INVESTIGATIONS ON THE SUITABILITY OF SHEEP AS A MODEL FOR ENDODONTIC REVITALISATION RESEARCH

A thesis submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy in Dentistry

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Signed statement

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Milad Al Taii

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I dedicate lovingly this thesis to my country Iraq, the cradle of civilization.

List of Abbreviations

µm/µg/µl	Micrometre / Microgram / Microliter
3D	Three Dimensions
AAC	Acellular Afibrillar Cementum
AAE	American Association of Endodontics
AEFC	Acellular Extrinsic Fibre Cementum
ALP	Alkaline Phosphatase
AM	Ameloblast
ARS	Alizarin Red Stain
BC	Blood Clot
BI	Bone-like Island
BMP	Bone Morphogenic Proteins
BMSCs	Bone Mesenchymal Stem Cells
C	Cementum
$Ca(OH)_2$	Calcium Hydroxide
$CaCl_2$	Calcium Chloride
Cb	Cementoblasts
CDJ	Cemento-Dentinal Junction
CEJ	Cemento-Enamel Junction
CHX	Chlorhexidine Digluconate
CI	Cementum-like Island

CIFC	Cellular Intrinsic Fibre Cementum
cm	Centimetre
CMFC	Cellular Mixed Fibre Cementum
Coll	Collagen
CPC	Cetylpyridinium Chloride
CT	Computer Tomography
D	Dentine
DAMT	Dentine Associated Mineralised Tissue
DAP	Double Antibiotic Paste
DAPI	4’,6-Diamidino-2phenlidole
DE	Dental Epithelium
DF	Dental Follicle
DFd	Degrees of Freedom for the Denominator
DFn	Degrees of Freedom for the numerator
DM	Dental Mesenchyme
DMP	Dentine Matrix Proteins
DMSO	Dimethyl-Sulfoxide
DP	Dental Papilla
dpi	Dot per inch
DPP	Dentine Phosphorprotein
DPSCs	Dental Pulp Stem Cells

DSP	Sialoprotein
DSPP	Dentine Sialophosphoprotein
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EK	Enamel Knot
EMSCs	Embryonic Mesenchymal Stem Cells
EO	Enamel Organ
ERM	Epithelial Rest of Malassez
FCS	Foetal Calf Serum
FGF	Fibroblast Growth Factor
G	Group
GIC	Glass-Ionomer Cement
GP	Gutta Percha
H and E	Hematoxylin and eosin
H_2O_2	Hydrogen Peroxide
HA/TCP	Hydroxyapatite / Tricalcium Phosphate
HEPES	(4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid))
HERS	Hertwig's Epithelial Root Sheath
IGFI	Insulin-like Growth Factor
IM	Intra muscular Injection
IMVS	Institute of Medical and Veterinary Science

K	Kilo
L	Litre
LARIF	Large Animal Research and Imaging Facility
M	Molar
MD	Mesio-distal
mg	Milligram
MI	Mineralised Island
MIM	Mineralisation Induction Medium (MIM)
ml/mm/mM	Millilitre/ millimetre/ milliMolar
MTA	Mineral Trioxide Aggregate
mTAP	Modified Triple Antibiotic Paste
NaOCl	Sodium Hypochlorite
NaOH	Sodium Hydroxide
NCPs	Non - Collagenous Proteins
NGF	Nerve Growth Factor
ns	Non significant
OD	Odontoblast
ODPCs	Ovine Dental Pulp Cells
PDGF	Platelet Derived Growth Factor
PDL	Periodontal Ligament
PDLSCs	Periodontal Ligament Stem Cells

PGA	Poly-Glycolic Acid
PLA	Poly-Lactic Acid
PLGA	Poly-lactic-co-Glycolic Acid
pNPP	p-nitrophenylphosphate
PPP or (PP)	Platelet Poor Plasma
PRP or (PR)	Platelet Rich Plasma
RGD	Arginyl Glycyl Aspartic Tripeptide
ROI	Region Of Interest
SAMRI	South Australia Health and Medical Research Institute
SCAP	Stem Cells from Apical Papilla
SD	Standard Deviation
SEM	Scanning Electron Microscope SEM
TAP	Triple Antibiotic Paste
TGFβ	Transforming Growth factor β
U/ml	Unit per millilitre
UV	Ultraviolet
v	Volume
VEGF	Vascular Endothelial Growth Factor
w/v	Weight per volume
x g	Times gravity
α-MEM	Minimum Essential Medium, α modification

Abstract

Revitalisation treatment of infected immature permanent teeth shows promising results by increasing root lengths, dentine wall thicknesses and further narrowing of the open apices. However, previous case reports and animal studies showed variation in the outcomes of revitalisation treatment protocols. In addition, histological studies showed that the healing tissues were different in pulp and dentine tissues. These variations could be due to differences in the type of trauma, infection history, treatment protocol, and animal model. Thus standardisation is required to investigate different aspects of revitalisation protocols to ensure more predictable outcomes.

Thus, the first objective of this project was to develop a standardised model for endodontic revitalisation research by examining the anatomy and histology of sheep teeth at different stages of development to find the most appropriate dental age to use for endodontic revitalisation research. Sheep at two-, four-, six-tooth and mature stages of development were investigated. Histology, standardised radiography and computed tomography have been used to evaluate and measure incisor root length, apical third dentine thickness and apex diameter of each tooth. During development, sheep at all dental ages showed small changes in incisor root lengths and major changes in the apical diameters and the dentinal wall thicknesses from the eruption time to maturation. Sheep appear to be a reliable animal model for endodontic revitalisation research. Each dental age has its advantages and disadvantages. The results of this research found that the two-tooth stage is the most appropriate dental age because permanent incisors are anatomically similar to human immature incisor teeth. In addition, the animals are readily available, small in size, easy to manage, and have a high growth rate.

The second objective of this project was to examine *in vivo* the response of two-tooth age sheep model to commonly used endodontic revitalisation protocol. To achieve this goal,

sheep incisor teeth were infected for four weeks, and then treated with a revitalisation protocol using blood clot as a scaffold. The changes in teeth diameter and the histology of the healing tissue were evaluated six months after treatment. The results showed some further root length development in the experimental teeth, significant increases in dentine wall thickness, and significant narrowing of the root apices of the experimental teeth compared with control teeth. There was also histological evidence of three or four distinct healing regions in the experimental teeth. Less mature tissue was observed coronally and more mature tissue was seen apically, suggesting that repair progressed from the apical to the coronal part of the root.

Revitalisation case reports and animal studies have identified difficulties in the stimulating bleeding during revitalisation treatment. Thus, the third objective of this project was to evaluate the suitability of platelet rich plasma (PRP) scaffold prepared using a simple protocol by examining its effectiveness on stimulating proliferation, migration and differentiation of cultured ovine dental pulp cells (ODPCs). The results showed culturing of ODPCs on both PRP and PPP scaffolds significantly increased the proliferation rate compared to groups without a scaffold. PRP scaffold had a significant stimulation effect on ODPCs proliferation compared to PPP. ODPCs migration rate was higher toward and inside PRP than PPP. Alkaline phosphatase activities of ODPCs cultured on PRP and PPP were significantly higher than the cells cultured without scaffold. ODPCs cultured on PRP scaffolds formed more mineralised nodules than PPP groups with and without the addition of mineralised induction medium. The addition of dentine discs to the scaffolds significantly reduced the activities of the cells. Seeding ODPCs with PRP and PPP in chemically cleaned roots showed migration and attachment of the cells to the dentinal walls with PRP group showing further attachment compared to PPP.

The results of these studies have shown that:

1- The sheep is a reliable animal model for endodontic revitalisation research.

2- The two-tooth stage is the most appropriate dental age endodontic revitalisation research.

3- Endodontic revitalisation treatment using Two-tooth age sheep showed a positive outcome with further development of the experimental teeth, and histological evidence of three or four distinct healing regions.

4- PRP scaffold prepared using a simple protocol of blood centrifugation can enhance proliferation, migration, and differentiation of dental pulp cells similar to complicated and long step protocols.

Chapter 1. Introduction

1.1 Introduction

Pulp infection of immature permanent teeth may develop secondary to caries (1, 2), trauma (3) or dental anomalies (4), which may eventually lead to necrosis of the entire pulp tissue and may spread further to the periapical tissues. Dental treatment for infected immature teeth with open apices is challenging. Deficiencies in dentine composition and the thin root dentinal walls make immature teeth more susceptible to root fracture. Furthermore, the divergent morphology of their apices makes traditional root canal treatment extremely difficult (5).

Traditionally, apexification has been the treatment of choice in immature teeth with necrotic pulps. Many materials have been employed to induce apical barrier formation. Calcium hydroxide has been used successfully since 1964 (6). However, it has some limitations. This treatment requires multiple visits with the associated possibility of recontamination of the canal during the treatment period, and dentine exposure to calcium hydroxide can cause changes in the physical properties of dentine which may lead to root fracture (7, 8).

Mineral Trioxide Aggregate has been used in treatment of immature infected teeth using one step technique. . A number of investigators have demonstrated a high success rate with this material (9-11). However, apexification using any protocol has the disadvantage of often arresting root development, which may result in a weak and fragile root.

In contrast, endodontic regeneration treatment has the potential to encourage further root growth and the maturation of radicular dentine. Endodontic regeneration techniques involve a range of approaches including: root canal revitalisation, postnatal (adult) stem cell therapy, pulp implants, scaffold implants, three-dimensional cell printing, injectable scaffolds, and gene therapy. Each one of these has its indications and limitations (12).

Current endodontic regeneration research is moving in two main research streams. The first is “cell-based” tissue engineering, which aims to recreate pulp and dentine tissues with three basic elements: stem cells, growth factors, and scaffolds (13, 14). In this approach, stem cells are added externally to generate a tissue which can replace the damaged pulp tissue. The second is a “cell-free” approach, which aims to create a suitable environment in the affected teeth to encourage ingrowth of vital tissues from the remnants of pulpal and periapical tissue to restore the damaged and missing tissues (15, 16). There is a debate about how to best describe the second treatment approach due to a lack of clear evidence indicating whether the process involved is tissue regeneration, repair, or a combination of both (17, 18). The “cell-free” approach has been referred to as revascularisation but the term revascularisation is often used to describe protocols for managing traumatised teeth, where the pulp tissues may remain intact and if severed, for example by avulsion, a replanted tooth may require only reestablishment of vascularisation to resume its growth. This possibly has different outcome if the pulp is infected and/or severely damaged (19, 20). More recently the cell-free approach has been referred to as revitalisation to describe the development of both new vital tissue inside the canal cavity and mineralised hard tissue on the canal walls. This terminology recognises that this tissue could be different from the original pulp parenchyma and dentine (21-23). In this thesis, the term ‘revitalisation’ is used to describe all of the cell-free approaches.

Chapter 2. Literature Review

2.1 Introduction

In order to understand the concepts of endodontic regeneration, revitalisation and pulp healing, it is important to consider normal dental tissue development, root formation, the cellular origin of odontoblasts and cementoblasts, dentine morphology, cementum and their variations, the elements required for the tissue development, and the factors affecting tooth development. Knowledge of tooth development in health will provide a basis for comparison with the tissues which develop after different revitalisation treatments in teeth.

2.1.1 Human tooth development

Human tooth development begins early in embryonic life through interactions between the dental epithelium and neural crest-derived mesenchymal cells (24). At approximately the fifth week of embryonic life, a band of epithelial cells in the developing jaws starts to thicken and grows downwards, forming a dental lamina which passes through five morphological stages during tooth development (**Figure 2.1).**

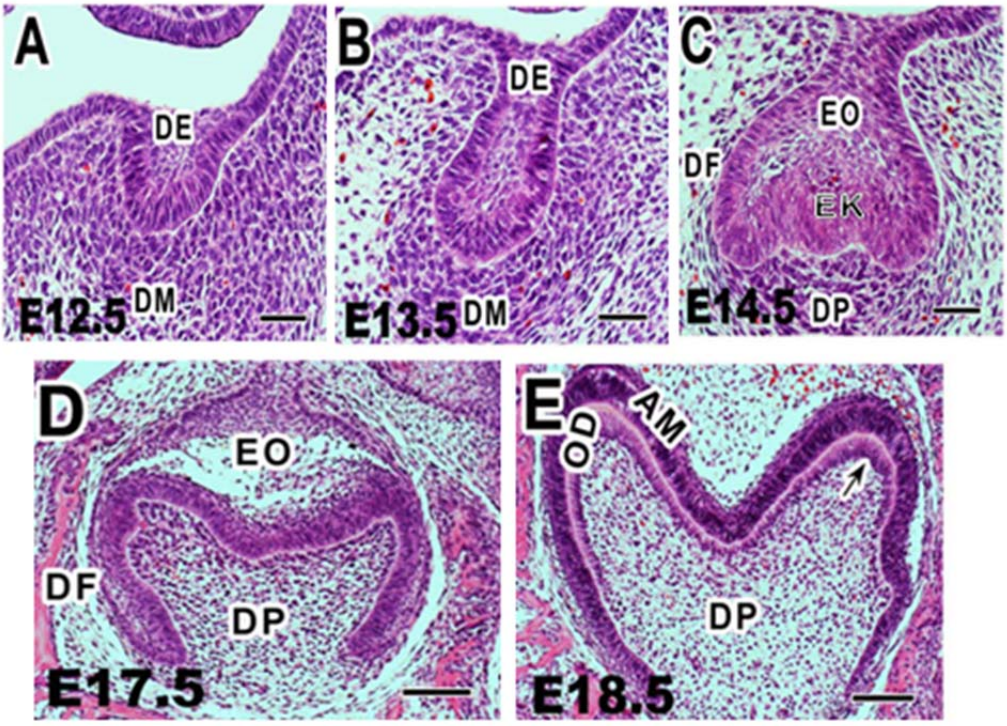


Figure 2.1 Tooth development stages (modified from Li et al. (25)).

A: Early bud stage.

B: Bud stage.

C: Cap stage.

D: Bell stage.

E: Late bell stage.

DE: Dental epithelium, DM: Dental mesenchyme, EO: Enamel organ, EK: Enamel knot, DP: Dental papilla, DF: Dental follicle, OD: Odontoblast, AM: Ameloblast.

The dental lamina first forms a tooth bud which continues to grow, forming a cap shape and then a bell shape (26, 27). During the cap and the following bell stages, a dense group of cells forms the enamel knot, which proliferates to become the enamel organ. The enamel knot cells determine the morphology of the tooth crown by their movement between the inner and outer enamel epithelial layers. The differentiation of the enamel organ stimulates connective tissue cells beneath the invagination of the dental epithelium to form the dental papilla, which is the beginning of the dental pulp (28). At the bell stage, enamel epithelial cells differentiate into ameloblasts, and subsequently the cells in the outer layer of the

dental papilla differentiate into odontoblasts (24). Once odontoblasts begin to deposit the dentine matrix, the ameloblasts begin depositing the enamel matrix. This process continues until the crown is fully formed (29). During tooth development a variety of molecules, e.g. bone morphogenic proteins (BMP)-2, BMP-4, transforming growth factor β (TGFβ), and fibroblast growth factor (FGF) act as induction signals for epithelial and mesenchymal tissue development (30-32).

2.1.2 Root formation

Root formation is the final stage of the tooth development. It begins at the cervical loop, where the external and internal enamel epithelium meets. Cells in this region proliferate to form Hertwig's epithelial root sheath (HERS), which acts as a template for root formation (33). HERS cells produce signalling molecules that stimulate differentiation of root odontoblasts (34, 35). At the time the mantle dentine is formed by the odontoblasts, the basement membrane beneath HERS breaks up and the deepest layers of the root sheath secrete a protein matrix known as the hyaline layer over the dentine. Hyaline helps the subsequent binding of cementum to dentine (36). The fragmentation of the epithelial root sheath stimulates the dental follicle mesenchymal cells to migrate and bind to the newly formed dentine, where they differentiate into cementoblasts and deposit cementum (37) (**Figure 2.2**). Root formation is continued after eruption of the tooth. When the root reaches its full length the apical area starts narrowing until only an apical foramen remains, through which blood vessels and nerves pass to the pulp (**Figure 2.3**). The remnants of HERS cells are known as Epithelial Cell Rests of Malassez (ERM), which remain in the periodontal ligament (PDL) as islands of cells surrounded by a basement membrane (38, 39). Although ERM may appear as quiescent structures in PDL, research suggest that they participate in cementum repair during root resorption (40-42).

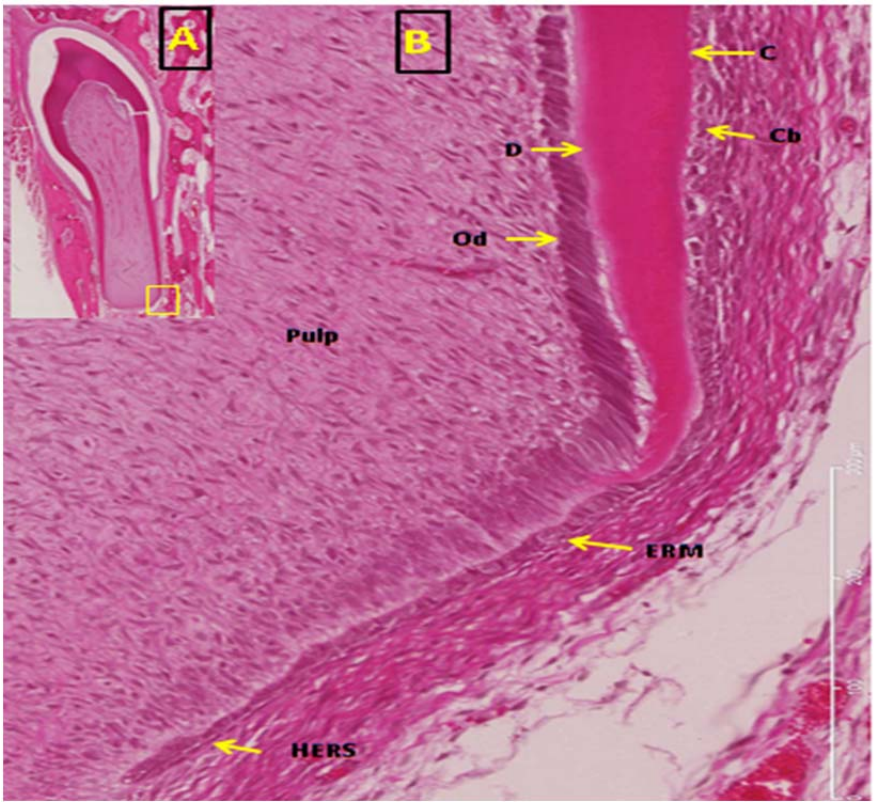


Figure 2.2 Root formation before eruption (sheep tooth)

Image (B) is a high magnification of the yellow rectangle in image (A).

D: dentine, Od: odontoblasts, C: cementum, Cb: cementoblasts, ERM: Epithelial Rests of Malassez.

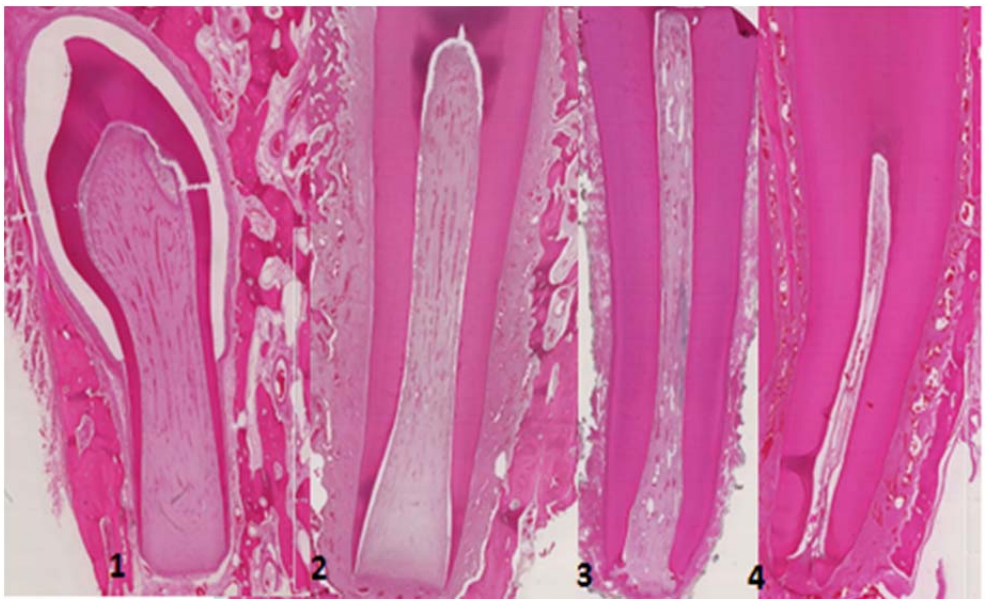


Figure 2.3 Root formation and developmental after eruption (sheep teeth).

Image shows root formation: (1) Before eruption, (2) after eruption with open apex, (3) further narrower of the apex, (4) mature root with narrow apex.

2.1.3 Dentine formation

2.1.3.1 Primary dentine formation

Primary dentine formation whether in the crown or the root occurs when the outer cells of dental papilla differentiate into odontoblasts and secrete extracellular matrix (ECM) (43). However, there are differences between crown and root dentine. The appearance of the odontoblasts in the crown and the root is different. Odontoblasts in the coronal area are columnar with a height of 50 - 60 μm, whereas at the apical region of the pulp they are more flattened (44, 45). In coronal dentine formation, odontoblast processes remain at the site of the dentino-enamel junction, while in the root they retract with the cell body away from the basal lamina. Furthermore, pre-dentine collagen fibrils are thicker and denser in the crown than the root (46). Thomas (34), and Tummers and Thesleff (47) suggested that the differences between coronal and radicular dentine result from the differences in the

mechanisms which induce crown dentine formation than those inducing root dentine formation.

2.1.3.2 Secondary and tertiary dentine formation

Secondary dentine forms after eruption of the tooth, and in response to the masticatory function and light thermal and chemical irritants (48). Although, secondary dentine possesses a similar structure to primary dentine, odontoblasts at the stage of secondary dentine production exhibit less synthesis granules than at the primary dentine production stage (43). Also, secondary dentine contains less and narrower tubules than primary dentine (49).

If the pulp is injured but the odontoblasts survive, they secrete a reactionary type of tertiary dentine (50). Unlike primary and secondary dentine, reactionary dentine matrix is not uniform in shape (51). These changes in the matrix possibly occur due to the acceleration in deposition, which depends on the severity of dentine damage and dentine thickness (52). In severe injury, causing localised death of odontoblasts, stem cells differentiate into odontoblast-like cells and lay down a reparative type of tertiary dentine (53). Dental pulp stem cells (DPSCs) differentiation into odontoblasts under the influence of signalling molecules such as TGFβ, BMP-2, -4, -7 and -11, dentine sialophosphoprotein (DSPP) and inflammatory mediators (prostaglandin E_1), which are released from the exposed pre-dentine, dentine or inflammatory cells (54-56).

