Mineral composition of hypothermally induced ankylosis in rat molars

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5. LITERATURE REVIEW

5. 1 PERIODONTIUM

5.1.1 Definition

The periodontium is defined as those tissues supporting and investing the tooth[1]. It comprises of the PDL, root cementum, bone lining the tooth socket (alveolar bone), and that part of the gingiva facing the tooth (dento-gingival junction)[2].

5.1.2 Periodontal Ligament

The PDL is a highly vascular and cellular connective tissue situated between the cementum covering the root of the tooth and the bone forming the socket wall. It ranges in width from 0.15 to 0.38 mm in humans, with its thinnest portion around the middle third of the root, showing a progressive decrease in thickness with age[2].

The PDL forms a link between the tooth and the bone, thus providing support to distribute multidirectional mechanical stresses such as masticatory forces[1]. It also acts as a sensory receptor for the masticatory system in aiding proper positioning of the jaws during mastication[3]. Finally, it is a cell reservoir for tissue homeostasis and repair／regeneration[2].

5.1.2.1 Composition

The structure of the PDL, like all fibrous connective tissues, comprises a fibrous matrix containing many differentiated cells and their precursors, unique vascular arrangement of blood vessels, a lymphatic system, and a highly specialized network of nervous elements [1, 4].

5.1.2.2 Cellular elements

The differentiated cells include synthetic cells (osteoblasts, fibroblasts and cementoblasts), resorptive cells (osteoclasts, fibroblasts, cementoclasts, odontoclasts), epithelial cells (epithelial cell rests of Malassez and endothelial cells), as well as miscellaneous connective tissue cells (mast cells, macrophages, neural cells etc)[1]. Odontoclasts have certain similar morphological features compared with osteoclasts. These cells show multinucleation, and usually have ruffled borders at resorption sites, clear zones, abundant mitochondria, scattered rough endoplasmic reticulum, and tartrate-resistant acid phosphatase (TRAP) activity. However, the odontoclasts have fewer nuclei and are smaller than the osteoclasts. Odontoclasts have very small or no clear zones in contrast to the well-developed clear zones of actively resorbing osteoclasts. This has been attributed to the difference in composition of the dental tissues when compared with bone[5]. Odontoclasts have not been determined to be osteoclast type cells. In addition, both the origin and differentiation system of odontoclasts are unclear although it is postulated that odontoclast cells may be derived from mononuclear precursor cells from the hematopoietic system, similar to osteoclast cells[6].

As the fibroblast is the principal cell type in the PDL, it plays a significant role in normal turnover, repair, and regeneration[7]. The fibroblasts of the PDL are characterized by their rapid turnover of collagen.

5.1.2.3 Extracellular matrix

The extracellular compartment consists mainly of well-defined collagen fibre bundles embedded in an amorphous background material, known as ground substance[2, 7]. Collagen is the principal protein found in PDL. Type I collagen is the major collagen type found and accounts for approximately 80% of PDL collagen. Type III collagen is the second most common collagen found. Both type I and type III collagen are uniformly distributed within the PDL[8].

The vast majority of collagen fibrils in the PDL are arranged in definite and distinct fibre bundles, and these are termed principal fibres. The extremities of collagen fibre bundles are embedded in cementum or bone. The embedded portion is referred to as Sharpey's fibres. Sharpey's fibres in primary acellular cementum are fully mineralized. Those in cellular cementum and bone are generally only partially mineralized at their periphery[2].

Oxytalan fibres occupy 3% of the volume of the PDL and extend the length of the ligament in an apico-occlusal direction. Unlike collagen fibres, oxytalan fibres do not attach to bone and teeth[8]. The oxytalan fibres are numerous and dense in the cervical region of the ligament and are hypothesized to act to regulate vascular flow in relation to tooth function. There are no mature elastin fibres in the PDL[9].

5.1.2.4 Origin of cementoblasts, osteoblasts & fibroblasts

It is not known whether distinct precursor cell lines exist for PDL fibroblasts, cementoblasts, and osteoblasts or whether these cells arise from a common precursor[2]. Classically, the dental follicle, with a possible contribution from the perifollicular mesenchyme, give rise to PDL fibroblasts, osteoblasts, and cementoblasts[10]. In rodents,

cementogenesis begins with the deposition of a matrix on the dentin surface by Hertwig's epithelial root sheath (HERS), disruption of the HERS, migration and organization by ectomesenchymal cells from the dental papilla, and their subsequent differentiation into cementoblasts[11]. A recent literature review supports the concept that cementoblasts producing both acellular extrinsic fibre cementum and cellular intrinsic fibre cementum are unique phenotypes that differ from osteoblasts [12]. It proposes a model that cells derived from HERS (Hertwig's epithelial root sheath) play an essential role in tissue development and maintenance. Cells descending from HERS may give direct rise to cells that form new cementum and PDL tissues, or play an indirect role by producing the necessary signalling molecules for cell recruitment and differentiation[2].

The precise origin of PDL fibroblasts, cementoblasts, and osteoblasts in mature tissues or the location of their progenitors is still unknown, and it is unclear whether they originate from a single, multipotential stem cell or from multiple different ancestral cells for the separate lineages. In unwounded periodontium, the PDL appears to be the principal source of PDL fibroblasts and also may contain specialized sites for other progenitor cells including the cementum-related part of the PDL for cementoblasts and the bone-related portion for osteoblasts[13]. Location for these progenitor cell populations of PDL include perivascular locations (i.e. adjacent to blood vessels), adjacent to cementum in the PDL and vascular channels in alveolar bone[14-16]. There is also limited cell kinetic evidence that a second-cell population located adjacent to the cementum in the PDL is a separate progenitor population[14, 16].

5.1.2.5 Physiologic regulation of the PDL space

The PDL has the capacity to maintain its width over time, despite being bordered by two hard tissues. The cells of the PDL itself have been demonstrated to have osteogenic abilities[17]. The expression of the bone phenotype must in some way be blocked; otherwise the PDL would undergo bone formation, mineralize across its width, and spontaneously ankylose[7]. There is a belief that the cells and connective tissue of the PDL possess the capacity to inhibit both osteogenesis of the periodontal space and progressive resorption of the connective tissue of the root[18].

Recent studies on biochemical characterization of PDL cells in vitro indicated that these cells have osteoblast-like properties. These include: synthesis and expression of alkaline phosphatase by PDL fibroblasts with a level comparable to that of small intestine, alveolar bone, and kidney [19]; production of an increased level of cAMP in response to parathyroid hormone [20]; and increased synthesis of bone-associated proteins in response to 1,25 dihydroxyvitamin D 3 [20].

An in vitro study on PDL cells has demonstrated that they are capable of forming mineralised nodules. Interestingly it was observed that the nodules displayed different morphological characteristics compared to mineralised tissue formed by bone cells in culture. Three dimensional nodules containing mineralized matrices were formed only when the cells were cultured in the presence of ascorbic acid and dexamethasone. They were composed of multilayered fibroblasts, and highly organised collagen fibrils with cross-banding patterns between the cell layers. The fibroblasts displayed morphological characteristics of PDL fibroblasts as seen in vivo. Mineral deposition with needle-like

crystals was initiated on collagen fibrils located in intercellular spaces of the upper cell layers and became increasingly heavier towards the bottom half of the nodules. X-ray microanalysis and electron diffraction analysis confirmed that mineral deposition contained calcium and phosphate in the form of immature hydroxyapatite. The nodules contained neither osteoblasts nor osteocytes, and had their own morphological organization and characteristics which differ from those formed by bone cells in culture. The authors speculated that the unique mineralised nodules may represent a different form of hard tissue, possibly acellular cementum[21].

More recently, real-time RT-PCR analysis of PDL cells under tensile strain showed upregulation of genes linked to the osteoblastic phenotype. Furthermore, the PDL cells were also found to constitutively express numerous osteotropic cytokines and growth factors[22, 23].

There is now evidence that populations of cells within the PDL, both during development and during regeneration, can secrete other molecules that can regulate the extent of mineralization and prevent the fusion of tooth root with surrounding bone, e.g. ankylosis. Among these molecules, a balance between the activities of bone sialoprotein and osteopontin may contribute to establishing and maintaining an unmineralised PDL region. Matrix Gla protein is also present in periodontal tissues. Based on its role as an inhibitor of mineralization, it may also act to preserve the PDL width[2]. At the genetic level, in vitro cell culture has shown that *Msx2* gene prevents the osteogenic differentiation of mouse PDL fibroblasts by repressing *Runx2* transcriptional activity[24].

Msx2, thus, may play a central role in preventing ligaments and tendons, in general, from mineralizing.

Overall, it seems that PDL is brimming with cells with osteogenic potential. There are regulatory factors within the PDL that play a role in the maintenance of the PDL space. Events such as injury and orthodontic tooth movement can result in change in the homeostasis and result in either maintenance of the PDL or mineral deposition leading to ankylosis. The factors that trigger the various events in periodontal width homeostasis during these events still need to be ascertained[2].

However, it is known that under certain conditions bone can be replaced by a functionally oriented PDL of normal structure and architecture, provided that the progenitors for PDL fibroblasts can repopulate the site. Masticatory function can accelerate the resolution of ankylotic areas and restoration of normal PDL width[25, 26].

5.1.3 Cementum

Cementum is a non-uniform, avascular, non-innervated, mineralized tissue covering the entire tooth root surface. The main function of cementum is to anchor the principal collagen fibres of the PDL to the root surface. It also has important adaptive and reparative functions, playing a crucial role to maintain occlusal relationships and to protect the integrity of the root surface[2, 27-29]. Cementum is also critical for the appropriate maturation of the periodontium, both during development as well as that associated with the healing and regeneration of periodontal tissues [29]. However, very little is known of the differentiation mechanisms of cementoprogentior cells and the cell dynamics during normal development repair and regeneration.

During growing and initial periodontal wound healing, new cementoblasts must be generated or recruited. It is likely that new cementoblasts take their origin in the same root related portion of the intact PDL. The precise origin and the molecular factors regulating new cell recruitment and differentiation are not known[27, 30].

5.1.3.1 Classification

Traditionally, cementum has been be classified based on the presence or absence of cells (cellular versus acellular) and the source of collagen fibres (extrinsic versus intrinsic)[2, 29]. There are three main types of cementum, which are: Acellular extrinsic fibre cementum; Cellular intrinsic fibre cementum; and Cellular mixed stratified cementum.

Acellular extrinsic fibre cementum (AEFC), also known as primary cementum, is found in the cervical half to two-thirds of the root, covering 40%-70% of the surface. It develops very slowly and is considered acellular as the cells that form it remain on the surface. The very high number of principal PDL fibres inserting into the AEFC (approximately 30,000 fibers/mm^2) shows its important function in anchoring the tooth to the PDL[2, 12]. The orientation of the Sharpey's fibres is subject to changes throughout life, due to post-eruptive tooth movement. These changes in orientation are reflected by individual AEFC layers that are interfaced by growth, resting, or incremental lines. AEFC grows very slowly, but at a fairly constant rate. The slow rate of formation, the absence of cementocytes, and the densely aggregated and parallel-oriented Sharpey's fibres account for the very uniform morphological appearance of the AEFC and make it a unique tissue[12].

Cellular intrinsic fibre cementum (CIFC) is characterised by intrinsic collagen fibres and the presence of cementoblasts entrapped in lacunae within the matrix they produce (cementocytes) [2]. CIFC is distributed along the apical third to half of the root, in furcation areas, old resorption sites and root fracture sites. The collagen fibrils of CIFC are intrinsic as they do not protrude from the cementum into the PDL space. Thus, CIFC has no direct function in tooth attachment. [12] CIFC has an important role as an adaptive tissue that maintains the tooth in its proper position and in the repair process of previously resorbed roots. This is due to its ability in being able to fill a resorptive defect in a reasonable period of time[2, 31]. The rapid speed of formation and the presence of cells and lacunae may be the reason why this cementum variety is less well mineralized than AEFC. The structural organization of the cementum matrix and the presence of cells in it give cellular intrinsic fibre cementum a bone-like appearance[2]. This cementum is also known as secondary cementum.

Cellular mixed stratified cementum (CMFC) is found in humans in the apical one- to two-thirds of roots and the furcations. This is comprised of a stratification of consecutively deposited, alternating layers of acellular extrinsic fibre cementum and cellular intrinsic fibre cementum. The intrinsic part of CMSC may exert an adaptive function, while the extrinsic part may contribute to tooth anchorage to the surrounding alveolar bone[12]. CMSC is not found in rodent molars but is always found in human teeth. The mineral density of CMSC is similar to bone[2].

5.1.3.2 Biochemical composition

The composition of cementum resembles that of bone. By volume, inorganic material comprises approximately 45%, organic material 33% and water 22%[32]. The principal inorganic component is hydroxyapatite. The hydroxyapatite crystals are thin and plate-like and similar to those in bone. Type I collagen is the predominant organic component, constituting up to 90% of the organic matrix. Other collagens associated with cementum include type III, a less cross-linked collagen found in high concentrations during development and repair/regeneration of mineralized tissues, and type XII, a fibril-associated collagen that binds to type I collagen and also to noncollagenous matrix proteins. The noncollagenous matrix proteins are similar in bone and cementum. They fill in the spaces between the collagen fibrils, regulate mineral deposition and impart cohesion to the mineralized layer[2].

5.1.4 Alveolar bone

Alveolar bone may be defined as the bone of the upper and lower jaws lining the sockets of the teeth. The presence of an alveolar bone along the entire tooth socket separates the support bone anatomically and functionally from the PDL[2]. Alveolar bone comprises the alveolar process, which is an extension of the basal bone of the jaws. The alveolar bone forms in relation to the teeth, but structurally it is similar to, and continuous with, the basal bone[33]. It is a mineralized connective tissue. By volume it is about 36% inorganic material, 36% organic, and 28% water. The mineral phase is hydroxyapatite, in the form of needle like crystallites or thin plates. 90% of the organic material is present as type I collagen. In addition, there are small amounts of proteins (e.g. osteocalcin, osteonectin, osteopontin and proteoglycans).

5.1.4.1 Composition

1) Compact (cortical) bone. These are the outer buccal, lingual and palatal cortical plates.
2) Cancellous (trabecular) bone. This occupies the central part of the alveolar process and consists of bony trabeculae with yellow marrow rich in adipose cells filling the intertrabecular spaces.
3) The alveolar wall. This bone lining the tooth socket is specifically known as bundle bone as it provides attachment for the PDL fibres[2, 34] . The presence of the bundle bone along the entire tooth socket separates the support bone anatomically and functionally from the PDL[34] .

5.1.4.2 Function

Alveolar bone is specialized for tooth support, with a rapid rate of turnover and lost in the absence of a tooth. This facilitates positional adaptation of teeth in response to functional forces and the physiological occurrences (such as mesial drift of teeth in humans and primates and bucco-distally in rodents) and tooth eruption that occur with the development of the jaw bones[1, 33, 34]. It also serves as a major reservoir of calcium[12].

5.1.4.3 Anatomy and microstructure

As a result of physiological tooth movement, the tooth socket is spatially oriented: the side of the socket in the direction of migration, is irregular and scalloped by numerous lacunae of various lengths and depths (the resorbing side), while the opposite side is regular and smooth (the apposition side). On the resorbing side of the tooth socket, as

part of bone remodelling, resorption, reversal and formation phases may be identified[34, 35].

The bundle bone consists of successive layers of coarse fibered woven bone fibres running parallel to the socket wall and arranged in lamellae[10]. It is thin and ranges between 100μm and 200μm in humans. The bundle bone may appear thicker on one side of the socket (the apposition side), but only its outer layer is functional, with the presence of regularly spaced cement lines which interrupts the course of the extrinsic fibres[34]. Embedded within this bundle bone, perpendicular to its surface, are the extremities (Sharpey's fibres) of the extrinsic collagen fibre bundles of the PDL. When bundle bone has reached a certain thickness and maturity, parts of it are reorganized into lamellar bone, with finer fibrils in its matrix[36].

The cortical plates and bone lining the alveolus meet at the alveolar crest. The cortical plates consist of surface layers (lamellae) of fine-fibre bone supported by Haversian systems. The trabecular bone occupying the central part of the alveolar process also consists of bone disposed in lamellae, with Haversian systems present in the larger trabeculae. Yellow marrow, rich in adipose cells, generally fills the intertrabecular spaces[2].

5.1.4.4 Osteogenic Cells

Bone formation, maintenance and resorption are regulated by osteoblasts, osteocytes, bone-lining cells and osteoclasts.

5.1.4.4.1 Osteoblasts

Osteoblasts are 15-30μm, cuboidal or columnar cells of mesenchymal origin that typically form a single layer that covers all periosteal or endosteal surfaces where bone formation is active. They are the most active secretory cells in bone and are primarily responsible for the production of the organic components of bone matrix[33, 37]. Their nucleus is round, and when active, their cytoplasm is filled with a prominent Golgi complex and abundant rough endoplasmic reticulum.

In areas of active bone remodelling, typical osteoblasts lay down bone matrix over a densely stained reversal line[34]. Active osteoblasts secrete type I collagen, the principal organic component of bone matrix, along with a variety of non-collagenous proteins which make up to 5 per cent of the organic matrix, including osteocalcin, phosphorylated glycoproteins, bone sialoproteins and proteoglycans. Expression of alkaline phosphatase by osteoblasts appears to reflect bone formation activity, although the precise function of this enzyme in bone is uncertain[37].

5.1.4.4.2 Osteocytes

This is the most abundant cell type in bone tissue. In mature bone, about 95% of total bone cells are osteocytes. Osteocytes are the only cell type to be embedded within the bone matrix[38]. Following maturation, osteoblasts may undergo apoptosis, become progressively encased in osteoid and then into mineralized matrix as osteocytes or remain on the bone surface as bone lining cells[3]. Osteoblasts that become osteocytes occupy spaces (lacunae) in bone and are defined as cells surrounded by bone matrix, whether mineralized or still part of the osteoid seam. Osteocytes are smaller cells than osteoblasts,

and have a decreased quantity of synthetic and secretory organelles, with the nucleus occupying a significantly larger proportion of the cell. A major feature of osteocytes is the presence of numerous and extensive cell processes that ramify throughout the bone in canaliculi and make contact, frequently via gap junctions, with processes from other osteocytes or with similar processes extending from osteoblasts or bone lining cells at the surface of bone[3]. The physiological function of the osteocyte with its 3D network throughout bone is hypothesized to be the regulation of the exchange of mineral ions between interstitial fluid and extracellular fluid, working with the bone-lining cells and maintaining a local mineral ionic environment that is suitable for bone matrix mineralization[38].

5.1.4.4.3 Bone-lining cells

The third cell type belonging to the osteoblast family, the bone-lining cells are also known as resting osteoblasts. Bone lining cells are about 1 micron thick with a 12-micron diameter, with a thin, flat nucleus and attenuated cytoplasm. They appear as a near confluent, flattened single layer over quiescent bone surfaces[37]. These cells have a reduced capacity for protein secretion with a relative paucity of organelles.

Bone-lining cells are derived from surface osteoblasts when they have completed their historical role as bone forming cells. The ultimate fate of bone-lining cells is presumable death by apoptosis[33]. They are connected to one another and to the osteocytes. During the quiescent period, bone lining cells, together with a 1μm underneath layer of osteoid, serve as a barrier to protect bone surfaces from inappropriate resorption by osteoclasts or other inflammatory cells[39]. These cells cover most quiescent bone surfaces in the adult

skeleton which are a primary site of mineral ion exchange between blood and adult bone. Together with osteocytes, bone lining cells and their connecting cell processes appear to form a homeostatic network of cells capable of regulating the plasma calcium concentration.

5.1.4.4.4 Osteoclasts

Osteoclasts are large, multinucleated, highly motile cells formed by the fusion of mononuclear precursor cells of hematopoietic origin[33].They are usually more than 100μm in diameter and normal osteoclasts contain up to 10 nuclei. They are the principal bone-resorbing cells, responsible for the degradation and removal of the inorganic and organic components of the bone matrix[39].

Osteoclasts are relatively sparsely distributed in bone. They represent the end point of a differentiation process and are not themselves capable of proliferation[37]. The most striking feature of osteoclasts is the presence of a clear zone in the peripheral cytoplasm of the cell that delineates a more central region of convoluted membrane infoldings and finger-like processes termed the 'ruffled border'. The clear zone is free of organelles and is also known as the 'sealing zone', as the plasma membrane in this region comes into tight apposition with the bone surface during resorption so the site of resorption is isolated and localized. Functionally, the ruffled border represents the resorbing apparatus of the cell. This 'ruffled border' allows it to achieve a high cell surface area where secretion of enzymes and uptake of matrix components can take place[39]. Resorption of bone occurs in a tightly sealed acidified extracellular matrix compartment as a result of

the combined actions of a variety of ruffled border membrane associated enzymes including a tartrate-resistant, acid adenosine triphosphatase[33].

5.1.5 Gingiva

Gingival epithelium has a protective role in resisting the insults produced by bacteria, chemicals, and trauma and plays a role in tooth attachment through the collagen fibre groups within the gingiva.

The free and attached marginal gingiva and sulcular epithelium are covered by a stratified squamous epithelium. Approximately 60-65% of the connective tissue compartment of healthy gingiva is occupied by collagen, with the individual fibrils organized into discrete fibre bundles. These gingival fibres provide tone and resistance to the free gingival margin and provide the most coronally positioned connective tissue attachment to the tooth surface[1].

5.1.6 Features of the periodontium unique to rodents

The rat has a cementum layer covering the enamel surface, also known as coronal cementum, which may be cellular-fibrillar, acellular fibrillar, or acellular afibrillar[40]. The absence of a secondary dentition may also predispose to the prevalence of root resorption observed in rat molars[41]. The teeth are monophyodont. Incisors and molar teeth are separated by a wide interdental space (diastema). The size of the molars decreases from M_1 to M_3. M_1 has 5 roots, M_2 four, and M_3 three in the upper jaw. Cementum covers one-third to one-half of the roots. The enamel folds are free of cementum[42]. The molar teeth of the rat migrates bucco-distally throughout its lifespan[34, 43, 44]. The mesial surface is predominantly formative while the distal is more resorptive[35]. The volume density of

cells (fibroblasts) in different parts of the PDL of rat maxillary molars does not vary, but the number of cell nuclei per mm^2 in the mesial root of the mouse first mandibular molar does[45].

Unlike the repair of resorption lacunae in human teeth with cellular intrinsic fibre cementum[31], in rodents, acellular cementum[6] or cellular cementum[46] can be deposited within these resorptive defects. Kimura hypothesized that this may be because cementoblasts in rats have a relatively high level of activity.

Therefore, certain aspects of cementogenesis and cementum biology (such as attachment mode of cementum to dentin, the rate of cementum apposition) seem to differ between rodents and large mammals, including humans[30]. Data derived from animal models must be used with caution before conclusions for human applications are drawn[11].

