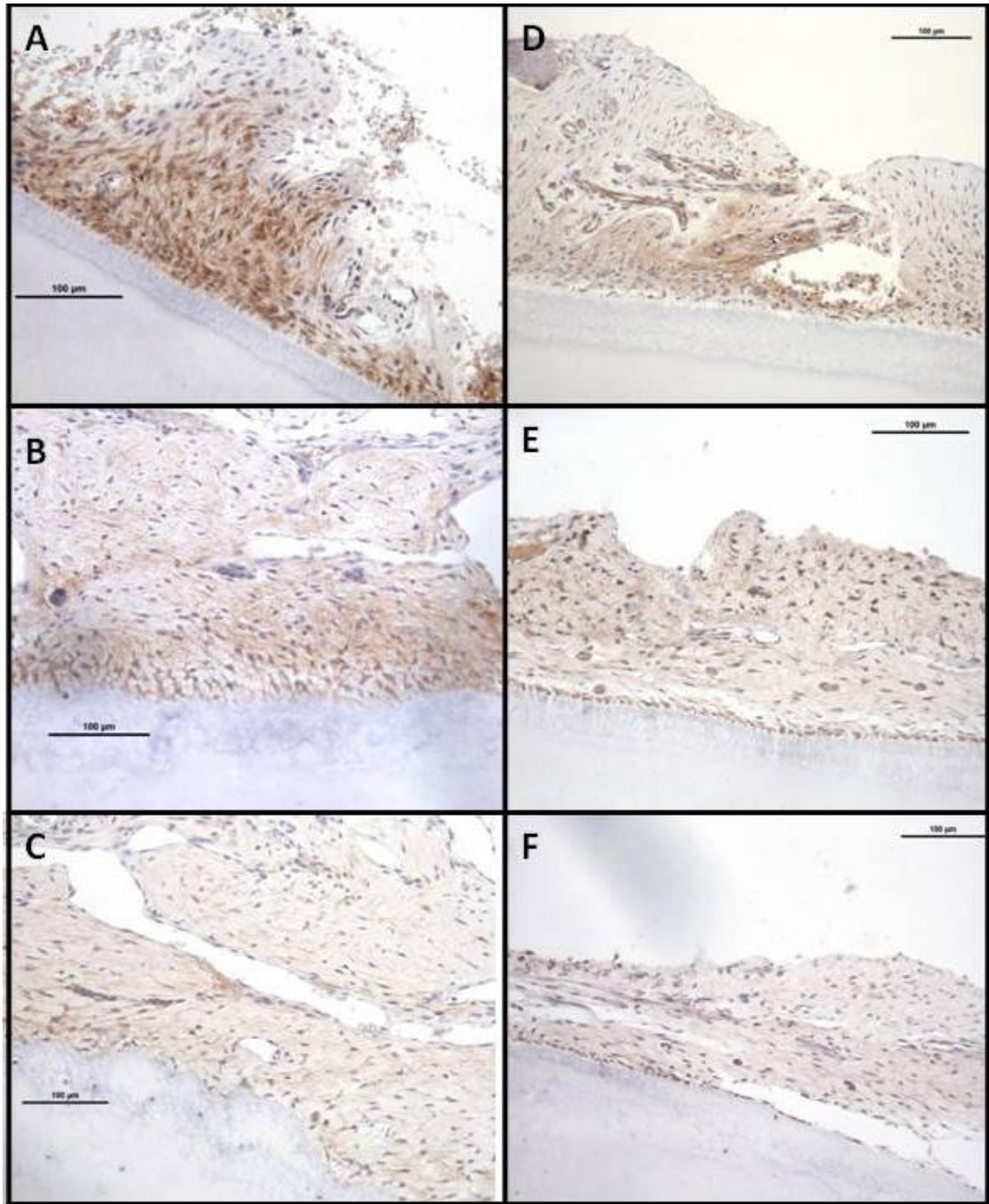
Figure 3.10 OPG staining across the length of the root surface at the cervical (A, D), middle (B, E), and apical (C, F) regions between two tooth specimens: one in the <5 minutes dry time group (A-C) and the other in the 30 minutes group (D-F). In the sample from same dry time group, the cervical region demonstrated the strongest staining.
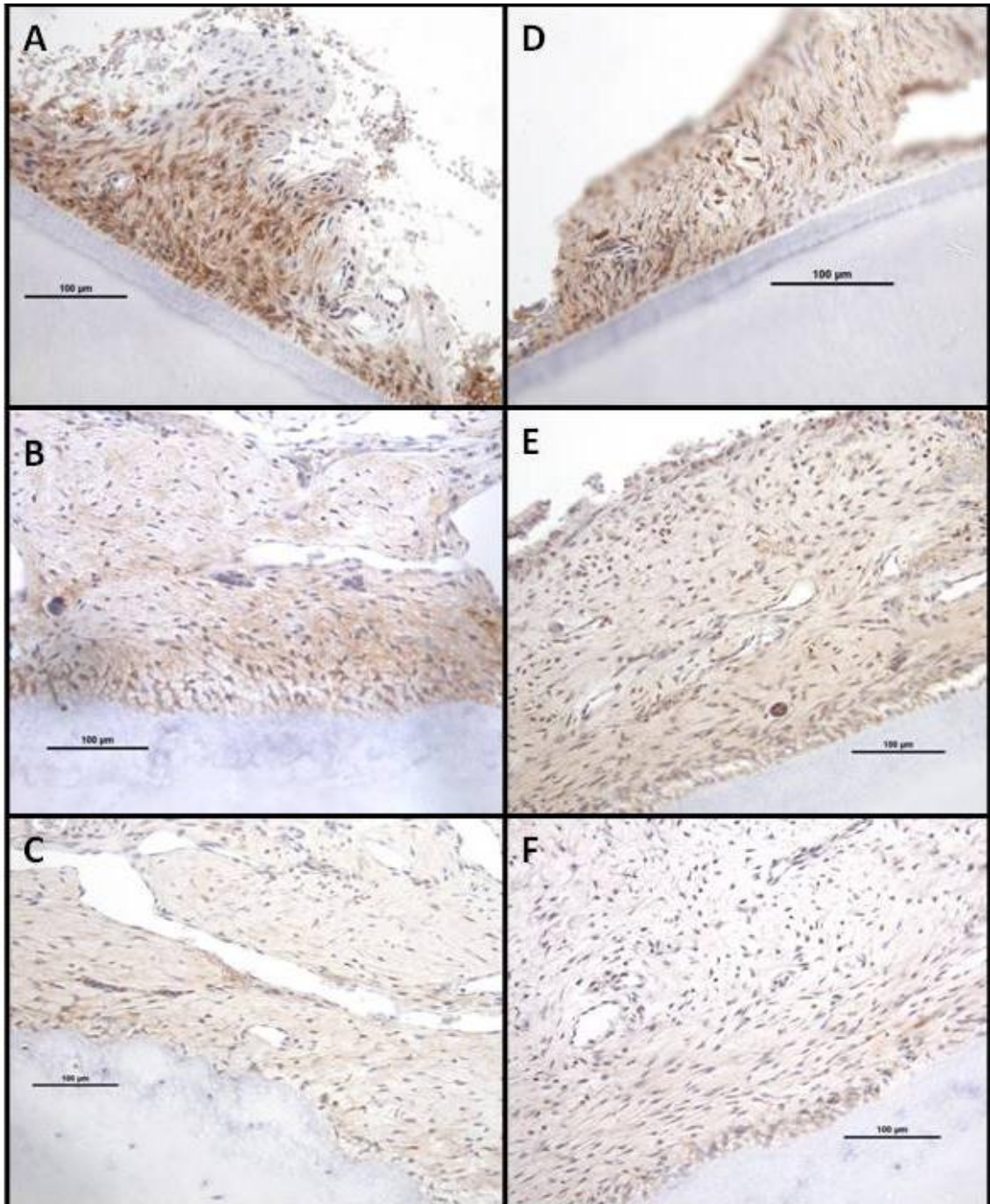
Figure 3.11 Specimen pictures comparing OPG immunostaining in the periodontal ligament along the root surface at the cervical (A, D), middle (B, E) and apical third (C, F) regions. The strongest staining was seen in the cervical regions. The apical regions (C,F) showed weaker immunostaining than the corresponding middle and cervical regions. (Pictures A-C = <5 minutes dry time; pictures D-F = 60 minutes dry time).

3.4.3. RANKL immunohistochemistry

Positive RANKL immunoreactivity within the periodontal ligament cells on the root surfaces was observed in some of the samples, with 5 out of the 27 samples showing no noticeable positive RANKL staining in two or more regions along the root surface. The cells that were consistently RANKL-positive on the root surface included cementoblasts and fibroblasts as well as the periodontal ligament fibres (Figure 3.12). Where there was positive immunoreactivity, the majority of the staining was observed within the extracellular matrix of the PDL. Cells which showed immunoreactivity were stained in the cytoplasmic region. Unlike for OPG staining, the epithelial cell rests showed negative staining for RANKL in majority of the specimens (Figure 3.13).
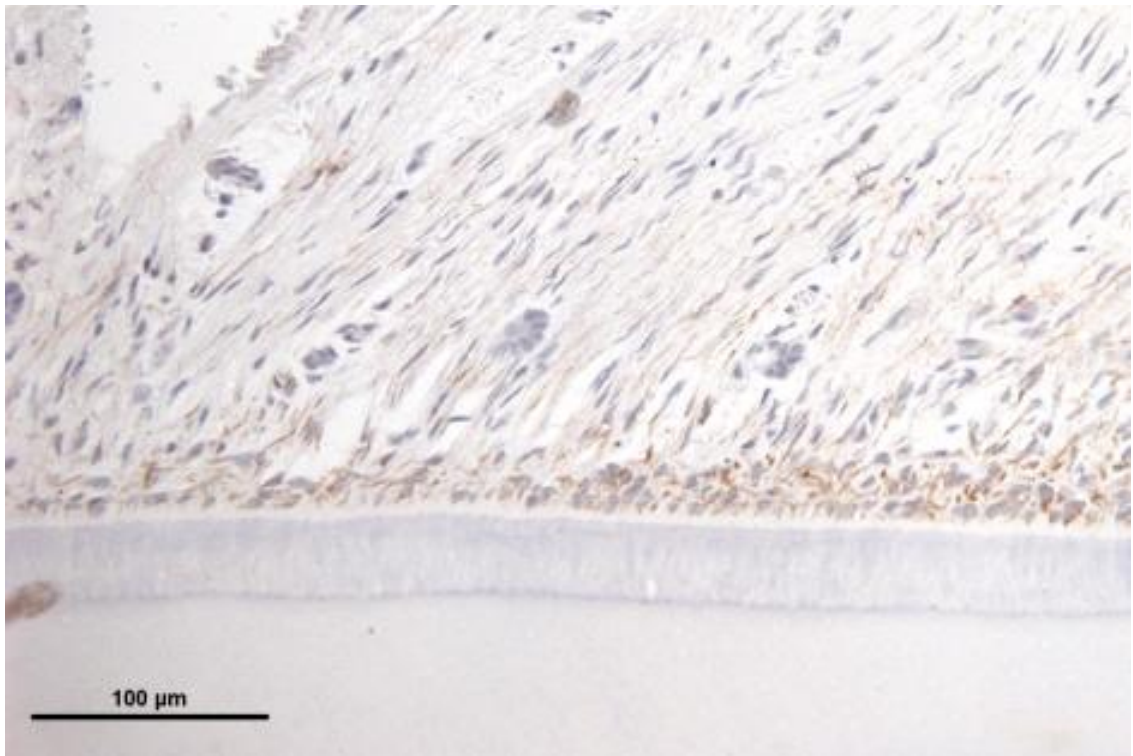
Figure 3.12 Positive RANKL immunostaining observed within the cementoblast layer and in periodontal ligament fibres.
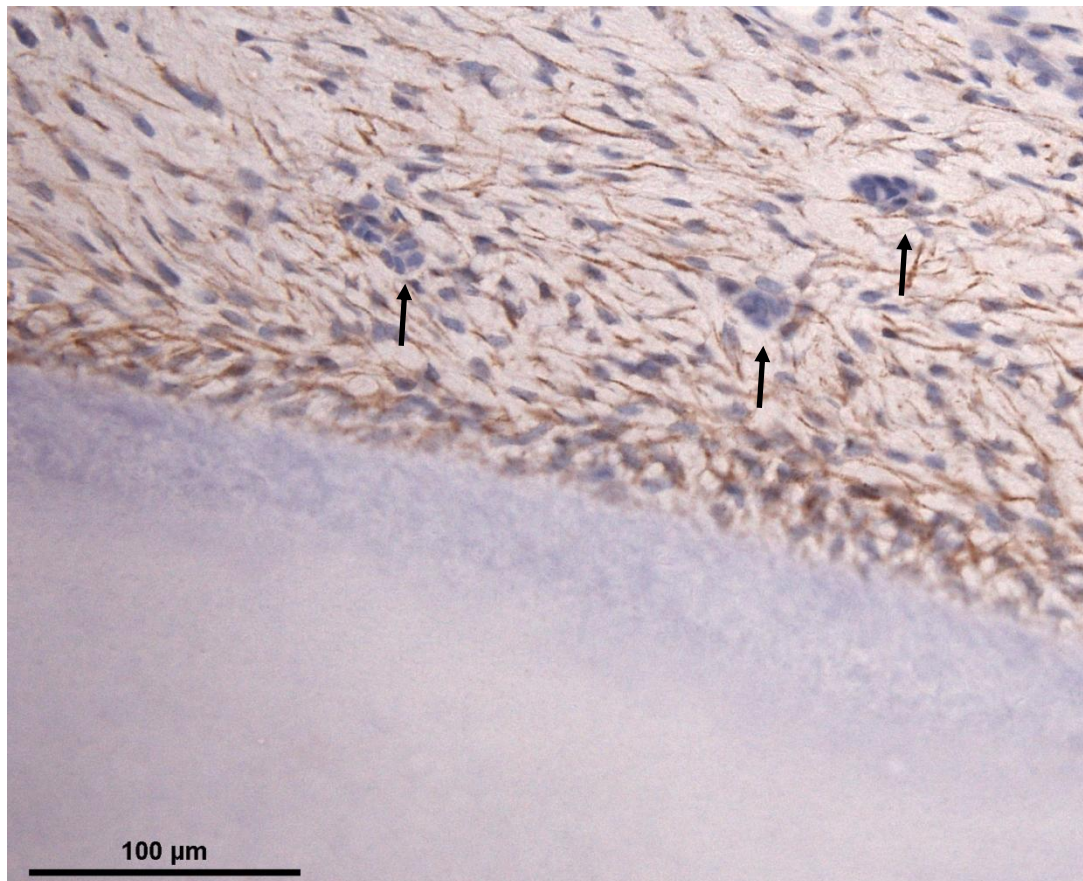
Figure 3.13 RANKL staining for epithelial cell rests and PDL. The epithelial cell rests (arrowed) in this <5 minutes dry time specimen showed negative RANKL stain. In contrast, the surrounding PDL fibres exhibited deep brown positive stain for RANKL.