Reparative dentine can be different in morphology from reactionary dentine. It may contain cells within its matrix resembling osteocytes within bone, as shown in Figure 2.4 (43, 57). Furthermore, non-collagenous proteins (such as osteoprotein, a protein usually found in bone rather than in dentine), may exist in the extracellular matrix. If this occurs, the reparative dentine can be regarded as an osteodentine (43) (**Figure 2.4**)

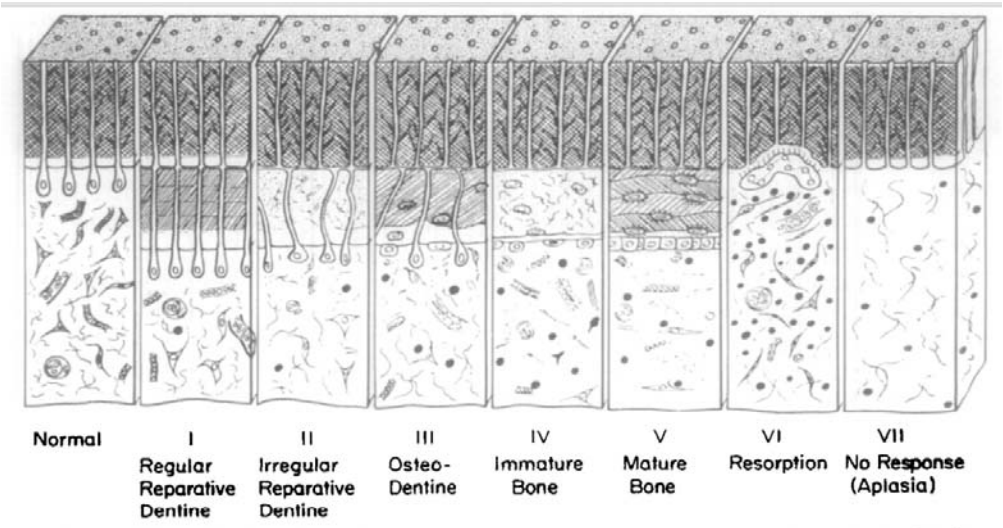


Figure 2.4 Dentine types.

Schematic drawing modified from Anderson et al. (57) showing reparative matrix deposited in response to the severity of central trauma to the pulp.

2.1.4 Cementum formation

Cementum is a mineralised tissue formed on the root surface. It sometimes overlaps the cervical region of the crown enamel. Cementum thickness increases toward the apex and may partially extend into the apical foramen (58). Histologically, cementum appears similar to bone. It may contain cells within lacunae and exhibits incremental deposition lines. Nevertheless, unlike bone, cementum does not contain nerve and blood vessels in its matrix (58). Cementum can be classified into acellular or primary cementum, and cellular or secondary cementum, according to its cellular component (59). Cementum can be further subclassified, into Acellular Afibrillar Cementum (AAC), Acellular Extrinsic Fibre Cementum (AEFC), Cellular Intrinsic Fibre Cementum (CIFC) and Cellular Mixed Fibre Cementum (CMFC) based on the existence and the direction of collagen fibres (58, 60, 61). AAC contains neither collagen fibres nor embedded cells, and usually forms as isolated patches on enamel and dentine at the cemento-enamel junction (58). To date, the origins of the cells which deposit this cementum have not been identified, but it has been hypothesized that epithelial mesenchymal cells differentiate into cementoblasts when they

come into contact with enamel matrix (62, 63). AEFC covers the cervical and middle third of the root. It was believed that AEFC produced by cementoblasts differentiated from mesenchymal cells of the dental follicle (64), but some research suggests that HERS cells or their products participate in acellular cementum formation (65-67). CIFC contains cementocytes entrapped in its matrix, and usually covers the apical third of the root and furcation area (58). Cellular cementum deposition occurs throughout life in a rhythmic way, and may extend to cover the primary cementum as the individual gets older (68, 69). This type of cementum has a repair function, and is therefore found at fracture and resorption sites (58, 70-72). In rare cases, CIFC consists of bundles of collagen fibrils and lacks cementocytes, and is therefore known as acellular intrinsic fibre cementum (AIFC) (58). At the apical portion of the root and furcation area, CIFC may overlay layers of AEFC or vice versa. This type of cementum, known as CMFC (73), is found at previously resorbed areas of the root as a reparative cementum (58, 74). Cellular cementum shares some similarities with bone unlike acellular cementum. An immunocytochemical study has shown that cementoblasts lining cellular cementum share many phenotypic characteristics with osteoblasts, but this does not apply to cementoblasts lining acellular cementum (75). In addition, research has shown expression of the epithelial factor (*distal - less gene -2),* by cementoblasts is associated with acellular cementum formation, while the expression of this gene is absent in the cells associated with cellular cementum formation (76). Thus, it is likely that acellular cementum has an epithelial origin, while cellular and reparative cementum has a mesenchymal origin (76, 77).

Cementum maintenance and regeneration are controlled by many factors. The exposure of dentine matrix and the release of ECM molecules stimulate differentiation of cementoblasts and induce cementogenesis (78). Signalling molecules like TGFβ, platelet derived growth factor (PDGF), BMP-2, 3, 4, and insulin like growth factor-1, have been reported to promote cell differentiation and subsequent cementogenesis during periodontal

tissue regeneration (60, 79-83). It has also been proposed that enamel related proteins released by HERS cells induce formation of acellular cementum (65, 84). There is also an assumption that HERS can transform into mesenchymal cells and differentiate into cementoblasts, but recent research found that HERS cells are able to express epithelial cell markers, but unable to express mesenchymal cell markers and no cell is able to express both epithelial and mesenchymal markers (85). Thus, HERS cells are unable to transform from epithelial to mesenchymal cells and differentiate into cementoblasts.

In conclusion, root development, and both dentine and cementum formation are integrated processes. Dental papilla, dental follicle, and HERS cells are all involved in the determining the size and the shape of the root. Cellular activities are coordinated through a system of signalling molecules. One shortcoming is that most theories concerning root development, dentine, and cementum formation are based on evidence from animal research, and animal teeth could differ significantly from human teeth, so caution is required in extrapolating findings in other species to humans.

2.2 Elements for revitalisation

2.2.1 Stem cells

Stem cells have the ability to divide continuously, producing progenitor cells which can differentiate into other types of cells or tissues (86). There are two main types of stem cells, embryonic mesenchymal stem cells, which are derived from the inner cell mass at the early stage of embryo development (blastocysts), and can give rise to any tissue in the body (termed pluripotent cells) (87), and postnatal stem cells which are tissue specific cells (multipotent cells), found in a quiescent state in stem cell niches in most tissues throughout the body (88, 89). Postnatal stem cells were first identified in bone marrow (90), and more recently different populations of stem cells have been found in oral tissues (91-93).

Postnatal multipotent stem cells, regardless of their tissue of origin, were initially called mesenchymal stromal cells, but were later termed mesenchymal stem cells (MSCs) (94). The International Society for Cellular Therapy (ISCT) proposed a set of minimal criteria required for the identification of MSCs. Firstly, MSCs must be able to adhere to plastic and form colonies with a fibroblast-like appearance when maintained in culture conditions. Secondly, they must express CD105, CD73, CD90, and lack CD45, CD34, CD14 and other negative markers. Thirdly, MSCs must be able to differentiate into osteoblasts, chondroblasts, and adipocytes *in vitro* in standard differentiation conditions (95).

In relation to this study, postnatal multipotent stem cells that can contribute to the tooth revitalisation process are worthy for further consideration.

2.2.1.1 Dental pulp stem cells (DPSCs)

The first postnatal stem cells of a dental origin were dental pulp stem cells (93). DPSCs have been successfully obtained from the extracted third molars (93), supernumerary teeth (96), crowns of fractured teeth (97), and from inflamed pulp (98). DPSCs stay in a quiescent state in specific niches in healthy pulps. During injury or trauma to the pulp, and the subsequent death of primary odontoblasts, signalling molecules are released into the ECM stimulating the differentiation of DPSCs into odontoblasts (50, 99). Further research has found that DPSCs have both a high proliferation rate and growth potential (91, 100). These cells can differentiate into odontoblasts, chondrocytes, adipocytes and neural-like cells under different conditions (91, 101, 102). When a suitable tooth-like environment is available, these cells tend to maintain their original character as pulp stem cells and differentiate into odontoblast-like cells (103, 104). Many animal studies have shown that transplantation of DPSCs with a suitable scaffold or a tooth slice can induce differentiation of the cells into odontoblast-like cells and subsequent formation of dentine and pulp-like

tissues (93, 105-107). Batouli *et al*. (108) cultured DPSCs *ex vivo* on a tooth slices and reported the formation of atubular dentine-like tissue which had cells trapped inside the matrix. However, evidence to support the conclusion that this dentine-like tissue is "real" dentine is lacking (23, 109). It would seem that intact dentine is essential for DPSC differentiation into odontoblasts and the subsequent production of mineralised tissues (110-112). However, *in vitro* culturing of DPSCs on root dentine discs has a lower proliferation rate than in culture medium (113). Removal of the smear layer from the dentine surface using ethylenediaminetetraacetic acid (EDTA) increases the adhesion of DPSCs to the exposed dentinal tubules, followed by the differentiation of these cells into cells with an odontoblast phenotype (114). Irrigation of the dentine surface with sodium hypochlorite (NaOCl) on the other hand, reduced cellular adhesion and differentiation (114). Inflammation of pulp tissue is another factor which can alter the properties of DPSCs. Alongi *et al.* (98) reported that although DPSCs exist in inflamed pulp tissue, they express less stem cell characters in comparison to DPSCs in normal pulp tissue, however they still have the capacity to form a pulp/dentine complex *in vivo*. Recently, Nakashima and Iohara (115) reported that DPSCs derived from young dogs had more potential for pulp regeneration and hard tissue deposition than those from aged dogs. These results suggest that differentiation of DPSCs into odontoblasts and deposition of dentine is affected by the specific environment, the status of the pulp, and the age of the pulp tissue.

2.2.1.2 Stem cells from apical papilla (SCAP)

Stem cells from the apical papilla are another source of MSCs for dental pulp revitalisation. Apical papilla exists in the immature teeth and is located apical to the epithelial diaphragm (116). The interactions between HERS cells and SCAP regulate root development and determine the root shape (117). SCAP are the primary source of odontoblasts that form root dentine and have a higher proliferation potential and

mineralisation rate than DPSCs (118, 119). Like other DPSCs, SCAP are positive to CD146 and CD34 surface markers (119, 120). Sonoyama *et al.* (120) confirmed the differentiation potential of SCAP into odontoblast-like cells and the formation of dentine-like tissue in *ex-vivo* research. The location of SCAP in the apical portion of the tooth, where there is a plentiful blood supply, allows them to survive even if the pulp is necrotic (116). Thus, when favourable conditions are available, SCAP may resume their role in revitalisation of the damaged pulp tissue in immature teeth (121-123). Lovelace *et al.* (124) found that an induced bleeding step in the revitalisation protocol increased the expression of MSCs markers in the canal, and suggested that a mechanical irritation to the periapical tissues lacerates the apical papilla and releases SCAP with the blood. A potential complication of laceration of the apical tissues is possible disruption between the apical papilla and HERS, which are both involved in guide root development (89). Alternatively, apical tissue stem cells can be stimulated to migrate to the root canal using signalling molecules (cell homing approach) (125, 126), which will be discussed later.

2.2.1.3 Periodontal ligament stem cells (PDLSCs)

Periodontal ligament stem cells are another possible source of stem cells during dental pulp revitalisation. PDLSCs like DPSCs express mesenchymal stem cell markers and have the capacity to differentiate into odontogenic, cementogenic, and osteogenic lineages under defined culture conditions (127, 128). These findings suggest that PDLSCs may participate in revitalisation of traumatised and infected immature teeth. *Ex-vivo* research has shown that when culturing PDLSCs subcutaneously in immunocompromised rodents, cementum-like and PDL-like tissues are formed (128, 129), and that culturing on de-cellularised odontogenic ECM results in differentiation into odontoblast-like cells (127). An immunohistology study reported that hard tissues formed after revitalisation in immature dogs teeth were different from dentine and have some similarities to cementum (109).

Similarly, the formation of cementum-like hard tissues on dentine walls after regeneration or revitalisation treatment procedures on immature teeth has also been demonstrated *in vivo* (130-133).

Extraoral MSCs, such as bone mesenchymal stem cells (BMSCs), may also be involved in the revitalisation process of immature teeth. Recently, Liao *et al*. (134) showed the presence of MSCs in human inflamed periapical tissue, which had the capacity to form a mineralised matrix *in vitro* and *in vivo,* which could be BMSCs. BMSCs, like pulpal and periapical tissue derived cells, can be stimulated in response to inflammation (135). Transplantation of bone marrow stem cells into amputated dental pulp in dogs induced pulp regeneration (136). *In vivo* research in dog teeth showed the formation of bone-like hard tissues after revitalisation treatment (21, 137). Zhou *et al*. (138) injected fluorescent labelled BMSCs intravenously into mice, which then migrated into periodontal PDL and pulp. The cells that migrated to the pulp exhibited similar properties to DPSCs. It is possible that BMSCs migrate to the pulp cavity under the effect of signalling molecules in the pulp, differentiate into odontoblast-like cells, but maintain some of their original characteristics as bone forming cells. Immunological studies have shown that some of the mineralised hard tissues formed after revitalisation, are similar to bone (109, 139). It is still unknown how the pre-treatment conditions of the pulp and periapical tissues affect the post-revitalisation outcomes with bone-like tissue.

2.2.1.4 Conclusion

The existence of stem cells within the dental pulp, at the apical area and in the PDL makes them a valid source of cells which can replace damaged odontoblasts and pulp cells.

2.2.2 Signalling molecules

There are many types of signalling molecules involved in a variety of regeneration and revitalisation, and repair processes. It is important to understand the source and effect of these signalling molecules on dental pulp cells and to appreciate how they are involved in pulp and dentine regeneration, revitalisation and repair.

2.2.2.1 Extracellular dentine proteins

During dentine formation, odontoblasts secrete pre-dentine, which is ECM rich in type I collagen fibrils. Pre-dentine also contains non-collagenous proteins (NCPs), which actively promote and control transformation of the matrix into dentine by mineralisation of collagen fibrils (140). Dentine matrix proteins (DMP) and DSPP are members of the NCPs family secreted by odontoblasts into the ECM (141).

DMP was first isolated from dentine and was believed to be specific for dentine (142), however a higher expression of it has since been found to exist in bone than in dentine (143). DMP is present in dentine, pre-dentine, odontoblasts and pulp cells (140, 141), while in cementum it is only present in cementocytes and the matrix surrounding their processes (141). Lack of the DMP gene results in: defective maturation of pre-dentine into dentine, hypomineralisation, expansion of the pulp cavity and abnormal dentinal tubules (144). It appears that DMP is involved in regulating odontoblast differentiation, dentine formation and mineralisation (140, 145). Matrix metalloproteinase-2 is a predominant protease protein in dentine matrix and proteolytically cleaves DMP to release a peptide (146), which is able to promote differentiation of DPSCs (147).

Application of a collagen scaffold impregnated with DMP to the exposed pulp of rat molars induced differentiation of odontoblasts and deposition of hard tissue at the exposed site (148).

DSPP is a widely distributed protein in teeth (149, 150), and a low level of DSPP is expressed in bone (151), kidneys (152) and salivary glands (153). Lack of DSPP in mice caused an increase of pre-dentine, irregular and hypomineralised dentine, and pulp exposures similar to that seen in cases of dentinogenesis imperfecta in humans (154) Proteolysis of DSPP produces dentine sialoprotein (DSP) and dentine phosphoprotein (DPP) in the ECM (155). DSP has been detected in odontoblasts, pre-dentine, reparative dentine, dental pulp, bone, osteocytes, cementocytes and cellular cementum, but not in acellular cementum, which suggests that the origin of acellular cementum is possibly different from cellular cementum (156-158). DPP is more abundant in dentine than DSP (159). It is believed that DPP binds to collagen fibrils to promote formation of hydroxyapatite mineral crystals. When the mineralisation process occurs, pre-dentine is converted to dentine (160, 161). *In vitro* studies have shown that DPP is important for the development and growth of hydroxyapatite mineral crystals (162, 163), and *in vivo* research has demonstrated that DSP regulates the initiation of the dentine mineralisation process while DPP is involved in the maturation of mineralised dentine (164).

2.2.2.2 Growth factors

Growth factors are peptides which bind to the surface receptors of stem cells and induce them to migrate, proliferate and/or differentiate (125). Many types of growth factors have been studied for pulp-dentine regeneration, and some of the most relevant growth factors are now described.

2.2.2.2.1 Transforming growth factor β

During the process of dentine formation, TGFβ is secreted into ECM by odontoblasts and remains in an inactive form through interactions with other dentine matrix components

(165). Demineralisation of dentine activates the TGFβ signalling cascade (166). Research has shown that TGFβ1 and TGFβ3 have a stimulatory effect on rat odontoblasts (167). TGFβ1 also stimulates DPSCs to regenerate dentine on exposed dental pulp in a dog model (166), however it lacked a similar induction effect when it was used to stimulate bone regeneration in alveolar jaw defects in dogs (168). Chan *et al*. (169) reported that different isoforms of TGFβ play crucial roles in regulating regeneration of damaged pulpal tissues (169).

2.2.2.2.2 Bone morphogenetic proteins

Bone morphogenetic proteins are another group of growth factors. Recombinant human BMP-2 promoted differentiation of DPSCs into odontoblasts *in vitro* and *in vivo* in a dog model (170, 171). Recombinant human BMP-2 and -4 induces odontoblasts differentiation and dentine formation over amputated canine pulps (172, 173). A similar effect has been observed for BMP-7 and BMP-11 on amputated pulps in many animal models (56, 174-176). Nakashima *et al*. (177) found that BMP-11 promotes differentiation of mouse dental papilla mesenchymal cells into odontoblasts.

2.2.2.2.3 Insulin like growth factor

Insulin like growth factor (IGF) contributes to the differentiation and proliferation of odontoblasts (178, 179). *In vitro* research has shown that IGF stimulates proliferation of HERS, which resulted in further development and elongation of the tooth root (180). Capping the exposed dental pulp in rat molars with methylcellulose gel impregnated with IGF induced differentiation of odontoblasts and dentine formation (181).

2.2.2.2.4 Vascular endothelial growth factor and fibroblast growth factors

Complete tooth revitalisation not only needs reparative dentine formation, but also fibrovascular support. Angiogenesis is an essential process for bringing nutrients and oxygen to the healing tissue, and providing a pathway for cells migration (182). Injured endothelial cells secrete vascular endothelial growth factor (VEGF) and FGF (182). Roberts-Clark and Smith (183) have shown the existence of these growth factors in the dentine matrix, suggesting that after injury or trauma to the pulp or dentine these proteins are released to contribute in pulp and/or dentine healing. Applying collagen scaffolds impregnated with FGF on amputated pulp in rat molars induced blood vessel formation and cell proliferation (184). An *in vitro* co-culturing of dental pulp fibroblasts with endothelial cells induced organization of endothelial cells into a network of tubular structures similar to the *in vivo* structure seen during angiogenesis (185). An *in vivo* study, implanting slices of teeth subcutaneously in mice, showed that density of microvessels in the slices was significantly increased when they were treated with VEGF (186).

2.2.2.2.5 Platelet derived growth factor

Platelet derived growth (PDGF) factor plays a significant role in cell proliferation and angiogenesis (187, 188). In the dentine matrix, concentration of PDGF is higher than VEGF and FGF (183). However, its effect on mineralisation is controversial, as some studies found that PDGF inhibits alkaline phosphatase activity of dental pulp cells in culture (189, 190), while *in vivo* research on laboratory animals showed that application of PDGF to the bone defect areas resulted in a significant increase in mineralisation and bone regeneration (191, 192). This discrepancy could be explained by the four isoforms of PDGF. Among them, PDGF-AB and BB stimulate the secretion of dentine sialoprotein by odontoblasts, while PDGF-AA has an inhibitory effect on the cells (193). Furthermore, the

activity of PDGF is dose dependent (194), as well as regulated by interaction with other growth factors such as TGFβ (195).

2.2.2.2.6 Nerve growth factor

Nerve growth factor (NGF) is another member of the growth factors family. Injury to the pulp in rats increases the expression of NGF (196). Arany *et al.* (197) reported that NGF can induce the differentiation of dental papilla stem cells into odontoblasts *in vitro*. These findings suggest that NGF may have a role in the pulp-dentine healing process.

2.2.2.3 Effects of signalling molecules on revitalisation

In infected, immature teeth the chance for a spontaneous regeneration or revitalisation is low. Therefore, exogenous growth factors may be required to stimulate the revitalisation or healing processes (197). Adding signalling molecules to tissue culture or to a tooth slice is a simple procedure, but the application of this concept in revitalisation treatment is much more complicated, as these molecules are rapidly degenerated at the required site and systemic administration may result in nonspecific distribution (125). In an attempt to induce dentine formation, BMP, IGF, FGF, and TGF have been applied to teeth with exposed pulps in animal models (174, 198-200). Histological studies have shown that the hard tissue formed in response to the signalling molecules was atubular and contained cells (14, 56, 136). Another approach is to add signalling molecules to the scaffolds. Kim *et al*. (126) filled extracted human teeth with collagen scaffolds impregnated with FGF, VEGF, and PDGF and implanted them subcutaneously in mice. Three weeks after implantation, pulp-like tissue developed inside these teeth (126).

Another problem in using exogenous growth factors for revitalisation treatment is that they may attract PDL cells, resulting in formation of PDL tissues inside the canal instead of pulp tissues, or may lead to complete canal calcification.

A recently published case series showed no significant difference in revitalisation radiographic results between the group treated with blood clot scaffolds and group treated with collagen scaffold impregnated with FGF (201).

2.2.3 Scaffold

The presence of a scaffold is another essential element for revitalisation treatment. Generally, a scaffold is a three dimensional replica of the ECM, and provides biological and mechanical support to the stem cells (12). An appropriate scaffold should create an environment which allows the cells to migrate, proliferate and differentiate (88). Scaffolds should be ideally biodegradable, and the rate of degradation has to coincide with the rate of tissue formation until all the scaffold structure has been replaced by ECM (202). Also, the scaffold has to degrade without releasing any toxic by-products (203).

In the current revitalisation protocols, a blood clot has been as a viable scaffold for tissue healing and repair (204, 205). Trauma to tissue that results in bleeding initiates a cascade of reactions leading to the formation of a blood clot (206). This scaffold acts as a temporary matrix for cellular migration and adhesion during tissue healing and repair (207). Success in using blood clot as a scaffold has been reported for revitalisation treatment (208-210), but difficulties with inducing bleeding inside the canal have been reported (1, 211, 212). This difficulty could possibly have been due to the use of inter-appointment medication which can stimulate a coagulation necrosis at the periapical area (213, 214). In addition, there are a limited amount of growth factors in blood clot (215), and an alternative scaffold could improve revitalisation treatment results.

2.2.3.1 Synthetic material scaffolds

Synthetic polymers have been used widely as scaffolds for regeneration. The most widely used synthetic polymer scaffolds for dentine and pulp regeneration are poly-glycolic acid (PGA), poly-lactic acid (PLA) and poly-lactic-co-glycolic acid (PLGA) (216-219). These scaffolds are polyester materials that degrade within the human body through hydrolysis. The degradation rate can be customised from several weeks to years (87, 220). They can be fabricated in the form of a sheet with various pore sizes, as a gel or as a more complex structure (218, 221). Hydrogels are another synthetic scaffold, which can easily be injected into the root by use of a syringe (222). The properties of these scaffolds have been improved by the addition of matrix metalloprotease and surface adhesion peptides (Arginyl-glycyl-aspartic tripeptide (RGD)), which enhanced cell adhesion, survival and mobility within the scaffold (223, 224). Although synthetic polymers have been used widely for tissue engineering, they may cause inflammation at the implantation site (225, 226).