There are species variations in the PDL. In mice, the width of the PDL was found to be uniform around the mesial root of the lower first molar in both young and old mice throughout the apico-occlusal length of the root[16]. The PDL volume in rats around buccal roots of the lower first molar also maintain a constant volume during distal drift[35].

5.2 DENTOALVEOLAR ANKYLOSIS

Dental ankylosis is defined as fusion of the cementum or dentine with alveolar bone[47].

The loss of the PDL in the ankylotic area, results in bony fusion between the tooth and the alveolar bone. The tooth is incapable of continued eruption and hence is unable to follow the normal vertical development of the neighbouring teeth resulting in infraocclusion and an incomplete development or the alveolar process[48]. Normal mesial drift of teeth may also be prevented as a result of ankylosis[49].

The development of ankylosis is a common complication when a traumatized tooth, e.g. avulsion or intrusive luxation[50], is replanted after prolonged extraoral dry storage[51, 52], storage in an unsuitable medium[53], or partial/complete removal of the periodontal membrane[51, 54]. Ankylosis can occur following autotransplantation[54], and has also been associated with disturbances of eruption[47, 55, 56].

5.2.1 Epidemiology

Ankylosis is most common in deciduous molar teeth, although it can also occur in permanent teeth, with the first molar being the most likely affected tooth[57]. It has also been reported that the incidence of ankylosis is twice as frequent in the mandible as the maxilla[48].

In a study on the prevalence of infraocclusion of primary molars in 1059 Swedish children aged 3-12 years with an even distribution between the age groups, the primary mandibular molars were affected more than 10 times as often as the maxillary molars[58].

5.2.2 Trauma

Teeth which have been traumatised, particularly if they have been avulsed or luxated, have a high incidence of ankylosis[59]. Andreasen[53] has reported that root resorption is a potential late complication following dental luxation injuries, with external root resorption much more common than internal resorption. External root resorption can be classified into three types: surface, inflammatory and replacement resorption. The latter type is characterised by direct contact between bone and tooth root, with gradual replacement of tooth hard tissue by bone. The author uses the term replacement resorption when describing the condition of ankylosis[53, 59].

5.2.3 Eruption

Biederman[47] defined three potential causes of cessation of eruption, these being physical obstruction (i.e. impaction), destruction or defect of the dental papilla and ankylosis. Presently, failure of eruption is considered to be due to either as a result of mechanical obstruction (idiopathic or pathological), or because of disruption to the eruptive mechanism[60].

5.2.3.1 Primary Failure of Eruption

The term "Primary failure of eruption" (PFE) was coined by Proffit and Vig (1981) in which non-ankylosed teeth fail to erupt fully or partially because of malfunction of the eruption mechanism.

The primary identifying characteristic is failure of an affected tooth to move along the eruption path that has been cleared for it. Involved teeth can erupt partially and then cease to erupt, becoming relatively submerged although not ankylosed. Only posterior teeth are

affected, so the result is a spectacular posterior open bite, and all teeth distal to the most mesial affected tooth also are affected. Patients with primary failure of eruption may or may not have teeth which have emerged from the alveolar bone.

PFE displays the following characteristics[55]:

1. Posterior teeth are more commonly involved than the anterior
2. Involved teeth may erupt into initial occlusion and then cease to erupt further, or mail fail to erupt entirely
3. Both primary and permanent molars may be affected
4. Involvement may be unilateral or bilateral
5. Involved teeth tend to become ankylosed
6. Application of orthodontic force in an attempt to bring the affected tooth into the arch leads to ankylosis rather than normal tooth movement
7. Condition tends to occur in isolation, with an absence of affected family members.

The aetiological basis for PFE is unclear. However, the observation that PFE can occur in families suggests that the developmental disturbance leading to PFE is heritable[61]. Recently, DNA testing conducted on four families, each with at least two members affected by non-syndromic PFE in successive generations, revealed that familial, non-syndromic PFE is caused by heterozygous mutations in the gene encoding the G protein-coupled receptor for parathyroid hormone and parathyroid hormone-like hormone (PTHR1). Three distinct mutations were identified in 15 affected individuals from four

multiplex pedigrees. In all cases, the mutations truncate the mature protein and should lead to premature proteolytic degradation of the precursor protein or to a functionless receptor, thus suggesting that haploinsufficiency of PTHR1 is likely to be the underlying principle of non-syndromic PFE[62].

All these factors present a diagnostic challenge to the clinician. In the absence of any clear genetic, pathological, or environmental factor being responsible for preventing the eruption of a permanent tooth, a definitive diagnosis of PFE might only be made retrospectively, following the failure of orthodontic extrusion to alter the position of an affected tooth or teeth[63].

5.2.3.2 Primary and Secondary Retention

The terms, 'primary retention' and 'secondary retention' have been used in conjunction with PFE. Primary retention of permanent teeth is an isolated condition associated with a localized failure of eruption but no other identifiable local or systemic involvement. Secondary retention on the other hand, is the unexplained cessation of eruption after a tooth has penetrated the oral mucosa without the evidence of a physical barrier in the path of normal eruption or as a result of abnormal position[64]

The aetiology of secondary retention is unknown. Ankylosis has been suggested as the main factor[48]. Scanning electron microscopy (SEM) and light microscopy (LM) investigations on secondary retained molars showed that ankylosis was present on all teeth. Ankylosis was generally localized to the bifurcation area and at the interradicular root surface. The authors concluded that ankylosis was and important factor in secondary

retention however they could not determine whether the eruption mechanism was disturbed before or after ankylosis began[65].

Other related factors reported in the literature are hypercementosis[66], primary failure of eruption[55], and genetic factors[67]. The biological mechanism involved in the development of ankylosis in cases of secondary retention has never been demonstrated, but a genetically determined or congenital gap in the PDL, a disturbance in the local metabolism, trauma or injury and tongue pressure during swallowing have been suggested[47, 48, 57].

Rare cases of spontaneous re-eruption after secondary retention have been reported in the literature[68, 69]. There has been no explanation for this unusual phenomenon. Histologic investigations have shown that in secondary retention, ankylosis is often restricted to few small areas of the root surface. It has been implied that, because of a constant bone remodelling of the alveolar process, the bony union could be resorbed. That would lead to release of the secondarily retained molar, and allow eruption to reach its normal occlusal level in contact with its antagonist[69].

5.2.4 Aetiology

The aetiology of ankylosis is largely unknown. Three causes of dental ankylosis were suggested by Biederman: congenital gaps in the PDL; local PDL trauma and disturbed local metabolism[47].

Congenital gaps in the periodontium could realistically only be an explanation for un-emerged primary dentition (primary retention), but ankylosed unmerging teeth are considered to be rare[57].

Local injury to the PDL, followed by ossification during the healing process may lead to ankylosis. Early experimental work did not produce ankylosis via direct pressure or trauma, although success was achieved using extraction and replantation. Thus trauma and excessive pressure were not considered to be likely causes of ankylosis [47]. However, traumatic injuries to the teeth which result in a defective PDL are associated with the development of ankylosis[70 53].

Prior to exfoliation of deciduous teeth, resorption of the root occurs first, followed by disappearance of the PDL. Disturbed local metabolism could cause the ligament to disappear before the root has resorbed sufficiently, as a result, the cementum and alveolar bone could potentially come into contact and thus lead to ankylosis[47].

Raghoebar et al[56] discussed the possibility of a disturbance of the interaction between normal root resorption and hard tissue repair as a potential cause of ankylosis. In their study of secondary retention of permanent molars, physiologic local root resorption was occasionally observed in normal teeth. This resorption was repaired by new cementum formation and the root shape was re-contoured. It was suggested that a disturbance of this repair process could occur whereby the usual cementoblasts are replaced by osteoblasts, with osteoid material being deposited within the resorption lacunae and the possible development of ankylosis. Molars usually exhibit the largest number of resorption areas, which may explain the prevalence of ankylosis in this tooth type compared to all other

permanent teeth. The authors conclude that a developmental problem within the PDL may be the reason this type of ankylosis occurs.

Kurol[58] performed a study of 138, 3-12-year-old siblings of 109 children with infraocclusion (secondary retention). The prevalence of infraocclusion was found to be 18.1%. When compared with the frequency in the total material (8.9%), the difference proved to be significant, supporting the hypothesis that there is a familial tendency in infraocclusion of primary molars. As there is a relationship between secondarily retained molars and ankylosis[56] familial tendency could be extended to ankylosis.

Homeostasis between the PDL fibroblasts and the bone cells lining the inner aspect of the alveolus has been proposed as one of the ways that the width of the ligament may be maintained[71]. It has been suggested that the cells of the PDL are able to inhibit osteogenesis, thus preventing ankylosis[18]. When homeostasis between the PDL cells and bone cells is interfered with, ankylosis results. This was accomplished in one study via the administration of the drug 1-hydroxyethylidene-1, 1-bisphosphonate (HEBP)[71]. The possible actions of this drug include inhibition of bone resorption, an increase in bone matrix formation, and a cytotoxic effect on the PDL fibroblasts. When HEBP was administered to experimental rats, a significant decrease in PDL width was noted, with ankylosis evident after thirty days[71].

Finally, temporary or permanent disruption of the nerve supply to a particular oral region has been suggested as a possible cause of primary and secondary retention of permanent teeth. Disruptions of this sort may be associated with herpes zoster and mumps infections, with spread of the virus along nerve branches. This hypothesis was suggested

particularly in cases where more than one permanent tooth exhibited primary or secondary retention[72].

5.2.5 Experimental Ankylosis

Experimental models of ankylosis have detailed study into the causes, histology and pathogenesis of this condition. Several methods have been utilized such as: trauma, extraction and replantation, thermal and chemical or pharmacological models. The common factor in these methods is the production of some kind of damage to the PDL tissue, whether direct or indirect.

5.2.5.1 Trauma

In a radiographic and histologic study on dogs, Parker et al[73], attempted to induce ankylosis by mechanically injuring the tooth root with a dental bur and then removing the teeth from occlusion and splinting them. Ankylosis was found in only one of the dogs. A similar protocol was used on monkey teeth in another study, once again no evidence of ankylosis was found[74]. In the same study, the authors also tried occlusal trauma, chemical trauma and luxation. Luxating the tooth to the point that it was mobile in all directions whilst still remaining within its socket was the only technique found to produce ankylosis.

5.2.5.2 Extraction and replantation

Experimental models have involved extraction of teeth, disturbance of the PDL cells by mechanical means or air drying, followed by replantation have consistently resulted in ankylosis formation[57,51, 52, 75].

5.2.5.3 Pharmacological models

Wesselink and Beertsen[71,26] attempted to disrupt the normal homeostasis between the PDL cells and those lining the alveolar bone of the tooth socket though the administration of the drug HEBP. HEBP treatment caused a significant decrease of the width of the PDL space which was influenced by time, Ankylosis started to occur after 30 days, predominantly in the interradicular areas and was more common around unopposed teeth. These results were also confirmed by Alatli et al[76].

5.2.5.4 Disruption of innervation

Berggreen et al[77] investigated the healing responses or the periodontium after denervation and replantation of teeth. In an experiment using ferrets, the authors produced denervation via axotomy of the inferior alveolar nerve on one side, with the opposite side serving as a control. The lower first premolars were then extracted and replanted. Histologic examination revealed that resorption of the roots of replanted teeth was greater on the innervated side, but the incidence of ankylosis was similar between the innervated (4) and the denervated side (6). They concluded that the sensory nerves promoted root resorption after pulpoperiodontal injuries but had less influence on the osteoblastic activity expressed by ankylosis.

In a more recent study by Fujiyama et al[78] however, ankylosis was reliably produced after transection of the inferior alveolar nerve in rats. The ankylosis, which was observed after six weeks, was found in the coronal region of the PDL, and it was also noted that root resorption was activated after denervation.

5.2.5.5 Thermal injury

Thermal injury as a means of inducing experimental ankylosis was utilised as early as the 1930s, where Gottlieb and Orban used electric diathermy on dog molars[74].

Wesselink et al[79] applied liquid nitrogen to the outer surface of the lower jaw of the mouse in order to freeze the periodontal tissues of the incisor tooth. When light and electron microscopic examination was carried out after this treatment, cell death within the periodontal tissue was noted. After 7-12 days following cold application, extensive root resorption and some ankylosis between the tooth and the alveolar bone were observed.

Tal and Stahl[80] subjected surgically exposed buccal alveolar plates of the first mandibular molar in 15 adult rats to an ultralow temperature using a cryoprobe, cooled to -81 degrees C for 5 seconds. Marked root resorption and reparative cementum were noted after five to seven weeks, some areas of ankylosis was also seen. However a similar experiment on dogs yielded no ankylosis[81].

Dreyer et al[46] refined the earlier methods of thermal insult using a cold stimulus in order to limit the associated injury to the PDL rather than the surrounding tissues. The technique consisted of the application of pellets of solid carbon dioxide (dry ice) continuously for a period of ten or twenty minutes to first molar crowns of rats, with a second group of animals subjected to three episodes of freezing. A further group also underwent mechanical trauma to the PDL. Histologically, two days after freezing, the teeth showed minor root resorption near the apex with only mild signs of fibre disorganisation and hyalinisation apparent within the PDL. By seven days, shallow

resorption lacunae, localised to the cervical and interradicular areas, were observed with associated multinucleated cells. Marked PDL disorganisation and extensive areas of hyalinisation were noted. After fourteen days, the resorption lacunae were larger although the multinucleated cells had reduced in number and signs of cementum repair were present. Active bone resorption was occurring at this stage. By twenty-eight days, active root resorption had ceased and repair of the lacunae was progressing.

The results of this study indicated that a longer freezing time, multiple freezing episodes and additional mechanical trauma generated more extensive injuries to the periodontal tissues. The tissue responses in the group with a single, short freezing episode were dominated by the root resorption and repair processes described above. However, in the groups which received multiple freezing episodes or single long episodes, ankylosis was often noted in the interradicular area and at times was quite widespread. The localisation of ankylosis in the interradicular region was suggested to be related to the difference in thermal conductivity when a stimulus is applied parallel or transverse to the dentinal tubules, as the alignment of dentinal tubules in the interradicular area paralleled the direction of application of the cold stimulus. The development of ankylosis was explained as possibly an effusive reparative response by the tooth and alveolar bone following periodontal tissue destruction by the freezing treatment [46].

The protocol of Dreyer et al[46] was used, with a single twenty minute freezing episode in a study of dentoalveolar ankylosis. It was shown to reliably produce ankylosis within the interradicular area of rats' molar teeth [82]. In this investigation, similar tissue reactions were reported. At seven days after freezing, shallow resorption lacunae were seen along

with some disorientation and hyalinisation within the PDL. These changes were more marked by fourteen days, and ankylosis was observed at this time. By 28 days, root resorption had ceased although ankylosis was still present. Repair of resorption occurred between 56 and 86 days, and ankylosis was noted to be widespread during this period.

Using the same model, Di Iulio et al[83] found a decreased prevalence of ankylosis. The ankylotic bone which was fused to the affected tooth appeared to be separated from the dentine by an intact cementum layer. The ankylotic material also appeared to become more solid with time, progressing from fine bony trabeculae with connective tissue interspersed in the 7 days observation group to solid bone in the 18, 21 and 28 day groups.

5.2.6 Pathogenesis

Ankylosis originates when enamel, dentine, or cementum becomes replaced by bone tissue as a direct extension from the alveolar bone. Pindborg[84] stated that ankylosis could only be established after preceding resorption of the dental hard tissues. In ankylosed teeth, active resorption processes ramifying deeply into dentine and newly formed bone were seen side by side.

In a literature review of ankylosis in permanent teeth, Jacobs[59] stated that ankylosis arose because of damage to the cells on the root side of the PDL. Cells from the bone side of the PDL then migrate and resorb tooth substance.

In a longitudinal histological study, fifty-eight teeth were replanted in four monkeys and six dogs[51]. Thirteen teeth were replanted without periodontal membrane, fifteen were dried in air for varying periods of time, and thirty were re-inserted into the alveolus immediately following extraction. Observation periods varied between 8 days and 33 months. Teeth replanted without periodontal membrane showed establishment of ankylosis was accomplished within 30 days. The teeth that were dried for short periods exhibited minor areas of normal periodontal attachment. In teeth replanted with vital periodontal membranes, the periodontal membrane was always reformed after replantation, ankylosis was never observed. Ankylosis was described as the process in which bone trabeculae, formed on the alveolar wall, gradually growing across the periodontal space and fusing with the root surface. These bone trabeculae then unite and form a solid plate directly upon the intact cementum or the previously resorbed cementum or dentin.

Andreasen could not find support for Löe's observations[52]. In his studies, the histological pattern of initial ankylosis in incisors of monkeys that were extracted and replanted after varying times of extraoral storage (0, 18 or 120 minutes) was observed. Follow up observation periods ranged from 2 to 8 weeks though results were mainly reported for the 2 week observation period in this paper. Two histological patterns of initial ankylosis were described at the 2 week period after 120 minutes of dry storage. In the cervical areas and on the labial and lingual surfaces, the most common finding was a complete mineralization of the entire PDL with the newly formed bone displaying few irregularly arranged osteocytes and no lamellae. On the remaining parts of the root surface, the initial ankylosis area consisted of a layer of bone deposited upon the root surface and

socket wall with an intervening soft connective tissue zone. In some areas bony bridges, formed along the Sharpey's fibre bundle, united these layers and demarcated soft tissue components. The thickness of bone deposition on the root surface was significantly more than the thickness of bone deposited on the alveolar socket wall. As no bone markers were used and only the single time point was measured, it was not possible to determine the direction and rate of this bone formation. The author suggested that the first type of initial ankylosis represented areas where both the cemental and alveolar parts of the PDL were avital; whereas the second type represented areas where only the cemental part of the PDL was avital (i.e., from dry storage). It was concluded that ankylosis after replantation is presumably a response in the periodontium to areas of the PDL and/or the root surface damaged by the extraction procedure or storage conditions prior to replantation. The data from this study also suggested a decrease in the incidence of ankylosis (as seen histologically) over time, as the observation period increased from 2 weeks to 8 weeks, in teeth which had been subjected to the lesser insult of immediate replantation.

Hammarström studied the initiation and progression of dentoalveolar ankylosis of replanted teeth and associated root resorption in monkeys[85]. Maxillary and mandibular lateral incisors were extracted and replanted after an extraoral period of 15 min or 1 hour. The observation periods varied from 2 days to 40 weeks. Irrespective of the length of the extraoral period, initial root resorption and minor areas of ankylosis were found 1 week after replantation. The initial ankylosis was not preceded by root resorption. The ankylosis observed in this group seemed to start with the formation of a bone-like tissue in the central part of the periodontal membrane. This central part then fused

with newly formed hard tissue on the root surface and socket wall. In teeth replanted after an extraoral period of 15 minutes the ankylotic area did not increase with increasing time after replantation. Instead the periodontal membrane was re-established, separating the root surface from the alveolar bone. In teeth replanted after an extraoral period of 1 hour, the initial ankylotic area increased with increasing time after replantation. At four weeks, two variations of ankylosis was observed; in monkeys given antibiotics the ankylosis was of the type that is not preceded by resorption; in the non-antibiotic monkey, there were some ankylotic areas preceded by resorption. Eight weeks and more after replantation, most of the PDL was replaced by bone covered by osteoblasts and occasional osteoblasts that were in continuity with the endosteal cells outlining the marrow spaces of the alveolar bone. The cementum and dentin were then gradually resorbed with increasing time after replantation. Based on the results it was suggested that root resorption associated with dentoalveolar ankylosis is initiated by endosteal osteoblasts and is thus a hormonally regulated process. Similar observations in the development of this replacement resorption leading to ankylosis, where the connective tissue cells of the PDL do not participate in its repair, were also noted in other studies[86, 87]. It has been suggested that the protective cells (cementoblasts) of the root surface are replaced by an osteoblastic type of cell that responds to the normal factors involved in bone remodelling i.e. parathyroid hormone[85, 88].

Histological evaluation of experimentally replanted teeth in monkeys, revealed two morphological types of ankylosis was present[70]. The first type of ankylosis was preceded by resorption of cementum and dentine and no cementum was found at the ankylosis site. In the second type, apposition of bone directly on the cemental surface had occurred,

without previous resorption of the cementum. The connections of the ankylosis to the surrounding alveolar bone consisted of either thin bony trabeculae or of wide bony areas.

Research on the distribution of periodontal epithelial cells and nerve fibres within the furcation of rat maxillary molar teeth[83] utilised the thermal injury technique as outlined by Dreyer [89]. Ankylosis was observed on several teeth. The ankylosis observed in the study was of the type where ankylotic bone which was fused to the affected tooth appeared to be separated from the dentine by an intact cementum layer. This is similar to the second type ankylosis as described above[70]. Resorption did not usually directly affect the area of ankylotic union, although it was often seen in adjacent sites. The ankylotic material appeared to become more solid with time, progressing from fine bony trabeculae with connective tissue interspersed in the 7 days observation group to solid bone in the 18, 21 and 28 day groups. The 18 days observation group was particularly notable for the fact that the entire furcation was filled with dense woven-bone type material and the only visible PDL was along the buccal and palatal roots. Interestingly, at 21 and 28 days, the ankylotic bone was quite solid, without connective tissue cells interspersed, but the contact area between it and the root surface was usually small. It was suggested that that may represent a regression of the ankylotic union similar to that noted by Hammarström et al[85].

5.2.7 Transient ankylosis and periodontal wound healing

The PDL contributes cells not only for its own repair but also to restore lost bone and cementum[2]. Melcher[18] proposed that cells of the PDL and their progeny possess the

ability to inhibit osteogenesis. If after injury to a portion of the ligament, the connective tissue cells which repopulate the wound are from the progeny of the PDL cells, normal PDL structure develops. If a portion of the wound is colonized from a source outside the ligament, ankylosis may occur. This hypothesis was supported by the observation of Line[87] who used labelled cells (as a marker of cellular activity) to observe the origin of cells from 3-31 days subsequent to an induced thermal injury to the periodontium. Within the ankylotic area, the bone marrow channels were widened in the early time periods which seemed to suggest the bone marrow areas as the major repopulating sites in the events preceding the onset of ankylosis.

Ankylotic fusion in experiment models can either be transient or permanent. In an in vivo study, ankylosis induced in rat molars' roots by means of surgical damage to the cervical vestibular bone, periodontal membrane and cementum of teeth was temporary and eliminated 21 days after surgery by resorption as seen histologically[68]. The resorption apparently started from intact parts of the periodontal membrane located apical and coronal to the injured area, with the removal of newly formed bone by osteoclasts. This suggested the importance of a vital PDL in initiating repair processes.

In Hammarström's[85] study on replanted monkey incisors, in the shorter extraoral time period of 15 minutes, a transient type of ankylosis was noted in the first weeks, characterized by the formation of an immature 'callus-like' hard tissue in the periodontal membrane of most teeth. This newly formed hard tissue seemed to regress with time, as it was not a common finding in the respective teeth after longer observation periods. Hammarström hypothesized that the viability of the remaining periodontal membrane

seemed to be critical for the regression of the ankylosis and its removal was similar in concept to the removal of calluses and the regaining of morphology and function after healing of a bone fracture.