All the specimens showed negative RANKL immunoreactivity in the pulpal region (Figure 3.14).
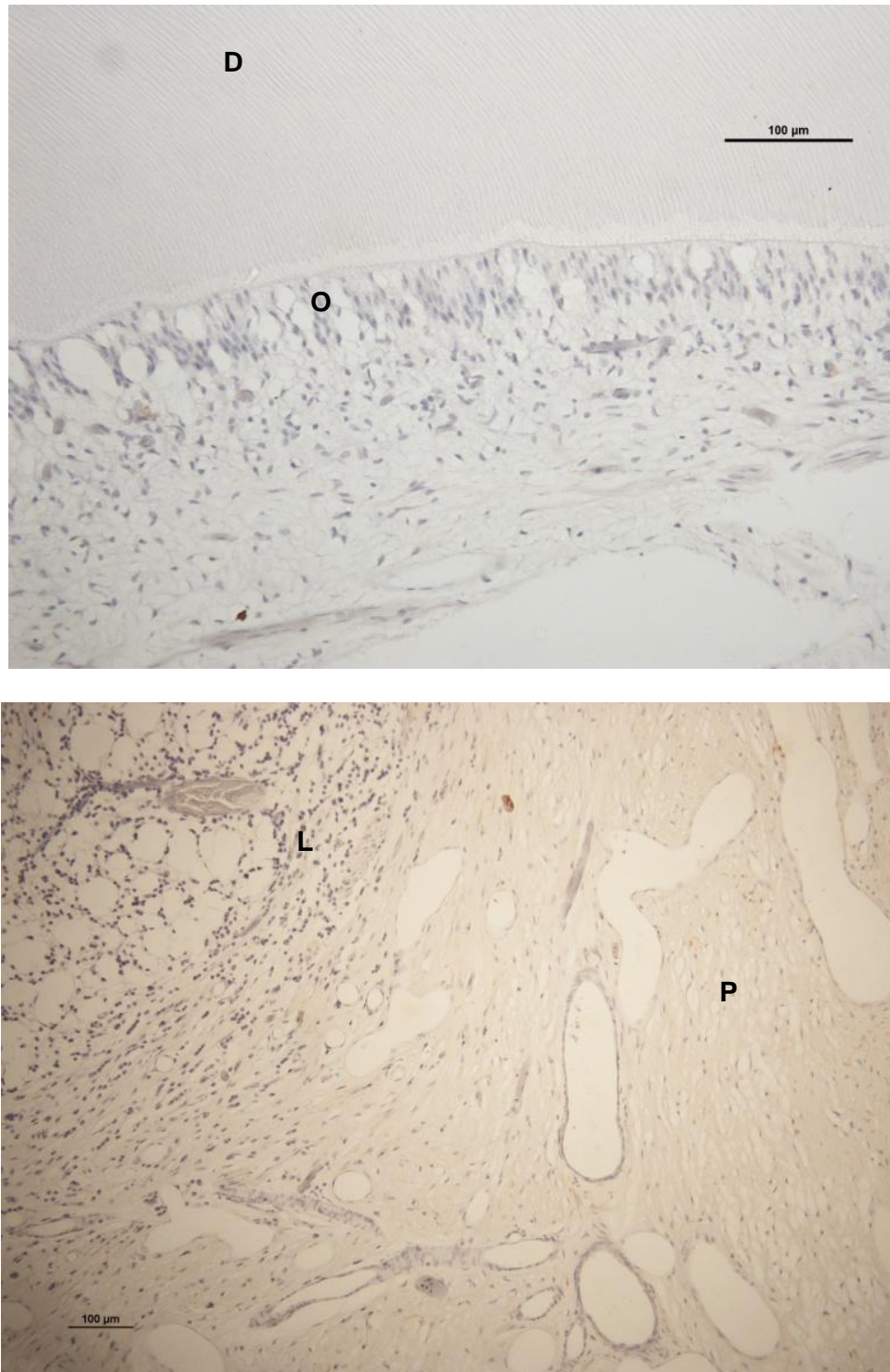
*Figure 3.14 RANKL staining within the pulp. Top picture: Odontoblast layer and pulp fibroblasts did not demonstrate any positive RANKL staining. Bottom picture: Carious specimen revealed that despite lymphocytic infiltration (**L**) in the pulp chamber, no RANKL immunoreactivity was observed within the pulp (**D**=dentine, **O**=odontoblasts, **P**=pulp).*

Comparing the different dry time groups, 5 of 8 specimens in the 60 minutes dry time group showed none or reduced RANKL immunoreactivity in the periodontal ligament cells across the length of the root surface compared to those in the <5 minutes or 30 minutes group (Figures 3.15, 3.16 and 3.17). Only 1 of 10 specimens and 1 of 9 specimens from each of the <5 minutes and 30 minutes dry time groups respectively showed weak or negative RANKL immunostaining.
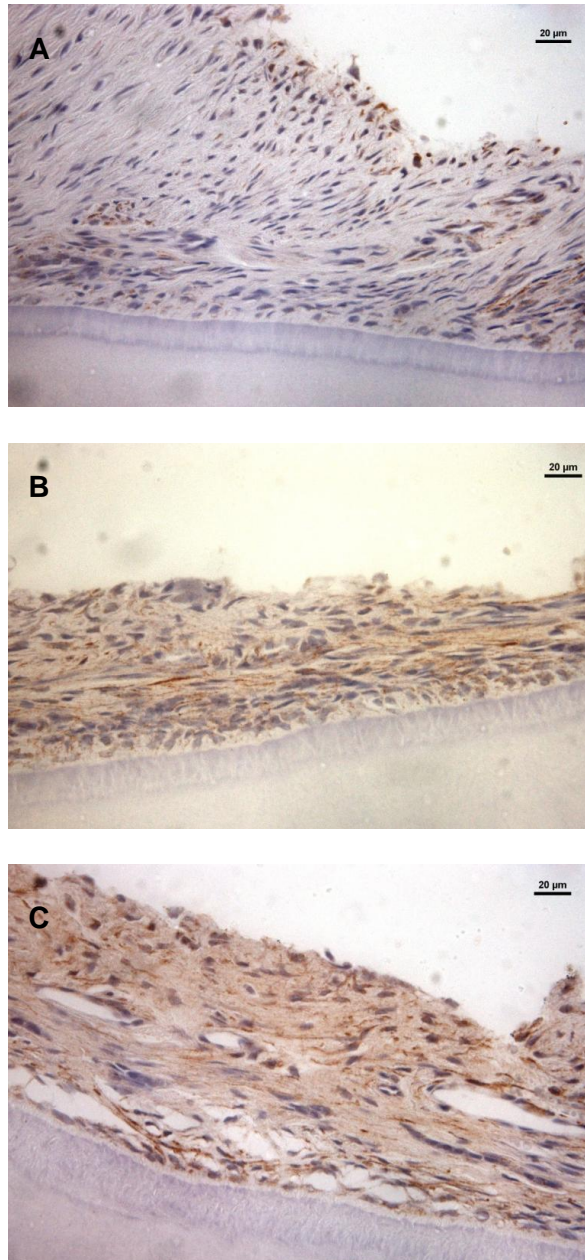
Figure 3.15 Images of the different regions (A: cervical, B: middle, C: apical) on the root surface of a specimen from the <5 minutes dry time group stained for RANKL.
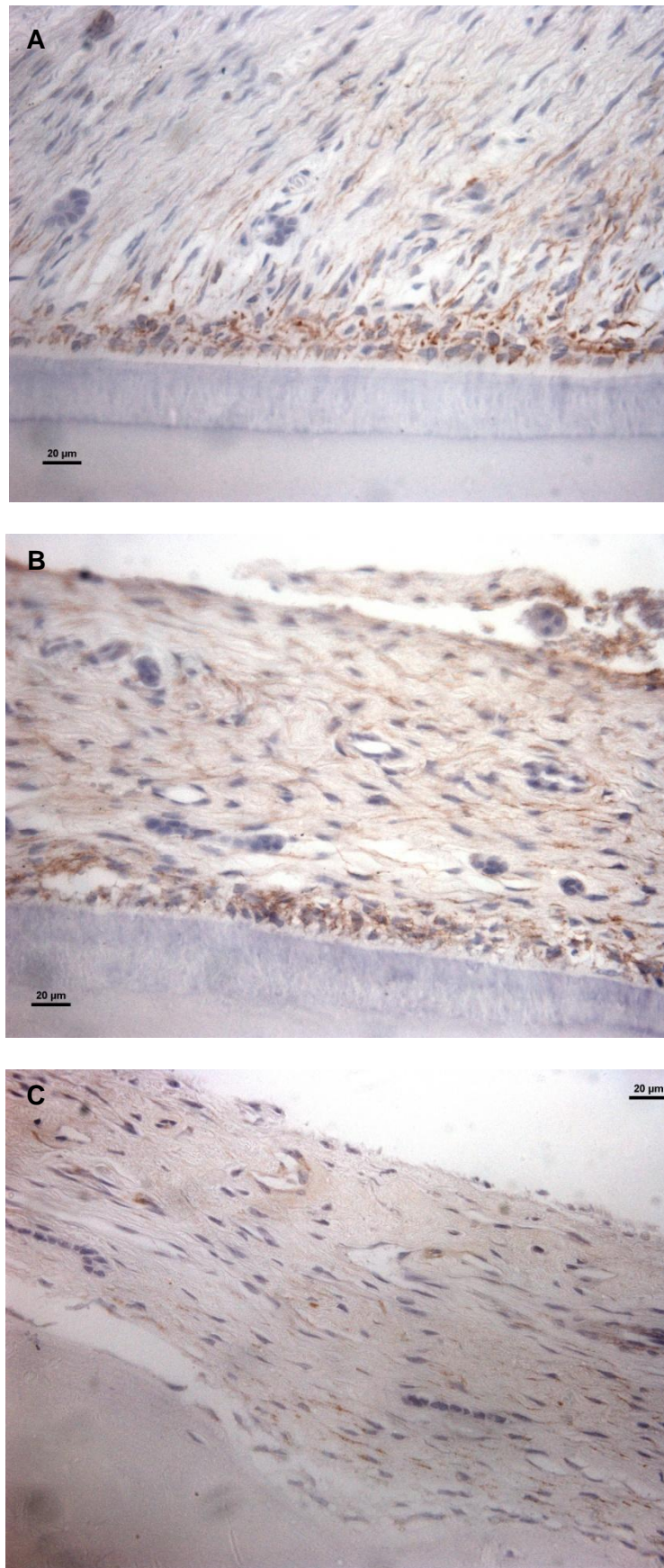
Figure 3.16 Images of the different regions (A: cervical, B: middle, C: apical) on the root surface of a specimen from the 30 minutes dry time group stained for RANKL.
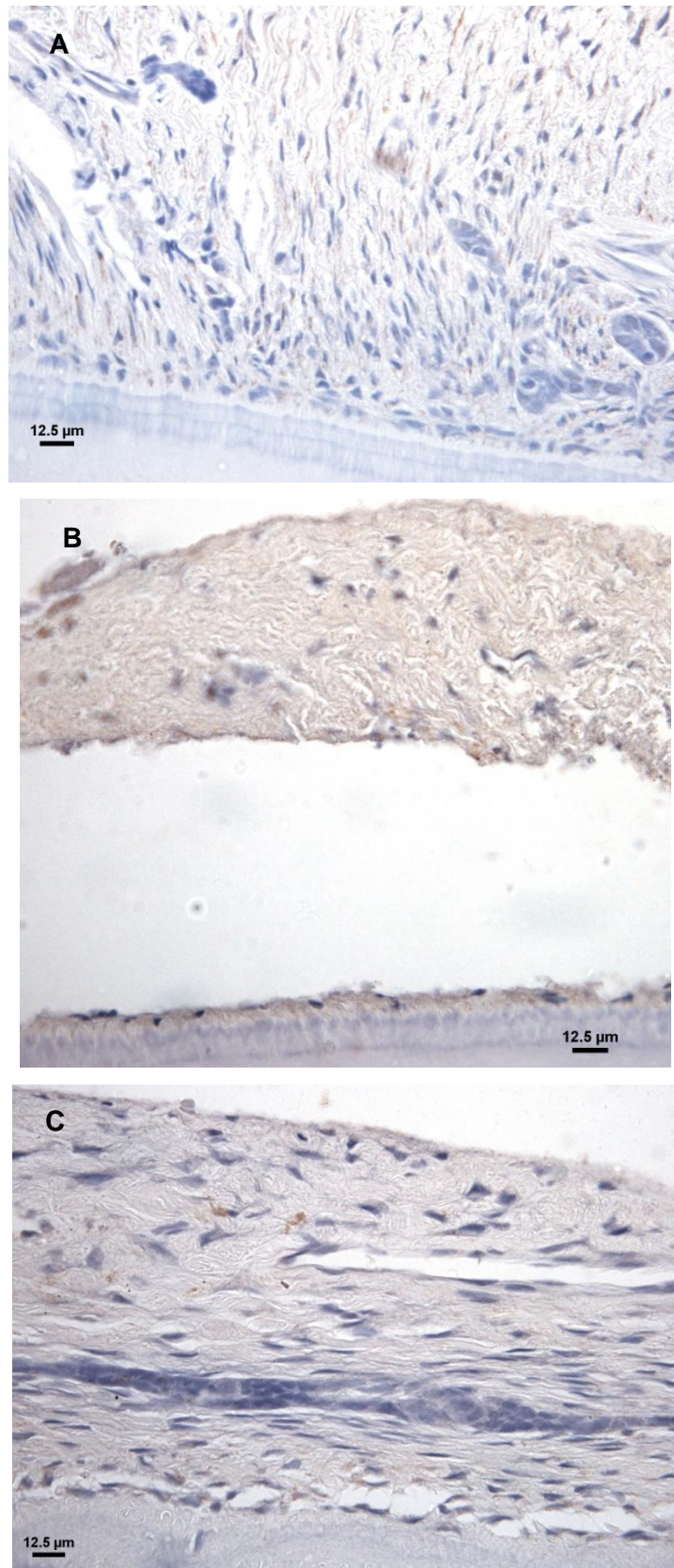
Figure 3.17 Images of the different regions (A: cervical, B: middle, C: apical) on the root surface of a specimen from the 60 minutes dry time group stained for RANKL. Comparing this figure and figures 3.16 and 3.17, note that RANKL immunostaining became weaker with increased extra-alveolar dry time.