Hydroxyapatite/tricalcium phosphate HA/TCP scaffolds have been developed as resorbable osteoinductive graft materials (227, 228). *Ex-vivo* research has shown that using this scaffold, DPSCs can differentiate into odontoblast-like cells and form dentine-like hard tissue (91, 93). Otaki *et al*. (229) found that mixing DPSCs with HA/TCP powder and transplanting subcutaneously in immunocompromised mice resulted in the formation of bone instead of dentine. In contrast, Batouli *et al*. (108), cultured DPSCs on HA/TCP, followed by a subcutaneous transplantation in mice which resulted in formation of organised tubular dentine (108). Using HA/TCP as a scaffold for revitalisation may result in an early apical or irregular calcification of the canal similar to the adverse effects which have been seen previously with parts of a Mineral Trioxide Aggregate (MTA) coronal seal pushed inside the canal (21, 212).

2.2.3.2 Natural material scaffolds

Natural scaffold materials should be bioactive, biocompatible and provide adequate support to the stem cells to survive and proliferate. Collagen is the most abundant protein in the body, and forms about 25 - 35% of total body protein (230). Collagen contains RGD (231), which facilitates cell attachment and migration (232, 233). Collagen based wound dressings, implants and scaffolds have been used to stimulate soft and hard tissue regeneration (234, 235). Gebhardt *et al.* (236) evaluated the survival rate of PDLSCs and DPSCs on collagen, polymer and calcium phosphate scaffolds. They found that polymer and collagen scaffolds had a significantly higher cell survival rate than calcium phosphate scaffolds (236). However, Huang *et al.* (113) reported that a collagen scaffold contracted by approximately 30% after seeding the stem cells on the scaffold, and such contraction of the scaffold could affect the healing process.

Silk fibroin is another biomaterial which has been proven to be useful as a scaffold for bone and cartilage regeneration (237, 238). Xu *et al.* (239) examined the suitability of silk scaffold for mineralised dental tissue formation by culturing rat tooth bud cells on the scaffold. They reported that these cells formed osteodentine on the scaffold, and the pore size and shape of the scaffold guided the mineralised tissue formation. Silk scaffold can be processed to have a specific degradation rate and porosity (240, 241). RGD peptides can be incorporated into this scaffold to enhance cell attachment and tissue formation (242, 243). However, research has been shown that both collagen and silk scaffolds material can stimulate inflammatory responses *in vivo* (242, 244).

In a blood clot, polymerisation of fibrinogen polypeptides form a three- dimension network of fibrin strands (245). Platelet, macrophages, neutrophils, and fibroblasts anchor to the fibrin strands to start the healing process (246, 247).

Autologous fibrin scaffolds like PRP have been used widely in medical and dental fields (205, 248, 249). PRP is the fraction of blood which contains a high concentration of thrombocytes (platelets) (250). Platelets are a source of several growth factors including PDGF, TGF-β, VEGF, EGF and IGF-I (250, 251). Using PRP as a scaffold is cost-effective and easily prepared from the patient's own blood, reducing the risk of infection and allergic reactions (252). Local injection of PRP into an injured area (for example, an injured tendon tissue) can initiate tissue regeneration and stimulate healing (253, 254). Culturing tendon cells on PRP and PPP scaffolds can synthesize considerable amounts of VEGF and Hepatocyte growth factor (255) and significantly increase cell proliferation (205, 255). PRP has been used for PDL regeneration (256), and alveolar ridge and sinus floor augmentation (257-259). The rationale of using PRP for bone regeneration was supported by evidence that PRP can enhance proliferation and mineralisation differentiation of MSCs (260, 261). The same effect was observed when PRP was used with PDLSCs and DPSCs (262-264). However, PRP fabricated using the methods described in these articles is effective only under specific concentrations, and some PRP preparation methods are slightly too complicated for clinical use (255, 263, 264). Recently, PRP has been used as a matrix for endodontic revitalisation instead of blood clots (23, 265). The protocol used to prepare PRP in these cases is simple and easy to apply in the dental clinic.

2.3 Factors affecting revitalisation outcomes

Case reports and studies have shown variable outcomes of revitalisation treatments. These variations could be the result of differences in disinfection protocols, the pulpal and periapical condition before treatment, apical diameters of treated teeth, and the barrier materials used to seal the orifices of the teeth.

2.3.1 Bacterial infection control

The success of revitalisation treatment is highly dependent on the eradication of bacterial infections from the root canal and radicular dentine. Total removal of the bacteria from the root canal is ideal but hard to achieve *in vivo*. A reduction in the bacterial load to a level that is not able to cause or maintain pathology is required. Unsurprising, there is a positive co-relation between bacterial free root canals and post-treatment healing of periapical lesions (266-268). Pulp regeneration and revitalisation processes only occur when bacterial infection and inflammation process are under control (267, 269). Bacterial infection causes the death of pulpal cells and demineralisation of dentine, processes that both lead to the release of cytokines (270) and growth factors (183, 271). Unfortunately, a high concentration of cytokines and growth factors like TGF can have adverse effects on stem cell differentiation (174, 272). Furthermore, cytokines stimulate the migration and activation of immune cells, which can cause a significant damage to the host tissue (273, 274). The choice of an optimised antimicrobial protocol is a vital step in the revitalisation treatment and can have a significant impact on the post treatment outcomes.

2.3.1.1 Mechanical debridement of the infection

Mechanical instrumentation of infected root canals in immature teeth is not recommended by the American Association of Endodontics (AAE) in revitalisation treatment (275) (**Table 2-1**). However, minimal instrumentation of the cervical and middle third of the root has been performed in animal revitalisation research (276-278) and in case reports of revitalisation treatment of infected immature teeth (279, 280). Mechanical cleaning of the wall may remove any remaining vital tissue in the canal and further weaken the walls. On the other hand, bacterial biofilm on the canal walls has an increase resistance to

antimicrobial agents compared to planktonic cells and needs to be mechanically disrupted to allow the antibacterial irrigants/medicaments to penetrate the biofilms and the infected tubules (281). If bacterial biofilms are left on the canal walls, they can induce apical periodontitis (282, 283). Disinfection of immature teeth is further complicated, as infection can extend faster and deeper in dentinal walls compared to mature teeth (284). Thus, only minimal mechanical instrumentation is advocated in the revitalisation treatment protocols for infected immature teeth (276-278).

2.3.1.2 Chemical debridement of the infection

Chemical debridement and disinfection of the root canals in the immature teeth by gentle irrigation with antimicrobial agents without causing damage to the host cells is a challenge. Ideally, root canal irrigants for revitalisation treatment should not irritate the vital tooth tissues, have no adverse effect on dentine, be active in the presence of blood, serum and proteins, and not interfere with the repair process (285). Intracanal disinfection is an essential step in the revitalisation process and various irrigants have been used (2, 286, 287). The most commonly used irrigant for revitalisation treatments is NaOCl (1, 288, 289). NaOCl is an effective antimicrobial and tissue dissolving agent (290) and has been used at a concentration range between 0.5 and 6%. The potential activity of NaOCl is proportional to its concentration (291, 292), exposure time (290), irrigation volume, and the temperature of the irrigant (21-37 ˚C) (293, 294). A high concentration of NaOCl (5-6%) is more effective in the elimination of a bacterial biofilm than a 3% concentration (295), and has been used in many revitalisation case reports (1, 2, 296). However, Spangberg *et al*. (297) found that a high concentration of NaOCl (5% or more) is more toxic to human HeLa cells (immortal cells) than a low concentration (0.5%) (297), while Trevino *et al*. (298) showed that the survival rate of SCAP exposed to 6% NaOCl was 74%. Another concern with the use of NaOCl as an irrigant is its effect on the dentine,

which may or may not be clinically relevant. It has been shown *in vitro* that exposure of dentine to NaOCl at concentration higher than 3% for 2 hours reduced the flexural strength and elastic modulus of the dentine (299). The changes in the dentine matrix due to the effect of high concentration of NaOCl can affect the attachment of the stem cells to the dentine (300). On the other hand, *in vivo* research found that the presence of dentinal tubules and lateral canals delay the effect of NaOCl. The effect of high concentration of NaOCl may not be as damaging clinically due to the buffering effect of dentine and the fact that intracanal inflammatory exudate and necrotic tissue can consume some available chlorine thereby diluting its effect (301, 302). Several endodontic studies report the effectiveness of using NaOCl with ultrasonic or negative pressure cleaning methods in elimination of bacterial and debris from the dentine walls (303-306), but there is a risk in the vitality of stem cell and pulp tissue by using this protocol.

Chlorhexidine digluconate (CHX) also has been used instead of NaOCl in some case reports (2, 296). *In vitro* and *in vivo* studies have found that the cleaning and antimicrobial effect of 2% CHX was inferior to 2.5% NaOCl (307, 308). CHX has a substantial antimicrobial activity because of its binding to the dentine, but its activity depends on the surrounding pH and is reduced in the presence of organic debris (309). In addition, CHX has no tissue dissolving effect (290), which means NaOCl is required to manage the organic debris that is present in regions inaccessible to conservative mechanical instrumentation.

Complete removal of the smear layers from root canal walls also necessitates the use of EDTA, which can remove the inorganic debris. In addition to smear layer removal, EDTA also exposes the entrapped growth factors in dentine (310, 311), increasing the attachment and differentiation of stem cells (114, 312). Trevino *et al.* (298) reported a 89% survival rate of SCAP after irrigation with 17% EDTA alone, and 74% for the irrigation protocol of

6% NaOCl and 17% EDTA, while 17% EDTA and 2% CHX protocol lacked any viable cell. Stem cell attachment to the dentine treated with a 6% NaOCl/17% EDTA protocol, is higher than cell attachment to dentine treated with 2% CHX/17% EDTA protocol (300). Recently, Martin *et al.* (313) has demonstrated that dentine conditioning with 17% EDTA promotes greater stem cells survival and differentiation than 6% NaOCl, and that a final irrigation of the canal with 17% EDTA can reverse the negative effect of NaOCl (313).

A final canal rinse with 17% EDTA has been recommended by the AAE for the revitalisation protocol (275). The use of 17% EDTA as a final irrigant after NaOCl showed encouraging outcomes in animal and human studies (137, 279). Nevertheless, low concentrations of EDTA could potentially be less harmful to the pulpal and apical papilla tissues, and it has been reported that low concentrations of EDTA (1 - 10%) are as effective in removing the smear layer as high concentration (290).

2.3.1.3 Root canal medication

Disinfection of the root canal in the immature teeth with dilute irrigants may not be enough to effectively eliminate bacterial contamination from the dentinal walls (314, 315), however full strength irrigants are toxic to stem cells and potentially have adverse effects on the dentine matrix (298). Thus, an additional step of an intracanal dressing containing antibiotics has been added to some revitalisation protocols to help eliminate any remaining bacteria in the canal (16). The effectiveness of a triple antibiotic paste (TAP) of ciprofloxacin, metronidazole and minocycline at concentration of 25 μg / ml each, against endodontic bacteria obtained from infected canals and periapical lesions has been shown *in vitro* (316). In addition, *in vivo* animal studies have revealed that irrigating the infected canals with 2.5% NaOCl followed by dressing with TAP, significantly eliminated bacteria from infected immature teeth (317, 318). Another animal study found that 1.25% NaOCl

was only able to disinfect 10% of the infected teeth, but a TAP dressing of these teeth increased the percentage of disinfected teeth to 70% (319). Several case reports and case series studies showed successful revitalisation outcomes after disinfection of immature teeth with TAP (1, 212, 320). Nevertheless, other clinical studies showed unfavourable discoloration of the treated teeth, which was associated with the use of minocycline in the TAP combination (3, 296, 321). To overcome this limitation, a number of alternative medications have been proposed, a modified TAP (mTAP) which replaces minocycline with cefaclor (322), eliminating minocycline to give double antibiotic paste (DAP) (286), or the use of calcium hydroxide ($Ca(OH)_{2)}$ paste (123, 323). TAP, mTAP, and DAP have all been shown to have a detrimental effect on the survival of SCAP at high concentrations, while $Ca(OH)_2$ promotes cell survival at all concentrations (324). $Ca(OH)_2$ mixed with 2% CHX gel was used as an intracanal dressing in a revitalisation case report with a successful outcome (279), however, care must be exercised in extrapolating the finding without a greater sample size. Bose *et al*. (325) compared the radiographic outcome of immature teeth which have been treated with revitalisation protocols using TAP, $Ca(OH)_2$, or formocresol as intracanal medicaments. Their results showed significant increases in the root lengths in both TAP and $Ca(OH)_2$ groups, compared to the control group which was treated with MTA apexification. The TAP group showed further increases in root thickness compared to the $Ca(OH)_2$ and formocresol groups (325). A case series of immature molars treated with revitalisation protocols using $Ca(OH)_2$ as the intracanal medicament, has shown progressive increase in root thickness and length from treatment time to the 9 - 10 months follow up period (123). Yassen *et al*. (326) compared the effect of $Ca(OH)_2$, TAP and DAP on dentine, and reported that all three medications caused degradation or demineralisation of the dentine after application for 1 - 4 weeks (327), which once again may or may not be clinically relevant.

2.3.2 Pulpal and periapical condition before treatment

Revitalisation treatment has been used for immature teeth with pulp necrosis due to caries (123), trauma (328), anatomical defect like dens evaginatus (329), and avulsion (323). The success rate of revitalisation treatment for necrotic pulps in teeth with an aetiology of caries and dens evaginatus (330) is higher than avulsion (331). It is possible that direct trauma to the periapical area causes damage to HERS and SCAP, which reduces the chances of the traumatised teeth to continue root development (323). In addition to the source of pulp necrosis, the history of pulp necrosis is another factor for the variation seen in treatment outcomes. Nosrat *et al*. (211) reviewed revitalisation case reports from 2004 - 2012, and reported that cases treated within six months or less of trauma or pulp necrosis are more likely to have successful outcomes. However, there have been some recently published case reports of a history of infection or trauma occurring for more than six months followed by revitalisation treatment showing favourable outcomes with increases in root lengths (328, 332, 333). In addition, there are case reports with a trauma history of less than six months in which no increases in root lengths occurred (320, 334). The differences in the outcomes of these cases may be due to the differences in the initial bacteria load, the type of infection, and the effect of these factors on the periapical tissues. When the bacterial infection load is severe before treatment, greater damage would be expected to any remaining pulp and the periapical tissues, which may then lead to unfavourable outcomes (335). A prospective study showed that post-treatment healing of infected teeth is not only affected by the presence of a periapical lesion but also the size of the lesion, the presence of the sinus tract, and the symptoms of the affected teeth before treatment (336). The presence of healthy vital tissues in the canals of immature teeth improved the revitalisation treatment outcomes even in the teeth that had periapical lesions before treatment (2, 320). A tooth with a thirty year history of trauma and presence of periapical lesion was successfully managed with a revitalisation protocol (333).

2.3.3 Apical diameter of the tooth

The size of the apical foramen of the infected or traumatised tooth is a critical aspect for determining whether it should be managed with traditional endodontic therapy techniques or by revitalisation treatment protocols. The dental trauma literature reports a positive relation between a large apex diameter and revascularisation success (19, 337, 338). Pulp healing of avulsed teeth was more often observed in teeth with apical diameters 1.1 mm or more than in teeth with lower diameters (19, 337). Murrary *et al.* (12) suggested enlargement of the apical diameter of immature necrotic teeth with narrow apices to 1 - 2 mm, to facilitate in growth of new tissue and blood vessels into the canal. A case report showed a successful outcome when the apices of teeth were enlarged up to 0.6 mm (339). Another *in vivo* study in a dog model showed successful ingrowth of blood vessels and revitalised tissues when the apices of teeth were enlarged to 0.7 mm (13). Angiogenesis of blood vessels in the affected teeth is an essential step for tissue revitalisation. The ingrowth of blood vessels can also provide a passage way for delivering stem cells (124).

2.3.4 Orifice barrier materials used in revitalisation

Mineral trioxide aggregate cement (MTA) has been used to seal the root canal orifices in almost all revitalisation case reports (16, 121, 122, 211, 340). MTA is biocompatible cement, able to set in the presence of blood, and can resist bacterial penetration (341-343). Both grey and the white MTA types have been reported to cause crown discoloration, when used to seal the access opening in revitalisation cases (296, 344). To avoid this adverse effect of MTA, glass ionomer cement (GIC) has been suggested as an alternative to seal the orifices of the canals (345). Another disadvantage of MTA placement at the cervical third of the root is that some fragments of MTA can be pushed further into the

canal (320), and result in the formation of a calcified bridge within the canal (332, 346), which prevents the cervical growth of the healing tissue, and the subsequent thickening of the dentinal walls cervically. Fracture of the root at the cervical area has been reported recently in a revitalisation case (347). Collagen wound dressings (e.g. CollaTape and CollaPlug (Integra LifeSciences Corp)) can be used over the blood clot to prevent MTA from overextending inside the root canal. Such treatment modifications have shown encouraging clinical outcomes (212, 320).

Case selection:

- Tooth with necrotic pulp and an immature apex
- Pulp space not needed for post/core, final restoration
- Compliant patient

Informed consent:

- Two (or more) appointments
- Use of antimicrobial(s)
- Possible adverse effects: staining of crown/root, lack of response to treatment, pain/infection
- Alternatives: MTA apexification, no treatment, extraction (when deemed nonsalvageable)
- Permission to enter information into AAE database (optional)

First appointment:

- Local anesthesia, rubber dam isolation, access
- Copious, gentle irrigation with 20 mL NaOCl using an irrigation system that minimizes the possibility of extrusion of irrigants into the periapical space (eg, needle with closed end and side vents, or EndoVac). To minimize potential precipitate in the canal, use sterile water or saline between NaOCl; lower concentrations of NaOCl are advised, to minimize cytotoxicity to stem cells in the apical tissues.
- Dry canals
- Place antibiotic paste or calcium hydroxide. If the triple antibiotic paste is used: (1) consider sealing pulp chamber with a dentin bonding agent to minimize risk of staining, and (2) mix 1:1:1 ciprofloxacin/metronidazole/minocycline (or, if esthetics are crucial, then consider a 1:1 mixture of ciprofloxacin/metronidazole).
- Deliver into canal system via lentulo spiral, MAP system, or Centrix syringe
- If triple antibiotic paste is used, ensure that it remains below the CEJ (to minimize crown staining)
- Seal with 3 to 4 mm of Cavit, followed by immediate restorative material, glass ionomer cement, or another temporary material
- Dismiss patient for 3 to 4 weeks

Second appointment:

- Assess response to initial treatment. If there are signs/symptoms of persistent infection, consider additional treatment time with antimicrobial, or alternative antimicrobial.
- Anesthesia with 3% mepivacaine without vasoconstrictor, rubber dam, isolation
- Copious, gentle irrigation with 20 mL of ethylenediamine tetraacetic acid, followed by normal saline, using a similar closed-end needle
- Dry with paper points
- Create bleeding into canal system by overinstrumenting (endo file, endo explorer)
- Stop bleeding 3 mm from CEJ
- Place CollaPlug/CollaCote at the orifice, if necessary
- Place 3 to 4 mm of white MTA and reinforced glass ionomer and place permanent restoration

Follow-up:

Clinical and radiographic examination:

- No pain or soft tissue swelling (often observed between first and second appointments)
- Resolution of apical radiolucency (often observed 6–12 months after treatment)
- Increased width of root walls (this is generally observed before apparent increase in root length and often occurs 12–24 months after treatment)
- Increased root length

Table 2-1 The American Association of Endodontics considerations for endodontic regeneration/revitalisation procedures (275)

2.4 Histological observation of revitalisation outcomes

Radiographic evidence from different case reports and animal studies have shown narrower of the root apices, increase in root lengths, and thickening of dentinal walls of treated teeth, all of which are similar to normal teeth development. However, reviewing the

histology of the available published revitalisation case reports (**Table 2-3)** and research in animal models (**Table 2-2**) revealed that the healing tissues are often different from dentine and pulp tissues. Recent revitalisation protocols have better bacterial infection control than older protocols (275), and reduction in post-treatment inflammation enhanced the healing process, which caused thickening of the walls and closure of the apices. Nevertheless, the histological outcomes of the treatment are still different from the normal dentine and pulp tissue. Furthermore, different treatment protocols have different effects on the histological outcomes. In general, post-treatment vital tissue can be categorised into the following:

1- Dentine associated mineralised tissue (DAMT)

2- Intracanal mineralised tissue (MI)

3- Intracanal soft tissue.

2.4.1 Dentine associated mineralised tissue (DAMT)

The formation of a reparative dentine or mineralised hard tissue with a tubular structure on the dentine walls after revitalisation protocols has been reported (132, 276, 277). Nevins *et al.* (276), utilising a monkey model, reported formation of reparative dentine on the walls of immature central incisors after a revitalisation protocol using collagen calcium phosphate gel scaffolds, and mentioned that remnants of lacerated pulp tissue survived and formed reparative dentine. Recently, Zhang *et al.* (132) tested a revitalisation protocol using a blood clot scaffold on immature anterior teeth of dogs and found that the tissue formed on the canal wall had a tubular structure similar to dentine in some sections. A similar protocol using the same animal model, showed the existence of odontoblasts on one side of the walls in one experimental tooth (21). Torneck *et al.* (278) found odontoblasts lining some areas of the canal wall in the presence of mild to moderate inflammation in

immature teeth previously cleaned mechanically and chemically. Another revitalisation study reported the presence of odontoblasts in the immature teeth after instrumentation and in the presence pulpal and periapical inflammation (277). Thus, dentine or tubular hard tissue found on the dentinal wall after revitalisation treatment is possibly produced by these cells, but it is still unknown if these cells were primary odontoblasts that survived, or differentiated odontoblasts from pulp stem cells. In addition, recent research tends to show less evidence of odontoblasts in the histological results after revitalisation treatment, compared to the results seen in older research (**Table 2-2)**. This is either due to the differences in the animal models or perhaps the newer treatment protocols may cause more damage to the odontoblasts and pulpal stem cells than the older protocols.

Formation of cementum-like tissue on dentine walls was also observed after various revitalisation protocols (**Table 2-2 and Table 2-3**). Case reports and animal studies have found that the DAMT observed was a cellular cementum-like hard tissue (23, 137, 347, 348), while in some teeth DAMT had an acellular cementum-like structure with few cell inclusions in the matrix (131). These mineralised tissues have been identified in histological sections as cementum-like tissues, due to the lack of vascular inclusion and tubular structure. However, reparative dentine morphology can look similar to cementum (43, 57). Furthermore, Yamauchi *et al*. (133) identified the mineralised tissue associated with dentine as cementum-like based on the histology, but after performing picrosirius red stain and immunohistochemical analyses (109) found that DAMT were different from dentine, cementum and bone. A recent immunohistochemical study suggested that the mineralised tissue formed on dentine walls after revitalisation treatment is likely to be cementum (139), though in this study they used mature teeth and enlarged the apices mechanically. Therefore, the outcomes of these teeth could be different from immature teeth.