Experiments in dogs[90], monkeys[52, 54, 85, 87, 91] and rodents [46, 71] have shown that when PDL cells are injured, removed from the cementum by mechanical means or displaced or altered under the influence of certain compounds, such as the bisphosphonate 1-hydroxyethylidene-1, ankylosis may occur. Bone tissue invades the PDL space and establishes a direct connection between the tooth and the wall of the alveolar socket. This non-resilient type of tooth support usually leads to loss of function and to resorption of the tooth root. The failure of homeostatic mechanisms to regulate PDL width in these non-physiological conditions, resulting in destruction of the PDL and subsequent ankylosis, suggests that the regulatory mechanisms for the maintenance of PDL width are expressed by PDL cells[86].

These experiments have also shown that in certain situations, localized ankylotic areas can be removed and the integrity of the ligament restored when PDL fibroblasts or their progenitors are allowed to gain access to the root and repopulate the area[3]. These cells may come from the adjacent PDL and perhaps also from endosteal spaces in the alveolar process but probably cannot differentiate from gingival cell populations. Invasion of PDL fibroblasts in an ankylosed site must be preceded by cells that have the capacity to resorb bone and/or cementum (such as osteoclasts). A new PDL space may be created at the cost of the cementum (and sometimes part of the dentin) and also of the alveolar process.

Shortly thereafter, this space is colonized by PDL cells that form new PDL fibres, new cementum and a new alveolar wall in which the fibres insert[26].

Lindskog & Blomlöf [92] analysed the different mineralized tissues around the dental root of the monkeys' incisors and premolars following treatment of different periodontal pathosis. Four distinctly different appearances of the mineralized tissue layers on the marginal dentine surfaces were described:

1. new cementum
2. Non-attached bone-like tissue. Even after accounting for occasional artifactual splits between the dentine and the bone-like tissue during the histo-technical procedure, it appeared that this bone-like tissue was likely only loosely apposed to the dentine surface. On occasion, an intervening layer of tissue debris was seen towards the root surface. This indicated that the mineralized tissue had formed not directly on the dentine surface, but within the periodontal space, close to the root surface and only appeared associated with the root after an extended healing period. It was hypothesized that this may have been formed by cells with an osteoblast phenotype from the pool of undifferentiated mesenchymal cells in the periodontal membrane.
3. partly attached bone-like tissue
4. ankylosis preceded by root resorption

He concluded that healing in the periodontal/root interface following periodontal therapy yielded several different mineralized tissues, depending on a number of host-specific and external factors. When the regenerative potential of the remaining periodontal connective

tissue is impaired or absent, a complete ankylotic fusion would occur. This was likened to a scar tissue formation. The pattern of the possible healing process within the PDL is summarised below (Figure 1).

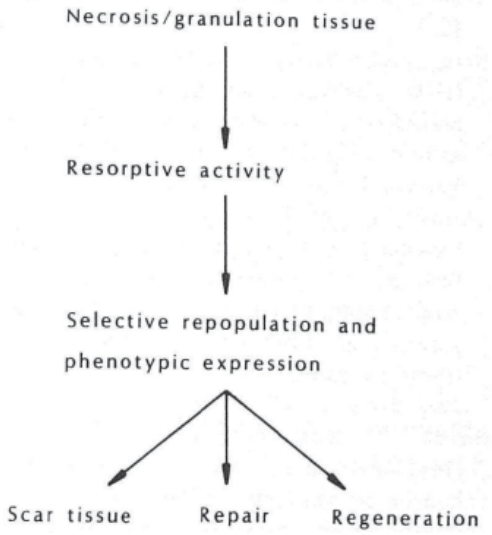

Figure 1. The temporal pattern of healing processes in the periodontal/root interface following periodontal therapy. The different mineralized tissues that are formed depend on a number of host-specific and external factors. Apart from scar tissue formation (ankylosis and epithelial down-growth), most of the mineralized tissues found on the root surfaces present evidence of a limited repair process. The only exception is new cementum, found commonly after cellular resorption on the root surface, for which the term regeneration should be reserved[92].

The nature of the signals that initiate neither this repair process nor the molecular mechanism associated with cellular interactions is clearly understood. In vivo studies have underlined the active role of PDL fibroblasts as regulators of PDL width[7]. In vitro studies have shown that PDL fibroblasts can inhibit mineralized bone nodule formation by rat bone stromal cells[93], and one mechanism may be through the secretion of

prostaglandins which inhibit bone formation[94]. Other cells also may contribute to the regulation of the PDL boundaries. The presence of the epithelial cell rests of Malassez may have a role in maintaining the width of the PDL space, possibly through secreting factors that promote bone resorption[95].

More recently, stem cell testing has shown that all cells of the PDL as well a pulp cells exhibit osteogenic potential. Cultured human PDL cells were subjected to a 12% uni-axial cyclic tensile strain and analysed the differential expression of 78 genes implicated in osteoblast differentiation and bone metabolism by real-time RT-PCR array technology. Genes that were up-regulated included BMP2, BMP6, ALP, SOX9, MSX1, and VEGFA all of which have been linked to the osteoblast phenotype.

This finding may imply that the connective tissue of the PDL may possess the capacity to inhibit osteogenesis[18]. Events such as tooth movement, trauma, pharmacological disturbances to homeostasis and thermal injury, may impair the inhibitory capacity and result in altering the balance toward the formation of mineral tissue i.e. ankylosis.

5.2.8 Spatial distribution of ankylosis

In Andreasen's extraction-replantation model of incisors in monkeys[52], the apical portion of the root in both maxillary and mandibular incisors showed a significantly more frequent occurrence of ankylotic sites compared with the cervical half.

Histological studies of ankylosis in molar teeth have found a high prevalence of ankylosis in the interradicular area, in both human[56, 65, 84] and rodent species[46, 71, 82].

Rygh & Reitan[84] noted in a histological study of replanted teeth in humans that they were nearly always ankylosed and that most of the bone uniting the alveolar process to the tooth was found in the bifurcation. Resorption of the root surface of replanted teeth was not repaired with cementum, but with bone deposition.

Dreyer et al[46] and Shaboodien[82], using a thermal model, found an increased prevalence of ankylosis in the interradicular site of the rats' molar. In histological studies on secondarily retained permanent molars (characterized by the cessation of eruption of the tooth after emergence) in humans compared with controls, the areas of ankylosis were observed mainly in the bifurcation and interradicular root surface[56, 65].

5.3 MINERAL COMPOSITION OF DENTAL HARD TISSUES

5.3.1 Calcium Phosphates

Calcium phosphates are the main constituents of the bones and teeth of vertebrates, inducing most hard tissues of humans. In biological hard tissue, calcium phosphate has two main functions. It provides structural stability to the skeleton by infusing the organic matrix of hard tissue to from a rigid structural material. Secondly, the mineral in bone acts as a storage site for calcium, inorganic orthophosphate, sodium, magnesium, carbonate and other ions. Thus it functions to aid the maintenance of ion homeostasis as well as a detoxifying depository to store ions unwanted in body fluids[96].

Hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, is the prototype of mature bone and tooth mineral, and makes up the primary component of inorganic material in biological hard tissue. It belongs to the apatite group, a family of minerals centred around calcium phosphate with a hexagonal crystal structure. The nature of this hexagonal lattice is that it can expand and contract with isomorphous substitutions resulting in the formation of many other apatite compounds[97]. The basic formula can be described as:

$$A_5(TO_4)_3Z$$

A can be calcium (Ca), strontium (Sr), lead (Pb), cadmium (Cd) or barium (Ba); T can be phosphorous (P), arsenic (As) or vanadium (V); Z can be hydroxyl (OH), fluoride (F) and chlorine (Cl). All these compounds have the same hexagonal structure. Other divalent cations, such as cadmium (Cd), magnesium (Mg), sodium (Na) and zinc (Zn) can

substitute for Ca in the hydroxyapatite structure. However these are smaller than Ca and cause shrinkage in the apatite lattice[97].

5.3.2 Mineralisation of bone

In general, the mechanisms proposed for the infusion of organic matrix of hard tissue with apatite mineral fall into 3 general categories:

A) Raising the saturation in localized volume levels that would cause spontaneous precipitation.

B) Providing substances which create nucleating sites or remove barriers to these sites

C) Removing or neutralising bone mineral inhibitors

All these mechanisms may take place separately or simultaneously, as well as intracellulary and/or extracellulary[96].

During mineralization, initial mineral deposits are deposited in the organic matrix of bone at discrete sites in, or on, the collagen fibrils. It had been hypothesized that the initial phase of bone mineral is not hydroxyapatite, but a precursor[98]. This precursor develops by hydrolysis, followed by transformation into a more apatic phase.

An unsolved problem in the formation of bone apatite is the nature of the precursor mineral phase. Electron diffraction on embryonic chick bone and developing fish fin has shown that the earliest mineral deposited was of a non-crystalline phase[96]. In vitro studies indicate the appearance of an unstable amorphous calcium phosphate (ACP) precursor to

precipitate hydroxyapatite. High voltage electron diffraction of the solid calcium–phosphate mineral phases in embryonic chick bone found that the solid mineral phase particles in the osteoid matrices of the sub-periosteal region of tibiae were principally those of poorly crystalline hydroxyapatite. Two distinct phases other than hydroxyapatite were also identified: brushite ($CaHPO_4 \cdot 2H_2O$) and beta-tricalcium phosphate ($Ca_3(PO_4)_2$) [99]. Octocalcium phosphate ($Ca_8H_2(PO_4)_6 \cdot 5H_2O$)[100, 101], along with Magnesium whitlocke ($Ca_9(MgFe)(PO_4)_6PO_3OH$) and a sodium and carbonate containing apatite [102, 103] have also been considered to be present at the beginning of mineralization of bone and teeth. Octocalcuim phosphate is slowly transformed over time into a heavily carbonated defective hydroxyapatite.

It was once believed that mature, post-embryonic bone mineral was a mixture of amorphous tricalcium phosphate (ACP) and hydroxyapatite (HA). However, a series of experiments on cortical bone of growing rats, rabbits and cows; detected a single phase of bone mineral. This was an apatite which was calcium deficient and containing both CO_3^{2-} and HPO_4^{2-} ions. The authors proposed a general formula to represent the mineral of cortical bone phase:

$$Ca_{8.3}(PO_4)_{4.3}(CO_3)_x(HPO_4)_y(OH)_{0.3}$$

$$\text{With } x + y = 1.7$$

Maturation and age related changes mainly involved decreases of the phosphate ion content which is replaced with carbonate ions while the calcium ions stayed constant. Carbonate and hydrogen phosphate ion contents were noted to vary from one animal to another, and within age of a given species[98, 104]. It is now accepted that mature, post-

embryonic apatite is a poorly crystalline, carbonate-containing analog of hydroxyapatite[105].

5.3.3 Elements and metals in the biologic system

As discussed previously, hydroxyapatite is vulnerable to isomorphic substitutions. The hydroxyapatite of biological hard tissues is not chemically pure, but contains elements that have been taken up from the tissue fluid during initial crystallisation and mineralization. Over time, the concentrations may change by additional uptake or substitution by other ions[27]. Common elements found in biological hard tissues include Mg, F, S and trace elements such as Cu, Zn and Na, these usually reflect dietary history. Other elements, e.g. Pb and Sr, can indicate exposure to environmental hazards.[106]. The amount of ions initially incorporated into the mineral phase reflects their concentration in the fluid environment during mineralization[27].

5.3.3.1 Magnesium

Mg is, following Ca, K, and Na, the fourth highest concentrated cation in the human body [107]. Mg has been implicated with the biomineralisation of bone and tooth and is present in various concentrations within dentine, bone, cementum and enamel [108-112]. Mg may influence biomineralization and mineral metabolism indirectly through bioactive factors such as enzymes[113]. Mg may independently influence bone mineral formation. Mg has been shown to bind to the surface of hydroxyapatite crystals and to retard the growth nucleation and growth of hydroxyapatite[114]. Mg has been demonstrated to compete with calcium for the same absorption site in hydroxyapatite[115]. Thus surface limited

magnesium may play a role in modulating crystal formation in the mineralization process. Crystal size can also be influenced by magnesium content. Crystal size increases as Mg content decreases[116] and vice versa[117]. That exact effect of these findings is still unknown and requires future research.

5.3.3.2 Fluoride

F is an essential trace element which is involved in the skeletal systems of teeth and bone. It has been the subject of extensive investigations in dentistry due to the well documented cariostatic effect.

F can substitute in the hydroxyapatite structure as either F^- or CO_3F^{3-}. If F ions substitute completely for hydroxyl ions on the hexagonal axis, fluorapatite is produced ($Ca_5(PO_4)_3F$). As a result, the substitution brings about a reduction in the volume of the unit cell, the lattice becomes more dense, and chemical stability is greatly enhanced[118].

5.3.3.3 Other elements

A number of trace elements associated with biological hard tissues have been detectable by electron microprobe analysis, in particular Cu, Zn, Na and S[119-121]. However, their influence on apatite distribution and significance has not been covered in any detail in the literature.

5.3.4 Calcium Phosphate Ratio (Ca/P)

The most common elements analysed in dental tissue are calcium and phosphates. Comparisons of Ca/P ratio's between different types of dentine have been used to compare mineralization as the Ca/P ratio, which indicates crystallization of

hydroxyapatite[122]. The molar Ca/P ratio for pure hydroxyapatite, calculated from the empirical formula, is 1.67 (Figure 2). If the theoretical precursors for hydroxyapatite are present in immature hard tissue, i.e. amorphous calcium phosphate, octacalcuim phosphate (Ca/P ratio 1.50 and 1.33 respectively), then the expected Ca/P ratio detected should be lower.

As outlined previously, ionic substitutions of calcium, phosphate or hydroxyl can occur in hydroxyapatite. This will result in altering of the Ca/P ratio. The substitution of Ca, by divalent cations such as Mg, Sr, Pb, Cd, Zn, Na or Ba, may result in reduction the Ca/P ratio. Environmental toxins such as Arsenic will increase the Ca/P ratio as it competes with phosphorous. Thus analytical chemistry of mineral tissue may help in the identification of phases present within a tissue sample.

Calcium Phosphate Phases

		Empirical formula	Molar Ca/P ratio
Dicalcium phosphate dihydrate	DCPD	$CaHPO_4 2H_2O$	1.00
Dicalcium phosphate	DCPA	$CaHPO_4$	1.00
Octacalcium phosphate	OCP	$Ca_8H_2(PO_4)_6 5H_2O$	1.33
β-Tricalcium phosphate	TCP	$Ca_3(PO_4)_2$	1.50
Whitlockite	Mg-TCP	$Ca_{3-v}Mg_v(PO_4)_2\ O \leq v \leq 2$	$3 - v/2$
Amorphous calcium phosphate	ACP	$Ca_9(PO_4)_6 xH_2O$	1.50
Hydroxyapatite	HAP	$Ca_{10}(PO_4)_6(OH)_2$	1.67
Defect apatites		$Ca_{10-y}(HPO_4)_{6-y}(OH)_{2-y}O \leq y \leq 2$	$10 - y/6$
Fluoroapatite	FAP	$Ca_{10}(PO_4)_6F_2$	1.67
Fluorohydroxyapatite	FHAP	$Ca_{10}(PO_4)_6F_z(OH)_{2-z}\ O \leq z \leq 2$	1.67

Figure 2. The potential phases of Calcium Phosphate and their relative molar Ca/P ratios[123]

Age related changes have been documented to have an effect on the Ca/P ratio. It is proposed that some of the potential phases may be amorphous calcium phosphate or octocalcuim phosphate [101]. These phases have a Ca/P molar ratio of 1.3 and 1.5 respectively (Figure 2). The Ca/P ratio would increase with age as the mineral matures towards the ideal hydroxyapatite, which has a Ca/P ratio of 1.67. A study on rat and bovine cortical bone by X-ray powder diffraction analysis found that the Ca/P ratio increased with age (Figure 3)[98]. This finding is in correlation with previous studies[124, 125].

Animals	Ca/P (at 900°C)
Rat	
at birth	1.51 ± 0.003
30 days	1.60 ± 0.003
1 year	1.65 ± 0.002
Bovine	
calf 2 months	1.65 ± 0.004
7 years	1.69 ± 0.010

Figure 3. Age related changes of Ca/P molar ratios in rat and bovine cortical bone [98]

Ca/P ratios of biological hard tissues have been analysed by various techniques and in various species. There is quite a varied range due to differences in analytical technique as well as type and age of specimen used. The table below outlines some of the results:

Author	Ca/P	Tissue	Calculated from:	Analysis	Species
Akesson (2004)[126]	1.90	Trabecular bone	Wt%	EDS	Human
Akesson (2004)[126]	1.74	Trabecular bone	Wt%	INAA	Human
Akesson (2004)[126]	1.77	Trabecular bone	Wt%	Chemical analysis	Human
Dempster (1980)[127]	1.70±0.04	Trabecular bone	W/W%	EDS	Sprague-Dawley Rats
Rohanizadeh (2000)[128]	2.1±0.1	Trabecular bone	Wt%	EDS	Sprague-Dawley Rats
Green (1970)[129]	1.57-1.67	Trabecular & cortico bone	Wt%	WDS	Rhesus Monkey
Tjaderhane (1995)[130]	2.0	Dentine	Wt%	EDS	Wistar Rats
Arnold (2007)[131]	2.1±0.21	Dentine	Wt%	EDS	Human
Humonon (1999)[132]	1.83 ± 0.06	Dentine	Wt%	WDS	Sprague Dawley Rats
Alvarez-Perez (2005)[133]	≈1.67	Cementum	At%	EDS	Human

Table 1. Ca/P ratios from literature search. Wt% = weight percentage, W/W% wet weight, At% = atomic weight percentage

5.4 X-RAY MICROSCOPY

To establish the content of the major constituents, Ca and P, traditional methods used, such as ashing studies, chemical analysis and X-ray diffraction requires the destruction of the material prior to analysis. An additional disadvantage is that they require larger amounts of material, and that each element must be analysed separately.

Electron probe X-ray microanalysis (EPMA) is an analytical technique that can provide qualitative and quantitative non-destructive elemental analysis of micron-sized volumes at the surface of materials. It involves the bombardment of the specimen with a beam of accelerated electrons. The electron beam is focused on the surface of a specimen using a series of electromagnetic lenses, and these energetic electrons produce characteristic X-

rays within a small volume of the specimen. The measurement of the wavelength (or energy) of each characteristic x-ray enables identification of elements present in the specimen (qualitative analysis). Measurement of how many x-rays of any type are emitted per second can indicate how much of the element is present (quantitative analysis).

Traditionally, there are 2 types of X-ray microanalyses commonly used:

1. Energy dispersive X-ray spectroscopy (EDS)
2. Wavelength dispersive X-ray spectroscopy (WDS)

A scanning electron microscope equipped with EDS electronically sorts and measures X-rays with respect to their energies. While WDS uses diffraction to sort X-rays by their wavelengths.

The possible application of X-ray microanalysis to dental research was first explored by Boyde *et al* (1961)[134], soon after the introduction of the first commercial electron probe in 1956. The majority of studies have attempted to measure the concentration profiles of calcium, phosphate, and trace minerals in both enamel and dentine caries lesions[135]. It has also been used in quantitative analysis of cementum and[136] developing enamel and dentine[131].

5.4.1 Production of Characteristic X-ray Spectra

5.4.1.1 Structure of an atom

Atoms make up the chemical elements. An atom is composed of three different particles:

- ***Protons*** - positively charged, reside in the centre of the atom called the nucleus.
- ***Electrons*** - negatively charged, orbit in a cloud around nucleus.
- ***Neutrons*** - no charge, reside in the nucleus.

The structure of a typical isolated atom is shown in Figure 4. The nucleus, consisting of protons and neutrons, is surrounded by orbiting electrons. These electrons are arranged in shells (or energy levels) which describes where electrons are located (i.e., a specific region around the nucleus). A nucleus can have seven shells, but more chemicals of medicinal importance contain electrons in the first four, which are labelled the K, L, M, and N shells. The K shell is the closest to the nucleus and the N shell is the farthest from the nucleus. Electrons in the outermost shells have higher energy than those in the inner shells. Thus if electrons were to fall from the outermost shells into the inner shells, energy would be released. When such electronic transitions occur, the energy is released as photons, such as X-rays[137].

Figure 4. A schematic diagram of inner electron shells[138]; characteristic X-rays are produced by transitions between these shells.

To produce an x-ray spectrum, a specimen is bombarded by primary electrons from a source, such as a scanning electron microscope. This causes an ejection of an electron from an inner shell of a sample atom. The resulting vacancy is then filled by an electron from a higher-energy shell in the atom. In "dropping" to a state of lower energy, this vacancy-filling electron must give up some of its energy, which appears in the form of electromagnetic radiation. The energy of the emitted radiation, then, is exactly equal to the energy difference between the two electronic levels involved. Since this energy difference is fairly large for inner shells, the radiation appears as x-rays. Thus, when excited by electrons of sufficient energy, every element in a sample will emit a unique and characteristic pattern of x-rays. The number of x-rays emitted by each element bears a more or less direct relationship to the concentration of that element [139].

These characteristic x-rays are named according to the shell in which the initial vacancy occurs and the shell from which an electron drops to fill that vacancy (Figure 5). For example, if the initial vacancy occurs in the K shell and the vacancy-filling electron drops from the adjacent shell (the L shell), a Kα x-ray is emitted. If the electron drops from the M shell-two shells away- the emitted x-ray is a Kβ x-ray.

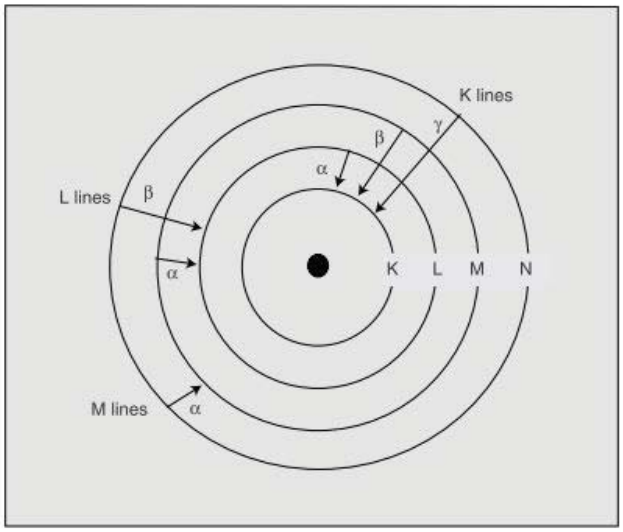

Figure 5. An illustration of the identification of characteristic x-rays according to the shell from which an electron drops to fill a vacancy.

5.4.1.2 Electron-Sample Interactions

As the primary electrons interact with the sample, they are scattered and spread. The volume in which the primary electrons interact with the sample is generally characterized as onion shaped (Figure 6). For spot analyses this sampling volume is around 1μm[139].