3.4.4. OPG vs RANKL immunohistochemistry

With an extra-alveolar dry time less than 5 minutes, the specimens showed similar staining intensity between OPG and RANKL along the three root surface regions in majority of the samples (Figure 3.18).
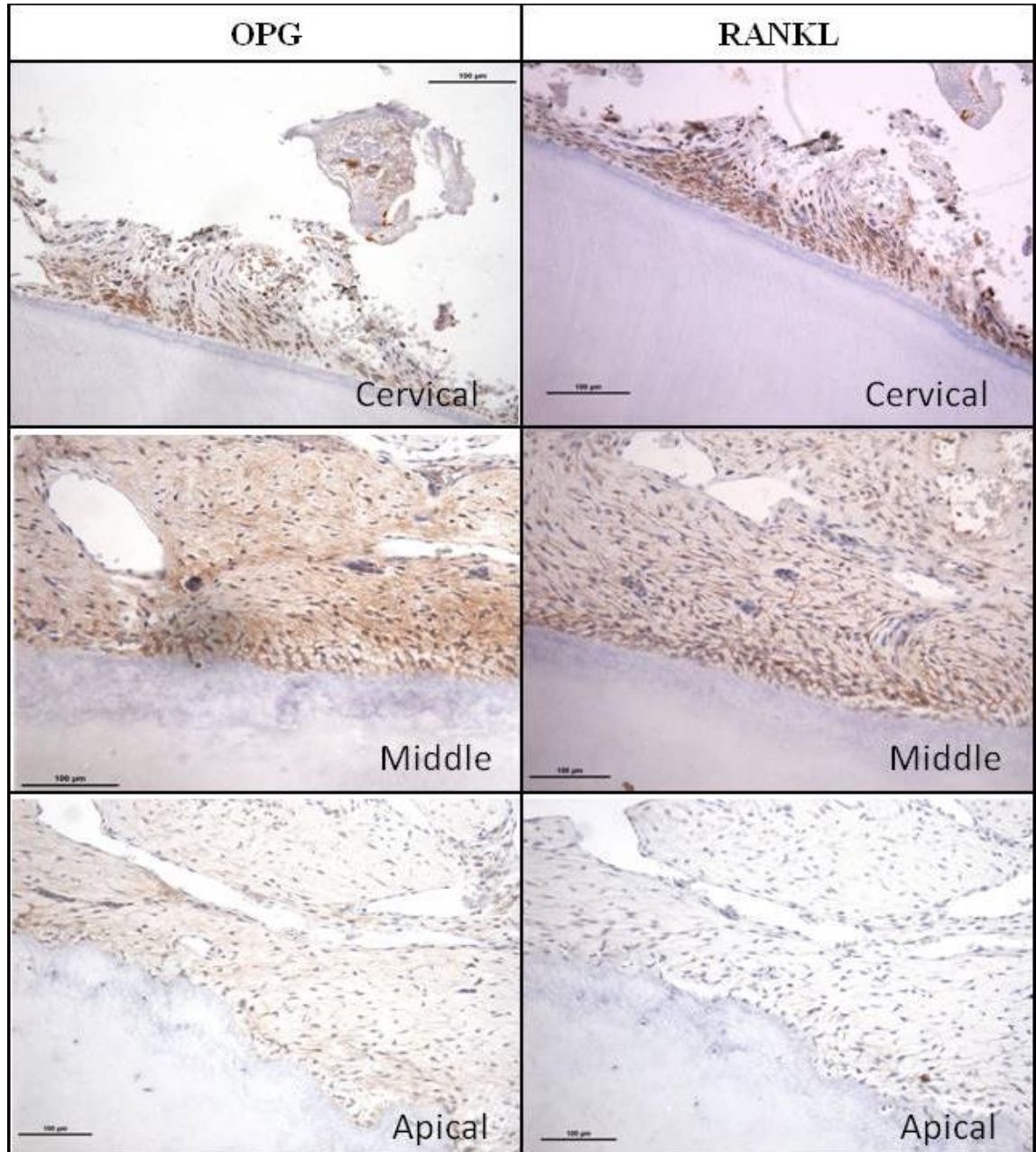
Figure 3.18 Comparison between OPG and RANKL staining in a tooth specimen that was exposed to an extra-alveolar dry time of less than 5 minutes. The staining intensities across the different root surface regions did not differ much between OPG and RANKL.

After 30 minutes extra-alveolar dry time, most specimens did not show any notable difference in OPG and RANKL staining intensity along the root surface (Figure 3.19).
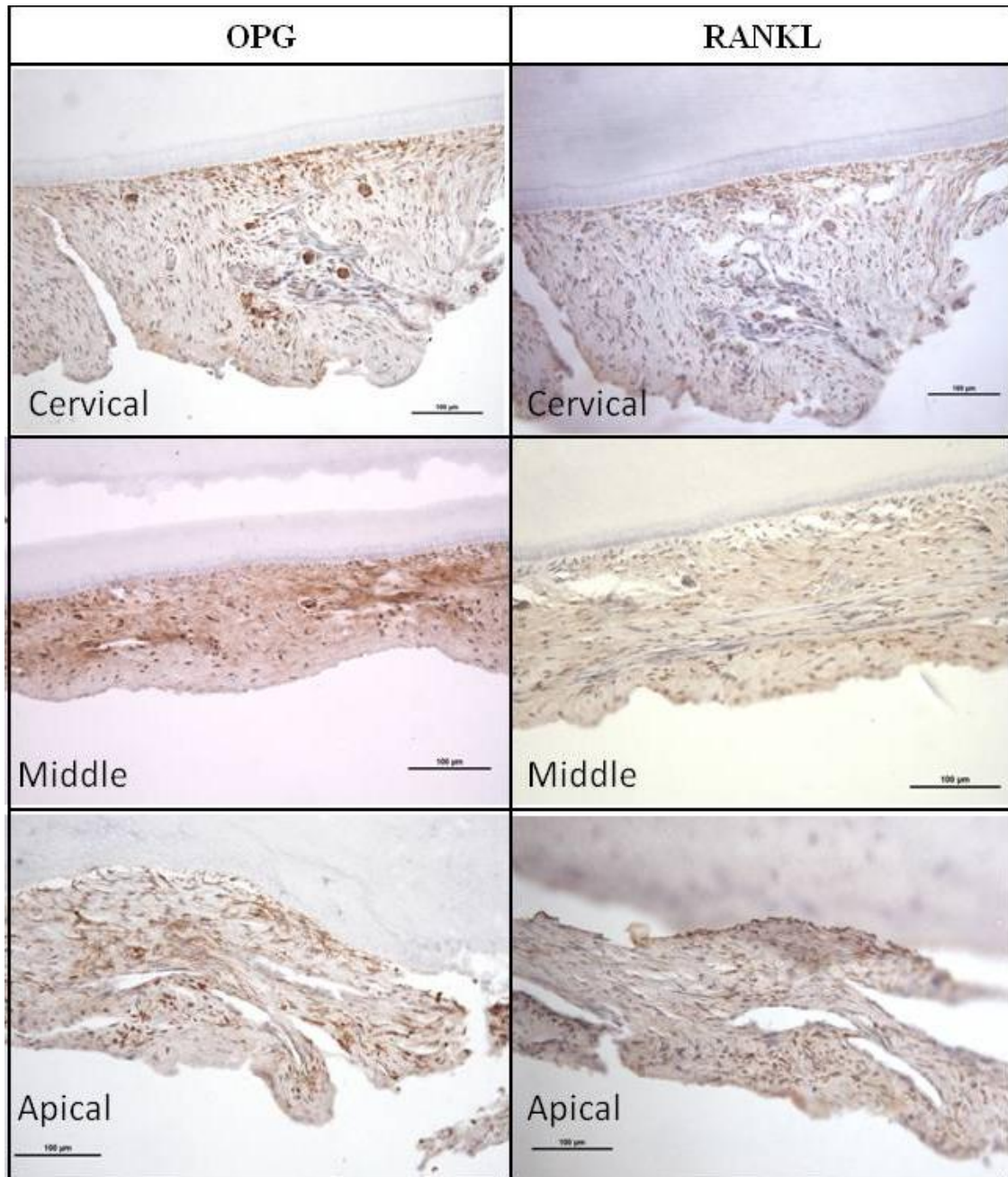
Figure 3.19 Comparison between OPG and RANKL staining in a tooth specimen that was exposed to an extra-alveolar dry time of 30 minutes. The staining intensities across the different root surface regions did not differ much between OPG and RANKL.