2.4.2 Intracanal mineralised tissue

Formation of mineralised islands (MI) in the canal space was observed after different revitalisation treatment protocols (**Table 2-2 and Table 2-3)**. MI on histology sections looked either bone-like (348) or cellular cementum-like (349). An immunohistochemical study (109) found that MI had some similarities to bone. Studies have found that these MI extend from the apex into the canal lumen (133, 306, 350). In some cases, the apical MI merged or was speculative to merge with the mineralised tissue on the dentinal walls (131). MI were also formed inside the canal coronally, close to the MTA orifice barrier (137, 348), and scattered MI were seen in the canal where parts of the MTA extended apically (350). Animal research has shown the formation of more MI in the canals treated with PRP scaffolds than in canals treated with a blood clot scaffold (131). A revitalisation case treated with PRP scaffold showed obliteration of the canal apically with cementoid/osteoid tissue (23). The origins and types of MI (dentine, cementum or bone) remain unknown, and if the pulpal and periapical condition before treatment effects the formation and type.

2.4.3 Intracanal soft tissues

The intracanal soft tissue seen after various revitalisation treatment protocols is fibrous connective tissue with variable amount of inflammatory cells and blood vessels (**Table 2-2 and Table 2-3**). It is hard to classify the revitalised soft tissues as pulp tissues in the absence of odontoblasts. Animal research suggests that the soft tissues probably generate from the PDL. Zhu *et al*. (137) reported that after revitalisation treatment of immature teeth, PDL-like tissues formed inside the canals, and inserted collagen fibres similar to Sharpey's fibres into the newly formed cementum-like DAMT. In another study by Zhu *et al*. (139), the tissues inside the canals were shown to express periostin protein (139), which

is a marker for PDL and periosteum (351). Conversely, Torabinejad *et al.* (265) reported the formation of pulp-like connective tissue in a human tooth treated with a revitalisation protocol using PRP scaffold. Odontoblast-like cells lining the dentine wall have been observed in a case report three and half weeks after treatment, but the case was an irreversible pulpitis, so it is likely that some residual odontoblasts remained in the pulp (352). Also, animal studies showed odontoblast-like cells lining the dentine walls six months or less after treatment (21, 277). Nevertheless, no case reports or animal studies show the existence of odontoblasts after longer evaluation periods. It is not known whether the tooth condition does not support the remaining odontoblasts, or if new healing tissues replace the odontoblasts with the healing tissue cells. It is also possible that mineralised tissues obliterate the entire canal (17), resulting in canal calcification similar to that reported in luxated and avulsed teeth (353, 354).

First author/year	Animal model	Canals or teeth/group	Pulp/ periapical condition	Treatment protocol	Data collection	Histology results
Ham, 1972 (355)	Monkey	9 immature anterior teeth	♦ Pulp tissues lacerated. ♦ Canals left open 3 days. ♦ Periapical radiolucency developed after 50-75 days.	► Canals filed with file size 110 ► Irrigation (chloramines 4%). ► Camphorated parachlorophenol on paper point sealed in the canal for 3 days./ repeated twice ► Blood clot scaffold ► Gutta percha (GP) orifices seal	Animals killed after: 120 day 148, day 165 day	● 4/9 partial bridge at the apex. ● Apical portion of the canal occupied by moderately dense connective tissue. ● Bone-like tissue extended 1.5 µm into the canal (BI) ● Cellular cementum cover the canal walls and extended coronally with the extension of the connective tissues. ● Some sections showed total calcified canal. ● Very little lengthening of the root.
Torneck, 1973 (278)	Monkey	Immature incisors 4 groups 2 teeth /group	♦ Pulp tissue removed. ♦ Walls filed ♦ Canal left open for: 14 days 49 days 88 days 92 days	► Instrumentation ► Irrigation (saline) ► Camphorated parachlorophenol on cotton pellet placed in the canal orifice. ► Accesses sealed with amalgam.	Observation periods following debridement: 14 days 35 days 49 days 63 days	**Results according to observation period**: ● 14 days: no apical closure, irregular bone-like deposit on walls, some sections showed odontoblasts on the walls in the tooth with less inflammatory tissue in the pulp canal. ● (35-49) days: apices still open, bone island (BI) at the apex, hard tissues (osteodentine, cementum or bone-like) on the walls, inflammatory tissue in the apical third, odontoblasts present in some areas. ● 63 days: apical lesions, pulp canal filled with pus, some root formation and apices narrower (deposit cementum or bone like tissues), and dentine resorption in some areas.
Myers,1974 (277)	Monkey	Anterior teeth 9 teeth/ blood clot (BC) 7 teeth/ BC + Gelfoam 4 teeth/ saline 16 teeth left empty *Some teeth were fully developed.	♦ Pulp tissue extirpated. ♦ Light instrumentation. ♦ Canals left open for 14 days	► Instrumentation. ► Irrigation NaOCl (5.25%). ► Apices of mature teeth enlarged ►Canals filled with: 1-Blood from the animal clotted with thrombin. 2-Blood with Gelfoam. 3- Saline.4- Left empty. ► Orifices sealed (materials are not mentioned)	Animals killed after: 2 weeks 4 Months 6 Months	● limited amount of tissue grow in the canal regardless of treatment protocol ● Immature teeth showed apical healing (4 and 6 months groups); continue root formation and apical bridging by cementoid-osteoid tissue. In three teeth odontoblasts survived and deposit dentine ● Over instrumentation caused inflammation and resorption.
Nevins, 1976 (276)	Monkey	**Group A:** 8 Immature Maxillary and Mandibular central incisors **Group B:** 8 Immature Max and Mad central incisors	Both groups the canals ♦ Instrumentation. ♦ Irrigation with saline. **Group A** canals left open to saliva for one week.	**Group A**: re-instrumentation and irrigation → filled with collagen calcium phosphate gel **Group B**: directly filled with collagen calcium phosphate gel. *One tooth left empty*. ► Orifices and coronal thirds sealed with short GP cones.	Animals killed after 12 weeks.	● Revitalisation with various soft and hard tissues. In some cementum, bone, reparative dentine lined the wall of the root canals. ● Remnants of pulp tissue produced dentine and osteodentine ● Root lengthening showed in one tooth. ● Resorption and inflammation found in some teeth of both groups. ● The empty control had tissue growth, inflammation, and resorption.

First author/year	Animal model	Canals or teeth/group	Pulp/ periapical condition	Treatment protocol	Data collection period	Histology results
Nevins, 1978 (356)	Monkey	6 animals Immature Maxillary and Mandibular Incisors.	♦ Total pulpectomy ♦ No infection	►21 teeth filled with collagen calcium phosphate gel ►6 teeth left empty (control). ► Orifices and coronal thirds sealed with short GP cones.	Radiograph each month. 3 animals killed after : 12 weeks 10 Months	● Control group: 3/6 contained uncalcified connective tissues apically. 3/6 had granuloma. ● Collagen gel group: 15/21 had hard and soft tissues: → 12 week group showed narrower of the canal space and apical foramen by cementum-like deposition on the walls. → 10 months group had more calcified tissue deposition (cementum-like or bone-like). 6/21 showed inflammation and resorption.
Thibodeau, 2007 (348) Wang, 2010 (21)	Dog	4 experimental groups. Each group has 12 immature premolar teeth	♦ Pulp tissue disrupts but not removed. ♦ Sponges soaked in plaque suspension sealed in the canals for 4 weeks	Disinfection protocol as following: →irrigation (1.25% NaOCl). →No instrumentation. →Triple antibiotic dressing for 4 weeks. Groups divided as following: ►G1: infection →disinfection. ►G2: infection →disinfection →blood clot. ►G3: infection →disinfection →collagen. ►G4: infection →disinfection →collagen + blood clot. All orifices sealed with MTA.	→Sacrificed: 3 months post treatment. →Histology analysis	→ Hard tissue on wall: 43.9% of all groups. 54% in G2andG4. →Apical closure: 54.9% of all groups. 59% in G2 and G4. 50% in G1andG3 →Vital tissue within canals: 29.3% of all groups. 36% in G2andG4. Healing tissues were cementum-like (cellular cementum) on the walls, bone-like (BI) in the canal space, bone or cementum like bridges at the apex and near MTA (G2-G4), and PDL-like tissues with some inflammatory cells. One case from G2 showed partial survival of pulp tissue and odontoblasts lining against one side of the walls. Bridge of cementum-like or bone-like (BI) under the MTA and at the apex found in G2 and G4.
Zhong, 2010 (132)	Dog	3 immature anterior teeth.	♦ Pulp tissue removed ♦ Plaque suspension sealed in the canals for 4 weeks. ♦ Apical periodontitis developed.	Treatment protocol: ►Irrigation (5.25% NaOCl and 17% EDTA). ►TAP (2 weeks). ►Blood clot scaffold ►Access seal MTA.	Animals sacrificed after 8 weeks.	● Granulation tissue in the lumen. ● Calcified tissue on the walls had a tubular structure like dentine in some sections; in other sections had cementum-like or bone-like structure.
Da Silva, 2010 (306)	Dog	40 immature premolars (80 canals). 3 experimental groups 28 canals/group	♦ Pulp tissue removed ♦ Canals left open 7days ♦ Apical periodontitis developed after 15-25 days.	►G1: Apical negative pressure irrigation using 2.25% NaOCl, canal orifices seal with MTA. ►G2: Irrigation with 2.25% NaOCl. →TAP (2 weeks). →MTA canal orifices seal. ►G3: no treatment (negative control).	Animals killed after 90 days.	●G1 and G2 →Canals showed connected tissue growth from PDL origin rich in fibroblasts and blood vessels with absence of inflammation. →Mineralised hard tissues on walls and BI inside the canal and extended from the apex. ●G3: absence of healing tissue, and resorption of dentine

First author/year	Animal model	Canals or teeth/group	Pulp/ periapical condition	Treatment protocol	Data collection	Histology results
Yamauchi, 2011 (109, 133).	Dog	immature premolars 4 experimental groups 12 canals/ group.	♦ Pulp tissue disrupt (without remove). ♦ Sponges soaked in plaque suspension sealed in the canals. ♦ Apical periodontitis developed after 3 weeks.	Disinfection protocol: →Irrigation with 2.5% NaOCl. →TAP (2 weeks). ►G1: blood clot ►G2: 17% EDTA wash the canal then blood clot scaffold. ►G3: bleeding the collagen scaffold inserted in the canals. ►G4: EDTA, blood, collagen scaffold All the canal orifices sealed with MTA.	All animals killed after 3 ½ months.	● Healing and mineralised tissues deposition were higher in the groups which had collagen scaffold than without groups, and the groups which treated with EDTA than without ●Histology showed mineralised tissues on the walls (DAMT), which were cementum-like, and bony islands (BI) in the lumen and extended from the apex. Immunohistology showed BI had some similarity to bone, DAMT were different from dentine, bone and cementum. Apical growth and closure was seen in the scaffold groups. Some teeth showed total closure of the apex.
Scarparo, 2011 (318)	Rat	Immature mandibular first molars: Experimental group: 18 molars.	♦ Pulp tissue removed ♦ Canals left open. ♦ Apical periodontitis 3 weeks.	►Exp. group: →Debris removed from canals (2mm from the apex using size 25 endodontic file. → Irrigation 2.5% NaOCl+ saline → TAP. Orifices sealed with cotton and amalgam.	Animals killed after each of the following period after treatment: 3 , 6 ,and 9 weeks	Half of the roots had cementum-like tissue on their apical portion. Some samples had connective tissue ingrowths in the canal. * Because of tooth fracture and loss of coronal sealing, the results of 9 weeks group did not consider.
Zhu, 2012 (137)	Dog	Mature premolars 8 roots/group	♦No infection	→Pulp tissues removed from the canals. →Apices enlarged into 0.8 mm. →Irrigation with 5.25% NaOCl + 17% EDTA. ►Blood clot group ►PRP group All the canal orifices sealed with MTA.	3 months post-treatment radiographs were taken, and the animals killed for histology analysis.	● One root from PRP showed apical periodontitis. ● Vital tissue found 8/8 blood clot group and 6/8 PRP group. Tissues inside the canal similar to PDL tissue (had Sharpey's fibres inserted in the cementum-like hard tissues. ● Cementum-like (cellular) hard tissue deposit on the root canal walls found in all teeth except one root from PRP. ● Bone-like island coronally close to MTA found in all teeth except two from PRP group. Some cases resemble cementum
Zhu, 2013 (131)	Dog	Immature premolars 10 canals/ group	♦ Pulp tissue disrupt (without remove). ♦ Plaque sealed in the canals. ♦ Apical periodontitis developed after 4 weeks.	Disinfection protocol: →Irrigation with 1.25% NaOCl. →TAP (2 weeks). ►Blood clot group ►PRP group All the canal orifices sealed with MTA.	90 days after treatment data collected for histology of type of healing tissues.	● Vital tissue in canals 90% in blood clot and 100% in PRP. Tissue in canal space consisted of fibrous connective tissue and blood vessels. ● Cementum-like hard tissue with few embedded cells deposit on the root canal walls found 50% in blood clot group and 60% in PRP. ● Bone-like island found (coronally and apically) in 50% in blood clot group and 60% in PRP. In some cases BI extended from apical region form brig or merge with DAMT.

First author/year	Animal model	Canals or teeth/group	Pulp/ periapical condition	Treatment protocol	Data collection	Histology results
Tawfik, 2013 (130)	Dog	Immature premolars. Evaluation period. G1 (1 week), G2 (3 weeks), G3 (3 months). Subgroups: BC/6 canals, BC + scaffold / 6 canals. Empty canal/6 teeth.	♦ Pulp tissue disrupt (without remove). ♦ Canals left open. ♦ Apical periodontitis developed after 2 weeks	Disinfection protocol: →Irrigation with 2.6% NaOCl. →TAP (3 weeks). ► Blood clot group ► Blood clot+ hydrogel with bFGF group ► Empty canal group All the canal orifices sealed with MTA.	Evaluation periods 1, 3 weeks, 3 months.	●Tissue inside the canal were connective tissue similar to PDL tissue with variable amount of inflammatory cells. ● Cementum-like hard tissue deposit on the root canal walls which occasionally had cellular inclusion in the matrix.
Torabinejad, 2014 (350)	Ferret	Immature canines. 12 teeth/BC 9 teeth/PRP	♦No infection	→Pulp tissues removed from the canals. →Irrigation with saline and 17% EDTA. ►Blood clot (**BC**) group ►PRP group All the canal orifices sealed with MTA.	Animals killed after 3 months. Histology analysis of healing tissues.	● Bone-like tissue (BI) ingrowth extended from apical region into coronal third in 9/9 PRP and 8/12 **BC** groups. ● Inflammatory cells found in 3/9 PRP and 7/12 **BC**. ● Resorption : 3/9 PRP ● MTA extended to the apical third: 4/12 MTA.
Zhang, 2014 (349)	Dog	Immature premolars. 18 canals/BC group. 18 canals/PRP group.	♦ Pulp tissue disrupted (without remove). ♦ Cotton balls soaked in plaque suspension sealed in the canals. ♦ Apical periodontitis developed after 3 weeks.	Disinfection protocol: →Irrigation with 3% NaOCl. →TAP (4 weeks). ► Blood clot group ► PRP group All the canal orifices sealed with MTA.	Histology analysis after 3 months.	● Pulp like tissue (had fibroblasts and blood vessels) in the canals of both groups which were not connected with apical PDL. ● Inflammatory cells found in PRP and **BC** groups. ● Cellular cementum-like (had many cellular inclusion (cementocytes-like cells)) tissues on walls found in both groups. ● A cellular cementum like tissues in canal space in 9/12 of PRP and 12/17 of **BC**.
Khademi, 2014 (357)	Dog	Immature maxillary incisors and mandibular premolars. **G1:** 20 teeth/**infected group** **G2** 10 teeth/ **Non infected** group	♦ Pulp tissue removed from both groups. **G1:** ♦ Cotton balls soaked in plaque suspension sealed in the canals. ♦ Apical periodontitis developed after 28 days..	Disinfection protocol: →Irrigation with 5.25% NaOCl.(**G1 and G2**) →TAP (28 days).(**G1 only**) ► Blood clot (both groups). All the canal orifices sealed with MTA.	Histology analysis after 3 and6 months (G1) G2 (3 months only)	● No significant differences in histology results were observed between G1 and G2, 3 months and 6 months groups. ● Mild inflammation in periapical tissues were observed in 60% G1 and 77% G2 after 3 months. ● Cementum-like and bone-like tissues on canal walls found in both groups.

Table 2-2 Histology outcomes of revitalisation research in animal models

Author / Date	Tooth	Pre- operative pulp condition	Pre-operative periapical condition	Treatment protocol	Follow up period	Outcomes
Nygaard-Östby, 1961 (358)	Seventeen teeth	Intact pulp, chronic pulpitis, partial and total pulp necrosis.	Periapical lesion in some teeth.	♦ Teeth with vital tissues in pulp canal → Pulp tissue removed from all teeth except three (apical tissue left intact). → Instrumentation and irrigation with EDTAC. →Blood clot scaffold at apical part of the root canals. ♦ Teeth with necrotic tissues in pulp canal → Tissue removed → Instrumentation and Irrigation with EDTAC. → Antibiotic and 4% formaldehyde dressing → 2nd visit/Blood clot scaffold at apical part of the root canals. ► Guttapercha and Kloroperka paste used to seal coronal part of the roots.	13 days - 31/2 years.	► Cases with complete pulp tissue removal: - After 13 days: inflammation and blood in pulp canal. - 35 days and over: the canal had connective tissue with fibre bundles run parallel to canal walls and blood vessels, which extended from the apex. -Cellular cementum deposited at the apical part of the canal. - Some teeth showed resorption areas which repaired with cellular cementum. - One case showed acellular cementum covering the canal walls and fibre bundles run in the canal and inserted in the cementum. ► Cases with partial pulp tissue removal: - After 13 days-35 days: normal vascularised fibrous connective tissue above the coronal fibrin clot. Over 35 days: cellular cementum deposition at apical foramen and occluded the canal coronally. - Resorbed dentine in some areas repaired with cementum. - Apical granuloma and necrotic tissue with dentine fragments in the canal was also seen in a case.
Nygaard-Östby, 1971 (280)	Maxillary teeth	**G1**: Intact pulp, chronic pulpitis (38 teeth) **G2**: pulp necrosis (12 teeth).	Not mentioned.	♦ **G1:** → Pulp tissue removed → Instrumentation and irrigation with EDTAC. →Blood clot scaffold at apical part of the root canals. ♦ Teeth with necrotic tissues in pulp canal → Tissue removed → Instrumentation and Irrigation with EDTAC. → 4% formaldehyde dressing → 2nd visit / Blood clot scaffold at apical part of the root canals. ► Gutta percha and Kloroperka paste used to seal coronal part of the roots.	9 days - 3 years	► **G1**: ● 7/37 showed complete repair: Canal filled with fibrous connective tissue, resorption healed by deposition of cellular cementum. Narrow canal and thick wall by deposition of cementum-like tissue ● 21/37 showed partial repair: Canal contained fibrous connective tissue apically and sometimes infiltrated with inflammatory cells. Coronal granulation tissue with degenerative changes in some cases. ► **G2**: ● 1/12 showed partial repair, and the rest showed no repair. ► **Cases with hard tissue deposit observed after 46 days and more.**

Author / Date	Tooth	Pre- operative pulp condition	Pre-operative periapical condition	Treatment protocol	Follow up period	Histology outcomes
Torabinejad, 2012 (265)	Maxillary right first premolar.	Necrosis.	Chronic periradicular periodontitis.	→ Necrotic pulp removed with large barbed broach →Irrigation: 5.25% NaOCl. →TAP for 22days. →Regeneration PRP clot. →MTA canal orifices seal.	14 months	- Pulp like connective tissues in the root canal.
Shimizu, 2012 (352)	Maxillary left central incisor.	Irreversible pulpitis	Normal periapical tissues.	→Minimal instrumentation →Irrigation with 5.25% NaOCl →CHO dressing (2 weeks) →Blood clot scaffold. →MTA canal orifices seal.	3 ½ week	- Loose connective tissue with collagen fibers filled more than half the canal space. - No inflammatory cells in the canal or periapical area. - Tissues in the canal appeared as extension of PDL. - layers of odontoblasts-like cells along predentine in apical canal - HERS like cells surround the root apex. - No hard tissue on the walls.
Martin,2013 (23)	Mandibular right first molar.	Pulp necrosis	Symptomatic apical periodontitis.	→Distal canal instrumented, not mesial canals. →Irrigation all canals with 5.25% NaOCl. →TAP for 4 weeks. →Patient return after 5 months canal irrigated with 5.25% NaOCl to remove the dressing. →Blood clot at mesial canals, BC + PRP to distal canal. →MTA canal orifices seal	25 months	- Periapical healing - Mineralised tissue occupied the entire apical third of distal canal. - Cementum-like tissue deposited on canal walls which was similar to cellular cementum apically. - Distal canal hard tissue had cementoid/osteoid-like structure with cementocyte/ osteocytes like cells. - Fibrous connective tissue and blood vessels in the canal. - No odontoblast-like cells found on the canal walls.
Shimizu, 2013 (347)	Maxillary left central incisor.	Necrosis	Chronic apical abscess	→ Irrigation 2.6% NaOCl. →CHO dressing (two weeks). → Irrigation 2.6% NaOCl to remove dressing. →Blood clot scaffold. →MTA canal orifices seal.	26 months	- Healing of apical lesion and closure of the apex. - Thickening of the walls - Increased in root length due to deposition of cementum-like tissue. - Apical portion of the canal was obliterated with mineralised tissue similar to cementum with incremental lines and cells in lacunae. - Necrotic tissue in the middle of the canal and between cementum and dentine. - Changed in dentine deposition pattern due to trauma before necrosis.

Author / Date	Tooth	Pre- operative pulp condition	Pre-operative periapical condition	Treatment protocol	Follow up period	Histology outcomes
Lin, 2014 (335)	Maxillary left central incisor.	Avulsed tooth with previously initiated root canal treatment.	Asymptomatic apical periodontitis.	- Previous history of instrumentation. - Irrigation 5.25% NaOCl →CHO dressing (11 days). → Irrigation 5.25% NaOCl to remove dressing. →TAP for 2 weeks. → Irrigation 5.25% NaOCl to remove dressing. →Blood clot scaffold. →MTA canal orifices seal.	16 months	- No increase in root length and thickening of the wall. - Dentine resorption areas at the coronal and middle of the root, and some areas repaired with cementum or bone like tissue. - Apical canal filled with necrotic tissues. - Bacterial biofilm on the walls.

Table 2-3 Revitalisation case reports with histology outcomes.

2.5 Animal models for revitalisation research

Different animal models have been used in endodontic revitalisation research to evaluate different treatment modalities. The preferred animal model for endodontic revitalisation research should reflect the same physiology and anatomy of human pulps. Clinically, the teeth should be easy to access and radiograph.

Non-human primates have been used in many endodontic revitalisation studies (276, 277, 356, 359) because of the similarities to humans in the morphology and histological structure of dental tissues, but these animals are expensive, not readily available and they can be difficult to manage (360). Dogs can provide a large number of teeth per animal for research studies, and have thus been used in many endodontic revitalisation studies to examine different treatment protocols (21, 306, 348). However, dogs are not only expensive, but are also seen as pets in many cultures, and there has thus been an increase in the objections to their use as an experimental animal.