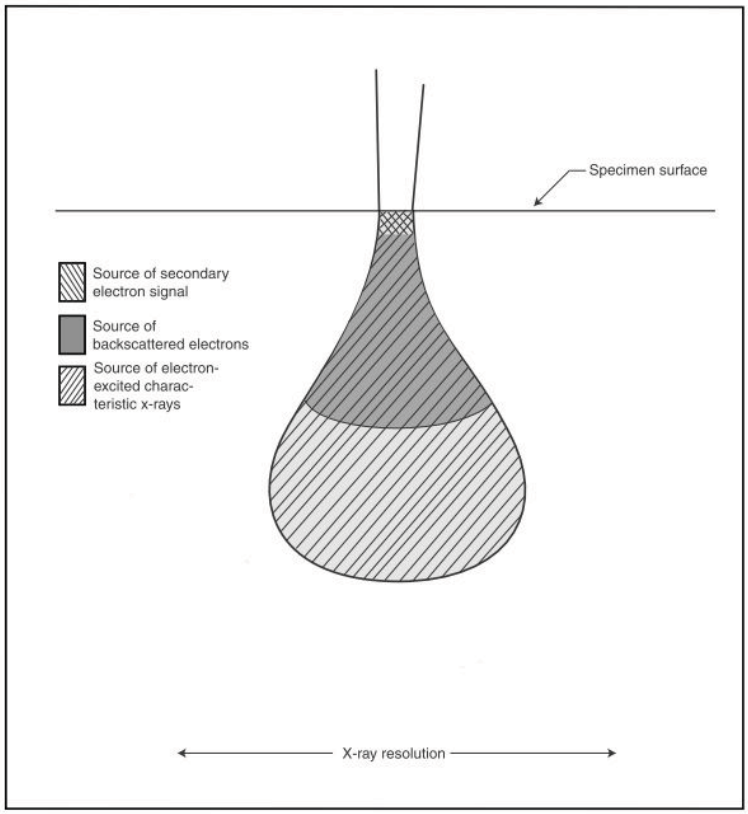

Figure 6. Generalized illustration of interaction volumes for various electron-specimen interactions. X-ray excited characteristic x-rays emerge from deepest within the sample (Adapted from[140])

Secondary electrons are created by the primary beam before it has a chance to spread. Because of the high density, they have high spatial resolution making them the most frequent choice for micrographic images. Secondary electrons carry little information about the elemental composition of the sample.

If the primary electron interacts with the nucleus of a sample atom, it may be scattered in any direction with little loss of energy. These backscattered electrons (BSE) are much more energetic than secondary electrons and so may escape from a greater depth within the sample. Therefore, compared to secondary electrons, the backscattered signal will not carry as much information about sample topography nor will it be as highly resolved in

space. The main influence on the strength of the BSE signal is the mean atomic number of the sample in the interaction volume. The higher the atomic number of an atom, the greater the positive charge of its nucleus and the more likely an interaction that produces a BSE. The BSE signal therefore carries some information about sample composition. Thus inorganic hard tissues can be contrasted from organic tissue in BSE imaging.

As outlined above, when an electron is ejected from an inner atomic shell by interaction with a high-energy electron beam, an electron in an outer shell "drops" into a vacancy in an inner shell. Each drop results in the loss of a specific amount of energy, namely, the difference in energy between the vacant shell and the shell contributing the electron. This energy is given up in the form of electromagnetic radiation-x-rays in the case of high-energy transitions involving inner shells. Thus the energy of the emitted radiation is characteristic for each element.

Converting these x-ray emissions to analysable data is the job of a series of electronic components (Figure 7) which, in the end, produce a digital spectrum of the emitted radiation. The x-ray photon first creates a charge pulse in a semiconductor detector; the charge pulse is then converted into a voltage pulse whose amplitude reflects the energy of the detected x-ray. Finally, this voltage pulse is converted into a digital signal, which causes one count to be added to the corresponding channel of a multichannel analyser. After a time, the accumulated counts from a sample produce an x-ray spectrum (Figure 8)

NOTE:
This figure is included on page 64
of the print copy of the thesis held in
the University of Adelaide Library.

Figure 7. Electronic components involved in energy dispersive microanalysis. Each emitted x-ray produces a charge pulse in a semiconductor detector. This current is converted first into a voltage pulse, then into a digital signal reflecting the energy of the original x-ray. The digital signal, in turn, adds a single count to the appropriate channel of a multi channel analyser (MCA)[141]

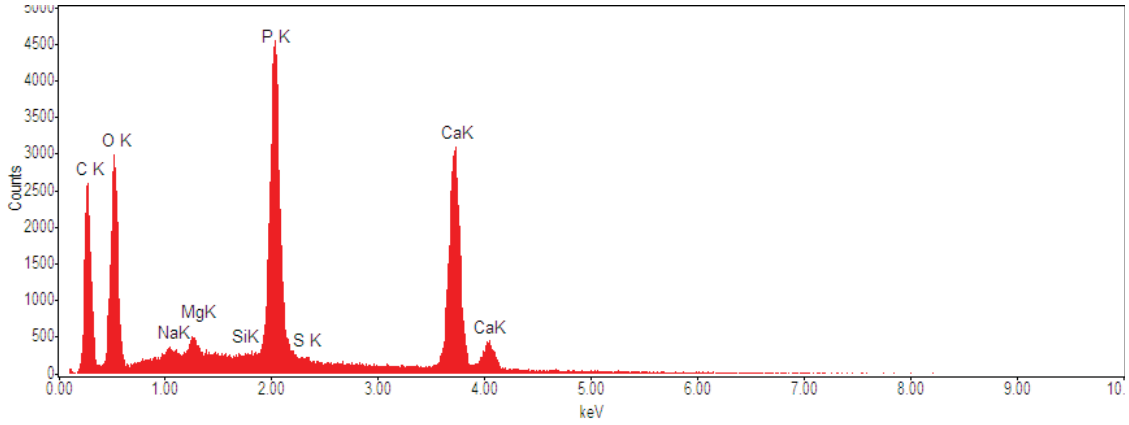

Figure 8. X-ray Spectrum. The dominant features of a typical x-ray spectrum include major spectral peaks superimposed on a broad background. Each peak is representative of the element and its characteristic x-rays caused by the transition of electrons between shells.

5.4.2 Energy-dispersive Spectrometer vs. Wavelength-dispersive analysis

The two EMPA techniques, EDS and WDS, both use the characteristic X-rays generated from a sample bombarded with electrons to identify the elemental constituents comprising the sample. Both techniques generate a spectrum in which the peaks correspond to specific X-ray lines and the elements can be easily identified.

Of the two methods, EDS is more commonly employed. EDS records the X-rays of all energies effectively simultaneously. Thus data collection and analysis with EDS is a relatively quick and simple process as the complete view of the X-ray spectrum is obtained with every EDS measurement. This allows for accurate qualitative analysis - to identify every elemental constituent present at each beam location. This analytical technique has a high spatial resolution and sensitivity, and individual analyses are reasonably short, requiring only a minute or two in most cases. Additionally, the X-ray microscope can function like a SEM and obtain highly magnified images of a sample. Rapid comparison of these elements can be made from the analysis of relative concentrations. Following elemental identification, one can proceed to quantitative analysis, in which the amount of each constituent present is determined and typically expressed as a concentration in terms of the mass (wt%) or atomic fraction (at%).

There are some limitations to EDS that an operator must be aware of. Firstly, the accurate detection of elements lighter than sodium (i.e. below 10 in atomic number) is impossible. Secondly, the energy resolution of the detector is poor. Each characteristic x-ray line is not detected as a sharp line, but as a broad peak. This can make it difficult to identify closely spaced lines on the x-ray spectrum e.g. hydrogen (H), helium (He) and lithium

(Li)[142]. Thirdly, there is a relatively large amount of electronic noise in the system which results in a low peak to background ratio compared to WDS, this can negatively affect the detectability of the analyzer.[139].

In WDS the spectrum is acquired sequentially as the full wavelength range is scanned. Although it takes longer to acquire a full spectrum, the WDS technique has much improved resolution compared to EDS. Generally, the strong points of WDS are where EDS performs badly – light element detection, peak separation and peak to background ratio[139]. WDS is capable of detecting elements lighter than Na. The combination of better resolution and the ability to deal with higher count rates allows WDS to detect elements at typically an order of magnitude lower concentration than EDS. WDS has better ability at detecting elements which contribute to less that 1% of the sample. The X-ray peaks are more distinct from the background in a WDS spectrum, resulting in a better accuracy and minimum detection limits that are at least ten times lower. In addition, due to the higher resolution of WDS, there is little ambiguity in peak identification. The disadvantage of WDS is that it can only detect one element at a time, making it more time consuming and less efficient. Thus the ideal method for quantification would be using a WDS detector.

5.4.4 Other considerations in X-ray Microanalysis

Ideal conditions for accurate qualitative and quantitative x-ray microanalysis include a sample which is homogenous, electrically conductive, flat and polished[137].

Compared to geological samples, biological samples are complex, heterogeneous in nature and structurally inhomogeneous[143]. As the main volume of interaction to produce

x-ray spectra occurs beneath the surface of a specimen (Figure), it may be difficult to ensure that the material being sampled is truly representative of the tissue.

An important consideration is whether or not the sample is electrically conductive. Biological and most mineral samples are not, and require a very thin coating of a conductive material (e.g., Carbon is commonly used, and applied by evaporation) to eliminate the charging - repulsion of incident electrons - that would otherwise occur and hinder analysis.

The samples must also be cleaned, to eliminate contamination that might interfere with the analysis (hydrocarbons from fingerprints or polishing compounds; diamond or alumina or other polishing materials). For quantitative analysis of thick sections using WDS and EDS, it is critical that the material have a mirror polish (1 micron or less final polish) and be in a mount so that this surface is at a known and constant angle to the electron beam[140]. If this is not so, the path length of the X-rays through the material at the takeoff angle will not be constant, and the key absorption correction will be incorrect. Biological hard tissues such as dentine are intrinsically uneven in density. This can interfere with the possibility to obtain an even, smooth surface, which is of great importance when preparing sections.

Thus element concentration values on biological hard tissues should be regarded as semi-quantitative, due to the limitations in preparation of the material. Where absolute quantification results maybe unobtainable, changes in values on a relative scale within the same tissue should not be under estimated[121]. The type of analysis most suitable for

qualitative and quantitative analyses may be influenced by the nature and preparation of the sample specimen.

5.6 CONCLUSIONS

The PDL is a highly vascular and cellular connective tissue situated between the tooth and alveolar bone that provides supportive, attachment, and sensory functions. Cells, vascular elements, and an extracellular compartment of matrix proteins and glycosaminoglycans provide unique biophysical functions that enable teeth of limited eruption to adjust their position while remaining firmly attached to the bony socket. The cells of the normal PDL include osteoblasts and osteoclasts on the bone side; cementoblasts on the root surface. Fibroblasts, epithelial cell rests of Malassez, macrophages, undifferentiated mesenchymal cells, neural elements, and endothelial cells in the body of the PDL; and cementoblasts on the root surface. The most interesting features of the PDL are its ability to adapt to rapidly changing applied force levels (e.g. mastication and orthodontic forces) and its capacity for renewal and repair. These characteristics are derived from a complex and heterogeneous grouo of cells all working together to maintain homeostasis[7].

The PDL has the capacity to maintain its width over time, despite being bordered by two hard tissues. The cells of the PDL itself have been demonstrated to have osteogenic abilities[17]. This has been reinforced in recent literature[19, 20]. Real-time RT-PCR analysis of PDL cells under tensile strain showed upregulation of genes linked to the osteoblastic phenotype. The PDL cells were also found to constitutively express numerous osteotropic cytokines and growth factors [22, 23]. In vitro studies have also shown the ability of PDL cells to form unique mineralised nodules which displayed different morphological characteristics compared to mineralised tissue formed by bone cells in culture [21].

There is a belief that the cells and connective tissue of the PDL possess the capacity to inhibit both osteogenesis of the periodontal space and progressive resorption of the connective tissue of the root; otherwise the PDL would undergo bone formation, mineralize across its width, and spontaneously ankylose [7, 18]. There is now evidence that populations of cells within the PDL, both during development and during regeneration, can secrete other molecules that can regulate the extent of mineralization and prevent the fusion of tooth root with surrounding bone, e.g. ankylosis. These include: a balance between the activities of bone sialoprotein and osteopontin; and Matrix Gla protein[2]. At the genetic level, in vitro studies have implicated that *Msx2* may play a central role in preventing ligaments and tendons, in general, from mineralizing[24].

Overall, it seems that the PDL is brimming with cells with osteogenic potential. There are regulatory factors within the PDL that play a role in the maintenance of the PDL space. Events such as injury and orthodontic tooth movement can result in change in the homeostasis and result in either maintenance of the PDL or mineral deposition leading to ankylosis.

Dental ankylosis is defined as fusion of the cementum or dentine with alveolar bone[47]. The loss of the PDL in the ankylotic area, results in a tooth incapable of continued eruption and hence unable to follow the normal vertical development of the neighbouring teeth. This results in infraocclusion and an incomplete development of the alveolar process[48].

There is evidence of a genetic component in ankylosis associated with primary and secondary retention[67, 144] as well as primary failure of eruption[55, 62]. Experimental models

involving injury to the PDL suggest that formation of ankylotic material within the PDL represents a physiological repair process. Essentially, the injury disrupts homeostasis of the PDL, triggers an osteogenic repair response and the ankylotic tissue merely represents scar tissue. Factors such as severity of injury and the viability of the surrounding PDL influence whether the ankylosis is resolved or continues to result in obliteration of the PDL[85, 92]. There is, however, uncertainty whether the osteogenic event is predominantly osteoblast driven[85], or cementoblast directed[92], or a consequence of osteogenic potential from PDL stem cells[145, 146]. The factors that trigger the various events in periodontal width homeostasis during these events still need to be ascertained[2].

PDL cells isolated from the root socket of Sprague Dawley rats have been demonstrated to form mineralised nodules in vitro[21]. The nodules displayed morphological characteristics different from bone mineralized nodules. X-ray microanalysis and electron diffraction analysis confirmed that mineral deposition contained calcium and phosphate in the form of immature hydroxyapatite. Currently, no studies have investigated the morphological and compositional characteristics of ankylosis. Such information may provide clues as to the origins of ankylosis

5.7 REFERENCES

1. Hassell TM. Tissues and cells of the periodontium. Periodontol 2000 1993;3.
2. Nanci A, Bosshardt DD. Structure of periodontal tissues in health and disease. Periodontol 2000 2006;40.
3. Beertsen W, McCulloch CAG, Sodek J. The periodontal ligament: a unique, multifunctional connective tissue. Periodontology 2000 1997;13.
4. Berkovitz BKB. The structure of the periodontal ligament: an update. Eur J Orthod 1990;12.
5. Gunraj MN. Dental root resorption. Oral surgery, oral medicine, oral pathology, oral radiology, and endodontics 1999;88.
6. Kimura R, Anan H, Matsumoto A, Noda D, Maeda K. Dental root resorption and repair: histology and histometry during physiological drift of rat molars. J Periodontal Res 2003;38.
7. Lekic P, McCulloch CA. Periodontal ligament cell population: the central role of fibroblasts in creating a unique tissue. Anat Rec 1996;245.
8. Mariotti A. The extracellular matrix of the periodontium: dynamic and interactive tissues. Periodontol 2000 1993;3.
9. Ten Cate AR. Development of the periodontium. In: Ten Cate AR, ed. Oral Histology: development, structure and function. St Louis: Mosby, 1994:228-243.
10. Ten Cate AR. Development of the tooth and its supporting tissues. In: Ten Cate AR, ed. Oral Histology: development, structure and function. St Louis: Mosby, 1994:57-79.

11. Grzesik WJ, Narayanan AS. Cementum and periodontal wound healing and regeneration. Crit Rev Oral Biol Med 2002;13.
12. Bosshardt DD. Are cementoblasts a subpopulation of osteoblasts or a unique phenotype? J Dent Res 2005;84.
13. McCulloch CA, Melcher AH. Cell migration in the periodontal ligament of mice. J Periodontal Res 1983;18.
14. Gould TR, Melcher AH, Brunette DM. Migration and division of progenitor cell populations in periodontal ligament after wounding. J Periodontal Res 1980;15.
15. McCulloch CA. Progenitor cell populations in the periodontal ligament of mice. Anat Rec 1985;211.
16. McCulloch CA, Melcher AH. Cell density and cell generation in the periodontal ligament of mice. Am J Anat 1983;167.
17. Roberts WE, Chase DC. Kinetics of cell proliferation and migration associated with orthodontically-induced osteogenesis. J Dent Res 1981;60.
18. Melcher AH. Repair of wounds in the periodontium of the rat. Influence of periodontal ligament on osteogenesis. Arch Oral Biol 1970;15.
19. Yamashita Y, Sato M, Noguchi T. Alkaline phosphatase in the periodontal ligament of the rabbit and macaque monkey. Arch Oral Biol 1987;32.
20. Nojima N, Kobayashi M, Shionome M, Takahashi N, Suda T, Hasegawa K. Fibroblastic cells derived from bovine periodontal ligaments have the phenotypes of osteoblasts. J Periodontal Res 1990;25.

21. Cho MI, Matsuda N, Lin WL, Moshier A, Ramakrishnan PR. In vitro formation of mineralized nodules by periodontal ligament cells from the rat. Calcif Tissue Int 1992;50.

22. Pinkerton MN, Wescott DC, Gaffey BJ, Beggs KT, Milne TJ, Meikle MC. Cultured human periodontal ligament cells constitutively express multiple osteotropic cytokines and growth factors, several of which are responsive to mechanical deformation. J Periodontal Res 2008;43.

23. Wescott DC, Pinkerton MN, Gaffey BJ, Beggs KT, Milne TJ, Meikle MC. Osteogenic gene expression by human periodontal ligament cells under cyclic tension. J Dent Res 2007;86.

24. Yoshizawa T, Takizawa F, Iizawa F*, et al.* Homeobox protein MSX2 acts as a molecular defense mechanism for preventing ossification in ligament fibroblasts. Mol Cell Biol 2004;24.

25. Andersson L, Lindskog S, Blomlöf L, Hedstrom KG, Hammarström L. Effect of masticatory stimulation on dentoalveolar ankylosis after experimental tooth replantation. Endod Dent Traumatol 1985;1.

26. Wesselink PR, Beertsen W. Repair processes in the periodontium following dentoalveolar ankylosis: the effect of masticatory function. J Clin Periodontol 1994;21.

27. Bosshardt DD, Selvig KA. Dental cementum: the dynamic tissue covering of the root. Periodontol 2000 1997;13.

28. Gonçalves PF, Enilson Antonio Sallum, Antonio Wilson Sallum, Márcio Zaffalon Casati, Sérgio de Toledo, Junior FHN. Dental cementum reviewed: development,

structure, composition, regeneration and potential functions Brazilian Journal of Oral Sciences 2005; Vol. 4.

29. Saygin NE, Giannobile WV, Somerman MJ. Molecular and cell biology of cementum. Periodontol 2000 2000;24.

30. Bosshardt DD, Schroeder HE. Cementogenesis reviewed: a comparison between human premolars and rodent molars. Anat Rec 1996;245.

31. Bosshardt DD, Schroeder HE. How repair cementum becomes attached to the resorbed roots of human permanent teeth. Acta Anat (Basel) 1994;150.

32. Berkovitz BKB, Holland GR, Moxham BJ, Berkovitz BKB. Oral anatomy, embryology and histology. 3rd edn. Edinburgh ; New York: Mosby, 2002:Pages.

33. Sodek J, McKee MD. Molecular and cellular biology of alveolar bone. Periodontol 2000 2000;24.

34. Saffar JL, Lasfargues JJ, Cherruau M. Alveolar bone and the alveolar process: the socket that is never stable. Periodontol 2000 1997;13.

35. Vignery A, Baron R. Dynamic histomorphometry of alveolar bone remodelling in the adult rat. Anat Rec 1980;196.

36. Thilander B, Rygh, P, Reitan, K. . Tissue Reactions in Orthodontics, in Orthodontics: Current Principles & Techniques. In: T.M. Graber V, R.L, Vig, K., ed. St Louis: Mosby, 2005:145-220.

37. Arnett TR. Update on bone cell biology. Eur J Orthod 1990;12.

38. Li X, Jee, WS. Integrated Bone Tissue Anatomy and Physiology, in Current Topics in Bone Biology. In: H. Deng L, YZ, ed. Singapore: World Scientific Printers, 2005:11-56.

39. Feng X, Zhou, H. Osteoclast Biology, in Current Topics in Bone Biology. In: Deng L, YZ, ed. Singapore: World Scientific Printers, 2005: 71-94.

40. Schroeder HE. Cementum, in the Periodontium. Berlin, Germany, 1986:23-128.

41. Reitan K, Kvam E. Comparative behaviour of human and animal tissue during experimental tooth movement. Angle Orthod 1971;41.

42. Hebel R, Stromberg, M.W. Osteology, in Anatomy of the Laboratory Rat The Williams & Wilkins Company, 1976:9-11.

43. King GJ, Latta L, Rutenberg J, Ossi A, Keeling SD. Alveolar bone turnover in male rats: site- and age-specific changes. Anat Rec 1995;242.

44. Shimpo S, Horiguchi Y, Nakamura Y*, et al.* Compensatory bone formation in young and old rats during tooth movement. Eur J Orthod 2003;25.

45. McCulloch CA, Melcher AH. Continuous labelling of the periodontal ligament of mice. J Periodontal Res 1983;18.

46. Dreyer CW, Pierce AM, Lindskog S. Hypothermic insult to the periodontium: a model for the study of aseptic tooth resorption. Endod Dent Traumatol 2000;16.

47. Biederman W. Etiology and treatment of tooth ankylosis. Amer J Orthod 1962;48.

48. Biederman W. The incidence and aetiology of tooth ankylosis. Am J Orthod 42:921-926, 1956.

49. Lamb KA, Reed MW. Measurement of space loss resulting from tooth ankylosis. ASDC journal of dentistry for children 1968;35.

50. Fuss Z, Tsesis I, Lin S. Root resorption--diagnosis, classification and treatment choices based on stimulation factors. Dent Traumatol 2003;19.

51. Löe H, Waerhaug J. Experimental replantation of teeth in dogs and monkeys. Arch Oral Biol 1961;3.

52. Andreasen J. A time-related study of periodontal healing and root resorption activity after replantation of mature permanent incisors in monkeys. Swedish Dental Journal 1980;4.

53. Andreasen J. Traumatic injuries of the teeth. Copenhagen: Munksgaard, 1981:Pages.

54. Andreasen J, Kristersen L. The effect of limited drying or removal of the periodontal ligament. Periodontal healing after replantation of mature incisors in monkeys. Acta Odontol Scand 1981;39.

55. Proffit WR, Vig KW. Primary failure of eruption: a possible cause of posterior open-bite. Am J Orthod 1981;80.

56. Raghoebar G, Boering G, Jansen H, Vissink A. Secondary retention of permanent molars: a histologic study. J Oral Path Med 1989;18.