After 60 minutes extra-alveolar dry time, 6 of the 8 samples that were analysed in this group showed considerable difference in staining intensity between OPG and RANKL across all 3 regions on the root surface (Figure 3.20). The remaining 2 samples exhibited similar staining intensity for both OPG and RANKL.
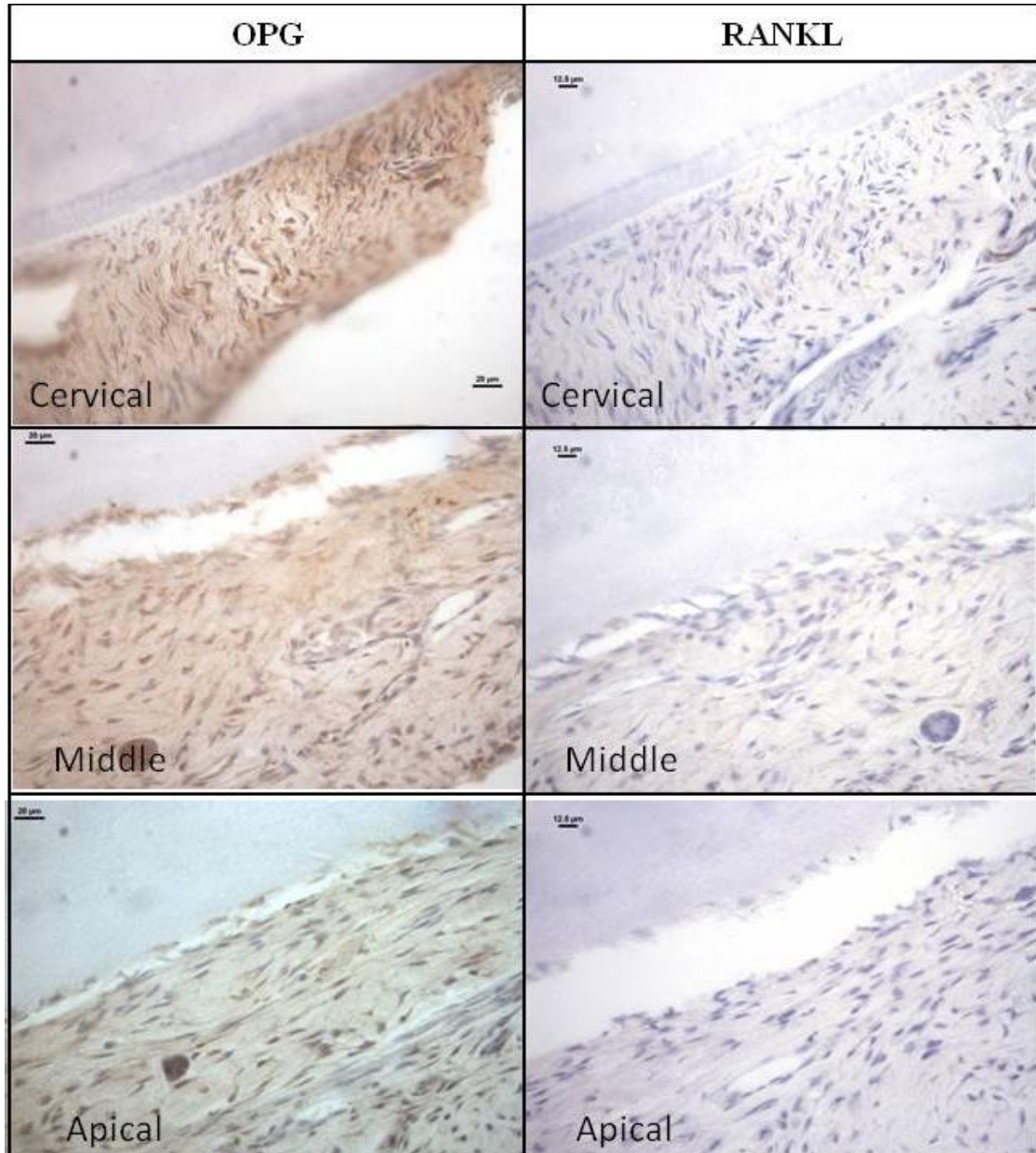
Figure 3.20 Comparison between OPG and RANKL staining in a tooth specimen that was exposed to an extra-alveolar dry time of 60 minutes. For this dry time group, there was considerable decreased RANKL staining intensity compared to that for OPG across the 3 root surface regions.

Overall, positive immunostaining for OPG was generally consistent across a majority of the specimens. For RANKL, a pattern towards weaker staining intensity with increasing extra-alveolar dry time was found.

A joint negative control (secondary antibodies raised in goat) was used for each tooth specimen that was being tested for the presence of OPG and RANKL. Neither OPG nor RANKL immunoreactivity was observed in the negative control experiments (Figure 3.21).
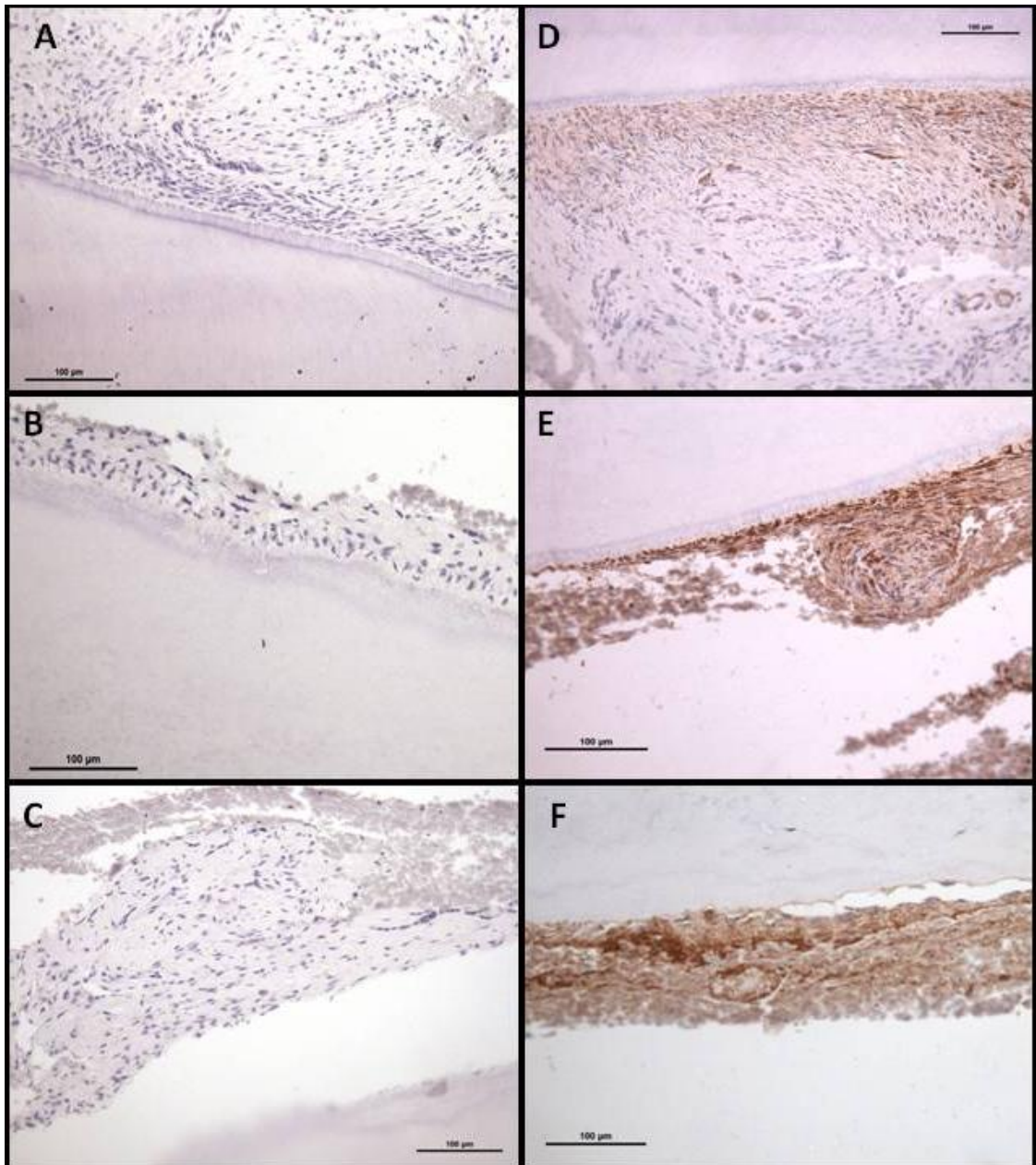
Figure 3.21 Negative controls of immunostaining at different regions (A: cervical, B: middle, C: apical). Compare these negative control images (left column, A-C) to the adjacent column images (right column, D-F) which show an example of positive OPG staining (D: cervical, E: middle, F: apical).

3.5. Semi-quantitative observations

3.5.1. Descriptive statistics

The median of the scores for the expressions of OPG and RANKL according to the dry times and regions were calculated as shown in Table 3.1 and summarised in Figures 3.22 and 3.23.

Table 3.1 Median of expressions of OPG and RANKL according to dry time and region.

Dry Time	Region		OPG expression	RANKL expression
5 minutes	Cervical	N	8	10
		Median (SD)	2.0 (0.7)	1.5 (1.1)
	Middle	N	8	10
		Median (SD)	2.5 (0.7)	2.0 (0.7)
	Apical	N	8	10
		Median (SD)	1.5 (1.0)	1.5 (0.8)
30 minutes	Cervical	N	9	9
		Median (SD)	2.0 (0.8)	2.0 (1.2)
	Middle	N	9	9
		Median (SD)	3.0 (0.5)	2.0 (1.2)
	Apical	N	9	9
		Median (SD)	3.0 (0.7)	1.0 (1.2)
60 minutes	Cervical	N	8	8
		Median (SD)	2.0 (0.8)	1.0 (0.6)
	Middle	N	8	8
		Median (SD)	2.0 (0.6)	1.0 (0.9)
	Apical	N	8	8
		Median (SD)	2.0 (0.5)	0.5 (0.7)
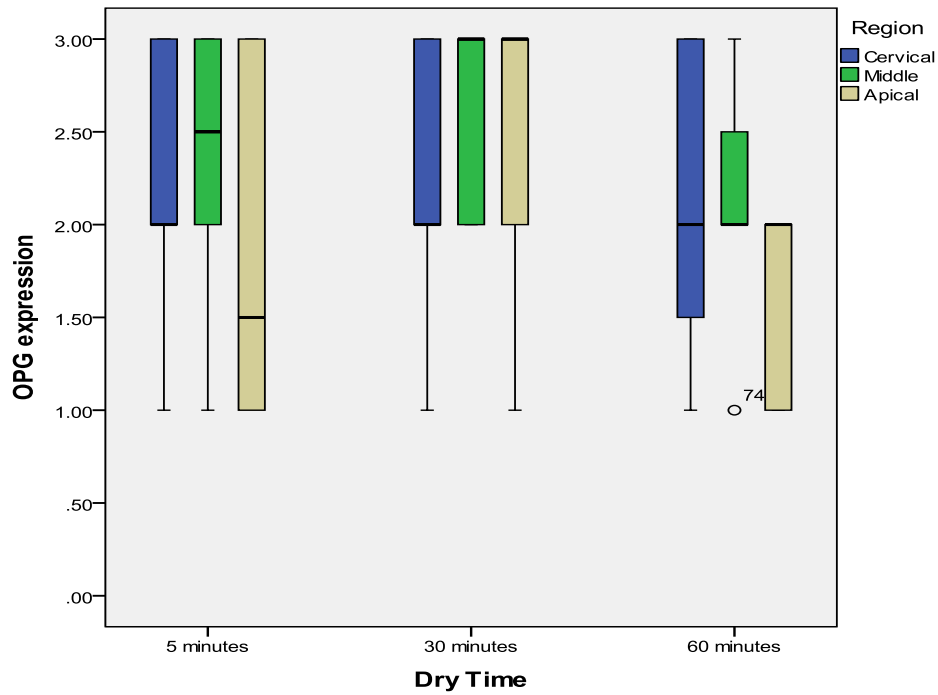
Figure 3.22 Boxplot showing the range and median scores for OPG expression according to location and extra-oral dry time (5 minutes n=8; 30 minutes n=9; 60 minutes n=8).
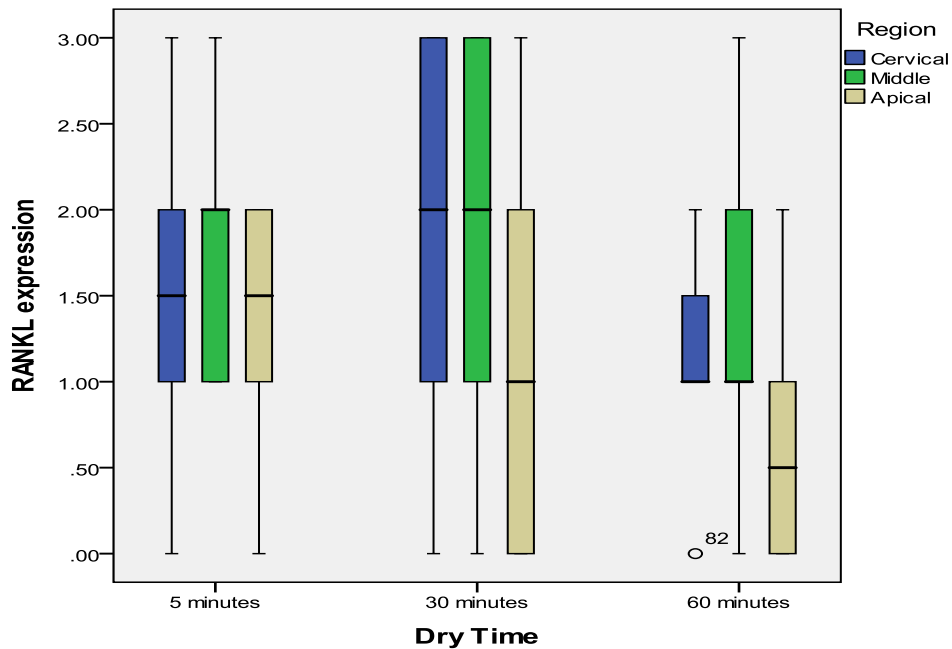
Figure 3.23 Boxplot showing the range and median scores for RANKL expression according to location and extra-oral dry time (5 minutes n=10; 30 minutes n=9; 60 minutes n=8).