Smaller animal models present some problems for endodontic studies. For instance, rodent incisor teeth are not suitable for this type of research because they are small, with wide-open apices and their growth is continuous (318, 361, 362). Scarparo *et al*. (318) studied the effect of canal disinfection protocols on revitalisation outcomes using rat molars. The observation periods were planned to be at three, six and nine weeks, but the crowns of the teeth started to fracture after six weeks, which meant longer studies could not be considered. Cats have also been used for regeneration research (363), but they are also common pets in many cultures. Finally, ferrets have been suggested as a promising animal model for regenerative endodontics but their pulpal anatomy is different from the human (364). A recent revitalisation study using ferrets showed unfavourable outcomes (350), while similar revitalisation protocols tested in dogs showed better outcomes (131, 137).

While small animals are still preferred for reasons of familiarity and cost, continual tooth eruption in the rodent and the vastly different teeth size and anatomy to humans, make them unsuitable models for revitalisation research (12), and in particular for research which requires long evaluation periods.

The urge for suitable larger animals encourages researchers to consider farm animals for laboratory studies. Pigs, as an alternative large animal, are similar to humans in physiology and anatomy, but pig teeth are smaller in size and different in shape to human teeth (365). In addition, pigs can grow to an unmanageable size and can be uncooperative animals in the laboratory (366).

2.6 Sheep as a model in dental research

Sheep are raised in nearly every country in large numbers and are used for research purposes. As the teeth are similar in size and anatomy to human, sheep have been used a variety of dental research as well as medical research. For example, sheep have been used in periodontics as a cadaver model to demonstrate different surgical methods (365). The similarities between human molars and sheep second premolars in size and histological appearance encourages the use of sheep as an animal model for guided tissue regeneration research, something which has been performed successfully (367). Sheep bones are similar to human bones in mineral composition, remodelling, and macroscopic structure (368, 369). Because of this, dental implant research has been conducted successfully on sheep (370-372). Some *in vitro* endodontic research studies have used sheep teeth to investigate canal irrigation protocols, dressing materials, and techniques in the management of traumatised immature teeth (373-375).

The usual method for determining sheep age is from the number of teeth present, usually from their incisors as shown in **Figure 2.5**. In over 90% of sheep breeds, permanent central

incisors (two-tooth age), erupt between 12 and 18 months of age, second incisors (four-tooth age) erupt between 18 and 26 months, permanent third incisors (six-tooth age) erupt between 24 and 36 months of age and permanent corner teeth (eight-tooth age) erupted at between 32 and 44 months (376).

Since the 1960s, reports describing the development, growth and eruption of sheep teeth have been published (377-379). These studies added valuable information about the stages of development of the tooth, cellular structure of the tooth, hard tissue formation, crown formation, and has shown their similarity to human teeth. However these studies lack information related to the root anatomy and histology after eruption, which is important information needed for endodontic revitalisation research.

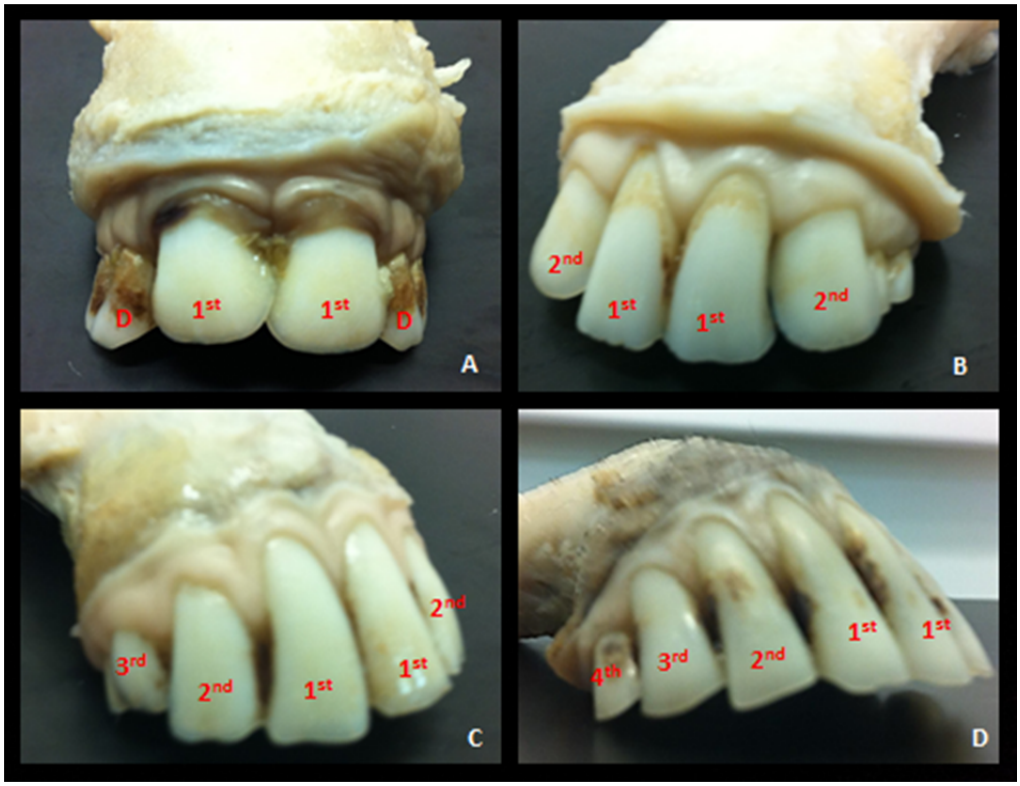


Figure 2.5 Sheep mandibles

A: Two-tooth age (First permanent incisors erupted, and the rest of incisors are deciduous (D)).

B: Four-tooth age (first and second permanent incisors erupted.

C: Six-tooth age (first, second and third permanent incisors erupted).

D: Eight-tooth age (first, second, third and fourth permanent incisors erupted.

2.7 Conclusion

Endodontic revitalisation of infected immature teeth is a management approach which allows ingrowth of vital healing tissues, which are not necessary pulp parenchymal tissue, to continue root development and hard tissue deposition on the weak dentinal walls. Revitalisation treatment protocols represent an improvement in the management of immature teeth with necrotic pulp, giving them a chance for further root development rather than the old protocols which left short and thin roots and subsequently prone to fracture. Current revitalisation treatment protocols have shown some successful outcomes, but possibly for only in the short term, as long term outcomes remain unpredictable. Many

aspects of these treatment protocols necessitate further investigation to improve results. It is of great importance to evaluate the condition of the pulp and periapical tissue before treatment, as this could be one of the key factors for the differences in the treatment outcomes. Another, aspect which needs further investigation is the canal disinfection protocols. Canal disinfection is a critical step in revitalisation protocols, because the persistence of the bacteria after treatment can jeopardise the whole treatment (335). However, the use of harsh disinfectant protocols and materials can also kill any remanent of pulpal vital cells and tissues, preventing the revitalisation process. Periapical irritation to create a blood scaffold, or the use of alternative scaffold, and their effect on the outcomes of the treatment also required further investigation. Furthermore, if MTA will be used as an orifice barrier, what are the strategies to avoid tooth discoloration? Formation of intracanal islands can make any further endodontic treatment difficult, but the reasons for their formation are yet unknown. Finally, total canal obliteration with mineralised tissue is possible after treatment, similar to what has been observed in avulsed teeth, but it is still unknown if this outcome is clinically acceptable or required further prophylactic endodontic treatment. All these issues are vital in the development of revitalisation treatment and a suitable animal model is also need.

Chapter 3. Standardisation of Sheep as a Suitable Animal Model for Endodontic Revitalisation Research

3.1 Introduction

Sheep have been used in many dental research studies (367, 371, 380) due to similarities in their teeth to those of humans in many anatomical and histological aspects (368, 369, 378, 381). Sheep are widely available, easy to handle and the cost of keeping and maintaining sheep is likely to be less than for other big animals, as sheep do not need to be kept in cages but can be released to fields.

There are obvious differences between sheep jaws and human jaws. Sheep do not have upper incisors, and the lower incisors bite against a hard toothless pad in the upper jaw. This difference should not affect the utilisation of sheep incisor teeth for revitalisation research. Sheep incisors are single-rooted teeth, easy to access and similar to human incisors in size and shape. Many studies have evaluated sheep incisors from early stages of development until eruption (377-379), but there is lack of information related to the root development, root diameter, histology and anatomy of incisor teeth from the stage of eruption until maturation. Therefore, in order to use sheep incisor teeth for revitalisation research, an investigation of the developmental stages of the root from eruption until maturation was required.

This part of the study involved a systematic investigation of incisor teeth roots at different developmental ages to identify the most suitable dental age for endodontic revitalisation research involving sheep incisors.

3.2 Part One: Evaluation of the anatomy and the histology of sheep permanent incisor root at the mature age (Full mouth age).

3.2.1 Aims

The aims of Part One of this study were to:

- Describe the anatomy and histology of incisor teeth in mature sheep.
- Investigate the superimposition of the anatomical structures on radiographic measurement of the incisor teeth.
- Investigate the reliability of imaging techniques in measuring root diameters of incisor teeth.

3.2.2 Material and methods

3.2.2.1 Animals

Seventeen mandibles of Merino sheep at the eight-tooth to full mouth ages were obtained from animals sacrificed at the completion of other studies following approved guidelines set by South Australia Pathology/Animal Ethics Committee (#ST25/12).

3.2.2.2 Fixation

Anterior segment of the mandible was removed as quickly as possible after death followed by fixation in 10% neutral buffered formalin (Australian Biostain, VIC, Australia) at 18° to 22°C for a minimum of ten days (**Figure 3.1**).

Figure 3.1 Sheep anterior segments of mandibles fixed in formalin.

3.2.2.3 Direct measurements

Sixteen teeth of each incisor type were extracted. The crowns of the teeth were sectioned at the cemento-enamel junction (CEJ) level under water cooling using a low speed hand-piece with a diamond disc. Root length was measured by introducing an endodontic file (size 10-30 K-file (SybronEndo, Glendora, USA)) into the root canal until the file tip was visible at the apex, as shown in **Figure 3.2**. The distance between the file tip and stopper was measured using digital callipers. Measurements were repeated twice and the average of the two measurements was taken. To replicate clinical endodontic procedures, 0.5 mm was subtracted from the measured length when determining root canal length.

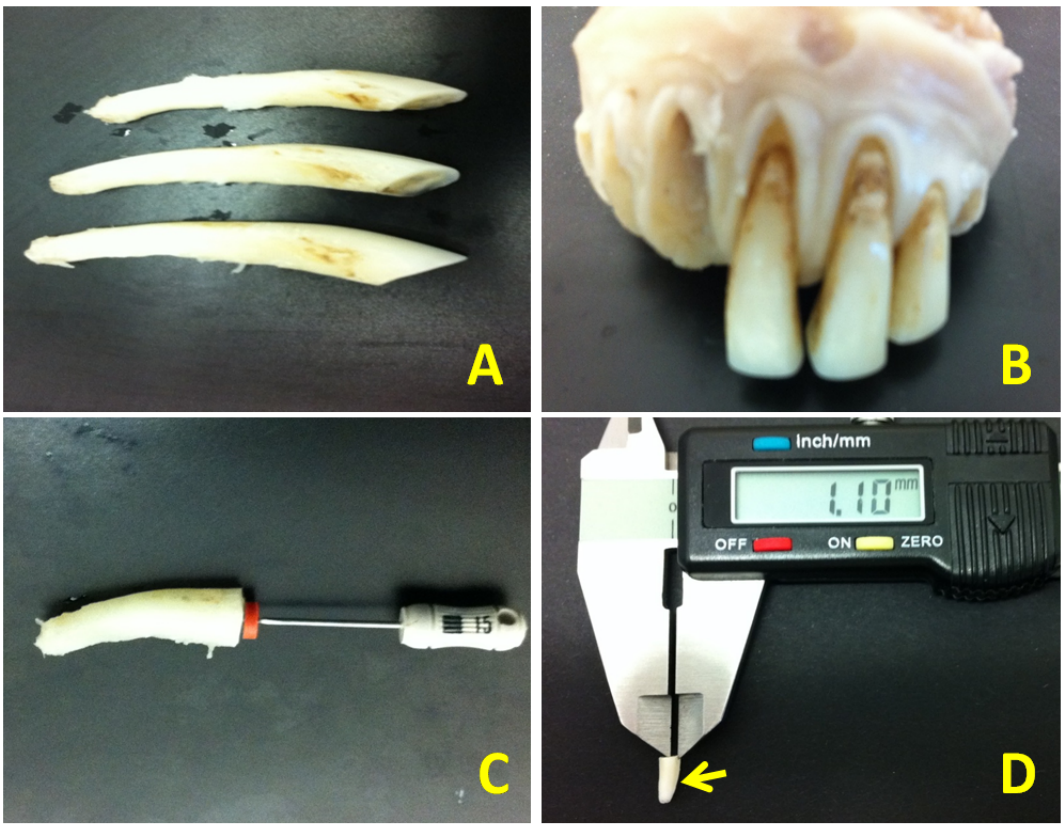


Figure 3.2 Direct measurement methods.

A and **B**: images showing teeth extraction.

C: measuring root length.

D: measuring root wall thickness.

Yellow arrow indicates the apical third of root.

The apical third of each root was sectioned and the mesial and distal root thicknesses of each piece were measured using digital callipers (**Figure 3.2D)**, and the average was considered as the direct apical third root wall thickness.

The apical diameter was measured by introducing an endodontic file (size 15-70) into the apical third segment after removal of the pulp content with a small file. The largest size file that passed passively through the apical foramen was recorded as the apical diameter.

3.2.2.4 Radiographs

Periapical-like radiographs were obtained using a Heliodent x-ray machine (Siemens) with ultraspeed occlusal films (Insight Kodak, Rochester, NY) using an exposure setting of 70 kV, 7 mA and 0.16 second. An occlusal radiograph was used, with a long cone tube at 90 degrees to the film plane. The mandible was seated with the occlusal plane horizontal. The film packet was placed on the occlusal surface of the teeth. A film holder designed especially for this study was used to ensure that the x-ray tube head, film and object were at the same position throughout the study (**Figure 3.3**).

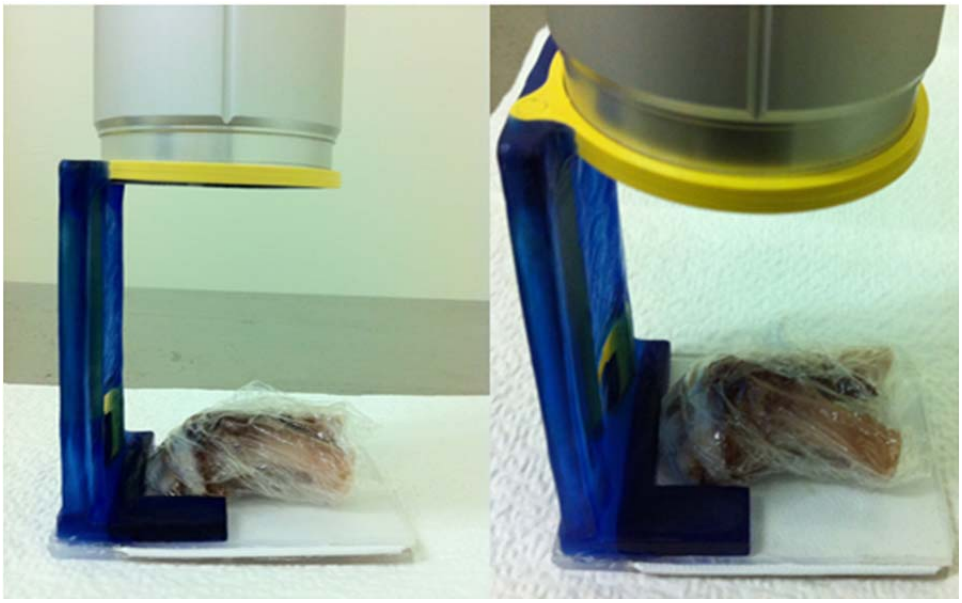


Figure 3.3 A sheep anterior segment of the mandible placed in the film holder designed for this study.

The images were digitised using an optical scanner (Epson, Australia), with a resolution of 1200 dpi and a 16 bit greyscale image type. The images were analysed using Image J software (Image J v 1.48, US National Institutes of Health, Bethesda, MD) to measure root canal length, apical third dentine thickness and apical diameter (**Figure 3.4)**.

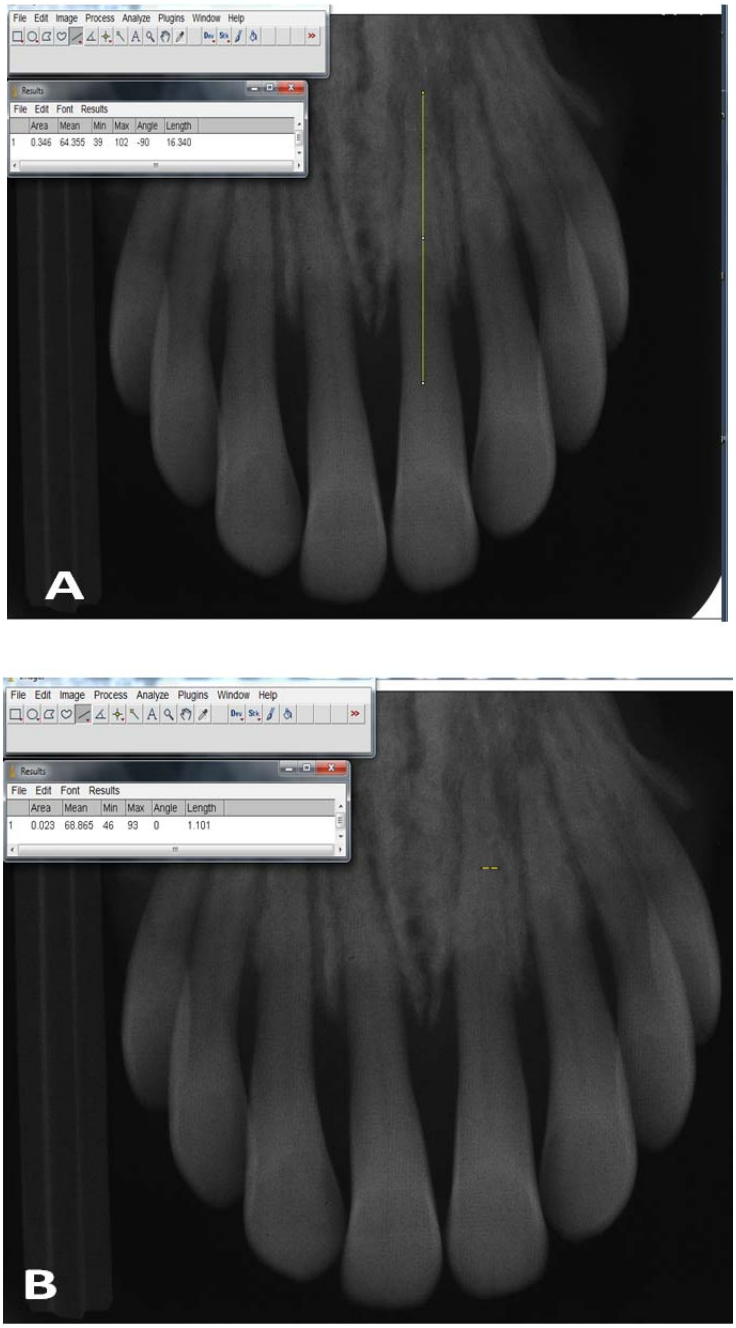


Figure 3.4 Measurements on radiographic image using Image J software.

A: measuring root length.

B: measuring apical root wall thickness.

3.2.2.5 Computer Tomography (CT)

High resolution CT images of mandible specimens were obtained using a live animal micro-CT scanner (SkyScan 1076, Kontich, Belgium). Images were taken with 100 kV and 90 μA using a 0.5 mm aluminium filter. Scanning width was 68 mm and 16 bits depth. The

distance between object to source and camera to source was 121 mm and 161 mm, respectively. When scanning, specimens were covered with a plastic film to prevent dehydration, placed in a scanner tube, and fixed with adhesive tape to the tube with the occlusal plane parallel to the horizontal plane.

Scans were imported to SkyScan NRECON software where three-dimensional reconstruction was performed. Region of interest (ROI) was defined as a square with a diameter that covered the whole sample vertically and horizontally. The files created by NRCON were analysed using Data viewer software. Images saved from Dataviewer were loaded to CTAn software (version 1.91.0-SkyScan) to make the necessary measurements. CT VOX software was used to create a 3D model.

3.2.2.6 Evaluation method reliability

High resolution CT scan is ideal for measuring small distances and examining structures but is hard to use on live animals. Dental radiography is the commonly used clinical method for *in vivo* research, but radiographic measurements are not always precise due to the superimposition of different anatomical structures on the site under investigation. To evaluate the reliability of radiographs and CT scans, measurements collected from the same teeth using radiographs and CT scans were compared to direct measurement.

To avoid biological hazards, sheep mandibles were fixed prior to radiography and CT scanning. After fixation, extraction of the teeth was difficult as they commonly fractured. First, third and fourth incisor teeth from five jaws were extracted successfully after fixation, and then radiographed, and scanned with micro-CT. Direct measurements were collected as described above, and the data was analysed to evaluate the relationship between radiographic and CT scan measurements.

3.2.2.7 Histology

Three fixed mandibles were decalcified in a solution prepared from a combination of EDTA and hydrochloric acid (**7.2.1 Appendix**). Radiographic analysis of each specimen was carried out weekly to determine the completion of decalcification. After decalcification, the specimens were washed in tap-water for approximately seven days to eliminate the decalcifying agent. The tissues were placed in 10% neutral buffered formalin before automatic processing in a Shandon Citadel tissue processor (Shandon Industries, Pittsburgh, Pennsylvania) (**7.3 Appendix**). Through this process the tissues were dehydrated in graded alcohols before being impregnated with paraffin wax. The tissues were then embedded in Surgipath EM400 wax (Leica Microsystems, Richmond, UK) and 7 μm thick serial longitudinal sections were then cut in a mesio-distal direction. The sections included the apical 3 mm and the entire surrounding periapical region. Approximately each 50th section was stained with hematoxylin and eosin (**7.4 Appendix**). The stained sections were imaged either using a slide scanner (NanoZoomer 2.0HT, Hamamatsu, Japan) or stereomicroscope (Olympus BX51, Leica, Germany). The images were saved using 24 bit colour image.

3.2.3 Statistics

The data was analysed for significant differences between direct, radiographic and CT scan measurements using repeated measurement one-way ANOVA followed by Sidak's multiple compression test. Statistical significance was set at $p < 0.05$. All statistical analyses were performed using Prism 6 Statistics software (GraphPad Software Inc., San Diego, CA, USA).

3.2.4 Results

3.2.4.1 Direct measurement results

Direct measurements of the root length, dentine thickness and apical diameter are presented in **Table 3-3-1**. The results showed significant differences in the root length between all the teeth ($p < 0.0001$), except the first and the second incisors ($p = 0.36$). Root length ranged from 13 - 16 mm in 79% of the evaluated first, second and third incisors. There were significant differences in dentine thickness mesio-distally (MD) between all the teeth ($p < 0.006$), except between the second and the third, the third and the fourth incisors. Dentine thickness (MD) ranged from 0.7 - 1.3 mm in 81% of the evaluated first, second and third incisors. There were significant differences in apical diameters between the first and the fourth, the second and the fourth incisors ($p < 0.02$). Apical diameter ranged from 0.2 - 0.45 mm in 98% of the evaluated first, second and third incisor teeth.