57. Biederman W. The problem of the ankylosed tooth. In: Spengeman WG, ed. Dental Clinics of North America, Symposium on Orthodontics. Philadelphia: W.B. Saunders, 1968.

58. Kurol J. Infraocclusion of primary molars: an epidemiologic and familial study. Community dentistry and oral epidemiology 1981;9.

59. Jacobs SG. Ankylosis of permanent teeth: a case report and literature review. Aust Orthod J 1989;11.

60. Raghoebar G, Boering G, Vissink A, Stegenga B. Eruption disturbances of permanent molars: a review. J Oral Path Med 1991;20.

61. Frazier-Bowers SA, Koehler KE, Ackerman JL, Proffit WR. Primary failure of eruption: further characterization of a rare eruption disorder. Am J Orthod Dentofacial Orthop 2007;131.

62. Decker E, Stellzig-Eisenhauer A, Fiebig BS*, et al.* PTHR1 loss-of-function mutations in familial, nonsyndromic primary failure of tooth eruption. American journal of human genetics 2008;83.

63. Ahmad S, Bister D, Cobourne MT. The clinical features and aetiological basis of primary eruption failure. Eur J Orthod 2006;28.

64. Raghoebar GM, Boering G, Stegenga B, Vissink A. Secondary retention in the primary dentition. ASDC journal of dentistry for children 1991;58.

65. Raghoebar G, Jansen H, Jongebloed W, Boering G, Vissink A. Secondary retention of permanent molars: an assessment of ankylosis by scanning electron and light microscopy. Br J Oral Maxillofac Surg 1992;30.

66. Humerfelt A, Reitan K. Effects of hypercementosis on the movability of teeth during orthodontic treatment. Angle Orthod 1966;36.

67. Bosker H, ten Kate LP, Nijenhuis LE. Familial re-inclusion of permanent molars. Clinical genetics 1978;13.

68. Andreasen JO, Skougaard MR. Reversibility of surgically induced dental ankylosis in rats. Int J Oral Surg 1972;1.

69. Raghoebar GM, van Koldam WA, Boering G. Spontaneous re-eruption of a secondarily retained permanent lower molar and an unusual migration of a lower third molar. Am J Orthod Dentofacial Orthop 1990;97.

70. Andersson L, Blomlöf L, Lindskog S, Feiglin B, Hammarström L. Tooth ankylosis. Clinical, radiographic and histological assessments. Int J Oral Surg 1984;13.

71. Wesselink PR, Beertsen W. Ankylosis of the mouse molar after systemic administration of 1-hydroxyethylidene-1,1-bisphosphonate (HEBP). J Clin Periodontol 1994;21.

72. Becktor KB, Bangstrup MI, Rolling S, Kjaer I. Unilateral primary or secondary retention of permanent teeth, and dental malformations. Eur J Orthod 2002;24.

73. Parker W, Frishe H. The experimental production of dental ankylosis. Angle Orthod 1964;34.

74. Rubin PL, Weisman EJ, Bisk F. Experimental tooth ankylosis in the monkey. Angle Orthod 1984;54.

75. Hellsing E, Alatli-Kut I, Hammarström L. Experimentally induced dentoalveolar ankylosis in rats. Int Endod J 1993;26.

76. Alatli I, Li J, Hammarström L. Ultimate tensile strength of PDL of molars in rats after 1-hydroxyethylidene-1,1-bisphosphonate injections. Journal of dentistry 1997;25.

77. Berggreen E, Sae-Lim V, Bletsa A, Heyeraas KJ. Effect of denervation on healing after tooth replantation in the ferret. Acta Odontol Scand 2001;59.

78. Fujiyama K, Yamashiro T, Fukunaga T, Balam TA, Zheng L, Takano-Yamamoto T. Denervation resulting in dento-alveolar ankylosis associated with decreased Malassez epithelium. J Dent Res 2004;83.

79. Wesselink PR, Beertsen W, Everts V. Resorption of the mouse incisor after the application of cold to the periodontal attachment apparatus. Calcif Tissue Int 1986;39.

80. Tal H, Stahl S. Healing following devitalization of sites within the periodontal ligament by ultralow temperatures. Journal of Periodontology 1986;57:735-741.

81. Tal H, Kozlovsky A, Pitaru S. Healing of sites within the dog periodontal ligament after application of cold to the periodontal attachment apparatus. J Clin Periodontol 1991;18.

82. Shaboodien S. Traumatically induced dentoalveolar ankylosis in rats. Orthodontics. Adelaide: Doctor of Clinical Dentistry Thesis, University of Adelaide, 2005.

83. Di Iulio DS. Relationship of epithelial cells and nerve fibres to experimentally induced dentoalveolar ankylosis in rats. Doctorate of Clinical Dentistry Thesis. Adelaide: University of Adelaide, 2007.

84. Pindborg JJ. Ankylosis of teeth, in Pathology of the dental hard tissues. Philadelphia: W.B Saunders, 1970:362-366.

85. Hammarström L, Blomlöf L, Lindskog S. Dynamics of dentoalveolar ankylosis and associated root resorption. Endod Dent Traumatol 1989;5.

86. Melcher AH. On the repair potential of periodontal tissues. J Periodontol 1976;47.

87. Line SE, Polson AM, Zander HA. Relationship between periodontal injury, selective cell repopulation and ankylosis. J Periodontol 1974;45.

88. Hammarström L, Lindskog S. General morphological aspects of resorption of teeth and alveolar bone. Int Endod J 1985;18.

89. Dreyer CW. Clast Cell Activity in a Model of Aseptic Root Resorption. Faculty of Health Sciences. Adelaide: PhD thesis, The University of Adelaide, 2002:402.

90. Karring T, Nyman S, Lindhe J. Healing following implantation of periodontitis affected roots into bone tissue. J Clin Periodontol 1980;7.

91. Nyman S, Gottlow J, Karring T, Lindhe J. The regenerative potential of the periodontal ligament. An experimental study in the monkey. J Clin Periodontol 1982;9.

92. Lindskog S, Blomlöf L. Mineralized tissue-formation in periodontal wound healing. J Clin Periodontol 1992;19.

93. Ogiso B, Hughes FJ, Melcher AH, McCulloch CA. Fibroblasts inhibit mineralised bone nodule formation by rat bone marrow stromal cells in vitro. Journal of cellular physiology 1991;146.

94. Ogiso B, Hughes FJ, Davies JE, McCulloch CA. Fibroblastic regulation of osteoblast function by prostaglandins. Cellular signalling 1992;4.

95. Lindskog S, Blomlöf L, Hammarström L. Evidence for a role of odontogenic epithelium in maintaining the periodontal space. J Clin Periodontol 1988;15.

96. Posner AS. The mineral of bone. Clinical orthopaedics and related research 1985.

97. Ergun C, Webster TJ, Bizios R, Doremus RH. Hydroxylapatite with substituted magnesium, zinc, cadmium, and yttrium. I. Structure and microstructure. Journal of biomedical materials research 2002;59.

98. Legros R, Balmain N, Bonel G. Age-related changes in mineral of rat and bovine cortical bone. Calcif Tissue Int 1987;41.

99. Lee DD, Landis WJ, Glimcher MJ. The solid, calcium-phosphate mineral phases in embryonic chick bone characterized by high-voltage electron diffraction. J Bone Miner Res 1986;1.

100. Termine JD, Posner AS. Infrared analysis of rat bone: age dependency of amorphous and crystalline mineral fractions. Science 1966;153.

101. Termine JD, Posner AS. Amorphous/crystalline interrelationships in bone mineral. Calcified tissue research 1967;1.

102. Driessens FC, Verbeeck RM. The dynamics of bone mineral in some vertebrates. Zeitschrift fur Naturforschung 1986;41.

103. Driessens FC. Probable phase composition of the mineral in bone. Zeitschrift fur Naturforschung 1980;35.

104. Legros R, Bonel G, Montel G, Balmain-Oligo N, Juster M. [Systematic study of the variations in mineral composition of different bones from various animals according to their localization]. Comptes rendus hebdomadaires des seances de l'Academie des sciences 1977;285.

105. Boskey AL. Current concepts of the physiology and biochemistry of calcification. Clinical orthopaedics and related research 1981.

106. Webster TJ, Massa-Schlueter EA, Smith JL, Slamovich EB. Osteoblast response to hydroxyapatite doped with divalent and trivalent cations. Biomaterials 2004;25.

107. Posner AS. Crystal chemistry of bone mineral. Physiological reviews 1969;49.

108. Wiesmann HP, Tkotz T, Joos U*, et al.* Magnesium in newly formed dentin mineral of rat incisor. J Bone Miner Res 1997;12.

109. Tsuboi S, Nakagaki H, Ishiguro K*, et al.* Magnesium distribution in human bone. Calcif Tissue Int 1994;54.

110. Terpstra RA, Driessens FC. Magnesium in tooth enamel and synthetic apatites. Calcif Tissue Int 1986;39.

111. Nakata TM, Stepnick RJ, Zipkin I. Chemistry of human dental cementum: the effect of age and fluoride exposure on the concentration of ash, fluoride, calcium, phosphorus and magnesium. J Periodontol 1972;43.

112. Neiders ME, Eick JD, Miller WA, Leitner JW. Electron probe microanalysis of cementum and underlying dentin in young permanent teeth. J Dent Res 1972;51.

113. LeGeros RZ, Kijkowska R, Bautista C, LeGeros JP. Synergistic effects of magnesium and carbonate on properties of biological and synthetic apatites. Connective tissue research 1995;33.

114. Bigi A, Foresti E, Gregorini R, Ripamonti A, Roveri N, Shah JS. The role of magnesium on the structure of biological apatites. Calcif Tissue Int 1992;50.

115. Aoba T, Moreno EC, Shimoda S. Competitive adsorption of magnesium and calcium ions onto synthetic and biological apatites. Calcif Tissue Int 1992;51.

116. Burnell JM, Liu C, Miller AG, Teubner E. Effects of dietary alteration of bicarbonate and magnesium on rat bone. The American journal of physiology 1986;250.

117. Boskey AL, Rimnac CM, Bansal M, Federman M, Lian J, Boyan BD. Effect of short-term hypomagnesemia on the chemical and mechanical properties of rat bone. J Orthop Res 1992;10.

118. Aoba T. The effect of fluoride on apatite structure and growth. Crit Rev Oral Biol Med 1997;8.

119. Barton NS, Van Swol RL. Periodontally diseased vs. normal roots as evaluated by scanning electron microscopy and electron probe analysis. J Periodontol 1987;58.

120. Sano T, Morohashi T, Amano H, Yamada S, Nakamura M. Distributions of magnesium and sulphur in defect layers of incisor dentine in magnesium-deficient rats. Arch Oral Biol 2005;50.

121. Hals E, Tveit AB, Totdal B. X-ray microanalysis of dentin: a review. Scanning Microsc 1988;2.

122. Arnold S, Plate U, Wiesmann HP, Stratmann U, Kohl H, Hohling HJ. Quantitative analyses of the biomineralization of different hard tissues. Journal of microscopy 2001;202.

123. Johnsson MS, Nancollas GH. The role of brushite and octacalcium phosphate in apatite formation. Crit Rev Oral Biol Med 1992;3.

124. Pellegrino ED, Biltz RM. Mineralization in the chick embryo. I. Monohydrogen phosphate and carbonate relationships during maturation of the bone crystal complex. Calcified tissue research 1972;10.

125. Meinke DK, Skinner HC, Thomson KS. X-ray diffraction of the calcified tissues in Polypterus. Calcif Tissue Int 1979;28.

126. Akesson K, Grynpas MD, Hancock RG, Odselius R, Obrant KJ. Energy-dispersive X-ray microanalysis of the bone mineral content in human trabecular bone: a comparison with ICPES and neutron activation analysis. Calcif Tissue Int 1994;55.

127. Dempster DW, Elder HY, Nicholson WA, Smith DA, Moss VA. Changes in bone mineral in experimentally induced rickets in the rat: electron microprobe and chemical studies. Calcif Tissue Int 1980;30.

128. Rohanizadeh R, LeGeros RZ, Bohic S, Pilet P, Barbier A, Daculsi G. Ultrastructural properties of bone mineral of control and tiludronate-treated osteoporotic rat. Calcif Tissue Int 2000;67.

129. Green LJ, Eick JD, Miller WA, Leitner JW. Electron microprobe analysis of Ca, P, and Mg in mandibular bone. J Dent Res 1970;49.

130. Tjaderhane L, Hietala EL, Larmas M. Mineral element analysis of carious and sound rat dentin by electron probe microanalyzer combined with back-scattered electron image. J Dent Res 1995;74.

131. Arnold WH, Gaengler P. Quantitative analysis of the calcium and phosphorus content of developing and permanent human teeth. Ann Anat 2007;189.

132. Huumonen S, Larmas M. An electron probe x-ray microanalytical study of dentine minerals in sucrose-fed or glucocorticoid-medicated rats. Calcif Tissue Int 1999;65.

133. Alvarez-Perez MA, Alvarez-Fregoso O, Ortiz-Lopez J, Arzate H. X-ray microanalysis of human cementum. Microsc Microanal 2005;11.

134. Boyde A, Switsur VR, Fearnhead RW. Application of the scanning electron-probe x-ray microanalyser to dental tissues. Journal of ultrastructure research 1961;5.

135. Ngo H, Ruben J, Arends J*, et al.* Electron probe microanalysis and transverse microradiography studies of artificial lesions in enamel and dentin: a comparative study. Advances in dental research 1997;11.

136. Rex T, Kharbanda OP, Petocz P, Darendeliler MA. Physical properties of root cementum: Part 4. Quantitative analysis of the mineral composition of human premolar cementum. Am J Orthod Dentofacial Orthop 2005;127.

137. Reed SJB. Electron microprobe analysis and scanning electron microscopy in geology. 2nd edn. Cambridge: Cambridge University Press, 2005:Pages.

138. Nelson PSA. Electron Shell. Tulane University, 30th September 2008.

139. Goodhew PJ, Humphreys FJ, Beanland R. Electron microscopy and analysis. 3rd edn. London ; New York: Taylor & Francis, 2001:Pages.

140. Goldstein J. Scanning electron microscopy and x-ray microanalysis. 3rd edn. New York: Kluwer Academic/Plenum Publishers, 2003:Pages.

141. Vaughan D. Energy Dispersive X-Ray Microanalysis - An Introduction. Wisconsin: NORAN Instruments, 1999:68.

142. Newbury DE. Standardless Quantitative Electron-Excited X-ray Microanalysis by Energy-Dispersive Spectrometry: What Is Its Proper Role? Microsc Microanal 1998;4.

143. Eshel A. Quantitative electron probe microanalysis of biological specimens: A theoretical analysis of some variables involved Micron 1974.

144. Raghoebar GM, Ten Kate LP, Hazenberg CA, Boering G, Vissink A. Secondary retention of permanent molars: a report of five families. Journal of dentistry 1992;20.

145. Melcher AH, McCulloch CA, Cheong T, Nemeth E, Shiga A. Cells from bone synthesize cementum-like and bone-like tissue in vitro and may migrate into periodontal ligament in vivo. J Periodontal Res 1987;22.

146. McCulloch CA, Bordin S. Role of fibroblast subpopulations in periodontal physiology and pathology. J Periodontal Res 1991;26.

147. Chang, A. Interradicular mineralised tissue adaptation in an aseptic root resorption model. Doctorate of Clinical Dentistry Thesis. Adelaide: University of Adelaide, 2008.

148. Komatsu K, Shimada A, Shibata T, Shimoda S, Oida S, Kawasaki K, et al. Long-term effects of local pretreatment with alendronate on healing of replanted rat teeth. J Periodontal Res 2000, 43(2):194-200.

149. Nishioka M, Shiiya T, Ueno K, Suda H. Tooth replantation in germ-free and conventional rats. Endod Dent Traumatol. 1998, 14(4):163-73.

6. STATEMENT OF PURPOSE

Ankylosis of a tooth stops its eruptive potential, leading to infra-occlusion of the affected tooth due to continued eruption of neighbouring teeth. An ankylosed tooth is incapable of tooth movement; eruptive, functional or orthodontic. In growing patients, progressive infraocclusion of teeth can produce significant aesthetic and functional defects. The aetiology of ankylosis is not known but, in all cases, there is a discontinuity in the PDL.

The aseptic root resorption model developed by Dreyer[46, 89] has been shown by Shaboodien[82] to produce ankylosis within the interradicular area of rat molar teeth at the day 14 and day 28 observation periods, persisting up to the last observation period of 86 days. However, using the same model, Di lulio[83] found a decreased prevalence of ankylosis. He also noted a change in the morphology of ankylosis, with a trend towards diminishing areas of ankylotic union in the later time periods. This suggested a resilient, dynamic nature of the periodontium in response to injury.

Utilising this model, an assessment of the morphology as well as elemental composition of ankylosis relative to alveolar bone, cementum and dentine at day 7, day 14, day 21 and day 28 observation periods would allow further characterisation of pathogenesis of ankylosis. Information on the site of mineral formation, morphology and composition of ankylosis may give clues as to the origin of this tissue. To date, no research has been published on the mineral composition of ankylosis, comparing it to that of neighbouring hard tissues.

7. ARTICLE 1

Mineral composition of hypothermally induced ankylosis in rat molars

Doctor of Clinical Dentistry (Orthodontics)

Article 1

Written in the format for submission to:

the Journal of Dental Research

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2009

ABSTRACT

There is a presumption that the ankylotic region formed after periodontal ligament (PDL) injury represents an unremarkable bony repair process. Essentially, the injury triggers an osteogenic repair response and the ankylotic tissue merely represents alveolar bone. There is uncertainty whether the oestogenic event is predominantly osteoblast driven, cementoblast directed, or a consequence of osteogenic potential from PDL stem cells. In this study, twenty-eight Sprague Dawley rats were divided into four groups of six animals, corresponding to one of four observation periods, and received a thermal insult as a continuous 20 minute application of dry ice to the crowns of their upper right maxillary molar. The appearance of ankylotic tissues was examined using backscattered electron images using a scanning electron microscope (SEM) equipped with an Energy Dispersive X-ray Analyser (EDS). The Ca, P, and trace elements contents were determined by EDS from four different hard tissue regions: ankylosis; bone; dentine and cementum, and the Ca/P ratios were calculated. Ankylosis was observed at days 14 and 21 in 3 rats and was not seen at day 28. No ankylosis was observed in the control teeth. BSE imaging revealed a focal type of ankylosis with central nodules of mineralized tissue forming within the PDL. The morphological features of ankylotic tissue differed to that of alveolar bone and dentine. Bridging between bone and dentine occurred with fine trabeculae which extended from the central mineralized nodule. EDS analysis showed that the ankylotic tissue was composed of major constituents Ca and P along with trace elements of Mg and Na. This was comparable to the surrounding alveolar bone, cementum and dentine. There was no statistically significant difference in the Ca/P ratios, Mg, and Na between ankylotic material and bone. Statistically significant differences was

evident in Mg content between ankylotic material compared to dentine and cementum. Na content was higher in dentine than in ankylotic material. The results of this study indicate that, histochemically, ankylotic material is similar to bone. However, the appearance of ankylotic material as centralised foci with a morphology different from bone suggest that ankylosis may originate from an osteoblastic phenotype originating within PDL.

INTRODUCTION

Ankylosis is a pathologic fusion of alveolar bone and tooth. Bone-like tissue invades the PDL space and establishes a direct connection between the alveolar wall socket and the root surface of a tooth (Andersson et al., 1984). Over time, the eruptive potential is stopped, leading to infra-occlusion of the affected tooth due to continued eruption of neighbouring teeth. An ankylosed tooth is deemed incapable of tooth movement - eruptive, functional or orthodontic. In growing patients, progressive infraocclusion of teeth can produce significant aesthetic and functional defects. The aetiology of ankylosis is not known, but in all cases, there is a discontinuity in the PDL.

Previous models for investigating ankylosis have involved an extraction-replantation model (Andreasen, 1980; Biederman, 1968; Hellsing et al., 1993; Löe and Waerhaug, 1961). Other animal models that have been used include: the use of a soldering iron tip into the prepared root canals of monkeys to induce thermal injury to the PDL cells (Line et al., 1974); cellular devitalisation of the PDL by freezing using liquid nitrogen (Tal and Stahl, 1986; Tal et al., 1991; Wesselink et al., 1986) and surgically induced damage to the periodontium (Andreasen and Skougaard, 1972). Chemical models using prolonged administration of bisphosphonates in mice have also been used to induce localized ankylosis in rats (Wesselink and Beertsen, 1994).

An aseptic root resorption model developed by Dreyer et al. (2000), involves hypothermic injury to the PDL via direct freezing of a rat molar tooth for 20 minutes. It has been shown to produce ankylosis within the interradicular area of rat molar teeth at day 14 and day 28 observation period (Chang, 2008; Di Iulio, 2007; Shaboodien, 2005).

The ankylosis produced from this technique was of the type where ankylotic bone which was fused to the affected tooth appeared to be separated from the dentine by an intact cementum layer. This is similar to the ankylosis as described by Andersson et al. (1984). Di Iulio (2007) observed that ankylotic material appeared to become more solid with time, progressing from fine bony trabeculae with connective tissue interspersed in the 7 days observation group to solid bone in the later groups. It was also noted that at 21 and 28 days, the ankylotic bone was quite solid, without connective tissue cells interspersed, but the contact area between it and the root surface was usually small. It was suggested that that may represent a regression of the ankylotic union similar to that noted by Hammarström et al. (1989). The thermal models for inducing ankylosis create site specific cellular death without significant structural alterations, and may be advantageous in studying periodontal healing.

Electron probe X-ray microanalysis is a powerful tool for studying mineral elements in dental hard tissues. It is a non-destructive technique which has the advantage of analysing the composition of well defined morphological areas with a sharply focused electron beam (spot analysis) aimed directly at a sample. This method allows qualitative and quantitative analysis of major and trace elements present within a sample (Ngo et al., 1997; Sanchez-Quevedo et al., 1998).

Hydroxyapatite makes up the primary component of inorganic material in biological hard tissues. The hydroxyapatite of biological hard tissues is not chemically pure, but contains elements that have been taken up from the tissue fluid during initial crystallisation and mineralization. Over time, the concentrations may change by additional uptake or

substitution by other ions (Bosshardt and Selvig, 1997). In addition to calcium (Ca) and phosphorous (P), small amounts of fluoride (F), magnesium (Mg), sulphur (S), copper (Cu), zinc (Zn) and sodium (Na) have been detected in dental hard tissues (Arnold and Gaengler, 2007; Rex et al., 2005; Sanchez-Quevedo et al., 1998; Selvig and Selvig, 1962; Tjäderhane et al., 1995; Webster et al., 2004; Wiesmann et al., 1997).