3.5.2. Findings relating to OPG

Key findings from the semi-quantitative analysis of OPG expression were:

- The greatest median for OPG expression was found in the middle and apical regions of the 30 minutes dry time group (median=3.0, SD=0.53 and 0.73 respectively) while the lowest was found in the apical region of the <5 minutes dry time group (median=1.5, SD=0.99).

- Statistical data revealed no significant difference for OPG expression between the <5 minutes, 30 minutes and 60 minutes extra-oral dry times at each region (cervical p-value=0.96, middle p-value=0.39, apical p-value=0.10, using a Kruskal-Wallis test).

- Comparing the different regions on the root surface, no statistically significant difference was noted for OPG expression at all extra-oral dry times (<5 minutes p-value=0.25, 30 minutes p-value=0.64, 60 minutes p-value=0.20, using a Friedman test).

3.5.3. Findings relating to RANKL

Key findings from the semi-quantitative analysis of RANKL expression were:

- The lowest median score for RANKL expression was found in the apical region of the 60 minutes dry time group (median=0.5, SD=0.74) while the highest median scores were in the <5 and 30 minutes dry time groups (median=2.0, SD=0.74 and 1.2 respectively).

- Statistical data revealed no significant difference for RANKL expression between the <5 minutes, 30 minutes and 60 minutes extra-oral dry times at each region (cervical p-value=0.40, middle p-value=0.45, apical p-value=0.25, using a Kruskal-Wallis test).

- Comparing the different regions on the root surface, no statistically significant difference was noted for RANKL expression at all extra-oral dry times (<5 minutes p-value=0.51, 30 minutes p-value=0.37, 60 minutes p-value=0.07, using a Friedman test).

3.5.4. Comparison between OPG and RANKL expressions

Key findings from the semi-quantitative comparison between OPG and RANKL expressions were:

- At <5 minutes and 30 minutes of extra-oral dry time, there was no significant difference between OPG and RANKL expressions at all regions (p>0.05, using a sign test).
- At 60 minutes of extra-oral dry time, there was a notable difference between OPG and RANKL expressions at all regions, with statistical significance found in the apical region (p=0.03 using a sign test) (Table 3.2).

Table 3.2 Summary of sign test for comparison of OPG and RANKL expression scores according to location and extra-oral dry time.

Dry time	Comparison between OPG and RANKL according to region	p-value (2-tailed)
< 5minutes	RANKL Cervical - OPG Cervical	0.687
	RANKL Middle - OPG Middle	1.000
	RANKL Apical - OPG Apical	0.687
30 minutes	RANKL Cervical - OPG Cervical	1.000
	RANKL Middle - OPG Middle	0.375
	RANKL Apical - OPG Apical	0.219
60 minutes	RANKL Cervical - OPG Cervical	0.063
	RANKL Middle - OPG Middle	0.125
	RANKL Apical - OPG Apical	0.031*

*denotes significance at p<0.05

3.6. Observation for preliminary confocal microscopy work

Images taken for sections stained for OPG and RANKL revealed positive immunofluorescence in the <5 minutes dry time teeth specimens (Figure 3.24A and B). Where positive immunofluorescence was observed, the distribution of both OPG and RANKL were found to be largely concentrated within the extracellular matrix of the PDL although OPG immunofluorescence was more diffuse within the matrix. There was similar immunofluorescence for OPG in the specimens from both the <5 minutes and 60 minutes dry time groups (Figure 3.24A and C) but RANKL expression had reduced noticeably from <5 minutes to 60 minutes dry time (Figure 3.24B and D) at the apical region.
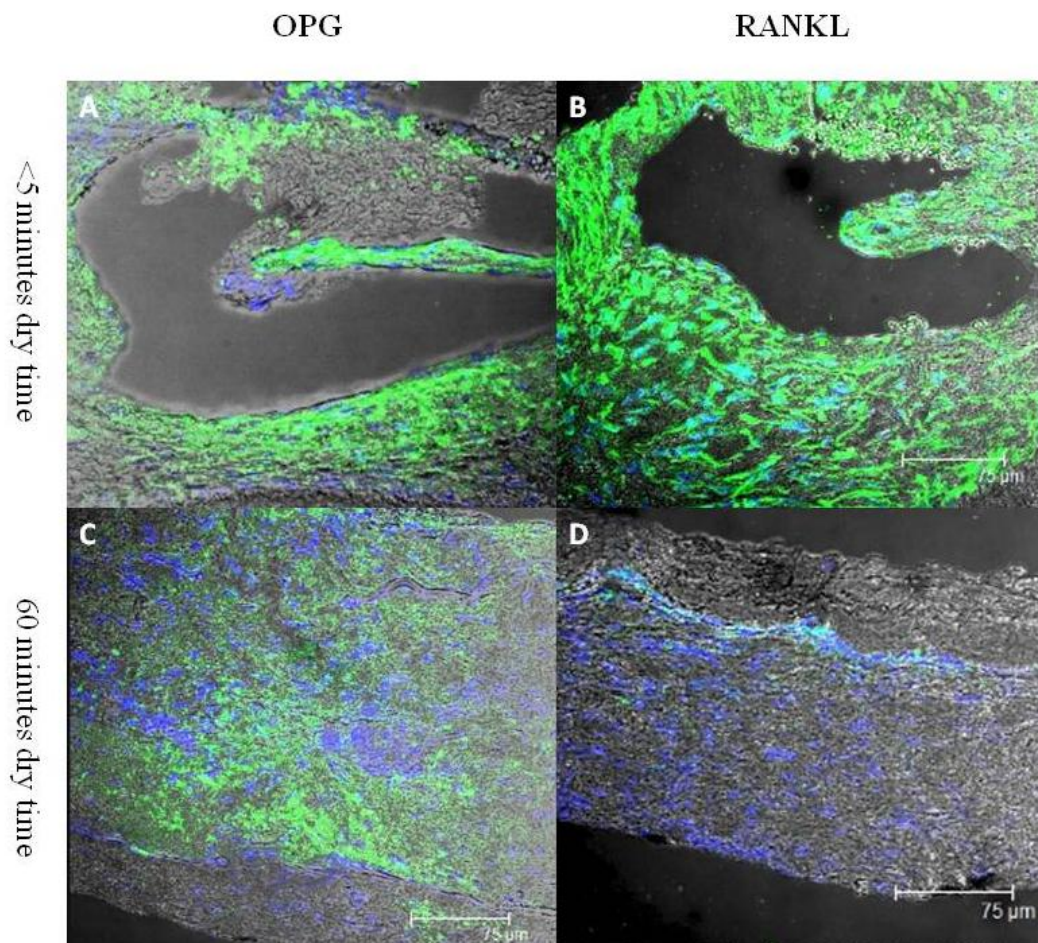
*Figure 3.24 Immunofluorescent-labelling for OPG/RANKL (green) and cell nuclei (blue). The images represent the distribution of OPG (**A, C**) and RANKL (**B, D**) in the apical region of the tissues on the root surface between specimens in the <5 minutes and 60 minutes dry time groups.*

To test the specificity of the antibodies, negative controls were carried out where the primary antibodies were omitted. The negative controls showed no staining for either OPG or RANKL (Figure 3.25).
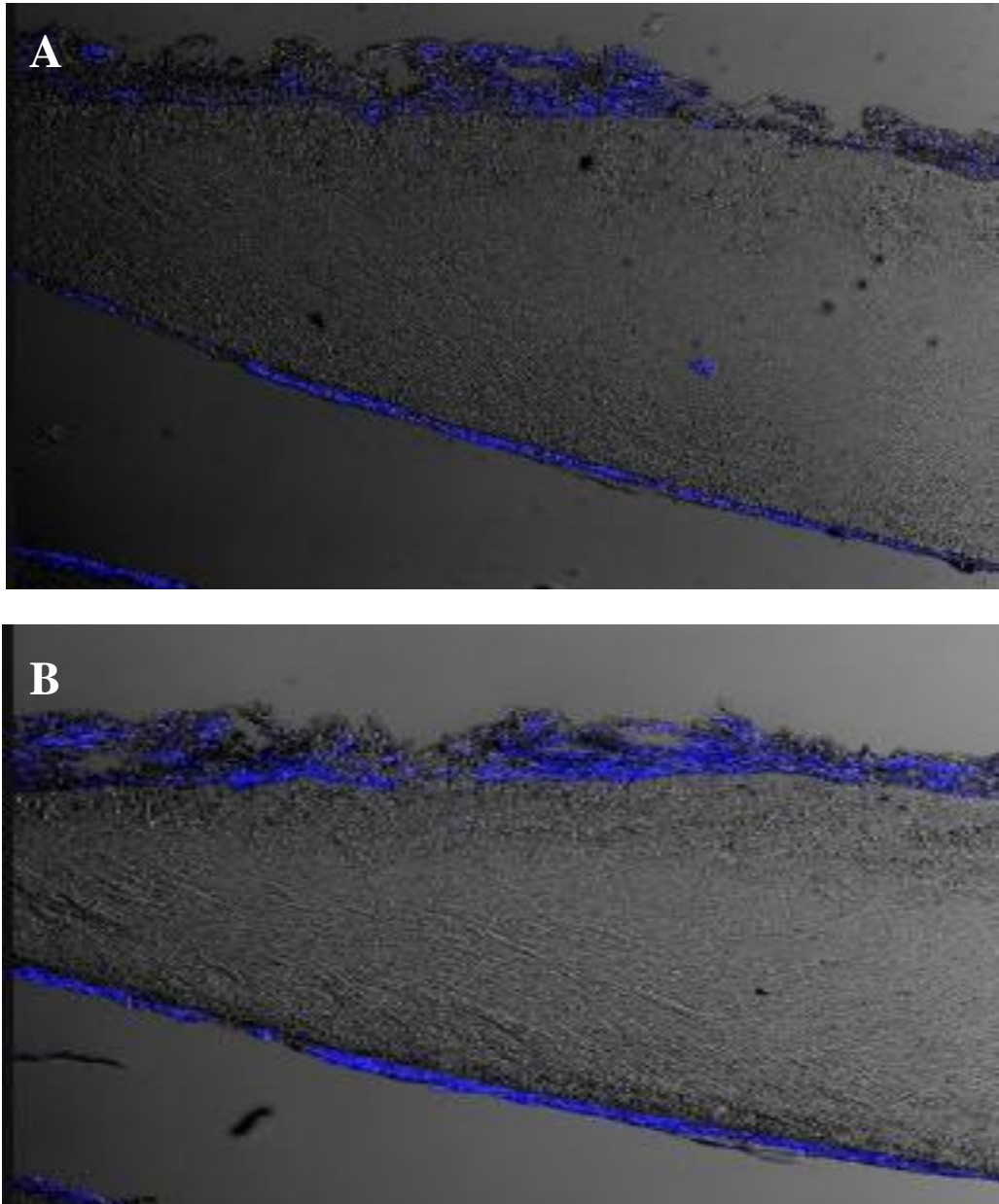
Figure 3.25 Negative controls demonstrating only blue TO-PRO®-3 nuclei staining and no positive green fluorescence for both specimens where OPG (A) and RANKL (B) primary antibodies were omitted.

4. DISCUSSION

4.1. Purpose of study

The expression of OPG and RANKL in tissues from the oral cavity have been documented in several *in vitro* and animal studies (Rani and MacDougall 2000; Hasegawa *et al.* 2002; Fukushima *et al.* 2003; Zhang *et al.* 2004). To date, no studies have established the distribution of these signalling molecules on the root surfaces of permanent teeth related to extra-alveolar storage times following dental extraction. This present study has demonstrated that both OPG and RANKL are expressed in the periodontal membrane tissues of non-resorbing permanent human teeth at the protein level.