3.2.4.2 Radiographic results

Radiographic measurements of the fourteen sheep jaws were larger than direct measurement in almost all the incisor teeth (**Table 3-3-1)**. The results showed the same significant differences in the root length between all the incisors ($p \leq 0.0003$), except between the first and the second incisors ($p = 0.19$), as was found with the direct measurement results. Radiographic root length ranged from 13 - 16 mm in 69% of the first, second and third incisors evaluated. There were significant differences in dentine thickness (MD) between all the teeth ($p < 0.001$), except between the second and the third incisors ($p = 0.38$), which is similar to the direct measurements results. Dentine thickness (MD) ranged from 0.7 - 1.3 mm in 89% of the evaluated first, second and third incisors. Apical diameters measurements showed significant differences between first and fourth incisors,

between second and fourth incisors ($p < 0.01$), which similar to the direct measurement results. Apical diameters ranged from 0.2 - 0.45 mm in 70% of the evaluated first, second and third incisors, which was a lower percentage of measurements in this range than with the direct measurement.

Method	Teeth	Average Root Length (mm) ±SD	Average Root Wall thickness/BL (mm) ±SD	Average Root Wall thickness/MD (mm) ±SD	Average Apex Diameter/BL (mm) ±SD	Average Apex Diameter/MD (mm) ±SD
Direct	1st Incisor N= 16	15.73 ± 1.20	1.39 ± 0.25	1.30 ± 0.3	-	0.33 ±0.05
	2nd Incisor N= 16	15.15 ± 1.12	1.19 ± 0.20	1.03 ± 0.21	-	0.31 ± 0.05
	3rd Incisor N= 16	13.90 ± 0.69	1.04 ± 0.14	0.98 ± 0.13	-	0.35 ± 0.08
	4th Incisor N= 16	11.93 ± 0.94	0.95 ± 0.17	0.79 ± 0.13	-	0.43 ±0.16
Radiograph	1st Incisor N=28	16.19 ± 1.14	-	1.23 ± 0.17	-	0.41 ±0.07
	2nd Incisor N=28	15.56 ± 1.20	-	1.04 ± 0.11	-	0.44 ± 0.08
	3rd Incisor N=28	14.24 ± 1.10	-	0.98 ± 0.12	-	0.50 ± 0.11
	4th Incisor N=28	10.63 ± 1.28	-	0.86 ± 0.08	-	0.54 ±0.17
CT scan	1st Incisor N=28	16.86 ± 1.42	1.30 ± 0.27	1.20 ± 0.25	0.78 ± 0.11	0.40 ±0.10
	2nd Incisor N=28	15.94 ± 1.20	1.13 ± 0.14	1.02 ± 0.18	0.79 ± 0.17	0.45 ± 0.10
	3rd Incisor N=28	14.30 ± 1.20	1.04 ± 0.19	1.00 ± 0.26	0.91 ± 0.26	0.45 ± 0.10
	4th Incisor N=28	11.40 ± 0.96	0.90 ± 0.17	0.83 ± 0.26	1.05 ±0.54	0.59 ±0.24

Table 3-3-1 Measurements of incisor teeth of the mature age sheep.

3.2.4.3 CT scan results

CT scans resulted in larger measurements than direct measurement in almost all of the evaluated teeth, as shown in **Table 3-3-1**. CT scan results showed a significant difference

in root length between first and second incisors ($p = 0.04$), and highly significant differences between all the other incisors ($p < 0.0001$). The percentage of first, second and third incisors which had root lengths in the mature age range from 13 - 16 mm on CT scan was 67%, which is similar to radiographic results. Apical root wall thickness (MD) results showed significant differences between all the teeth ($p < 0.05$), except second and third incisor apical root wall thicknesses were not significantly different ($p = 0.99$). Direct and radiographic measurements also showed no significant differences between second and third incisors in root wall thickness (MD). Similar to the direct and radiograph results, apical root thickness (MD) ranged from 0.7 - 1.3 mm in 85% of the evaluated first, second and third incisors. Apical diameter (MD) results showed no significant differences between first, second and third incisors, which is the same as the direct measurements. It also showed significant differences between all the incisors and the fourth incisor ($p \leq 0.03$). As with the radiographic results, the CT apical diameter (MD) measurements ranged from 0.2 - 0.45 mm in 76% of the evaluated first, second and third incisors. CT scans of the mature sheep incisors showed that the root apex consisted of three anatomical structures: a major apical foramen; intermediate dilatation; and minor foramen **(Figure 3.5)**. The distance between the major foramen and minor foramen ranged from 0.5 - 1 mm.

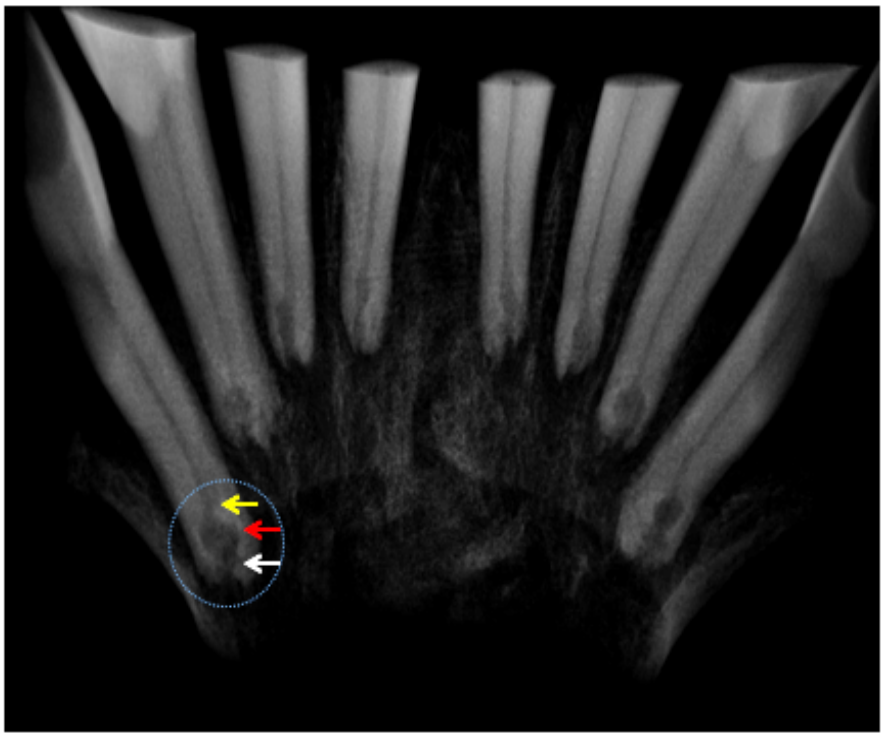


Figure 3.5 Sheep incisors, using the three dimensions CT imaging.

Apical areas of mature incisors. Blue circle indicates a high magnified of the apical area, yellow arrow indicates the major apical foramen, red arrow indicates the intermediate dilatation and white arrow indicates the minor foramen.

3.2.4.4 Evaluation methods reliability

The results showed no significant differences between direct, radiographic and CT scan measurements of the first incisor root lengths. The same results were found for the third incisors. Radiographic measurements of fourth incisor root lengths and apex diameters were significantly different than CT scans and direct measurements, while there were no significant differences between direct and CT scans in root length measurements (**Figure 3.6**, **Figure 3.7**).

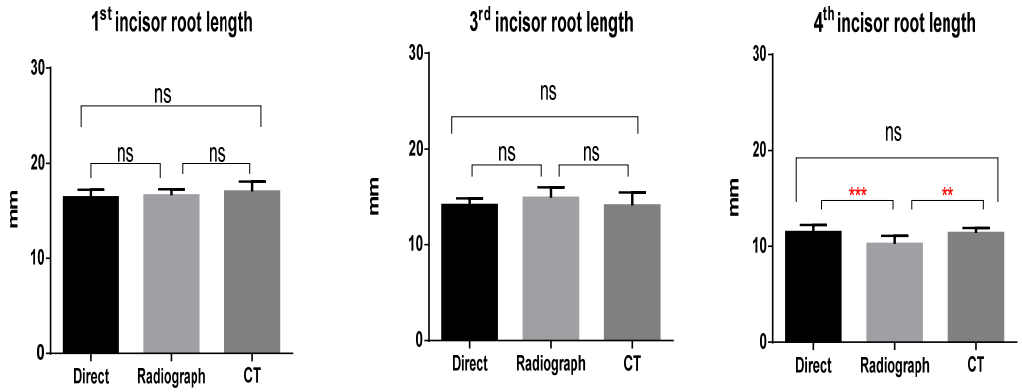


Figure 3.6 Comparison between sheep incisor root lengths.

Lengths evaluated using direct, radiograph and CT scan methods. Results shown are means ± SD.

*p < 0.05, **p < 0.01, ***p < 0.001.

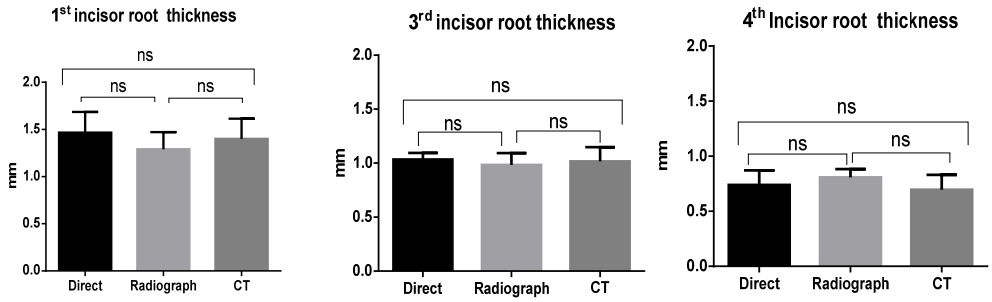


Figure 3.7 Comparison between sheep incisors apical root wall thicknesses.

Measured made using direct, radiograph and CT scan methods. Results shown are means ± SD.

*p < 0.05.

Apical diameter results showed no significant differences between the measurements collected using the three methods for first incisors. Third incisor radiographic measurements were significant larger than the direct measurements. Fourth incisor radiographic measurements also were significantly larger than direct and CT scan measurements (**Figure 3.8)**.

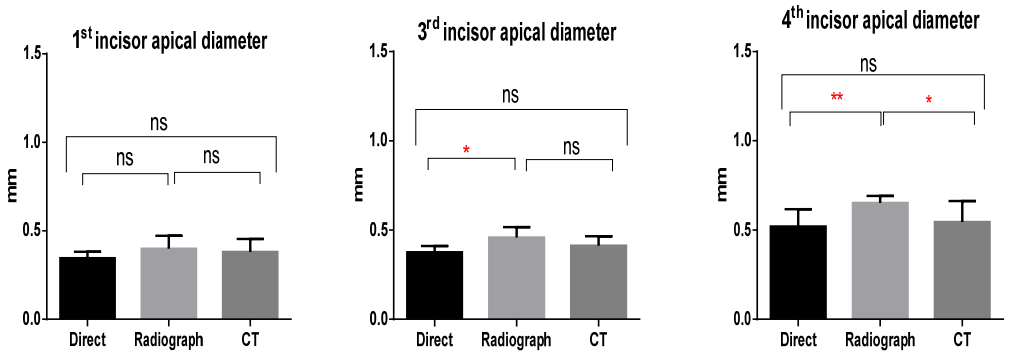


Figure 3.8 Comparison between sheep incisor apices.

Apices diameters evaluated using direct, radiograph and CT methods. Results shown are means ± SD.

*p < 0.05, **p < 0.01, ***p < 0.001.

3.2.4.5 Histology results

The histology of the teeth and surrounding structures showed well developed pulp tissues with odontoblasts lining dentine walls, thick dentinal walls, narrow pulp canals, closed apices and normal periodontal ligaments (**Figure 3.9-Figure 3.11**).

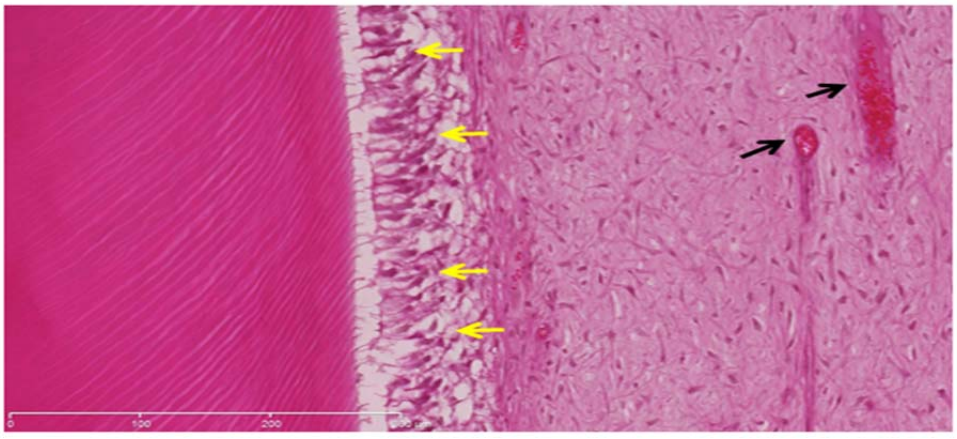


Figure 3.9 High magnification of a section through the pulp.

Yellow arrows indicate odontoblasts lining dentinal wall. Black arrows indicate blood vessels inside the pulp.

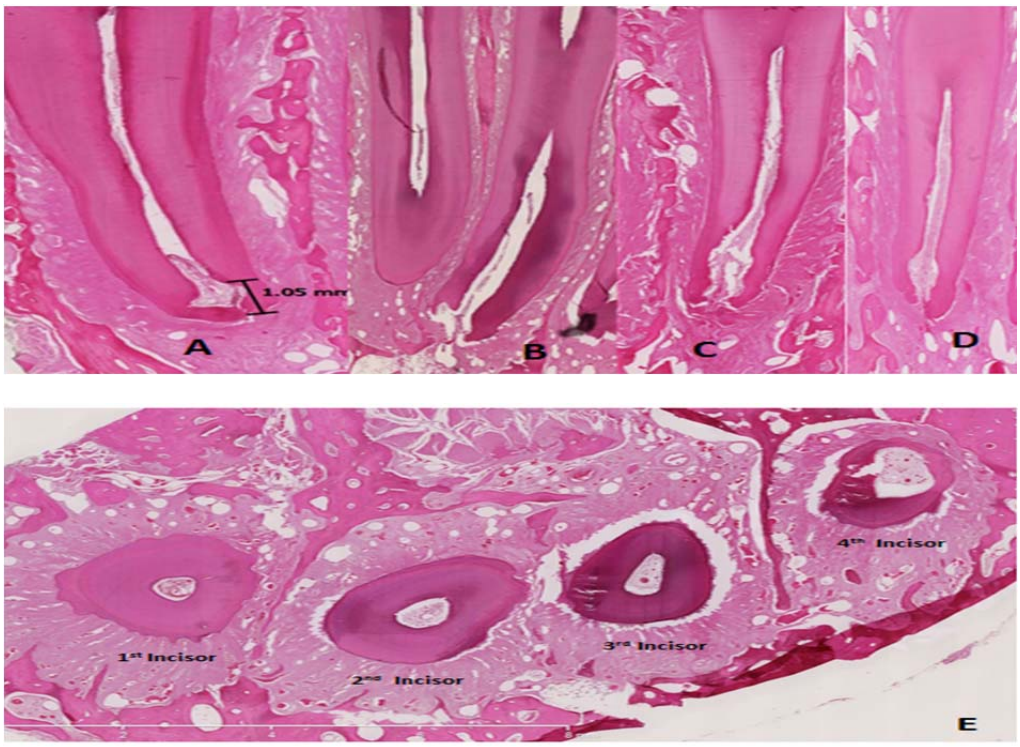


Figure 3.10 Histological sections full mouth sheep teeth.

A: Left first incisor.

B: Right second incisor.

C: Third incisor,

D: Fourth incisor.

E: Transaxial section showing apical third of all incisors.

All incisors at this age have narrow canal, thick root walls, and well developed apices.

The histological sections of all incisors at the mature age showed an apical area with three anatomical features: The major foramen consisted of dentine and pulp tissue and cementum on the external root surface. The intermediate dilatation was divided into two parts; the coronal section had dentine adjacent to the pulp followed by cementum, while the apical section had cementum d without any dentine layer. The minor foramen is formed by the outer cementum (**Figure 3.11)**.

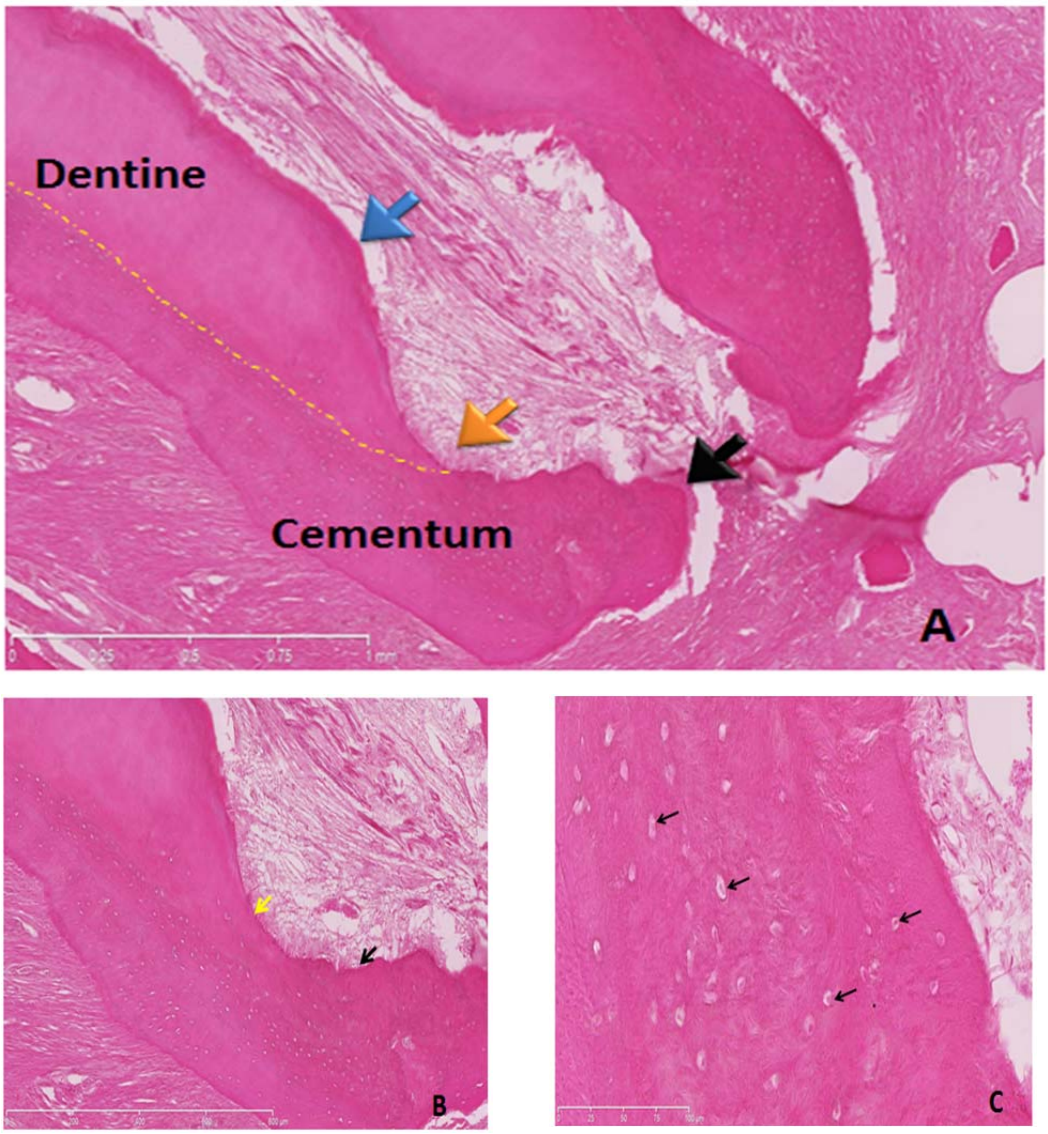


Figure 3.11 Histology sections showing apical area.

A: Blue arrow indicates major foramen, yellow arrow indicates intermediate dilatation, and black arrow indicates minor foramen. Interrupted line indicates margin between dentine and cementum.

B: Intermediate dilatation, yellow arrow indicates dentine, black arrow indicate cementum.

C: High magnification of cementum lining of the dentine in the intermediate dilatation, black arrows indicates cementocyte cells.

3.2.5 Discussion

Sheep teeth have been used for testing a variety of endodontic materials and techniques (374, 382), but in order to be utilised for endodontic revitalisation research, further investigation and standardisation of the teeth and their surrounding structures was required.

Thurley (378) examined sheep incisor teeth development, mechanism of eruption and evaluated crown length and structure. In addition, comparative data to that for human teeth was reported, but, there was insufficient information relating to sheep root anatomy and histology to be able to confidently use sheep as a model for endodontic revitalisation research.

This study evaluated the root length of sheep incisors using direct measurement, as well as measurements from radiographs and CT scans. The results showed that more than 65% of the evaluated sheep incisors had root lengths ranging 13 - 16 mm, which is very similar to the human incisor root lengths (383) (**Table 3-2).**

A - Human	AVERAGE (mm)	RANGE (mm)
Max 1st Incisor N = 398	13	6.3 - 20.3
Max 2nd Incisor N = 295	13.4	9.6 - 19.4
Max Canine N = 321	16.5	10.8 - 28.5
Mand 1st Incisor N = 226	12.6	7.7 - 17.9
Mand 2nd Incisor N = 234	13.5	9.4 -18.1
Mand Canine N = 316	15.9	9.5 - 22.2
B - Sheep		
1st Incisor N = 16	15.7	13.3 - 17.8
2nd Incisor N = 16	15.2	12.9 - 16.8
3rd Incisor N= 16	13.9	12.7 -15
4th Incisor N = 16	12.1	11.2 - 13.3

Table 3-2 Root lengths of anterior teeth

A: Root length in human teeth (modified from Woelfel's dental anatomy (383)).

B: Sheep teeth direct measurement of root length.

Bellucci and Perrini (384) evaluated dentine thickness at 4 mm level from the apex in 165 anterior human teeth. In the current study, a similar method was used to measure root thicknesses at the apical third of sheep incisors which showed that 82% of the evaluated

teeth dentine thickness (MD) ranged from 0.7 - 1.75 mm, which corresponded closely to that reported for humans (384) (**Table 3-3)**.

A - Human	AVERAGE (mm)	RANGE (mm)
Max 1st Incisor N = 398	13	6.3 - 20.3
Max 2nd Incisor N = 295	13.4	9.6 - 19.4
Max Canine N = 321	16.5	10.8 - 28.5
Mand 1st Incisor N = 226	12.6	7.7 - 17.9
Mand 2nd Incisor N = 234	13.5	9.4 -18.1
Mand Canine N = 316	15.9	9.5 - 22.2
B - Sheep		
1st Incisor N = 16	15.7	13.3 - 17.8
2nd Incisor N = 16	15.2	12.9 - 16.8
3rd Incisor N= 16	13.9	12.7 -15
4th Incisor N = 16	12.1	11.2 - 13.3

Table 3-3 Dentine thickness of anterior teeth.