There is a presumption that the ankylotic region formed after PDL injury represents an unremarkable bony repair process (Hammarström et al., 1989; Lindskog and Blomlöf, 1992). Essentially, the injury triggers an osteogenic repair response and the ankylotic tissue merely represents alveolar bone. There is, however, uncertainty whether the osteogenic event is predominantly osteoblast driven (Hammarström et al., 1989), or cementoblast directed (Lindskog and Blomlöf, 1992), or a consequence of osteogenic potential from PDL stem cells (McCulloch and Bordin, 1991; Melcher et al., 1987). This gives 3 potential sources of ankylotic tissue and potential differences in composition. In particular, we are interested in the mineralization profile of the repair material and the literature provides scant reference to the mineral content.

The aims of the present study were to (1) observe PDL adaptation subsequent to thermal insult within the interradicular region and (2) to compare the mineral profile of alveolar bone, dentine, cementum and the ankylotic tissue; by investigating calcium (Ca), phosphorous (P) and other trace element concentrations using energy dispersive x-ray spectrometry (EDS) element analysis. The relative differences in elemental composition may provide insight as to the origins of ankylotic material.

Null hypotheses are proposed: (1) A single, prolonged thermal insult to a rat molar has no effect on mineralized tissue formation and composition within the periodontium and (2) ankylotic tissue is similar to alveolar bone.

MATERIALS AND METHODS

This experiment was approved by the Ethics Committee of The University of Adelaide under ethics number M-054-2006.

Twenty-eight eight week old Sprague Dawley rats were randomly divided into four groups of seven animals corresponding to one of 4 observation periods i.e.: t_1= 7 days, t_2= 14 days, t_3= 21 days, t_4= 28 days. At t=0 days, six animals in each group received a continuous 20 minute application of dry ice pellets of (CO_2 at -81^{0}C, BOC Gases, Adelaide, Australia), under anaesthesia, to the crowns of their upper right first maxillary molar. The left maxillary first molar served as a control. One rat from each group did not undergo thermal injury and was a sham for the experiment. All rats were given two sequential bone labels; calcein 5mg/kg and alizarin red 30mg/kg, administered intraperitoneally 8 days apart. The timing of the labels was such that all rats were euthanised 2 days after the last label.

The four groups of seven animals were sacrificed via CO_2 asphyxiation at 7, 14, 21, 28 days respectively after the application of the dry ice. The maxilla was dissected out, trimmed, and fixed in 70% ethanol. Tissue dehydration and defatting was performed prior to embedding in methylmethacrylate. Ten micron serial, coronal sections were cut with a Reichert-Jung microtome (Leica Microsystems GmbH Wetzlar, Germany), through the furcation region of the upper first molar teeth. For every 3 out of 10 sections: the first was kept unstained and undecalcified; the second was stained with Von Kossa/haematoxylin & eosin; and the third was decalcified and stained with haematoxylin & eosin. Unstained

sections were viewed under fluorescence, while transmitted light microscopy was used for the other sections. Following initial analysis, the unstained, un-decalcified sections were de-coverslipped, by soaking in xylene, and carbon coated.

These sections were investigated with a Philips XL30 FEG Scanning Electron Microscope (SEM) at 15kV using the backscattered electron detector (BSE). Qualitative and quantitative elemental analysis was carried out by EDS with an EDAX DX4 detector (EDAX, USA). Measurements were taken under the following conditions: acquisition time of 100 live seconds, accelerating voltage 10kV, take-off angle of 35^0, count rate of 1800-2000 counts per second, dead time of 20-30% and working distance 10mm. In each rat, three spot measurements, approximately 1µm in volume, were obtained in each region of interest: ankylosis, bone, cementum and dentine for both the experimental and control molars (Figures 1 and 2). The weight percentage of Ca, P and other detected elements was analyzed after a ZAF (atomic number, absorption and fluorescence) correction procedure that converts the elements to a fraction of 100% of the chemical global composition. *(For information on acquisition time and number of measurements – refer to Appendix 9.3)*

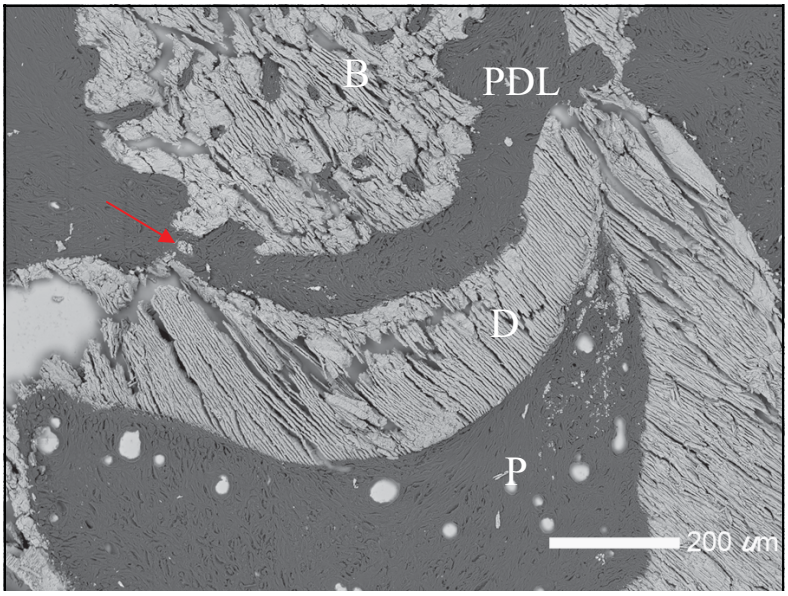

Figure 1. BSE image of a specimen. Ankylosis is indicated with an arrow. B=Alveolar bone, PDL=periodontal ligament, D=dentine, P=pulp

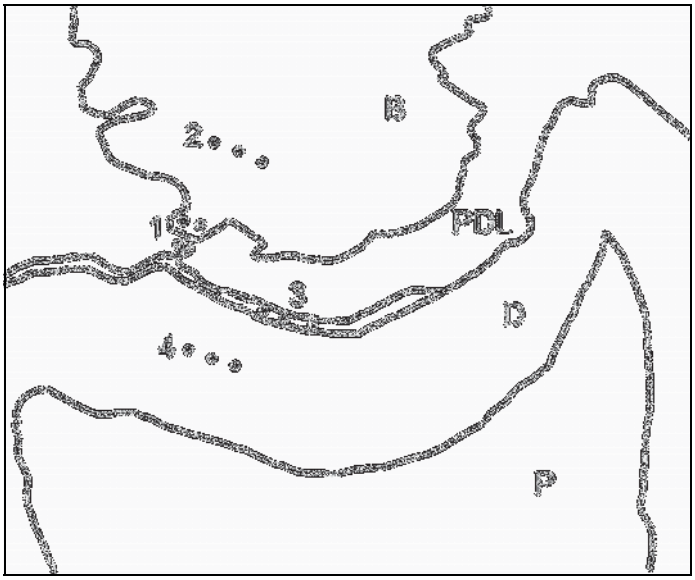

Figure 2. Schematic presentation of the sites of measurements of the specimen in Fig. 1. (1) Ankylotic material, (2) Alveolar bone, (3) Cementum, (4) Dentine

Statistical analysis was performed using SAS Version 9.2 (SAS Institute Inc., Cary, NC, USA). In assessing relative differences in minerals between teeth and across regions in the experimental rats, linear mixed effects models were fitted to the data. In the models side (left, right), region (bone, cementum, dentine, and ankylosis) and the interaction between side and region were included as fixed effects. A random animal effect was included in all the models so as to adjust for the dependence in results from the same animal. Where the interaction between side and region was found to be non-significant (i.e. $p > 0.05$), a second model excluding the interaction term was fitted to the data. *Post Hoc* tests were employed where interactions between side or region were significant ($p<0.05$).

RESULTS

Histological Observation

Ankylosis was morphologically defined as the physical union between the alveolar bone and furcal root surface that extended across the entire width of the PDL space. Of the 28 rats, there were three experimental animals which developed definite ankylosis on their upper right first molars (Table 1). Ankylosis was not seen in any of the sham rats or in any of the control molars of the treated rats.

Time Period	Number of animals with ankylosis in furcation	Rat ID
7 days	0	-
14 days	2	3,7
21 days	1	2
28 days	0	-

Table 1. Distribution of ankylosis in experimental rats (Chang, 2008)

Seven days after thermal injury, no ankylosis was detected in the treatment and control teeth. Small resorption lacunae were noted in the furcal region of the treatment molars. The adjacent alveolar bone surface was also more irregular than the control side, with resorption lacunae more prevalent.

At day 14, a definite ankylosis was seen in two treatment rats, (Figures 3, 4, 5, 6 and 7). When present, fusion between the bone and root was only present in a few sections, suggestive of a focal nature. In the first rat featuring ankylosis, examination of the unstained section under a fluorescence microscope (Figure 3) revealed what appeared to be ankylotic bridging across the PDL. The presence of alizarin red bone labels suggested that rapid mineral apposition had occurred recently. BSE imaging of the de-coverslipped

slide (Figure 4) showed that the ankylosis seemed to start with formation of bone-like tissue in the central part of the PDL with thickening of mineralized tissue extending from both the bone and root surface into the PDL. The bone and root surfaces adjacent to these extensions were irregular and characteristic of resorption. In the second rat, a layer of unmineralised matrix was seen around the area of ankylosis and along its immediate connecting bone and root surfaces (Figure 5). Similar to the first rat, the ankylotic material consisted of a distinct centralised nodule with finger like extensions extending from the alveolar bone wall (Figure 7). Blood vessels within the PDL were seen in close proximity to the ankylotic tissue (Figure 6). Also observed, were signs of rapid repair of the resorption lacunae, with cellular cementum-like material observed along the root surface.

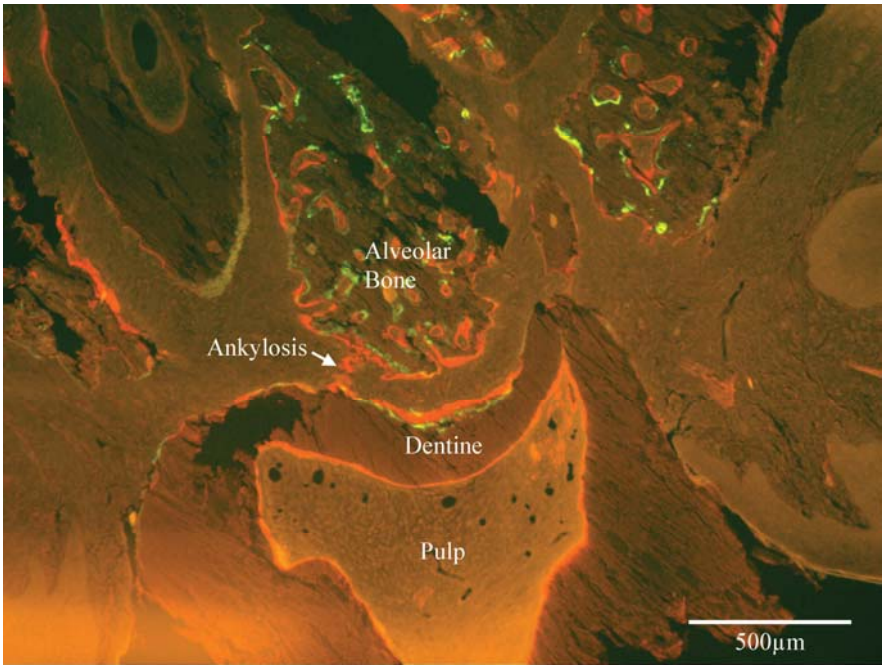

Figure 3. Ankylosis at day 14, unstained. Calcein and alizarin red labels. Focal type of ankylosis with rapid apposition across the periodontal ligament space, characterised by a concentration of alizarin red (white arrow).

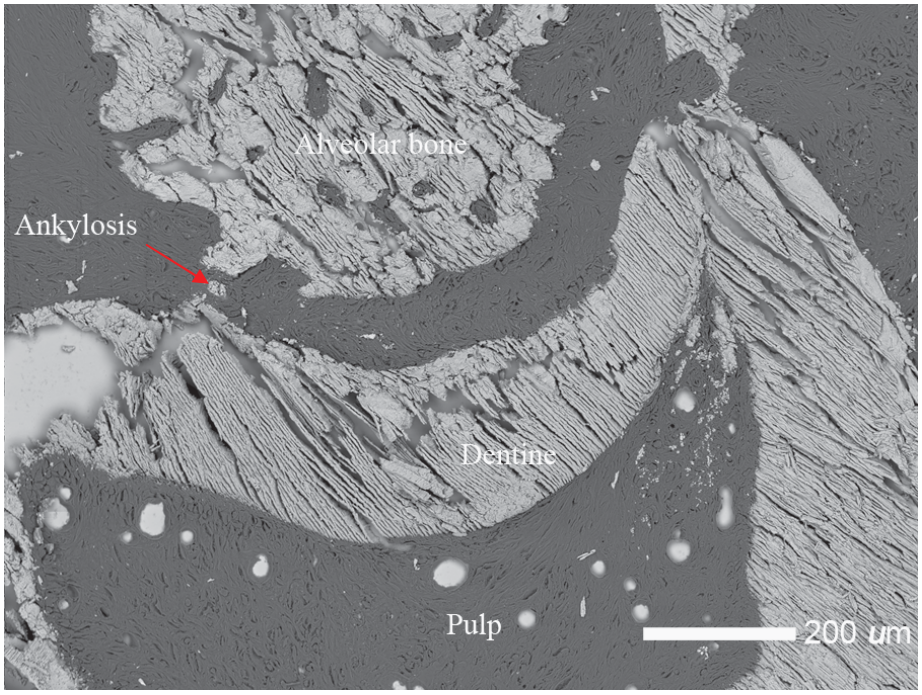

Figure 4. Ankylosis at day 14, BSE image of Figure 3. BSE imaging reveals the ankylotic material to be of a focal type of ankylosis (red arrow), characterised by a centralized nodule within the PDL with reactive tissue extending from the root surface and bone side.

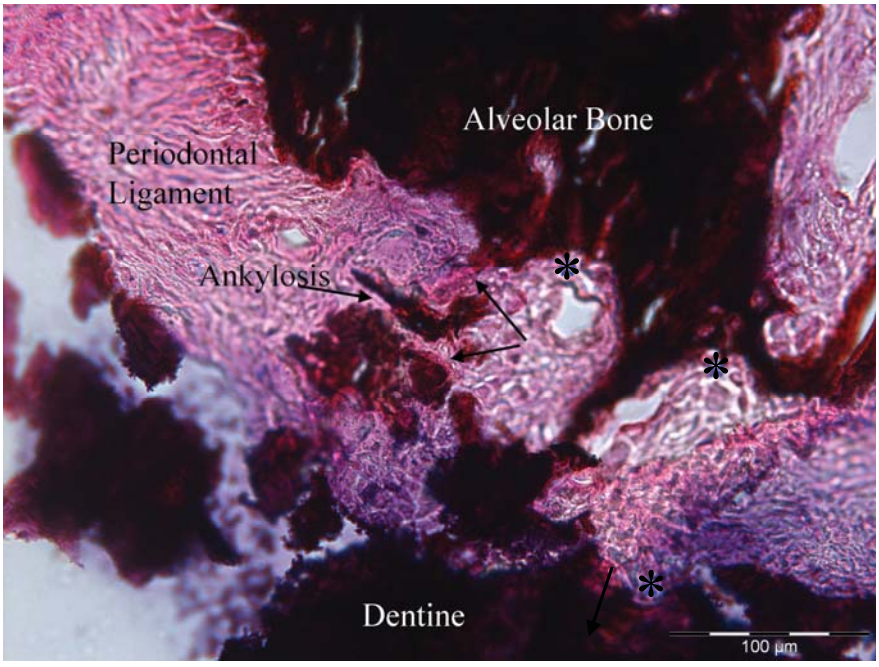

Figure 5. Ankylosis at day 14, VK/H&E stain. Deposits of unmineralised matrix along the bone, root surfaces and in the body of the ankylotic region (arrows). Ankylotic extensions occur adjacent to irregular surfaces characteristic of resorption along the bone and root surface (asterisks).

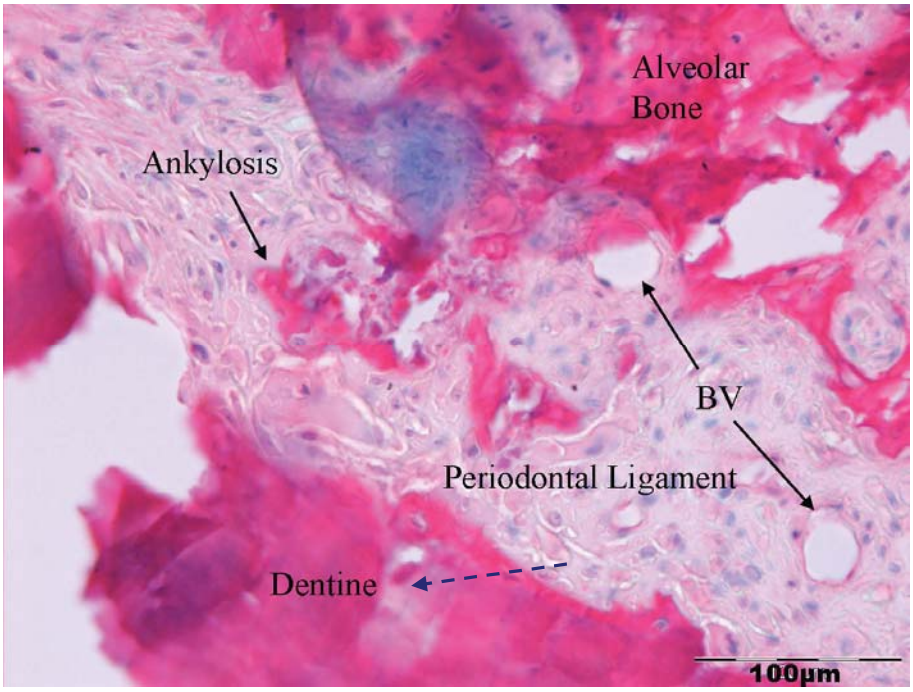

Figure 6. Ankylosis at day 14, H&E stain. Ankylosis with increased cellularity and vascularity in the periodontal ligament. Cellular cementum-like tissue (broken arrow) on the root surface. Adjacent section to Fig 5. BV=Blood vessels

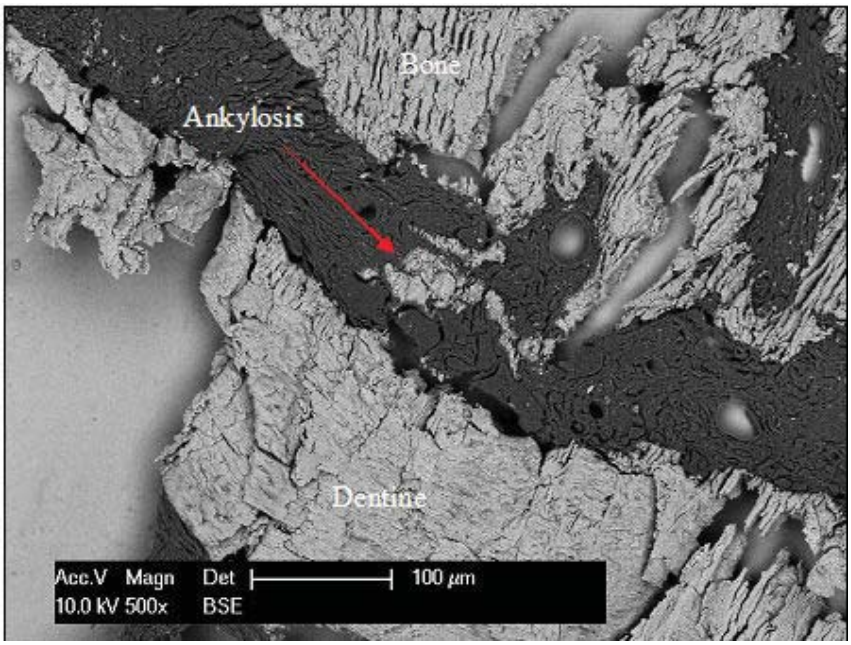

Figure 7. Ankylosis at day 14, BSE image. Ankylosis appearing as a central nodule within the PDL similar to Figure 4. Finger like extensions are visible, extending from the alveolar wall.

Twenty one days after thermal injury, definite ankylosis was seen in 1 treatment rat (Figures 8 and 9). Some common features were also noted in the pattern of ankylosis as seen in day 14. Once again, a centralised nodule featured, although larger in size and almost extending across the entire width of the PDL. Finger-like extensions connected the focal ankylotic region to the adjacent bone and root surfaces. A layer of unmineralised matrix lined the peripheral surfaces of the ankylotic region and between its bone and root connection in the VK/H&E section (Figure 8). Cellular cementum-like material noted along the root surface suggested a repair response had occurred. Only calcein labels were visible in the unstained section (Figure 9). The labels were concentrated along the peripheries of the ankylotic body, and the immediately adjacent bone and root surfaces. This suggested that the ankylosis may have been a result of an over exuberant mineralized tissue apposition, which may have occurred initially as a part of a repair response to resorption along the root surface.

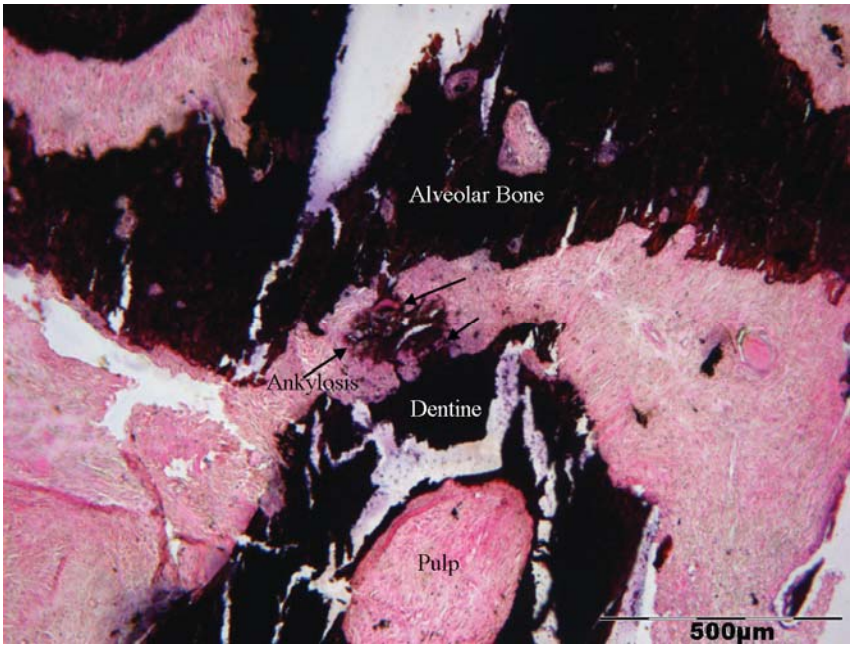

Figure 8. VK/H&E stain of ankylosis at 21 days. Ankylosis with deposits of unmineralised matrix (unlabeled arrows) along its peripheries.