Extra-oral dry times were the main variable for this study. The duration of dry period for the avulsed tooth has been found to be the most significant prognostic factor for periodontal ligament healing in multiple clinical studies (Andreasen *et al.* 1995; Schatz, Hausherr and Joho 1995; Chappuis and Von Arx 2005). In a large clinical study, Andreasen and co-workers found that replanted teeth exposed to less than 5 minutes of extra-oral dry time prior to replantation healed significantly better than those with extra-alveolar time of 5-20 minutes (Andreasen *et al.* 1995). In agreement, other studies also reported increased occurrence of replacement resorption with extended extra-alveolar dry time of 30 minutes or more following replantation of avulsed teeth (Schatz, Hausherr and Joho 1995; Chappuis and Von Arx 2005). Several experimental studies have demonstrated that replacement resorption is a result of desiccation and subsequent necrosis of the periodontal ligament cells (Soder *et al.* 1977; Andreasen 1981b; Blomlof *et al.* 1983a; Andersson, Bodin and Sorensen 1989). The viability of PDL cells have been found to be essential in repopulating the surface of the root of the replanted tooth and contributing to favourable periodontal ligament healing (Blomlof and Otteskog

1980; Andersson, Bodin and Sorensen 1989). The effect of drying on the survival and proliferative ability of human PDL cells had been tested *in vitro* by Lekic *et al* (1996), who found that there was a significant reduction of clonogenic capacity in PDL cells subjected to 30-60 minutes of dry storage. The longer the extra-alveolar dry time, the higher the occurrence of root resorption, with significant decrease in periodontal cell vitality after 30 minutes of dry time (Andreasen *et al.* 1995). Based on findings from various studies, a number of published guidelines for dental traumatology recommend a cut-off of 60 minutes extra-alveolar dry time as a prognostic factor for PDL healing (Gregg and Boyd 1998; Flores *et al.* 2007; AAPD Council 2011). Therefore, to provide a range of cell viability, extra-alveolar dry times of <5, 30 and 60 minutes were chosen.

Since this study was focused on assessing OPG and RANKL molecules expressed within the PDL and cementum, it was necessary to identify the sections which would maximise the amount of remnant PDL available on the root surface. Monkey studies have demonstrated the occurrence of root resorption at the curved areas of the teeth where the luxation forces would have compressed the PDL, namely the buccal and palatal/lingual surfaces (Andreasen 1980a, 1980b). These resorption sites have been thought to be due to the damage to the PDL cells sustained during the extraction and replantation procedures. Furthermore, these monkey replantation studies reported that subsequent replacement resorption and further cell damage was found to occur more commonly in the apical half of the root than the cervical half (Andreasen 1980a). More recently, one study examining the topography of the root surface of extracted human premolars showed lower amount of remnant PDL at the apical and cervical than the middle third root regions (Haas *et al.* 2008). Histological analysis of multiple transverse sections at the cervical, middle and apical root regions of all four extracted teeth in this same study by Haas and co-workers (2008) could not demonstrate the presence of

continuous PDL circumferentially on the root surface. Thus in this study, only the longitudinal sections revealing the proximal surfaces were used. To verify that the sections would have sufficient PDL tissue for analysis, serial longitudinal sections using haematoxylin and eosin staining were performed to identify a range of sections which would contain the tissue of interest on the root surface. The root for each tooth section was divided into three main regions for analysis: cervical, middle and apical third. This was to investigate if the expressions of OPG and RANKL varied along the length of the root. In addition, this would allow a better insight of OPG and RANKL distribution in the different cell types such as gingival fibroblasts, cementoblasts, periodontal ligament fibres and epithelial cell rests.

4.2. Experimental approach

4.2.1. Sampling

As current clinical guidelines recommend that avulsed teeth should be replanted, using such teeth would be unethical. Furthermore, it would be outside the researcher's control how long the tooth had been stored dry prior to presentation. Therefore, a human teeth extraction model was chosen to resemble tooth avulsion in this study. Haas and co-workers (2008) had shown that there was a similar amount of remaining PDL on human teeth that had undergone extraction and those which had avulsed following trauma. However, they noted that the distribution of the PDL on the root surface between avulsed and extracted teeth samples were not similar; with more remnant PDL on the palatal than the labial surface for avulsed teeth but not for extracted teeth. Nevertheless, they recorded the amount of remnant PDL on the extracted and avulsed teeth to be greater on the proximal surfaces than the other surfaces (Haas *et al.* 2008). This finding was in agreement with a previous monkey study which found that the convex areas,

namely the labial and lingual surfaces of the root suffered the most damage and loss of periodontal ligament tissue during extraction (Andreasen 1981b).

The sample was collected from a fit and well paediatric population who had undergone extraction of permanent teeth under general anaesthetic in the Leeds Dental Institute (LDI) or Leeds General Infirmary. The extractions were carried out by different operators who were either postgraduate trainees or staff of the Child Dental Health Department of LDI. Usually, extraction of permanent teeth in this population would be due to poor long-term restorability, molar-incisor hypomineralisation and compensating extraction of maxillary first permanent molars where mandibular first permanent molars were to be extracted. The presence of caries in the coronal portion of the crown will have no effect on the periodontal membrane as long as the cementum is intact and thus prevent inflammation travelling from the pulp to the periodontal space. Teeth with gross caries extending into the pulp were excluded as early periapical inflammation may affect the apical PDL region. Collecting extracted teeth from the paediatric population was beneficial as the occurrence of periodontal disease is very rare. Teeth which fulfilled the criteria as described in sections 2.2.1.2 and 2.2.1.3 were eligible for use in this study. All teeth were extracted in the presence of the investigator (KST) to ensure that teeth which exceeded 60 seconds for luxation or mishandled at the PDL region prior to collection in the specimen pot were not included in this study. This was to ensure there would be minimal damage to the PDL on the teeth used in this study. Due to time limitation and lack of suitable sample available from the Tissue Bank, a total of 10 teeth per dry time group, 30 teeth in total, were used in this study.

Careful handling of the teeth following extraction was important. The teeth were neither handled forcefully at the root(s) nor wiped against any surface to prevent any tissue

loss. A standardised collection method by the investigator after the extraction was carried out to prevent potential loss of remnant tissue on the root surface of these extracted teeth. These teeth were left to air-dry in the specimen pot with no lid on to replicate the surrounding temperature and humidity of real-life situations. This was similar to the method used in a monkey study for storage of the teeth during the extra-oral period (Schwartz, Andreasen and Andreasen 2002).

4.2.2. Tooth type

Ideally, single-rooted teeth samples would be practical substitutes to resemble the anterior teeth, which in real-life are the most at-risk for dental trauma. However, both single-rooted and multiple-rooted teeth were used in this study because it was uncommon for single-rooted permanent teeth to be extracted in the children's dental department general anaesthetic list, and therefore difficult to collect a sufficient sample size. In view of the guidelines set out by the UK National Clinical Guidelines (Davies, Harrison and Roberts 2008), premolar extractions for the purpose of orthodontics were preferably carried out by the patients' own general dental practitioner whenever possible, and hence such teeth were not commonly available from the Tissue Bank. Other single-rooted teeth which were extracted for reasons other than orthodontics would be due to external root resorption or deep caries resulting in periapical inflammation, thus not suitable for this study. Nevertheless, it is very unlikely that the number of roots each tooth had would have a significant impact on the response of OPG and RANKL signalling molecules in the periodontal ligament with different extra-oral dry times in this study. To standardise the final sections as much as possible, only the most lateral proximal surfaces of the roots in the longitudinal sections were considered for all teeth. Furcation areas for the first permanent molars were not used for analysis.

4.2.3. Immunohistochemistry

4.2.3.1. Tissue preparation

Although frozen sections require less preparation time, they are inferior in microscopic morphology and less amenable to room temperature storage compared to FFPE sections. Several studies have successfully identified OPG and/or RANKL on FFPE decalcified tooth sections (Rani and MacDougall 2000; Lossdorfer, Gotz and Jager 2002; Crotti *et al.* 2004). Occasionally, antigen retrieval may be required on FFPE sections because of the formation of protein cross links which can occur during the fixation and processing of samples and thus alter the target antigen such that it cannot be recognised by the antibody specific for it. Antigen retrieval treatment would help to remove these protein cross links. However, for this present study, the antigen retrieval trial carried out prior to finalising the protocol showed that there was acceptable positive immunoreactivity for both OPG and RANKL in the tooth sections without antigen retrieval treatment. No antigen retrieval was preferred since it resulted in maintenance of morphological detail and less introduction of artefact to the section without excessive non-specific staining.

4.2.3.2. Indirect labelling method

The direct method involves the use of a labelled primary antibody to react directly with the tissue section. The indirect method is a 2-layer protocol where the primary antibody is unlabelled whilst the secondary antibody is labelled. This gives the indirect method greater sensitivity through increased signal amplification where two or more secondary antibodies can attach to the primary antibody. The advantages of the indirect method are that it allows more flexibility as it can use unlabelled primary antibody with any type of labelled secondary antibody (e.g. horseradish peroxidase, fluorescein isothiocyanate, tetramethylrhodamine isothiocyanate) and it enables a negative control to be carried out.

There are different types of label/marker(s) available:

- Enzyme markers – including horseradish-peroxidase (HRP)

- Fluorescent markers - including fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC)

Although fluorescent markers provide a more stable signal amplification, the disadvantages of fluorescent markers include the use of a special microscope to view the stained sections, the tendency for the markers to fade overtime thus requiring a special anti-fade mounting medium, fairly prompt viewing under the microscope following completion of staining, and the need for refrigerated storage at 4ºC (Polak and Van Noorden 2003).

4.2.3.3. Immunoperoxidase labelling

The immunohistochemical method with horseradish peroxidase labelling was used in this study for several reasons: (1) it provides detailed information on the location and distribution of the signalling proteins on the root surface, (2) it is relatively less technique-sensitive and demanding than other methods, (3) slides can be stored easily with the stain still present thus providing a long shelf-life and enabling slides to be viewed several years later, (4) no special equipment is required for viewing the specimens. The final reaction product of the horseradish peroxidase and DAB chromogen is a brown precipitate. Chromogenic staining has less non-specific background staining compared to that of immunofluorescence but involves more blocking stages. The major advantage of DAB staining is that the stain is not sensitive to light such as in the case for immunofluorescence and therefore the DAB stains will not fade overtime.

Other techniques which quantify protein expression more accurately such as RT-PCR could be considered but this would usually involve pulverising the tissues and cells to calculate the amount of a particular molecule or protein. As such, there would be a loss of spatial information. Although the RT-PCR technique gives a quantitative outcome, it measures the expression of proteins at the mRNA level which does not give any idea about translational information. Some of the literature have found a poor correlation between mRNA and protein expressions in various studies (Greenbaum *et al.* 2003; Guo *et al.* 2008) but the exact reason for this is still unknown as the transcription and translation biological processes are poorly understood.

4.2.4. Semi-quantitative analysis in immunohistochemistry

The quantification of protein expression with immunohistochemistry has always been a subject of debate because of the lack of reproducibility and standardisation (Taylor and Levenson 2006). Variation in expression analysis could easily arise from any source such as fixation conditions, specimen handling, reagents, detection techniques and interpretation of results. The amount for immunoreactivity can be assessed in relation to the extent, proportion and/or intensity of staining. If the staining was confined within individual cells, the percentage of stained cells over an area of standardised field could be performed. However, for this study, the staining was largely in the stroma such as among the periodontal ligament fibres. Hence, only the intensity of staining for each specimen slide could be assessed against the positive control at each staining batch. The regions of interest comprised of three divided areas (cervical, middle and apical) along the root surface which had at least 100μm of periodontal ligament tissue from the cementum surface. Scoring for the staining intensity in this study was used on a scale of 0 to 3 (Chuang *et al.* 2009) and the investigator was trained by an experienced researcher in the field of immunohistochemistry. This method of analysis is termed

"semi-quantitative" because it only offers a relative and not absolute quantification of the protein expression. Admittedly, the nature of this assessment method is subjective although such scoring system in immunohistochemistry has shown statistically significant correlations with clinical outcomes in breast carcinoma receptor studies previously (Taylor and Levenson 2006; Walker 2006).

Despite the widespread use of computerised image analysis to improve quantification variability, DAB produces a brown colour precipitate which only follows the Beer-Lambert law (absorbance is proportional to the thickness and concentration of the sample) at lower intensities (van der Loos 2008). Therefore, the use of automated systems which measure the absorption of colour to determine the amount of staining in this case would not be valid and thus not meaningful if the staining were of darker intensity. Furthermore, antigen-antibody reactions are essentially not stoichiometric and any attempts of true quantification would be inaccurate (Walker 2006).

4.3. Method reproducibility

The Bland-Altman plot was used to assess the intra-examiner agreement on scoring the staining intensity (see Figure 3.3, section 3.3). To ensure reasonable consistency and reliability of the investigator, the scores for 20% of the sample (6 teeth, 36 sites) were repeated a month after the first set of scores was recorded. A total of 27 of the 36 readings were identical. Consequently, the semi-quantitative scoring system was consistently applied by the examiner. The Bland-Altman plot constructed from the two sets of scores showed an acceptable correlation with all but three of the readings falling within the 95% confidence interval.

4.4. Statistical analysis

Owing to a small sample size and the variables were presented in discrete data form, the statistical tests undertaken were of non-parametric in nature. For this reason, the statistical comparisons involved the use of medians rather than means.

The Kruskal-Wallis test was used to compare the samples across the different extra-oral dry times at each location. One of the assumptions in the Kruskal-Wallis test is that the data of all the groups are homogenous. The test for homogeneity was first carried out for this study and it revealed no significant differences in the variances for each group. Then, the Kruskal-Wallis test was performed to compare if there was any effect of the different extra-oral dry times on the expressions at each location. If any significant differences were found, a post-hoc analysis could be performed to identify which two groups differed significantly from each other. For this study, no statistical significance was found between the dry time groups at each location. One of the reasons for this may be due to the small sample size. Non-parametric tests are less sensitive than parametric tests in detecting an effect of the independent variable on the dependent variable.