A: Dentine thickness in human anterior teeth (384).

B: Sheep teeth dentine thickness.

Green and Brooklyn (385) measured the apical foramen diameters of anterior human teeth using a stereomicroscope. They found that the average diameter of the apical foramen in maxillary anterior teeth was 0.4 mm, while Morfis *et al* (386) found that the average apical foramen diameter in human maxillary incisors was 0.28 mm using scanning electron microscopy (**Table 3-4)**. Our radiographic and CT scans results showed that more than 70% of first, second and third incisors sheep teeth have major apical foramen diameters (MD) between 0.2 - 0.45 mm (**Table 3-4)**.

A- Human	AVERAGE (mm)	SD
Max Incisors	0.29	0.12
Mand Incisors	0.26	0.19
B- Sheep		
1st Incisor	0.41	0.07
2nd Incisor	0.44	0.08
3rd Incisor	0.50	0.11
4th Incisor	0.54	0.17

Table 3-4 Apical diameters of anterior teeth.

A: Human teeth apical diameters (386).

B: Sheep teeth radiographic mesio-distal apical diameters.

These results suggest that sheep teeth are comparable to human teeth, and are therefore are an appropriate anatomical model for endodontic revitalisation research.

For *in vivo* endodontic revitalisation research, a radiograph is the easiest and the most common way of evaluation of the treated teeth. For standardisation purposes, a holder was designed for this research to keep the x-ray tube, film, and jaw in the same position throughout the experiment. After finishing the clinical part of the experiment, the tissues are often required for histological analysis, preventing direct measurements of the teeth, thus requiring other ways of measurements. To examine the reliability of data collection methods, data collected from radiographs and CT scans were compared to the direct measurements. The results showed that CT scanning is an effective method for evaluation for all incisor teeth. Radiographs were also effective for all teeth, except the fourth incisors. This could be due to its position at the corner of the jaw where its image sometimes overlaps the third incisor, making it difficult to measure the dimension precisely. However, our results suggest high resolution CT scans could replace the direct

measurements, saving both time and the number of animals needed for evaluation of endodontic revitalisation protocols.

The present study showed that the apical part of incisor teeth in mature age sheep have a major apical foramen followed by a small dilatation and then a minor foramen. The distance from the major apical foramen to the root apex ranged between 0.5 mm and 1.5 mm. At this distance, pulp tissues are associated with cementum except the area close to the major foramen (**Figure 3.11)**. A portion of the intermediate dilatation walls are covered by cementum. Although there is a difference in the apical area anatomy between sheep teeth and human teeth, it has been reported that in human maxillary incisors there is between a 0.2 - 3.8 mm distance from the root apex to the cemento-dentine junction (CDJ), and pulp tissue in this region is covered by cementum only (387, 388).

Previous research models for endodontic regeneration or revitalisation research (e.g. dogs and ferrets) have similar differences in the apical anatomy (364, 389, 390), and this has not prevented the use of these animals for research (21, 306, 350, 391). Even though the differences between human teeth and sheep teeth requires further investigation, there is no reason to believe that this difference would affect the suitability of the sheep teeth as a comparable model to human teeth for endodontic regeneration or revitalisation research.

3.3 Part Two: A suitable sheep dental age to be utilised for regenerative endodontic research.

3.3.1 Aims

The aims of this part of the study were to:

- Describe the anatomy and histology of sheep incisors at each developmental age.
- Validate the effectiveness of imaging techniques in measurement of sheep incisors at each age.
- Specify the most appropriate dental developmental stage for *in vivo* endodontic regeneration or revitalisation research.

3.3.2 Materials and Methods

3.3.2.1 Animals

Dental development of the sheep is traditionally described in terms of the pattern of eruption of the permanent incisors:

- Two-tooth with first permanent incisors erupted (12-18 months of age).
- Four-tooth with first and second permanent incisors erupted (18-26 months of age).
- Six-tooth with first, second, and third permanent incisors erupted (24-36 months of age).

Merino sheep mandibles were obtained from animals sacrificed at the completion of other studies. The collected specimens included fourteen jaws at two-tooth stage and ten jaws from animals at each of the four-tooth and six-tooth stages (total number = 34 mandible). The teeth at each developmental stage were fixed in 10% neutral buffered formalin (Australian Biostain, VIC) at 18° to 22°C followed by examination using radiographs; CT scans and histology using the same protocols as for Part One of this study.

3.3.2.2 Direct measurement

Teeth from four sheep jaws at each age were extracted after radiography and CT scans, and from one jaw without radiographs or CT scan. Because most of the evaluated teeth were immature and their apical diameters were bigger than endodontic file sizes, digital callipers were used to measure root lengths, root wall thicknesses and apical diameters (**Figure 3.12**). Apical foramen size was calculated by subtracting the wall thickness form the apical diameter rather than measured directly.

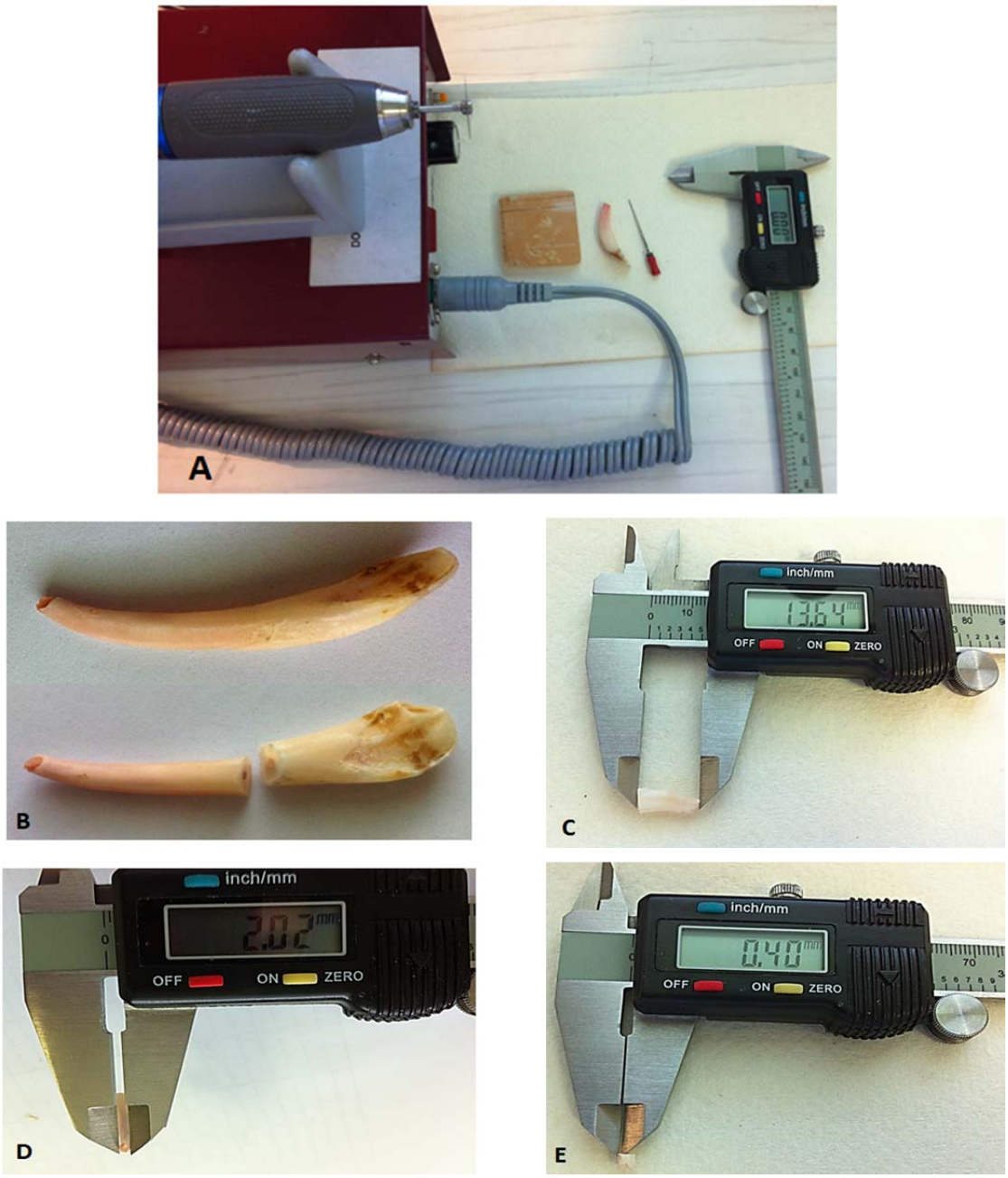


Figure 3.12 Direct measurement methods.

A: Experiments setting.

B: tooth sectioning.

C: Measuring root length.

D: Measuring root apical diameter.

E: Measuring apical third root thickness.

3.3.3 Results

3.3.3.1 Two-tooth age

3.3.3.1.1 Direct measurements

Direct measurement of sheep permanent first incisors at two-tooth age showed that the teeth have relatively thin root walls (**Table 3-5**).

3.3.3.1.2 Radiographic measurements

Radiographic measurements of sheep permanent incisors at the two-tooth age gave similar results as the direct measurements for the root thicknesses, while root length and apical diameter were slightly less (**Table 3-5**).

3.3.3.1.3 CT scan measurements

CT scans measurements were similar to the direct measurements. All measurements showed that permanent first incisors at this age have very thin dentine walls and wide apices (**Table 3-5)**.

1st Incisor Data Collection Methods	Average Root Length (mm) ± SD	Average Root Thickness/BL (mm) ± SD	Average Root Thickness/MD (mm) ± SD	Average Apex Diameter/BL (mm) ± SD	Average Apex Diameter/MD (mm) ± SD
Direct N = 9	15.62 ± 0.95	-	0.57 ± 0.09	-	2.22 ± 0.28
Radiograph N = 26	15.01 ± 0.85	-	0.57 ± 0.10	-	1.99 ± 0.40
CT Scans N = 20	15.39 ± 1.01	0.62 ± 0.08	0.60 ± 0.07	2.75 ± 0.35	2.22 ± 0.22

Table 3-5 Measurements of permanent first incisors of two-tooth age sheep.

3.3.3.1.4 Evaluation methods reliability at two-tooth age

Comparison by repeated measurements ANOVA between direct, radiograph and CT measurements of four jaws at two-tooth age showed no significant differences between all measurements (**Figure 3.13**).

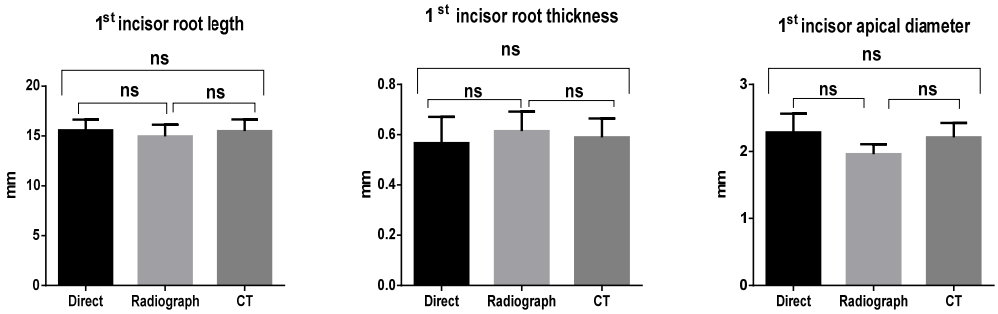


Figure 3.13 Comparison between incisor measurements

Root lengths, root thicknesses, apical diameters of sheep first incisors of two-tooth age measured using direct, radiograph and CT measurements. Results shown are means ± SD. $p < 0.05$.

3.3.3.1.5 Histology results

Histological examination of permanent first incisors showed a fibrovascular pulp tissue and thick dentine walls coronally. The apical third of the root was at an early stage of development, having very thin dentine walls lined by a continuous layer of columnar cells and an open apex (**Figure 3.14**, **Figure 3.15**).

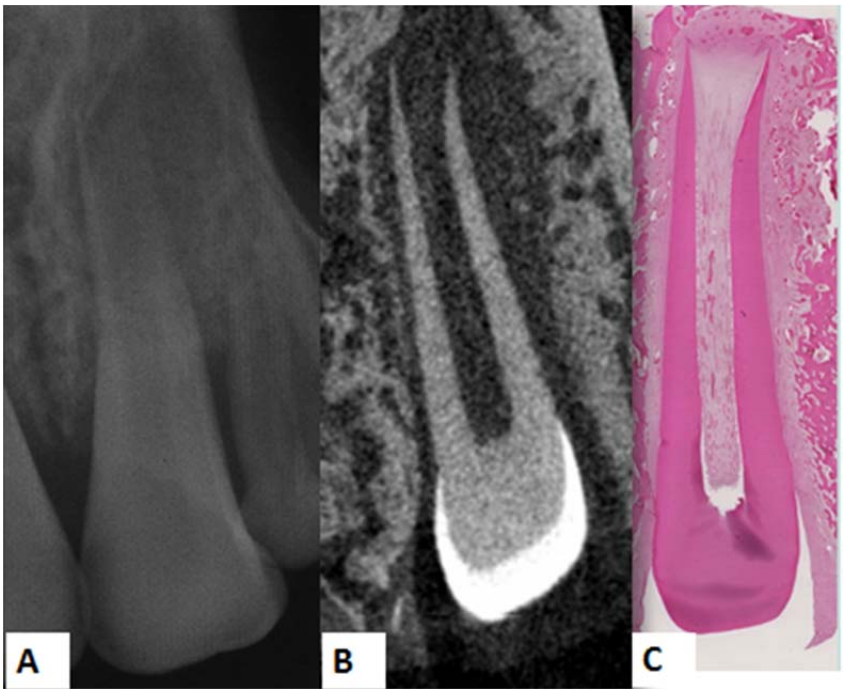


Figure 3.14 Two-tooth sheep.

A: Radiograph.

B: CT section.

C: Histology section.

Images showing wide canals, thin apical third root walls and open apices of incisor teeth.

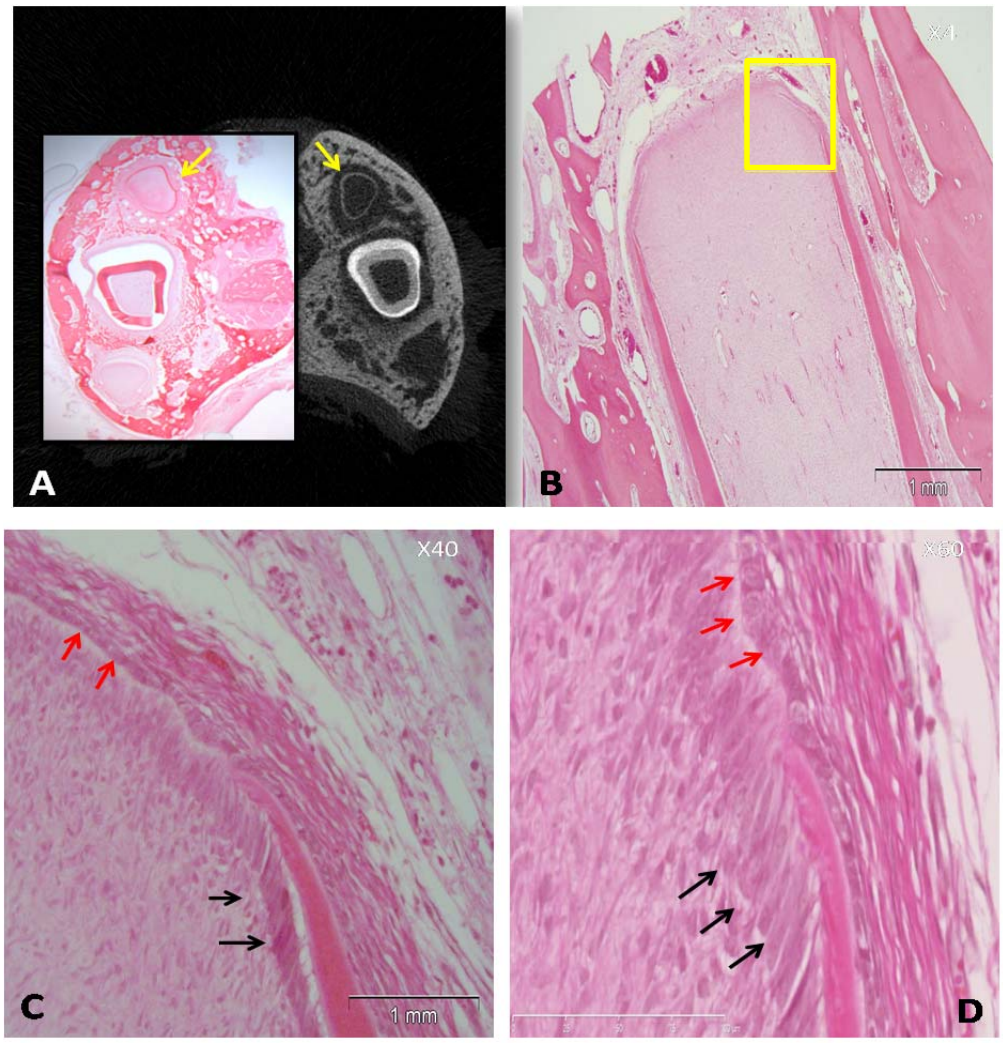


Figure 3.15 Two-tooth age.

A: Tranaxial section left histology and right CT, yellow arrows indicate first incisor apices.

B: Histology section showing apical third of the root with fibrovascular pulp tissue, odontoblast layer lining the wall and epithelial diaphragm shaping the pex.

C and **D**: Magnified images of area indicates by yellow rectangle in image (B), black arrows indicate Odontoblasts, red arrows indicate epithelial diaphragm.

3.3.3.2 Four-Tooth age

3.3.3.2.1 Direct measurements

Direct measurements of sheep first incisor teeth at four-tooth age showed longer roots, thicker root walls and narrower apices than at the previous age. Second incisors at this age had short roots, thin root walls and open apices (**Table 3-6**).

3.3.3.2.2 Radiographic measurements

First and second incisors measured shorter on radiograph than the direct measurements, and apical diameters were narrower on radiograph, while radiographic root thickness measurements were larger by than direct measurements (**Table 3-6)**.

Methods	Teeth	Average Root Length (mm) ± SD	Average Root thickness/BL (mm) ± SD	Average Root thickness/MD (mm) ± SD	Average Apex Diameter/BL (mm) ± SD	Average Apex Diameter/MD (mm) ± SD
Direct	1st Incisor N= 8	16.37 ± 1.46	-	0.84 ± 0.19	-	0.91 ± 0.47
	2nd Incisor N= 8	13.25 ± 0.70	-	0.49 ± 0.12	-	1.96 ± 0.34
Radiograph	1st Incisor N=20	16.13 ± 0.98	-	0.94 ± 0.11	-	0.74 ± 0.23
	2nd Incisor N=20	12.92 ± 0.89	-	0.54 ± 0.12	-	1.57 ± 0.41
CT scans	1st Incisor N=20	16.32 ± 1.09	0.96 ± 0.14	0.90 ± 0.15	1.35 ± 0.36	0.83 ± 0.36
	2nd Incisor N=20	13.32 ± 0.89	0.59 ± 0.22	0.50 ± 0.11	2.54 ± 0.49	1.79 ± 0.40

Table 3-6 Incisor teeth measurements of four-tooth sheep.

3.3.3.2.3 CT scans measurements

CT scans measurements of first and second incisors were similar to the direct measurements. The measurements of first incisors at the four-tooth age showed further changes in all the root diameters than at two-tooth age, as shown in **Table 3-6,** and the major changes were seen in the root thickness and apex diameters (**Table 3-3-7** and **Figure 3.16**). The apices of the first teeth were narrower than the previous age; however, six out of the twenty evaluated first incisors still had wide apices with a mesio-distal diameter greater than 0.9 mm.

Teeth	% Change Average Root Length	% Change Average Dentine thickness/BL	% Change Average Dentine thickness/MD	% Change Average Apex Diameter/BL	% Change Average Apex Diameter/MD
1st Incisor	6 %	36 %	33 %	51 %	63 %

Table 3-3-7 Approximate changes in first incisors CT measurements between two-tooth and four-tooth age sheep.

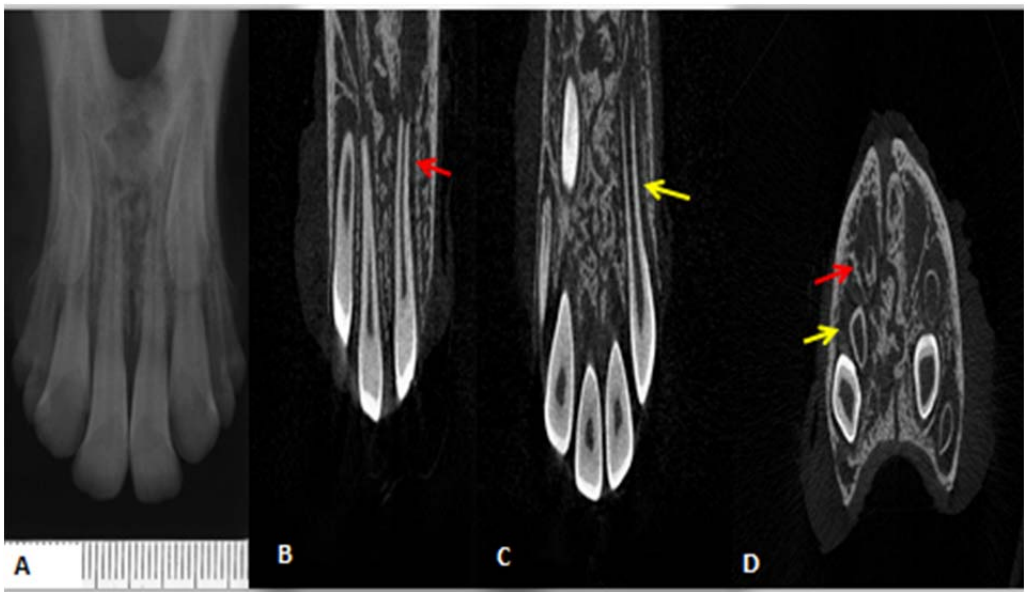


Figure 3.16 Four-tooth age sheep.

A: Radiograph showing first incisors with thick walls and narrow apices comparing to the second incisors with thin walls and wide open apices.

B: and **C**: CT scan coronal sections showing first incisor and second incisors.

D: Transaxial section showing teeth apices.

CT images showing first incisor with thin walls and wide open apex and second incisor apex is still wide and at developing stage. Minor foramen has not developed yet.

Red arrows indicate first incisor, yellow arrows indicate second incisors.

3.3.3.2.4 Evaluation methods reliability at four-tooth age

Comparison between direct, radiograph and CT scans measurements of incisor teeth from four mandibles (four-tooth age) showed no significant differences between all the measurements of the first incisors. The same results were found for the second incisors (**Figure 3.17**).