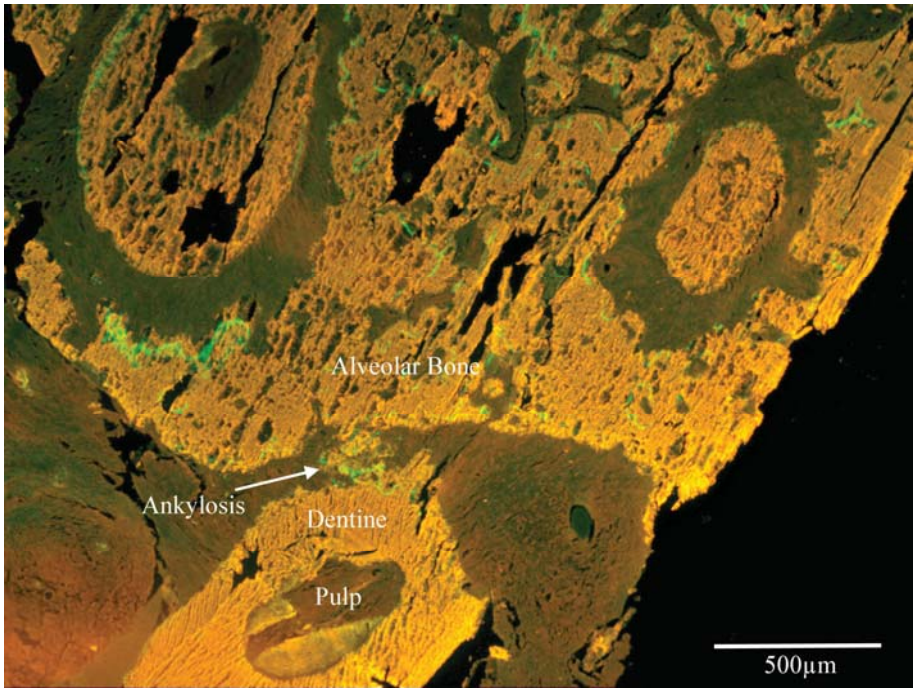

Figure 9. Ankylosis at 21 days, unstained, fluorescence microscope image. Focal region of ankylosis. Active mineral apposition present on the surface of the ankylotic body and along its immediately connecting bone and root surfaces.

Serial, sections of ankylosis at day 21 were examined with BSE. This created a tomographic view of the ankylosis from a mesial to distal direction (Figure 10-1, 2 and 3). The ankylotic material initially appeared as centralized, nodules within the PDL (Figure 10-1). In the successive section the ankylotic body appeared as multiple smaller nodules coalesced together to form a single larger mass (Figure 10-2). No structural organisation was observed in the ankylotic body in comparison to the tubular structure seen in dentine as well as alveolar bone, which consists of successive layers of coarse fibered woven bone fibres running parallel to the socket wall and arranged in lamellae. Concurrent slides showed an increase in volume of the central mass with fusion to the surrounding tissues. The finger-like whorls became thicker and formed definite ankylotic

bridging. This emphasizes the 3-dimensional focal nature of ankylosis within the PDL space.

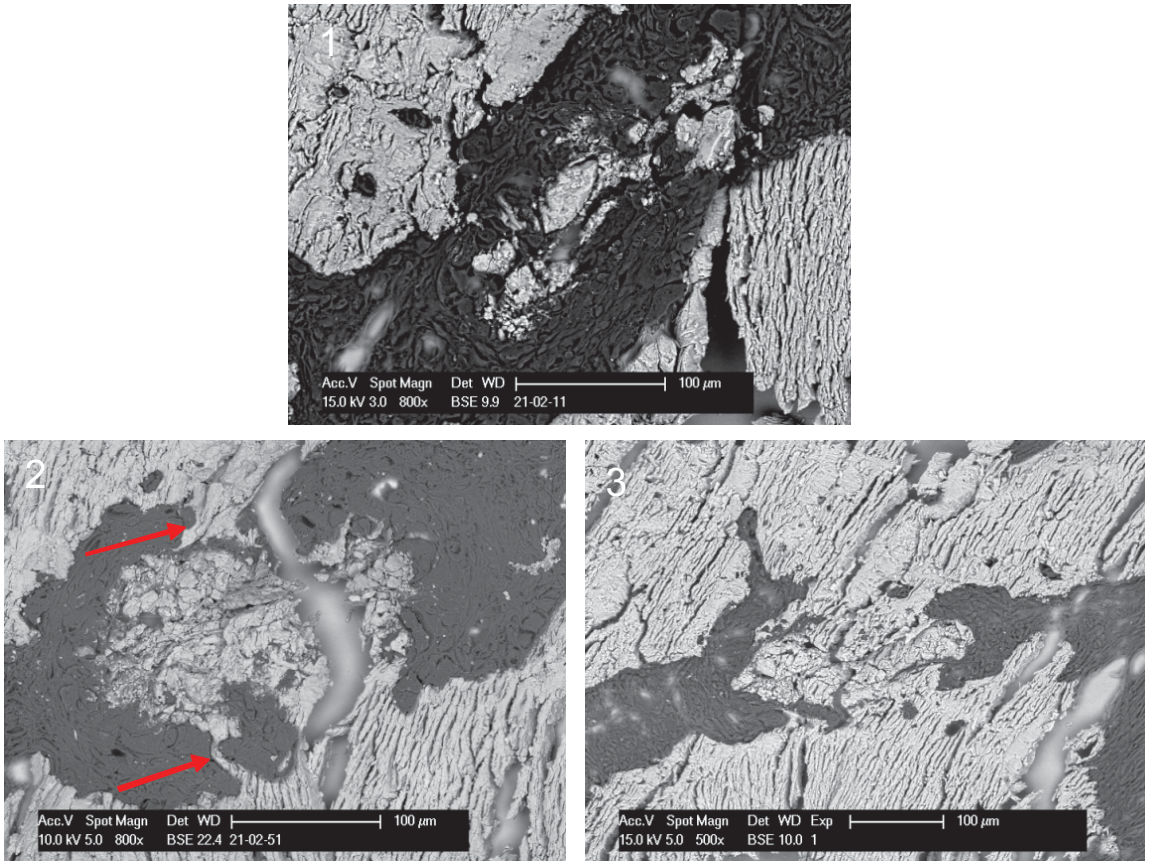

Figure 10. BSE Serial sections of Ankylosis at day 21. A tomographic view from mesial of the furcation towards the distal. (1) Mesial section of furcation shows small mineralized nodules in the central part of the periodontal membrane. (2) Mid furcal section shows an enlargement of the central nodular mineralization. The central mineralized tissue fuses with hard tissue on the surfaces with finger-like extension from the root and alveolar bone (red arrows). (3) The following distal section displays a much more definite bridging. Here the lack of structural organisation of ankylosis is evident compared to tubular nature of dentine and lamellar organisation of alveolar bone.

X-Ray Microanalysis

Initial qualitative analysis was performed with spot analyses of all hard tissues. EDS data (Figure 11) showed strong peaks for Ca and P and low peaks for Mg and Na. This was also similar for bone, cementum and dentine in all rats.

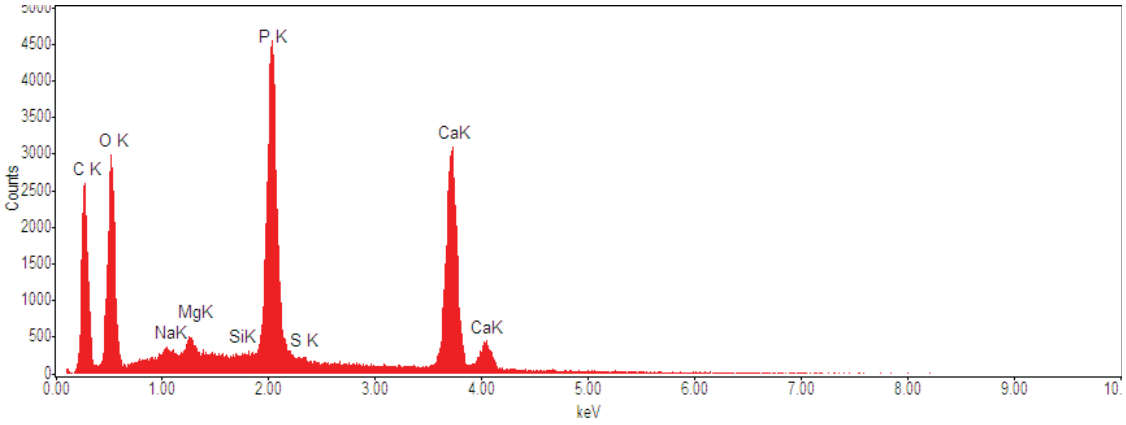

Figure 11. EDS spectrum of a spot scan in ankylotic tissue. Strong peaks were shown of Ca, P, O and C, with lower peaks for Na and Mg. Similar spectrums were obtained for all other tissue types in all the rats studied.

Colour Mapping

Colour mapping of the detected elements in a slide featuring ankylosis showed that Ca, P and the trace elements were found in ankylosis as well as the surrounding calcified tissues such as bone and dentine (Figure 12).

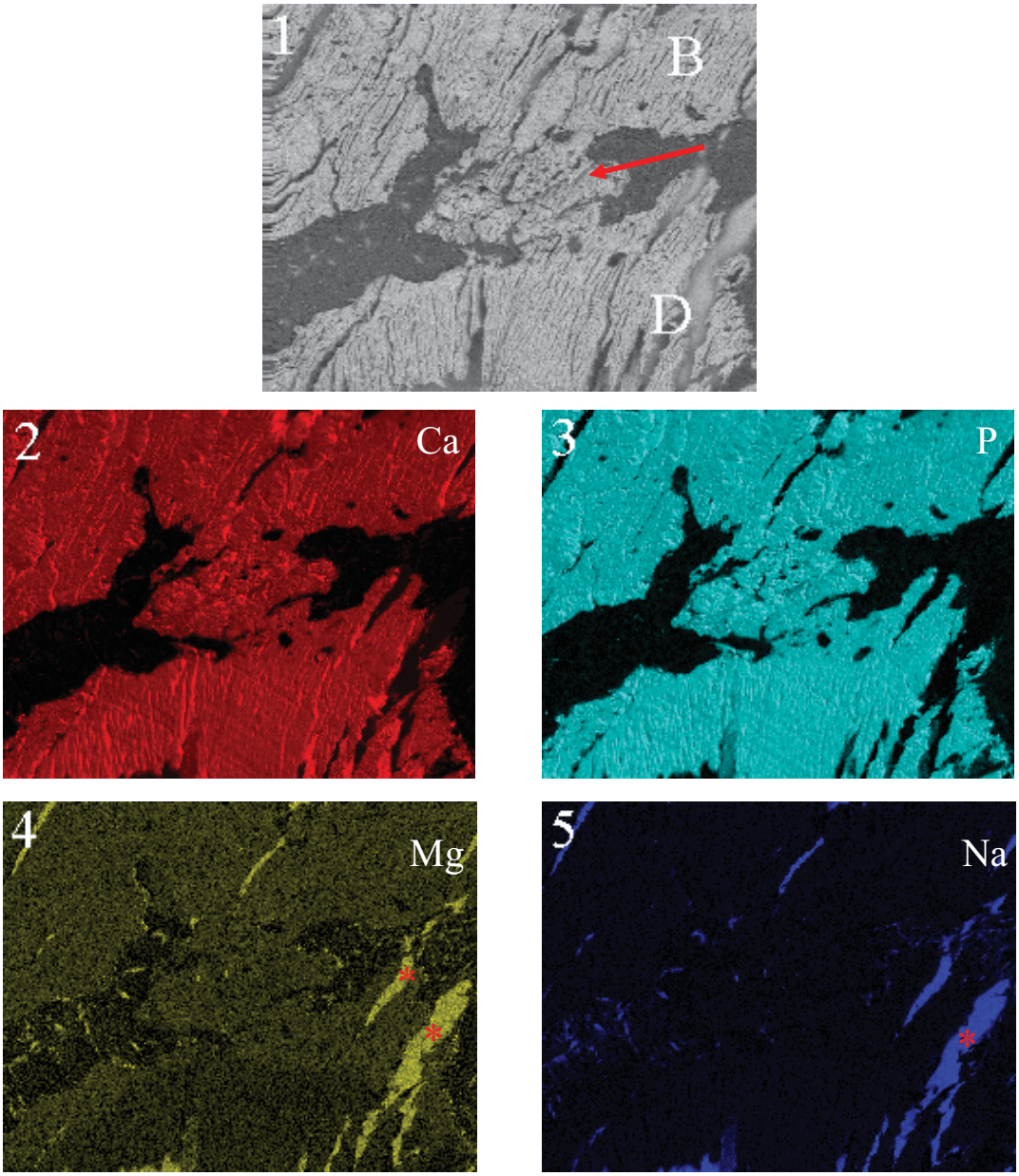

Figure 12. Colour mapping by wt% of Ca, P, Mg and Na. 1. BSE image of ankylotic area (red arrow). B=alveolar bone, D=dentine. 2. Calcium distribution as characterised by red. The more intense the colour, the higher the weight percentage of the element. 3. Phosphorous. 4. Magnesium, 5. Sodium. Asterix (*) indicates tears in the section, exposing the underlying glass slide for x-ray analysis.

Quantitative analysis

The mean Ca/P ratios as well as Mg and Na concentrations of bone, cementum and dentine, in experimental molars were compared to the control molars (Figure 13). A linear fixed effect model revealed that there was no significant interaction effect between sides (treatment vs. control) for each region (i.e. bone, cementum and dentine) for Ca/P ratio (P=0.81), Mg (P=0.32) and Na (P=0.38). Thus there was no significant difference of Ca/P ratio as well as trace mineral concentrations between the experimental molars and the control molars.

As the interaction term was not statistically significant, a second linear mixed effects model excluding the interaction term was fitted to the data. This allowed interpretation of the main effects of regions. The second linear mixed effects model showed that there was evidence for a significant difference between the four regions for Ca/P ratios (P=0.0182), Mg (P<0.0001) and Na (P<0.0001). *Post Hoc* tests were then used to compare the regions (Figure 14).

Comparing ankylosis, bone, cementum and dentine, the Ca/P ratios, as revealed by spot analyses, ranged from an average 1.85 to 2.00. Ankylosis did not differ significantly to bone (P=0.17), cementum (P=0.67), and dentine (P=0.076). Post hoc tests did reveal, however, that the mean Ca/P ratio was significantly higher in bone compared to cementum (P=0.002).

Mg concentration in ankylosis was not significantly different to that of bone. Ankylosis did have a significantly lower concentration to cementum (P=0.01) but was higher compared with dentine (P<0.0001). When comparing the other regions, the concentration

of Mg was higher in cementum than bone (P=0.0002) while dentine was lower than both bone (P<0.0001) and cementum (P<0.0001).

The Na concentration of ankylosis appeared slightly higher than bone and cementum; however, this was not statistically significant (P=0.08 and P=0.07 respectively). Dentine displayed significantly higher concentrations of Na compared to ankylosis (P=0.03), bone (P<0.0001), and dentine (P<0.0001).

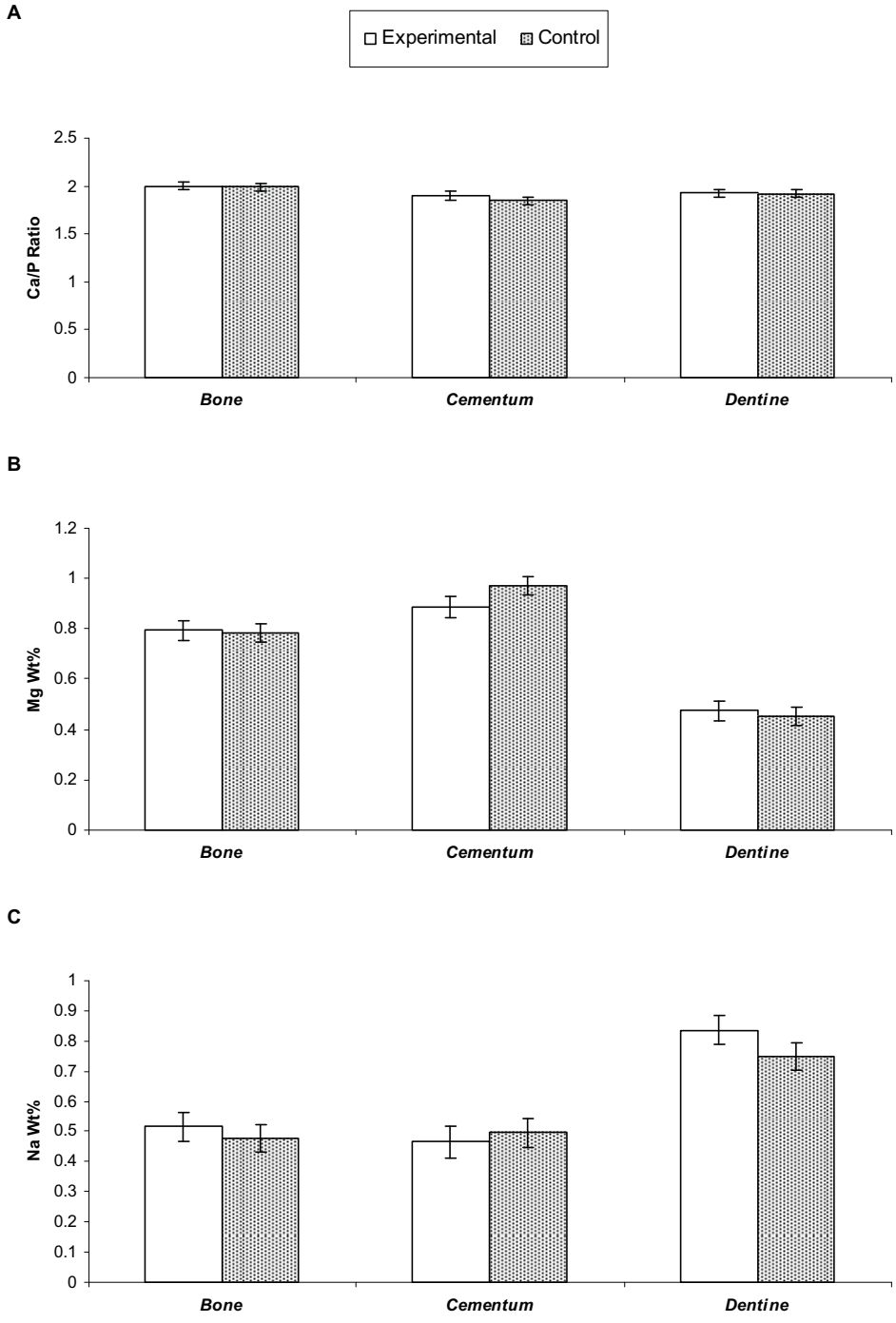

Figure 13. Comparisons of Ca/P ratio (A), Mg (B) and Na (C) concentrations (Wt %) in different hard tissues (bone, cementum, and dentine) between the experimental and control teeth. Values are means ± Std error. No statistically significant differences were detected between the experimental and control teeth.

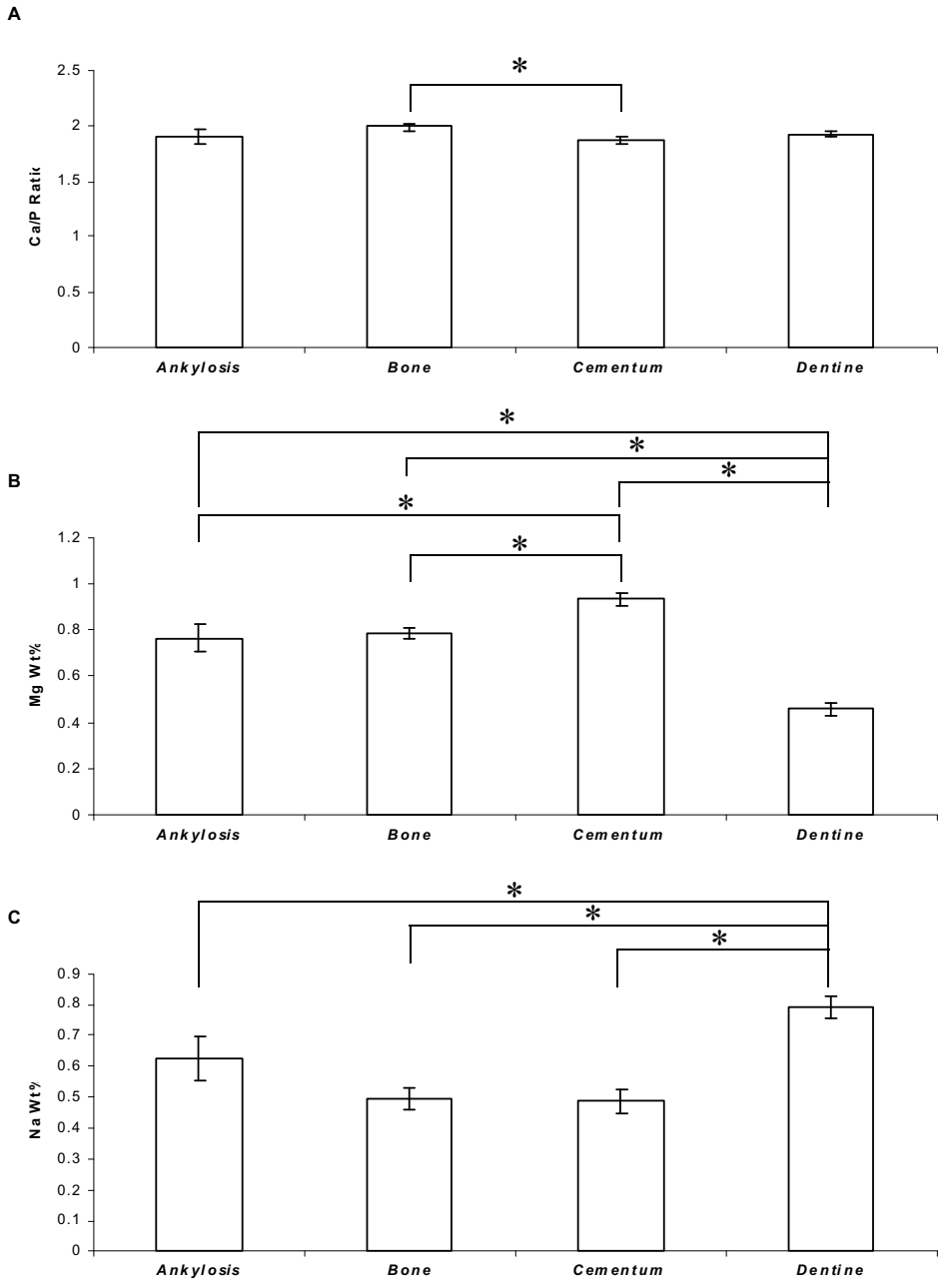

Figure 14. Adjusted mean values of Ca/P (A), Mg (B) and Na (C) concentrations (Wt %) in different hard tissues (ankylosis, bone, cementum, and dentine) of the experimental and control teeth. Values are means ± Std error. Linear mixed effects model followed by *post-hoc* testing was used for multiple comparisons (*P<0.05)

The Ca/P ratio of bone in the sham rats was compared over time (Figure 15). Qualitatively, no major differences were seen from day 7 to day 28. Due to the small number of rats (n=4), no valid statistical analysis was able to be applied to the data.