For comparing the three different locations at each dry time duration, the Friedman test was used as it involved measures of expression of OPG and RANKL at different regions from the same tooth sample. This would be followed by Wilcoxon signed rank test if any significant differences were found following the Friedman test. In this study, the Friedman test revealed no statistical significant difference for both OPG and RANKL expressions between the three regions on the root surface. Due to the small sample size, it was not possible to draw firm conclusions.

To test for difference in OPG and RANKL expressions for each dry time group at each location, the sign test was performed. As the expressions were measured on an ordinal scale and the sample size for each comparison was too small to consider normality in the distribution, the non-parametric sign test was used instead of the paired student T-test or Wilcoxon signed rank test. Statistical significant difference between OPG and RANKL expressions were found in the 60 minutes dry time group at the apical region (p=0.03) while the p-value for the cervical region showed close to significance at p=0.06. To validate and strengthen these statistical findings, future studies using a bigger sample size of 20-30 teeth per group and parametric statistical tests would be advisable.

4.5. Null hypothesis

The null hypothesis for the main research question was established at the beginning of the study (see section 2.3). From the statistical analysis, there were no significant changes in the expressions of either OPG or RANKL with different extra-oral dry times and therefore, the null hypothesis could not be rejected. This indicated that extra-oral dry time duration of up to 60 minutes did not change OPG expression significantly. Similarly, RANKL expression was also not significantly affected by the extra-oral dry times.

As a secondary research question, statistical analysis for difference between OPG and RANKL expressions at each location in every dry time group was performed, where the null hypothesis tested was no difference between OPG and RANKL expressions. There was statistical significance found in the 60 minutes dry time group at the apical region and therefore the null hypothesis in this case was rejected.

4.6. Qualitative findings

The immunoreactions in this study for similar cell types in some samples were not of uniform intensity along the whole root surface. This could possibly be due to the fact that different cells may express varying levels of OPG and/or RANKL at different stages of activity and that individual variation can play a role as well. An ultrastructural study by Sahara and co-workers suggested that individual odontoclasts within the same specimen could have different phases of activity and hence demonstrate varying immunoreactivity (Sahara *et al.* 1994).

The decrease in immunoreactivity with increasing dry times could likely be explained by the degradation of the OPG and RANKL molecules with time due to cell death. From this study, RANKL immunoreactivity decreased more markedly compared to that of OPG. This study did not look at dry times beyond 60 minutes but presumably, OPG immunoreactivity would decrease overtime as well. One of the possible explanations as to why RANKL degraded faster than OPG could be that the presence of OPG may result in a shorter RANKL half-life following OPG-RANKL binding internalisation (Tat *et al.* 2006). The stability and degradation of the OPG and RANKL proteins could also be related to the size and number of subunits of the protein. However, the stability of OPG and RANKL proteins with dry time and the extent to which the primary antibodies interfere with normal OPG-RANKL interactions have not been studied.

4.6.1. Findings related to OPG

Positive OPG stain was found along the cervical, middle and apical third regions within the periodontal membrane tissue, with especially consistent strong staining within the cementoblast layer. The cementoblast layer directly adjacent to the root surface had been shown to be essential in the process of root resorption in a monkey study, where

the decreased number of vital cementoblasts was associated with the occurrence of replacement resorption (Andreasen 1981b). This study also compared the extent of damage to periodontal ligament cells at different distances from the root surface and found that damage to the innermost cementoblast layer contributed significantly to the development of root resorption (Andreasen 1981b). Another experimental study by Andreasen showed that increased extra-alveolar period and removal of the periodontal ligament led to the progression of replacement resorption (Andreasen 1981a). These studies suggested that the presence of an intact and viable periodontal ligament, especially the cementoblasts on the root surface, was essential in preventing root resorption and maintaining homeostatic control of the PDL. Further work focusing on signalling proteins in the PDL revealed OPG to be expressed in human periodontal ligament cell cultures (Sakata *et al.* 1999). Multiple studies postulated OPG to be the factor in PDL that inhibited osteoclastogenesis and maintained the periodontal ligament space (Shimizu, Inoyama and Tagami 1996; Kanzaki *et al.* 2001; Hasegawa *et al.* 2002). Another study found that PDL cells obtained from non-resorbing deciduous or permanent teeth expressed abundant OPG (Fukushima *et al.* 2003). This was consistent with the findings of the present study which used non-resorbing permanent teeth. Furthermore, this study found that OPG expression was observed to be stronger in the cervical than the middle or apical regions within the same sample. The abundance of OPG in the periodontal membrane would inhibit RANK-RANKL binding and any subsequent osteoclastic/odontoclastic activation. This might explain why replacement resorption was found to occur less commonly at the cervical half compared to the apical half of replanted teeth in a monkey study (Andreasen 1980a). These findings seem to reinforce the role of OPG in the homeostatic function of PDL in suppressing RANKL-induced resorption of the root surface.

Within the pulp, the non-carious specimens had positive OPG immunoreactivity only within the odontoblastic layer. However, in the presence of inflammation within the coronal pulp, there was positive OPG immunoreactivity in the pulp chamber fibroblasts as well. This was in agreement with Kuntz and co-workers who also had similar outcomes following immunohistochemistry between carious and non-carious permanent human teeth samples (Kuntz *et al.* 2001). The presence of OPG in the pulpal cells was previously demonstrated in a cell culture study by Sakata *et al* (1999) who also found that the expression of OPG was influenced by cytokines positively. Overall, these findings seem to point that OPG could be regulated during inflammatory processes which might then lead to resorption of mineralised dental tissue.

There was no statistically significant difference in OPG expression with different extra-oral dry times at all regions along the root surface, suggesting that dry time may not have an effect on OPG expression. The maximum duration of dry time (i.e. 60 minutes) used in this study could be insufficient to induce a change in OPG expression. Inflammatory cytokines such as interleukin-1β and tumour necrosis factor-α stimulated by the endotoxin lipopolysaccharide in human PDL cell cultures were shown to affect OPG expression in one study but it took up to 48 hours for this change of expression to be detected (Wada *et al.* 2004). Compared to real-life dental trauma situations where the avulsed tooth surface would likely be contaminated by soil debris or dirt at the time of the avulsion, this study used a clean specimen pot to store the teeth dry immediately following extraction, thus reducing chances of contamination and a subsequent increase in inflammatory stimulus which might be necessary to stimulate any alteration in OPG expression.

4.6.2. Findings related to RANKL

Unlike that for OPG, immunoreactivity for RANKL was strongly positive (grade 3 in two or more regions) along the root surface in only 6 of 27 teeth while the majority of the sample showed weak or no immunoreactivity. The presence of RANKL mRNA was detected in periodontal ligament tissue of human deciduous teeth (Kanzaki *et al.* 2001; Lossdorfer, Gotz and Jager 2002) but not in that of non-resorbing permanent teeth in some studies (Sakata *et al.* 1999; Fukushima *et al.* 2003). However, none of the studies have investigated the presence of RANKL on a protein level. Detection of the RANKL protein but not the mRNA might indicate that the protein could have a slow turnover rate and the mRNA expression is inhibited by the activity of the protein. Future research could explore this relationship between mRNA and protein expressions by carrying out both mRNA and protein level investigations for RANKL from the same tissue sample. The finding of RANKL antigen in the PDL of some of these non-resorbing permanent teeth in this study suggests that RANKL might have another function other than osteoclasts/odontoclasts activation.

In the 60 minutes extra-oral dry time group, 5 of 8 specimens had weak or no RANKL immunoreactivity in the periodontal ligament cells across the length of the root surface. In a clinical situation, the effect of increased dry time would almost certainly result in replacement resorption of the replanted tooth (Andreasen *et al.* 1995). Numerous studies have reported the presence of RANKL in odontoclasts at the resorptive PDL front of shedding human deciduous teeth, thus suggesting the role of RANKL in dental root resorption (Lossdorfer, Gotz and Jager 2002; Fukushima *et al.* 2003). Extrapolating theoretically from these studies and their findings, RANKL might be responsible for replacement root resorption in dental trauma and therefore should increase in expression with increased dry time. However, this study had shown that the opposite, where the

overall observed expression of RANKL was lower with 60 minutes extra-oral dry time compared to that in the <5 and 30 minutes group. This finding suggested that with increasing dry time, there was a reduction in RANKL expression in the PDL. Comparing across the locations, the statistical results indicated that RANKL expression was reduced especially at the apical region with the p-value of 0.07 approaching significance.

One reason for the reduced RANKL expression with increased dry time could be that inflammatory mediators such as interleukins or endotoxins (Palmqvist *et al.* 2002; Wada *et al.* 2004) may be necessary to initiate marked RANKL regulation. In the pulp of a shedding primary tooth, cytokines and other complement factors produced by inflammatory cells were thought to prompt the degradation of odontoblasts and activate odontoclast formation (Sahara *et al.* 1994). RANKL has been shown to be expressed by activated T-cells (Kong *et al.* 1999; Schoppet, Preissner and Hofbauer 2002) and also by macrophages in a study which looked at areas of bone loss from failed implants in humans (Crotti *et al.* 2004). In this study, the teeth were kept in relatively clean conditions and not replanted. The lack of necrotic tissue or debris on the PDL would not be able to stimulate the inflammatory process needed to subsequently alter RANKL expression.

In the 60 minutes dry time group, the apical region in particular demonstrated an overall reduced RANKL expression compared to other regions (p=0.07, approaching significance) and to OPG expression (p=0.03, statistically significant). In a monkey study, there was a higher occurrence of replacement resorption in the apical half compared to the cervical half of replanted teeth observed (Andreasen 1980a), thus suggesting that a higher RANKL expression would be expected for root resorption to

occur if RANKL was indeed responsible for root resorption. One possible explanation for this opposite finding in this study could be that OPG and RANKL found within the periodontal membrane might be responsible for osteoclastic activation on the alveolar bone front and not on the root surface. Hence, in this instance, the lower expression of RANKL compared to OPG would allow osteoblasts from the alveolar bone to deposit bone and slowly replace the root. However, this hypothesis warrants more robust studies to validate this observation.

Despite the postulated link between RANKL regulation and root resorption, Zhan and co-workers who investigated the effect of different storage media on osteoclastogenic potential of human PDL cells did not detect any RANKL mRNA even in the presence of osteoclast-like cells in the culture. They proposed that osteoclastogenesis was likely related to the secretion of cyclooxygenase-2 and interleukin-1β in a RANKL-independent manner and that the potential for PDL cells to induce osteoclastogenesis should be considered as an important factor along with PDL cell viability in tooth avulsion (Zhan *et al.* 2012). This seemed to suggest that the effect of extended extra-oral dry times on root resorption might not be regulated by RANKL from the tissue on the PDL and cementum of the avulsed/extracted tooth. However, this study by Zhan and co-workers did not demonstrate that these osteoclasts formed in the PDL cell cultures resulted in tooth root resorption. More research would be necessary to confirm if this osteoclastogenic process in the PDL was indeed directly responsible for root resorption.

4.6.3. Comparison between OPG and RANKL expressions

In this present study, the difference in OPG and RANKL expressions was statistically significant or close to significance in only the 60 minutes of extra-oral dry time group at the cervical (p=0.06) and apical (p=0.03) regions, where median RANKL expression

scores were less than that of OPG. This finding suggested that OPG/RANKL regulation could be triggered significantly after an extended period of extra-oral dry time between 30-60 minutes. This could lend an explanation to the observation of increased risk of replacement resorption seen clinically when avulsed teeth had been left dry for more than 30 minutes prior to replantation (Kinirons *et al.* 2000). The balance between the levels of OPG and RANKL could be the key to regulating root resorption in a manner similar to that seen in bone resorption in chronic periodontitis and orthodontic tooth movement (Wara-aswapati *et al.* 2007; Yamaguchi 2009). If indeed so, a change in the ratio of OPG to RANKL would upset the homeostatic balance of the PDL and prompt a cascade of events influencing mineralised tissue resorption. However, further research is warranted in this area as we cannot exclude that the process of progressive replacement resorption from extensive drying of the PDL could be induced by more complex mechanisms other than just OPG/RANKL regulation.

4.7. Clinical significance of findings

This present study has shown that although OPG expression on the tissue of the root surface had remained unchanged with increased extra-alveolar dry time, RANKL showed a trend towards decreased expression. Furthermore, the differences found between the expressions of OPG and RANKL at the different regions on the root surface in this study might explain as to why replacement resorption had been observed clinically to occur more commonly at the apical than the cervical regions. These suggest that OPG and RANKL proteins within the PDL are expressed differently along the root surface and that their expressions can be affected by extended extra-alveolar dry times to influence the occurrence of replacement resorption following replantation of the avulsed tooth.