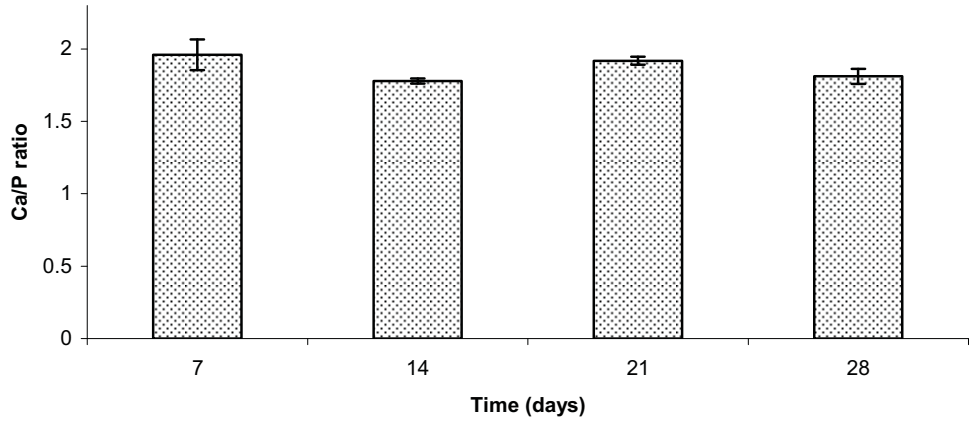

Figure 15. Mean Ca/P ratios of alveolar bone in shams rats over time. Values are means ± Std error.

Discussion

Ankylosis, as observed in this study, was characterised by a focal region of mineralisation within the centre of the PDL. This focal region exhibited morphological characteristics which differed to that of bone and dentine. Ankylosis presented as a single nodule (Figures 4 and 7) or multiple nodules (Figure 10) which was accompanied by reactive mineral deposition along both the alveolar socket wall as well as the tooth root surface. Where bridging had occurred, it appeared as finger-like trabeculae extending from the bone and root surface (Figure 10). The findings from this study suggest that focal nodules within the PDL may have been the initial nidus in the development of ankylosis.

Where ankylosis was established, the ankylotic body was nodular in shape and connected by finger-like extensions to the bone and root surface, and lined by a rim of unmineralised matrix, with a concentration of bone labels in situ. This supports the findings of Hammarström who observed that ankylosis started with the formation of bone-like tissue in the central region of the PDL, which then fused with newly formed hard tissue on the root surface and socket wall (Hammarström et al., 1989). The central location of the ankylotic material is suggestive of the fact that the mineralised deposit may originate from cells of osteogenic nature within the PDL.

Following hypothermal injury to the PDL, the formation and presence of calcified bodies with quite varied morphology have been observed in this study. Similar calcified bodies have mainly been noted in tooth replantation studies (Hammarström et al., 1989;

Lindskog and Blomlöf, 1992). Calcified particles have also been observed in the pressure zone during tooth movement in rats (Nakamura et al., 1996; Nakamura et al., 2003). These calcified bodies were visible at day 7 and still evident at day 21. Nakamura (2003) concluded that the periodontal reaction and calcified body formation during tooth movement was a self defence response to a non-infectious inflammatory reaction. Thus the initial formation of calcified bodies may be a physiological response to injury.

Animal experiments have shown that when damage and disruption of the PDL cells occurs by mechanical means, thermal injury or is altered by pharmacological agents, formation of areas of necrotic tissue and granulation tissue are provoked (Andreasen, 1980; Andreasen and Kristersen, 1981; Dreyer et al., 2000; Hammarström et al., 1989; Karring et al., 1980; Line et al., 1974; Nyman et al., 1982; Wesselink and Beertsen, 1994). These areas, then evoke an array of both destructive and reparative processes in the periodontium (Lindskog and Blomlöf, 1992). The healing processes of the PDL have been compared with the callus formation after fracture of a bone (Hammarström et al., 1989). After initial inflammation has subsided, a provisional callus of immature bone and cartilage is formed on the bone surface. Some osteoblasts seem to arise from transformed fibroblasts in the granulation tissue. Subsequently, this provisional callus is strengthened by increased mineralization and widening of the initial bony trabeculae. Under optimal conditions this callus then regresses with time and a virtually perfect reconstruction of the bone is accomplished. In the PDL, the 'consolidation of a callus' occurs when a direct connection, by mineralised tissue, between the tooth and wall of the alveolar socket has been established.

It has been well documented that localised ankylotic areas can sometimes be removed and the integrity of the PDL restored. The formation of mineralised tissue within the PDL after injury can be regarded as an immature "callus-like" hard tissue. With time, a normal periodontal membrane would be then re-established in the same way as the periosteal tissues regain their morphology and function after healing of a bone fracture (Hammarström et al., 1989). Regardless of the nature of the damage, a key factor in the re-establishment of a healthy PDL is the viability of the surrounding periodontal membrane (Beertsen et al., 1997; Lindskog and Blomlöf, 1992; Melcher, 1970; Nanci and Bosshardt, 2006).

The origins and nature of the cells producing calcified bodies in the PDL are largely unknown. Experimental evidence supports the concept that the PDL is a repository of cells involved in the formation of cementum, PDL itself and alveolar bone (Karring et al., 1993). However, the nature and precise locations of these progenitor cells remain to be determined (Nanci and Bosshardt, 2006). However, possible locations for these progenitor cell populations of PDL include perivascular locations near cementum and vascular channels in alveolar bone (Gould et al., 1980; McCulloch and Melcher, 1983; McCulloch, 1985). There is also limited cell kinetic evidence that a second-cell population located adjacent to the cementum is a separate progenitor population (Gould et al., 1980; McCulloch and Melcher, 1983).

PDL cells isolated from the root socket of Sprague Dawley rats have been demonstrated to form mineralised nodules in vitro (Cho et al., 1992). The nodules displayed morphological characteristics different from bone mineralized nodules. In the current

study, the ankylotic nodules displayed a lack of structural organisation compared to that of alveolar bone and dentine. This feature, along with the initial presentation within the PDL and within vicinity of blood vessels (Figure 6), suggest the possibility that ankylosis is a consequence of recognised osteogenic potential of the PDL cells. Recently, real-time RT-PCR analysis of PDL cells under tensile strain showed upregulation of genes linked to the osteoblastic phenotype. Furthermore, the PDL cells were also found to constitutively express numerous osteotropic cytokines and growth factors (Pinkerton et al., 2008; Wescott et al., 2007).

EDS x-ray microanalysis was used to compare the mineral profiles of ankylosis, bone, cementum and dentine. Comparison of the Ca/P ratio, which indicates crystallization of hydroxyapatite, have been used to compare mineralization of dental hard tissues (Arnold et al., 2001). X-ray microanalysis revealed high peaks of Ca and P and comparison of the Ca/P ratio of ankylosis to that of bone, cementum and dentine, showed that the ratio was similar with no statistical difference between the various hard tissues. The Ca/P ratios found in this study compare favourably to that of rat dentine reported by others (Huumonen and Larmas, 1999;Tjäderhane et al., 1995). This suggests that ankylotic mineral is similar to that of a biological apatite.

Magnesium as the fourth highest concentrated cation in the human body (Posner, 1969), has been implicated in the biomineralisation of bone and tooth and is present in various concentrations within dentine, bone, cementum and enamel (Terpstra and Driessens, 1986; Tsuboi et al., 1994; Wiesmann et al., 1997). Reported concentrations of Mg in cementum range from 0.5-0.9% (Nakata et al., 1972; Neiders et al., 1972). This compares

favourably to the findings in this study. The Mg concentration of ankylosis was similar to that of bone, but was statistically different to that of dentine and cementum. Further observations of Figure 12b 14b shows a trend in that the Mg concentrations of bone and cementum are similar when compared to dentine. This agrees with the notion that the composition of cementum is more similar to bone tissue than to dentine. (Bosshardt and Selvig, 1997; Gonçalves et al., 2005)

Sodium has been detected as a trace element in dental hard tissues (Barton and Van Swol, 1987; Hals et al., 1988; Sano et al., 2005). However, its influence on apatite distribution and significance has not been covered in any detail in the literature. Na concentration, in the present study, was shown to be higher in dentine than ankylotic tissue, bone and cementum. There was a trend for Na concentration to be higher in ankylosis as compared to bone; however, this was not statistically significant. The amount of ions initially incorporated into the mineral phase reflects their concentration in the fluid environment during mineralization (Bosshardt and Selvig, 1997), thus relative differences in trace elements suggests that the ankylotic body may have formed initially within the PDL, separate from alveolar bone and cementum.

Age related changes have been documented to have an effect on the Ca/P ratio. It is proposed that some of the potential initial phases of mineralized hard tissue may be amorphous calcium phosphate or octocalcium phosphate (Termine and Posner, 1967). These phases have a theoretical Ca/P molar ratio of 1.3 and 1.5 respectively. The Ca/P ratio would increase with age as the mineral matures towards the ideal hydroxyapatite,

which has a Ca/P ratio of 1.67. A study on rat and bovine cortical bone by X-ray powder diffraction analysis found that the Ca/P ratio increased with age (Legros et al., 1987). This finding is in correlation with previous studies (Meinke et al., 1979; Pellegrino and Biltz, 1972). In the present study, no differences in Ca/P ratio was observed over time (Figure 15). This may be due to the short observational period. Previous literature reported on small changes of approximately 2.5% in the Ca/P ratio over a period of 1 and 7 years. Thus the changes may not be evident from the limited data.

The experimental protocol of the current study was designed to standardise the thermal insult intervention over each observation time group in an attempt to produce consistent results in each of the animals. However, only three of the 24 experimental animals displayed definite ankylotic bridging. It would be expected that most, if not all animals from each group should either develop ankylosis or otherwise remain unaffected. Three additional animals, at day 21, also showed partial changes within the PDL *(please refer to appendix 9.5 for images)*. The changes included: large amounts of cellular cementum-like deposits on the root surface; finger-like extensions extended from the surface of the alveolar bone and mineralized bone-like tissue in the central part of PDL.

Compared to previous studies employing a similar technique (Di Iulio, 2007; Shaboodien, 2005) there were fewer numbers of rats with ankylosis. Dreyer (2002) suggested that a sufficient length of time is required for a thermal stimulus, when applied to a tooth, to produce changes in the surrounding tissues. In that study, a 10 minute application of dry ice was considered long enough to reliably produce resorptive changes, whereas a single 20 minute application or multiple freezing episodes were suggested for

the production of ankylosis. Severity of insult has been positively correlated with incidence of ankylosis (Hammarström et al., 1989). Although the freezing time used in the current study fulfilled these guidelines, other factors may have been involved: including variations in the thickness of the dentine and enamel; and inconsistency in size of the dry ice pellets. Further refinement of the technique will enable future research in the thresholds of the PDL and its responses to hypothermic insult. A freezing time longer than 20 minutes or multiple freezing episodes should be considered in order to achieve more consistent ankylosis.

CONCLUSION

The results of this study show that ankylosis formed in response to hypothermal injury has similar mineral profile to bone and thus has the characteristics of biologic apatite. The morphology of ankylosis, as observed in BSE imaging and histological slides, differs from alveolar bone and dentine. There is some evidence to suggest that ankylosis may be due to the osteogenic potential of the PDL cells.

The following null hypotheses are rejected: (1) A single, prolonged thermal insult to a rat molar has no effect on mineralized tissue formation and composition within the periodontium and (2) ankylotic tissue is similar to alveolar bone.

REFERENCES

Andersson L, Blomlöf L, Lindskog S, Feiglin B, Hammarström L (1984). Tooth ankylosis. Clinical, radiographic and histological assessments. *Int J Oral Surg* 13:423-431.

Andreasen J (1980). A time-related study of periodontal healing and root resorption activity after replantation of mature permanent incisors in monkeys. *Swedish Dental Journal* 4:101-110.

Andreasen J, Kristersen L (1981). The effect of limited drying or removal of the periodontal ligament. Periodontal healing after replantation of mature incisors in monkeys. *Acta Odontol Scand* 39:1-13.

Andreasen JO, Skougaard MR (1972). Reversibility of surgically induced dental ankylosis in rats. *Int J Oral Surg* 1(2)*:*98-102.

Arnold S, Plate U, Wiesmann HP, Stratmann U, Kohl H, Hohling HJ (2001). Quantitative analyses of the biomineralization of different hard tissues. *J Microsc* 202(Pt 3)*:*488-94.

Arnold WH, Gaengler P (2007). Quantitative analysis of the calcium and phosphorus content of developing and permanent human teeth. *Ann Anat* 189(2)*:*183-90.

Barton NS, Van Swol RL (1987). Periodontally diseased vs. normal roots as evaluated by scanning electron microscopy and electron probe analysis. *J Periodontol* 58(9)*:*634-8.

Beertsen W, McCulloch CAG, Sodek J (1997). The periodontal ligament: a unique, multifunctional connective tissue. *Periodontology 2000* 13:20-40.

Biederman W (1968). The problem of the ankylosed tooth. In: Dental Clinics of North America, Symposium on Orthodontics. WG Spengeman editor. Philadelphia: W.B. Saunders.

Bosshardt DD, Selvig KA (1997). Dental cementum: the dynamic tissue covering of the root. *Periodontol 2000* 13:41-75.

Chang A (2008). Interradicular Mineralized Tissue Adaptation In An Aseptic Necrosis Model. Doctorate of Clinical Dentistry Thesis. Adelaide, University of Adelaide.

Cho MI, Matsuda N, Lin WL, Moshier A, Ramakrishnan PR (1992). In vitro formation of mineralized nodules by periodontal ligament cells from the rat. *Calcif Tissue Int* 50(5)*:*459-67.

Di Iulio DS (2007). Relationship of epithelial cells and nerve fibres to experimentally induced dentoalveolar ankylosis in rats. Doctorate of Clinical Dentistry Thesis (thesis). Adelaide, University of Adelaide.

Dreyer CW, Pierce AM, Lindskog S (2000). Hypothermic insult to the periodontium: a model for the study of aseptic tooth resorption. *Endod Dent Traumatol* 16:9-15.

Gonçalves PF, Enilson Antonio Sallum, Antonio Wilson Sallum, Márcio Zaffalon Casati, Sérgio de Toledo, Junior FHN (2005). Dental cementum reviewed: development, structure, composition,regeneration and potential functions *Brazilian Journal of Oral Sciences* Vol. 4(No.12)*:*651-658

Gould TR, Melcher AH, Brunette DM (1980). Migration and division of progenitor cell populations in periodontal ligament after wounding. *J Periodontal Res* 15(1)*:*20-42.

Hals E, Tveit AB, Totdal B (1988). X-ray microanalysis of dentin: a review. *Scanning Microsc* 2(1)*:*357-69.

Hammarström L, Blomlöf L, Lindskog S (1989). Dynamics of dentoalveolar ankylosis and associated root resorption. *Endod Dent Traumatol* 5:163-175.

Hellsing E, Alatli-Kut I, Hammarström L (1993). Experimentally induced dentoalveolar ankylosis in rats. *Int Endod J* 26(2)*:*93-8.

Huumonen S, Larmas M (1999). An electron probe x-ray microanalytical study of dentine minerals in sucrose-fed or glucocorticoid-medicated rats. *Calcif Tissue Int* 65(3)*:*223-5.

Karring T, Nyman S, Lindhe J (1980). Healing following implantation of periodontitis affected roots into bone tissue. *J Clin Periodontol* 7(2)*:*96-105.

Karring T, Nyman S, Gottlow J, Laurell L (1993). Development of the biological concept of guided tissue regeneration--animal and human studies. *Periodontol 2000* 1:26-35.

Legros R, Balmain N, Bonel G (1987). Age-related changes in mineral of rat and bovine cortical bone. *Calcif Tissue Int* 41(3)*:*137-44.

Lindskog S, Blomlöf L (1992). Mineralized tissue-formation in periodontal wound healing. *J Clin Periodontol* 19(10)*:*741-8.

Line SE, Polson AM, Zander HA (1974). Relationship between periodontal injury, selective cell repopulation and ankylosis. *J Periodontol* 45(10)*:*725-30.

Löe H, Waerhaug J (1961). Experimental replantation of teeth in dogs and monkeys. *Arch Oral Biol* 3:176-184.

McCulloch CA, Melcher AH (1983). Cell density and cell generation in the periodontal ligament of mice. *Am J Anat* 167(1)*:*43-58.

McCulloch CA (1985). Progenitor cell populations in the periodontal ligament of mice. *Anat Rec* 211(3)*:*258-62.

McCulloch CA, Bordin S (1991). Role of fibroblast subpopulations in periodontal physiology and pathology. *J Periodontal Res* 26(3 Pt 1)*:*144-54.

Meinke DK, Skinner HC, Thomson KS (1979). X-ray diffraction of the calcified tissues in Polypterus. *Calcif Tissue Int* 28(1)*:*37-42.

Melcher AH (1970). Repair of wounds in the periodontium of the rat. Influence of periodontal ligament on osteogenesis. *Arch Oral Biol* 15:1183-204.

Melcher AH, McCulloch CA, Cheong T, Nemeth E, Shiga A (1987). Cells from bone synthesize cementum-like and bone-like tissue in vitro and may migrate into periodontal ligament in vivo. *J Periodontal Res* 22(3)*:*246-7.

Nakamura Y, Tanaka T, Kuwahara Y (1996). New findings in the degenerating tissues of the periodontal ligament during experimental tooth movement. *Am J Orthod Dentofacial Orthop* 109:348-354.

Nakamura Y, Tanaka T, Noda K, Shimpo S, Oikawa T, Hirashita A, et al. (2003). Calcification of degenerating tissues in the periodontal ligament during tooth movement. *J Periodontal Res* 38:343-50.

Nakata TM, Stepnick RJ, Zipkin I (1972). Chemistry of human dental cementum: the effect of age and fluoride exposure on the concentration of ash, fluoride, calcium, phosphorus and magnesium. *J Periodontol* 43(2)*:*115-24.

Nanci A, Bosshardt DD (2006). Structure of periodontal tissues in health and disease. *Periodontol 2000* 40:11-28.

Neiders ME, Eick JD, Miller WA, Leitner JW (1972). Electron probe microanalysis of cementum and underlying dentin in young permanent teeth. *J Dent Res* 51(1)*:*122-30.

Ngo H, Ruben J, Arends J, White D, Mount GJ, Peters MC, et al. (1997). Electron probe microanalysis and transverse microradiography studies of artificial lesions in enamel and dentin: a comparative study. *Adv Dent Res* 11(4)*:*426-32.

Nyman S, Gottlow J, Karring T, Lindhe J (1982). The regenerative potential of the periodontal ligament. An experimental study in the monkey. *J Clin Periodontol* 9(3)*:*257-65.

Pellegrino ED, Biltz RM (1972). Mineralization in the chick embryo. I. Monohydrogen phosphate and carbonate relationships during maturation of the bone crystal complex. *Calcif Tissue Res* 10(2)*:*128-35.

Pinkerton MN, Wescott DC, Gaffey BJ, Beggs KT, Milne TJ, Meikle MC (2008). Cultured human periodontal ligament cells constitutively express multiple osteotropic cytokines and growth factors, several of which are responsive to mechanical deformation. *J Periodontal Res* 43(3)*:*343-51.

Posner AS (1969). Crystal chemistry of bone mineral. *Physiol Rev* 49(4)*:*760-92.

Rex T, Kharbanda OP, Petocz P, Darendeliler MA (2005). Physical properties of root cementum: Part 4. Quantitative analysis of the mineral composition of human premolar cementum. *Am J Orthod Dentofacial Orthop* 127(2)*:*177-85.

Sanchez-Quevedo MC, Nieto-Albano OH, Garcia JM, Gomez de Ferraris ME, Campos A (1998). Electron probe microanalysis of permanent human enamel and dentine. A methodological and quantitative study. *Histol Histopathol* 13(1)*:*109-13.

Sano T, Morohashi T, Amano H, Yamada S, Nakamura M (2005). Distributions of magnesium and sulphur in defect layers of incisor dentine in magnesium-deficient rats. *Arch Oral Biol* 50(8)*:*715-25.

Selvig KA, Selvig SK (1962). Mineral content of human and seal cementum. *J Dent Res* 41:624-32.

Shaboodien S (2005). Traumatically induced dentoalveolar ankylosis in rats. Adelaide, Doctor of Clinical Dentistry Thesis, University of Adelaide.

Tal H, Stahl S (1986). Healing following devitalization of sites within the periodontal ligament by ultralow temperatures. *Journal of Periodontology* 57:735-741.

Tal H, Kozlovsky A, Pitaru S (1991). Healing of sites within the dog periodontal ligament after application of cold to the periodontal attachment apparatus. *J Clin Periodontol* 18(7)*:*543-7.

Termine JD, Posner AS (1967). Amorphous/crystalline interrelationships in bone mineral. *Calcif Tissue Res* 1(1)*:*8-23.

Terpstra RA, Driessens FC (1986). Magnesium in tooth enamel and synthetic apatites. *Calcif Tissue Int* 39(5):348-54.

Tjaderhane L, Hietala EL, Larmas M (1995). Mineral element analysis of carious and sound rat dentin by electron probe microanalyzer combined with back-scattered electron image. *J Dent Res* 74(11):1770-4.

Tsuboi S, Nakagaki H, Ishiguro K, Kondo K, Mukai M, Robinson C, et al. (1994). Magnesium distribution in human bone. *Calcif Tissue Int* 54(1):34-7.

Webster TJ, Massa-Schlueter EA, Smith JL, Slamovich EB (2004). Osteoblast response to hydroxyapatite doped with divalent and trivalent cations. *Biomaterials* 25(11):2111-21.

Wescott DC, Pinkerton MN, Gaffey BJ, Beggs KT, Milne TJ, Meikle MC (2007). Osteogenic gene expression by human periodontal ligament cells under cyclic tension. *J Dent Res* 86(12):1212-6.

Wesselink PR, Beertsen W, Everts V (1986). Resorption of the mouse incisor after the application of cold to the periodontal attachment apparatus. *Calcif Tissue Int* 39:11-21.

Wesselink PR, Beertsen W (1994). Ankylosis of the mouse molar after systemic administration of 1-hydroxyethylidene-1,1-bisphosphonate (HEBP). *J Clin Periodontol* 21:465-71.

Wiesmann HP, Tkotz T, Joos U, Zierold K, Stratmann U, Szuwart T, et al. (1997). Magnesium in newly formed dentin mineral of rat incisor. *J Bone Miner Res* 12(3):380-3.