The detection of both OPG and RANKL in the periodontal ligament tissue and not in the dentine or cementum could possibly support the fact that the any remnant viable PDL on the root surface is crucial to the prevention of ankylosis and subsequent resorption. Pending more in-depth research, it might be possible to apply a suitable substance/medicament on the root surface of the avulsed tooth as well as in the avulsed tooth socket which could specifically target the OPG/RANKL mechanism to slow down the rate or inhibit replacement resorption prior to replantation. Careful handling of the tooth to preserve as much vital PDL on the root surface following avulsion would likely be the key to maximising the effects of such medicaments.

4.8. Conclusion

OPG and RANKL were found to be expressed in cementoblasts and periodontal ligament fibroblasts on the root surface of non-resorbing permanent teeth that were exposed to less than 5 minutes of extra-oral dry time. There was stronger OPG expression observed in the cervical than the middle or apical regions, and RANKL expression was noticeably lower in the apical region although these observations were statistically not significant. There was also no statistically significant difference in the immunoreactivity of OPG and RANKL among the different dry time groups despite an observed trend towards lower RANKL expression after 60 minutes of extra-oral dry time. The median expression scores for RANKL were either lower or similar to that for OPG at every region and dry time, with RANKL signalling significantly weaker than OPG only after 60 minutes of dry time in the apical region. This suggests that the extra-oral dry time duration of 60 minutes and beyond could trigger an upset in the balance of OPG and RANKL levels at the apical region of the periodontal membrane. However, future improved studies would be necessary to validate these findings *in vivo*.

4.9. Limitations

Several limitations in this study included the relatively small sample size and the types of teeth collected. As there was also demand for tooth specimens from other researchers from the Tissue Bank, the number of teeth available per research project was restricted. Furthermore, the lack of single-rooted teeth available within the study period necessitated the use of other readily available permanent molar teeth may differ with single-rooted teeth. To date, we are not aware of any specific research in this area. However, several animal studies have demonstrated increased chance of ankylosis where teeth have been more difficult to extract (Andreasen 1980a, 1980b).

Due to time and logistic restrictions, the specimens could not all been immunostained in a session. There would be inevitably minor variation between batches of immunostaining despite following the protocol strictly and carrying out the necessary controls for each run.

The use of immunoperoxidase-labelling made it difficult to quantify the expression of the antigens more objectively. To overcome these limitations, the use of a larger sample size and RT-PCR or fluorescence *in situ* hybridisation techniques together with immunohistochemistry could be performed. This also would provide information about the correlation between the observations at both the protein and mRNA levels and help confirm the research results more objectively.

4.10. Future research

Based on the preliminary work carried out for confocal microscopy, the initial observation found that RANKL expression was noticeably reduced in the 60 minutes tooth specimens compared to the those in the <5 minutes dry time group while OPG

expression remained similar in both dry time groups. This seemed to be in agreement with the overall finding for the HRP-labelling immunohistochemistry that was carried out albeit only four teeth were used for this preliminary immunofluorescence work. Although the distribution of the immunoreactivity for OPG and RANKL were observed to be similar between the HRP-labelling and immunofluorescence components in this study, the magnification for the HRP-labelling under light microscopy was insufficient to define the detailed location of the immunostains. Using the protocol for confocal microscopy, future work could involve producing consecutive sections stained with fluorescein dye and allow more defined spatial distribution of these signalling proteins. The serial images generated could then be combined to reconstruct a 3-dimensional view of the root surface and help further understanding of the spatial distribution of OPG and RANKL.

The use of RT-PCR or *in situ* hybridisation along with immunocytochemistry on cells cultured from the root surfaces of teeth exposed to the appropriate dry times could also be performed to investigate the expressions of OPG and RANKL at both the mRNA and protein levels within the same study. This would give some information about the production and regulation of these proteins (i.e. excess protein may inhibit corresponding mRNA production). In addition to RT-PCR quantification for mRNA, double-labelling techniques in immunocytochemistry to quantify the ratio of OPG and RANKL within the same specimen would also be of value in determining the effect of dry times on the balance of this ratio.

These future studies could employ the use of *in vivo* animal model. For the *in vivo* experiments, the teeth could be extracted and stored dry for different duration prior to replantation. These animals would later be sacrificed at different time points post-

replantation and evaluated histologically. Further experiments could also focus on investigating the effect of different storage media (e.g. milk, saliva, saline) or other variables on OPG/RANKL expression.

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LIST OF APPENDICES

APPENDIX A: DREC TISSUE BANK APPLICATION

New Item | Edit Item | ✗ Delete Item | Alert Me	
Title of project	Expression of Osteoprotegerin (OPG) and Receptor Activator for Nuclear-factor-κ-B Ligand (RANKL) on Root Surface After Different Extra-oral Dry Times
Application number	261110/KT/49
Lead applicant surname	Tang
Lead applicant first name	Kok Siew
Lead applicant title	Dr
E-mail address	dnkst@leeds.ac.uk
Telephone number	07702042553
Co-applicant name(s) (must include supervisor, indicated by a '*', if main applicant is a student)	Dr. P. Day*, Dr X. Yang*, Mr A. High
What samples do you require and how many?	This is a pilot study as the expressions of OPG and RANKL on root surfaces have not been investigated in the area of dental traumatology. Research in the fields of tooth resorption in orthodontic movement [1] and primary tooth exfoliation [2] has demonstrated variable expression of OPG and RANKL. Tissue samples required for this study are permanent teeth with or without caries and no evidence of root pathology. Earlier studies [3-5] have suggested approximately 10 teeth per group may give statistical significant results where possible. 10 freshly extracted teeth are required for the first phase of this study. A histological analysis investigating the amount and site of periodontal ligament remaining following extraction in a convenient human sample found 54-58% of root surface periodontal ligament remaining on these extracted teeth [6]. Freshly extracted teeth are necessary for this study because we are attempting to identify protein molecules expressed by periodontal membrane cells on root surface, thus making it important that any remaining soft tissue on the root surface is not scraped or removed during handling of the extracted tooth. Furthermore, duration of exposure of the extracted tooth to air needs to be controlled as per the protocol in this study. This study is therefore asking for 10 freshly extracted teeth from the tooth bank.

Inclusion criteria for tooth selection:

  - Permanent teeth extracted intact without fracture of roots.
  - The teeth must have no root caries or root resorption.

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Brief outline of aims and objectives and experimental protocol in lay language	Tooth avulsion (complete dislodgement of tooth from its socket) accounts for up to 18% of dental trauma to the permanent dentition. The current treament modality is to immediately replant the tooth as soon as possible to increase the chance of survival of the periodontal membrane on the root surface [1-3]. However, this is usually difficult to achieve and therefore, storage of the avulsed tooth in appropriate mediums is important to ensure the cells do not become necrotic from desiccation. The length of time of the tooth out of the socket and the type of storage medium (eg dry, milk, saline, saliva) affect the prognosis and healing of the cementum and periodontal ligament cells [4]. The main role of the periodontal ligament is to protect the root against physiologic processes of resorption and deposition of the alveolar bone [5]. Following replantation, the periodontal ligament could either heal by surface resorption where the root surface is eventually repaired by formation of new cells, or by replacement resorption where the root is permanently replaced by the surrounding bone [3]. Unless immediately replanted, most of the avulsed teeth would demonstrate progressive root resorption and inevitably end up in extraction [6]. The phenomena of root resorption in deciduous teeth during exfoliation and in tooth moevement for orthodontics have been reported [7-8] and are found to be regulated by the molecular signalling system of osteoprotegerin (OPG) and receptor-activator of nuclear-factor-kappa B Ligand (RANKL). The effect of OPG activation results in prevention of formation of bone-absorbing cells (osteoclasts) whereas the effect of RANKL results in the opposite. The roles of OPG and RANKL in root resorption of replanted tooth have not been reported. This study aims to establish this association, if any, and also to quantify the expression of these signalling molecules under different dry times. This research will be done in 2 phases using permanent teeth that are extracted intact without fracture of roots and with no root pathology. The first phase would involve the use of immunohistochemistry and immunofluorescence to localise the OPG and RANKL on the root surface and detect for presence of these molecules after a dry time of less than 5 minutes. Should the first phase show positive presence of OPG and RANKL, the next phase would be focused on using immunohistochemistry and immunofluorescence in localising the expression of OPG and RANKL on root surface after extra-alveolar dry times of 30 minutes and 60 minutes. References: 1. McIntyre, J., Lee, J., Trope, M., Vann Jr, W. 2009. Permanent tooth replantation following avulsion: Using a decision tree to achieve the best outcome. Ped Dent 31, 137-144. 2. Wright, G., Bell, A., McGlashan, G., Vincent, C., Welbury, R. 2007. Dentoalveolar trauma in Glasgow: an audit of mechanism and injury . Dent Traumatol 23, 226-231. 3. Trope, M. 2002. Root resorption due to dental trauma. Endod Topics 1, 79-100. 4. Andreasen, J., Borum, M., Jacobsen, H., Andreasen, F. 1995. Replantation of 400 avulsed permanent incisors. 4. Factors related to periodontal ligament healing. Endod Dent Traumatol 11, 76-89. 5. Lindskog, S., Pierce, A., Blomlof, L., Hammarstrom, L. 1985. The role of the necrotic periodontal membrane in cementum resorption and ankylosis. Endod Dent Traumatol 1, 96-101. 6. Kenny, D., Barrett, E. 2001. Pre-replantation storage of avulsed teeth: Fact and fiction. J Calif Dent Assoc. 7. Yamaguchi, M., Aihara, N., Kojima, K., Kasai, K. 2006. RANKL increase in compressed periodontal ligament cells from root resorption. J Dent Res 85, 751-756.

8. Fukushima, H., Kajiya, H., Takada, K., Okamoto, F., Okabe, K. 2003. Expression and role of RANKL in periodontal ligament cells during physiologic root-resorption in human deciduous teeth. Eur J Oral Sci 111, 346-352.

Is this work part of an undergraduate project	No
Is this work part of a postgraduate project	Yes
If 'Yes' give the degree title	Professional Doctorate in Clinical Dent
Intended use outside the Institute	No
If 'Yes' please give details	
Where will the work be carried out?	All laboratory work will be undertaken in the Oral Biology laboratory at the Leeds Dental Institute.
How will the tissue be stored?	The freshly extracted teeth will be stored dry in a container for the necessary duration as assigned accordingly. Following this, the teeth will be fixed, decalcified and prepared on slides for immunohistochemistry and immunofluorescence. All tissue samples will be labelled and stored in the Oral Biology laboratory throughout the duration of the study.
How will the tissue be disposed of?	All tissue samples will be disposed of as per other extracted teeth into the clinical waste bins.
How is the proposed work to be funded?	The project will be funded by the Dept of Paediatric Dentistry, LDI.
How was the work scientifically reviewed	The work will be internally reviewed by the Dept of Paediatric Dentistry, LDI.
How will results be disseminated	The results will be presented at a paediatric dental conference and thereafter published in a peer-reviewed dental journal.
Submit? Only tick box if you are ready to submit. Don't forget to click 'OK' to save	Yes
Decision (for office use only)	Accepted
Attachments	261110_KT_49protocolv3.docx

Created at 06/12/2010 10:26 by Kok Tang
Last modified at 24/05/2011 13:06 by Julie McDermott

APPENDIX B: TISSUE BANK APPROVAL

Date:	Tue, 24 May 2011 13:07:23 +0100 [24/05/2011 13:07:23 BST]
From:	Julie McDermott <J.K.McDermott@leeds.ac.uk>
To:	"'Tang, Kok Siew'" <dnkst@leeds.ac.uk>
Cc:	Gail Douglas <G.V.A.Douglas@leeds.ac.uk>, Peter Day <P.F.Day@leeds.ac.uk>, Xuebin Yang <X.B.Yang@leeds.ac.uk>
Subject:	Tissue Bank application - 261110/KT/49

Dear Kok,

I am pleased to inform you that your above Tissue Bank application has been approved by the Dental Research Ethics Committee.

NB: Please be aware that 20 teeth cannot be supplied all at once and they will be made available to you as and when they become available.

With best wishes for the success of your project.

Kind regards,

For and on behalf of Professor Gail Douglas

DREC Chairman

APPENDIX C: TOOTH SAMPLE INFORMATION SHEET

No.	Toothbank no.	Tooth type	Dry time	Caries / non-caries	Decalcification	
					Start	End
1						
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APPENDIX D: SCORING OF STAINING INTENSITY DATA SHEET

Tooth sample	Location	OPG staining grade	RANKL staining grade
1	Cervical		
	Middle		
	Apical		
2	Cervical		
	Middle		
	Apical		
3	Cervical		
	Middle		
	Apical		
4	Cervical		
	Middle		
	Apical		
5	Cervical		
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	Apical		
6	Cervical		
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	Apical		
13	Cervical		
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	Apical		
14	Cervical		
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15	Cervical		
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	Apical		
16	Cervical		
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	Apical		
17	Cervical		
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	Apical		
18	Cervical		
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	Apical		
29	Cervical		
	Middle		
	Apical		
30	Cervical		
	Middle		
	Apical		