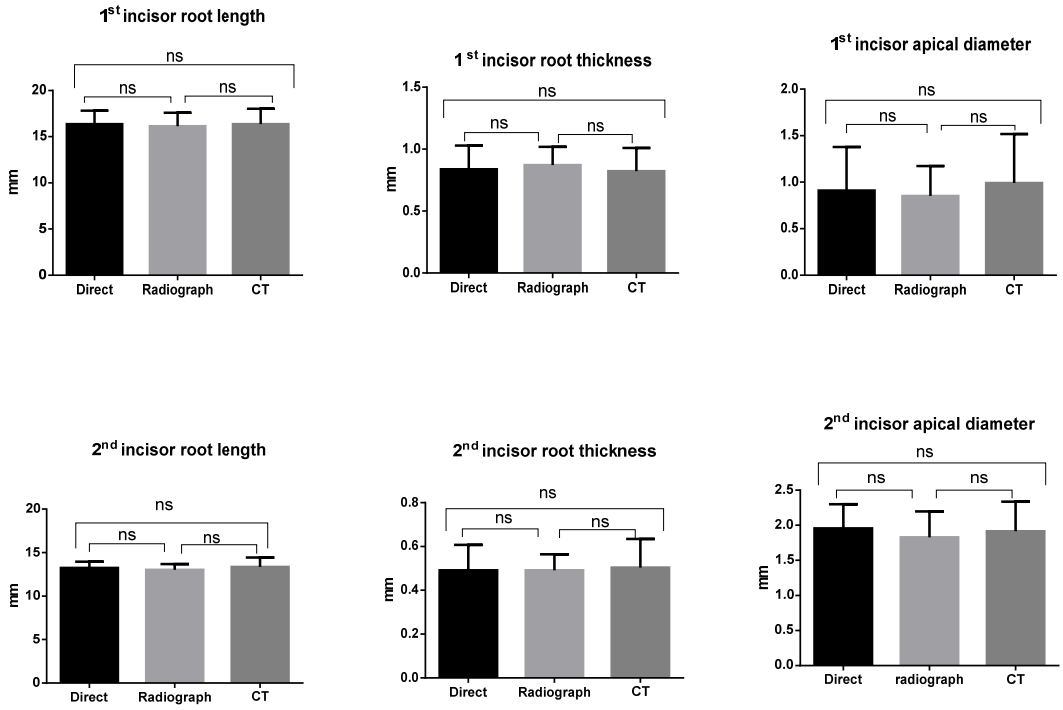


Figure 3.17 Comparison between incisor teeth measurements of four-tooth age.

Measurements presented are direct, radiograph and CT scan measurements of sheep incisor root lengths, root thicknesses and apical diameters of four-tooth age. Results shown are means ± SD. $p < 0.05$.

3.3.3.2.5 Histology results

The histology of first incisor teeth showed that the pulp had matured further, with thicker dentinal walls and narrower apex compared to the previous age. Second incisors compared to the first incisors, had wide canals, thin walls and open apices (**Figure 3.18**).

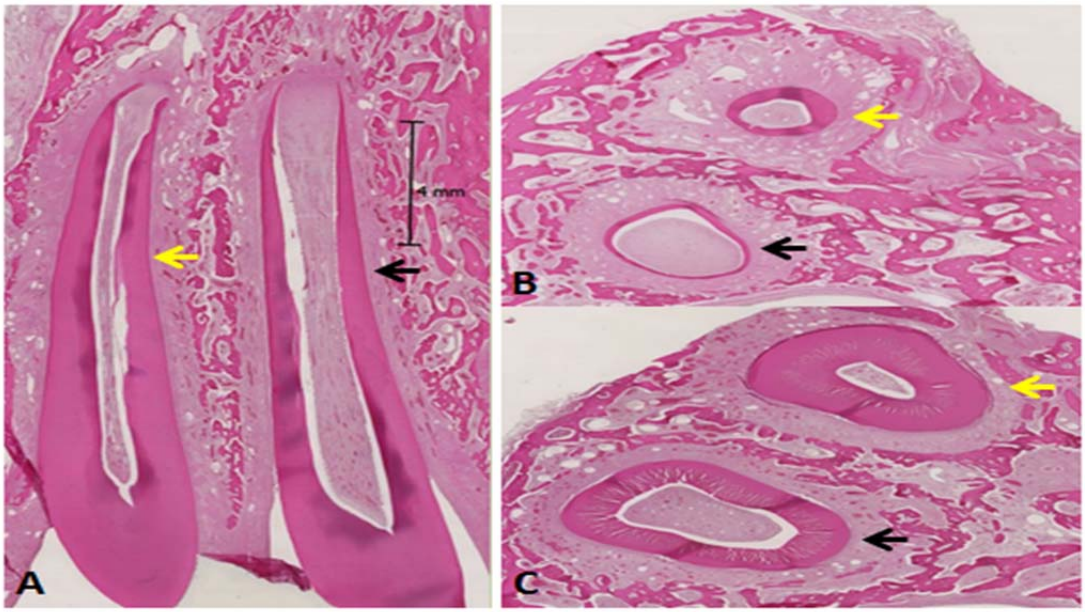


Figure 3.18 Four-tooth age histology sections.

A: Coronal section.

B: Transaxial section showing teeth apices.

C: Transaxial section at the root coronal third.

All the sections showing first incisor with thick walls and developing apex. Second incisor has thin apically walls and very wide open apex.

Yellow arrows indicate first incisor, black arrows indicate second incisors.

3.3.3.3 Six-tooth age

3.3.3.3.1 Direct measurements

Direct measurement results of first and second incisor teeth of six-tooth age showed longer roots, thicker apical root walls and narrower apices than at the previous ages. Third incisors at this age had short roots, thin apical root walls and open apices, as shown in **Table 3-8**.

3.3.3.3.2 Radiographic measurements

Radiographic results of incisor teeth at six-tooth age were similar to the direct measurements except for the lengths of the first and the third incisors being lower on radiographic measurement (**Table 3-8**).

3.3.3.3.3 CT scan measurements

CT scan measurements of all incisors were similar to the direct measurement, except for the first incisor root lengths, which were less than the direct measurements, as shown in **Table 3-8**.

Methods	Teeth	Average Root Length (mm) ± SD	Average Root thickness/BL (mm) ± SD	Average Root thickness/MD (mm) ± SD	Average Apex Diameter/BL (mm) ± SD	Average Apex Diameter/MD (mm) ± SD
Direct	1st Incisor N= 8	17.88 ± 1.10	-	1.02 ± 0.05	-	0.58 ± 0.25
	2nd Incisor N= 8	15.59 ± 1.24	-	0.75 ± 0.12	-	1.00 ± 0.35
	3rd Incisor N= 8	13.38 ± 1.34		0.57 ± 0.16		1.54 ± 0.32
Radiograph	1st Incisor N=20	16.83 ± 1.27	-	1.03 ± 0.15	-	0.6 ± 0.10
	2nd Incisor N=20	15.30 ± 1.24	-	0.75 ± 0.13	-	0.94 ± 0.16
	3rd Incisor N=20	12.84 ± 0.99		0.53 ± 0.14		1.43 ± 0.19
CT scans	1st Incisor N=20	16.80 ± 1.43	0.103 ± 0.06	0.94 ± 0.07	1.05 ± 0.32	0.61 ± 0.16
	2nd Incisor N=20	15.76 ± 1.34	0.76 ± 0.16	0.71 ± 0.11	1.62 ± 0.48	0.97 ± 0.26
	3rd Incisor N=20	13.02 ± 1.71	0.52 ± 0.12	0.50 ± 0.09	2.15 ± 0.5	1.52 ± 0.3

Table 3-8 Measurements of incisor teeth at six-tooth age.

CT scan measurements of first and second incisors showed changes in root length and apical root thickness at this age compared to the previous dental age. The most obvious changes were seen in the apical diameter (**Table 3-9**), with about half of the evaluated second incisors still having a mesio-distal apical diameter greater than 1 mm.

Teeth	% Change Average Root Length	% Change Average Dentine thickness/BL	% Change Average Dentine thickness/MD	% Change Average Apex Diameter/BL	% Change Average Apex Diameter/MD
1st Incisor	3 %	7 %	4 %	22 %	26 %
2nd Incisor	15 %	23 %	29 %	36 %	40 %

Table 3-9 Changes in CT scan measurements of incisor teeth between four-tooth and six-tooth age

3.3.3.3.4 Evaluation methods reliability at six-tooth age

Comparison between direct, radiographic and CT scan measurements of the teeth from four jaws (six-tooth age) showed no significant differences between all the measurements of the first, the second and the third incisors, as shown in **Figure 3.19.**

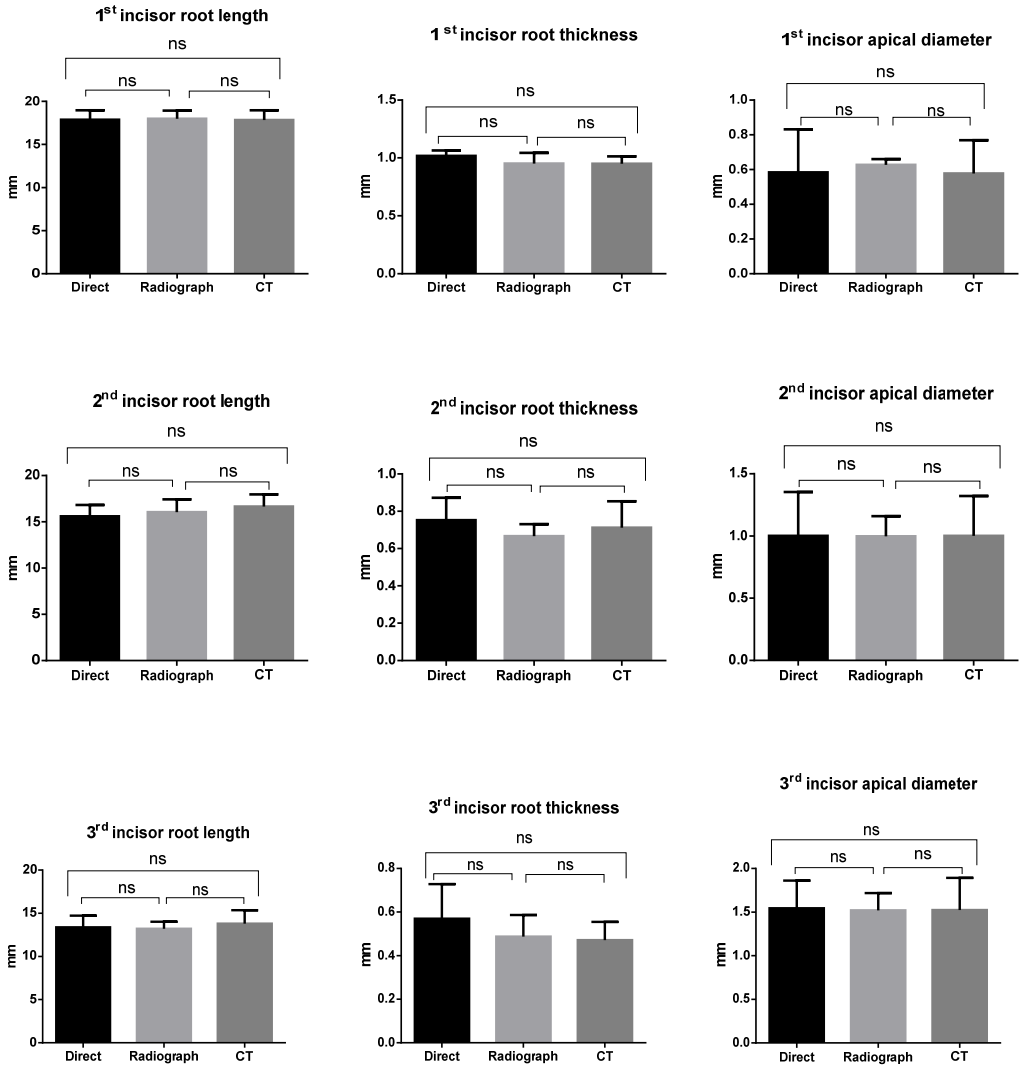


Figure 3.19 Comparison between incisor measurements of six-tooth age.

Measurements presented are direct, radiograph and CT measurements of sheep incisor root lengths, root thicknesses of six-tooth age. Results shown are means ± SD. $p < 0.05$.

3.3.3.3.5 Histology results

The histology of sheep teeth at this age compared to previous ages revealed more maturation of first incisors, narrower apices, thicker dentine walls and the initial development of the apical dilatation and minor foramen by deposition of cementum (**Figure 3.20** and **Figure 3.21**).

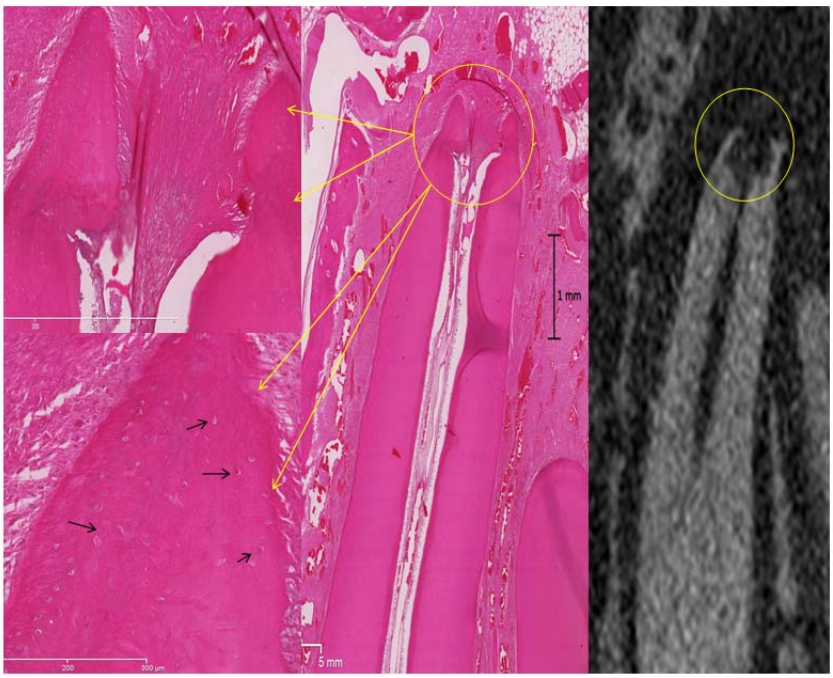


Figure 3.20 Six-tooth sheep model first incisor.

CT scan (right side image), histology coronal section (middle image), high magnification images of area indicated by the yellow circle (left side images) showing a developing root tip by deposition of cementum. Black arrows indicate cementocytes.

The second incisors had thicker root walls, thinner canal spaces and narrower major foramina than at the previous ages. Intermediate dilatations and minor foramina had not developed at this stage. The third incisors had open apices, thin apical root walls and wide pulp canals (**Figure 3.21** and **Figure 3.22**).

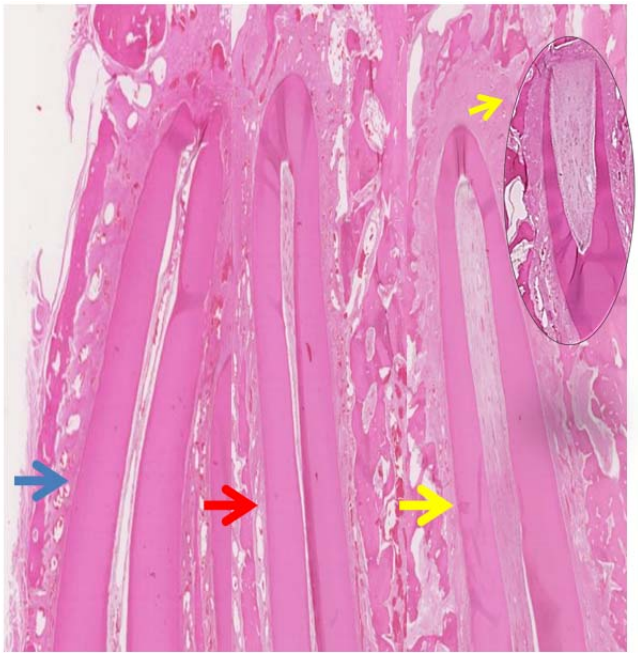


Figure 3.21 Six-tooth age histology sections.

Image showing incisor root canals and apices. Third incisor has thinner walls, wider and open apex comparing to the first and second incisors. Blue arrow indicates first incisor, red arrow indicates second incisor and yellow arrows indicate third incisor canal and apex (oval image).

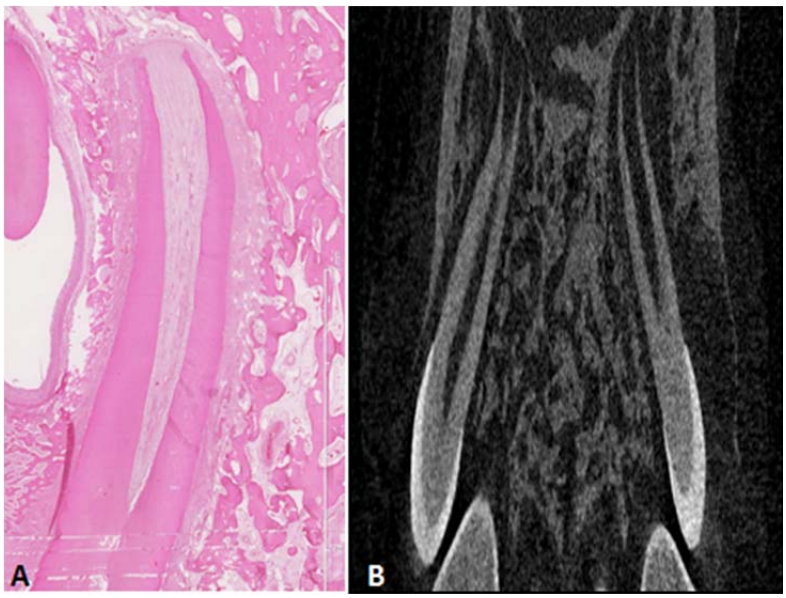


Figure 3.22 Sheep third incisor tooth.

A: Histology sections.

B: CT scan section of third incisor showing a wide pulp canal, thin dentine walls apically, and a wide apex.

3.3.3.4 Incisor teeth CT measurement changes from eruption to maturation age

Comparison between CT scan measurements of incisor teeth from the eruption to the maturation age showed changes in all the diameters. The major changes were seen in the apical diameters and the apical root wall thicknesses, as shown in **Table 3-10**.

Tooth	% Change Average Root Length	% Change Average Root Wall Thickness/BL	% Change Average Root Wall Thickness/MD	% Change Average Apex Diameter/BL	% Change Average Apex Diameter/MD
1st Incisor	9 %	52 %	50 %	72 %	82 %
2nd Incisor	16 %	48 %	51 %	69 %	75 %
3rd Incisor	9 %	50 %	50 %	57 %	71 %

Table 3-10 CT scan measurement changes of first, second and third incisor from the eruption to the maturation ages. Mature age CT scan measurements were from Part One (Table 3-3-1)

3.3.4 Discussion

According to the findings in Part One, the size and the anatomy of sheep incisor teeth are comparable to human anterior teeth and show promise as being suitable for endodontic investigations including endodontic revitalisation and regeneration.

The age of the sheep is usually estimated from the number of erupted permanent anterior teeth (376) but in order to use sheep for endodontic research information is required on the stages of root development, the suitability and limitations at each developmental stage.

On the basis of the findings, the permanent first incisor teeth of two-tooth age sheep have open apices and thin canal walls and would thus be suitable for endodontic revitalisation research. It is easy to radiograph the right and left incisors in one occlusal film, in order to estimate the root length, apical root wall thickness and apical diameter. An exception to this occurs when the crown of the second incisor covers the apex of the first incisor. At this stage it is sometimes difficult to obtain precise measurements. All CT scan sections (coronal, sagittal and transaxial) can be used to measure the diameters of the teeth but in the present study only transaxial sections were used for the purpose of standardisation. Because incisor roots are straight at this age, the histological investigation was relatively easy. Both coronal and sagittal sections are easy to obtain and can give all the necessary information about the root from the cemento-enamel junction area to the apex.

Sheep at this age are small and readily available from farms. The only limitation with this model is that each sheep supplies only two teeth. However, permanent first incisors of the sheep at this dental age have very wide apices and very thin walls which will change by 82% and 50% respectively (**Table 3-10)** as the animal reaches maturation. The large expected changes means only a reasonably small sample size is required to give statistical power to a study.

Four-tooth age sheep were slightly harder to find. Intraoral radiographic assessment of first incisor apical third root wall thickness and apex diameter was sometimes difficult to measure due to the thick mandible bone. The apical diameter is narrow and in older sheep apical third curves disto-lingually. Furthermore, the crown of the third incisor can cover the apex of the second incisor; making it hard to measure the diameters of the second incisor apices with radiographs. A specific angulation was required and each tooth then needs to be radiographed separately, which would take additional time during surgery. As the curvature of the root increases, the transaxial CT scan becomes the most precise way to

measure root length, apical root wall thickness and apex diameter. At this age the apex diameters of the first incisors could be over 0.9 mm, giving the opportunity to use all the four incisors for endodontic revitalisation research within the same animal.

Animals at six-tooth age are less readily available and the size of the animal is considerably bigger than the four-tooth age, making management in theatre more difficult. The limitations with radiographs and CT scans are similar to the four-tooth age model. Furthermore, the mandible of the sheep at this age is more convex, and in some cases the first incisor roots curve disto-lingually. The third incisor teeth are near the corner of the mandible, making histology sectioning harder. The advantage of using this age is that in addition to the third incisors, second incisor teeth can be used for regeneration research since their apex diameters could be 1 mm. In some endodontic regeneration or revitalisation research on dog teeth, the apical foramens of mature teeth were enlarged to simulate teeth with open apices (137, 392). If a similar protocol was applied, it might be possible to use all the six permanent incisors in the same animal.

In all the evaluated ages the results showed no significant differences between the measurements collected using direct, radiographic and CT scans methods of first, second and third incisors. Therefore, radiographs and CT scans are both reliable methods for data collection using the settings presented in the method section.

By reviewing the three immature ages and the mature age (full mouth), it is obvious that the tooth maturation occurs within approximately 6 - 12 months after eruption. During this period, no major change occurs in the root length (**Table 3-10**), but changes do occur in the apical root walls and apical area. Apical area development is finished by closure of major foramen which is accompanied by development of an intermediate dilatation and the minor foramen. The distance from the major foramen to the root apex is approximately 0.5 mm to 1 mm. In human maxillary incisors, there is between 0.2 - 3.8 mm distance from the apical

foramen to the tooth apex, which is covered by cementum (387, 388). Thurly (378) reported that in some cases sheep roots can elongate by 1 or 2 mm, possibly to compensate for tooth wear. Similarly in human teeth, secondary cementum deposition can occur on the root apices due to crown wear (393), trauma to the PDL (394, 395) and as a response to tooth functional needs (69). Thus, despite the apical morphological differences between human and sheep teeth, it seems that cementum deposition occurs in a similar way in response to functional demanded.

3.3.5 Conclusion

On the basis of the need for a reliable animal model to investigate various aspects of endodontic revitalisation protocols, and the findings from the current investigations, sheep appear to be a promising and reliable model. In this study, three sheep developmental dental ages were assessed. Each one of these ages can be used to evaluate various issues related to endodontic revitalisation treatment. The two-tooth age showed the most favourable criteria making this age the most suitable for *in vivo* endodontic revitalisation research.

Chapter 4. A Study of an Endodontic Revitalisation Protocol in a Sheep Model

4.1 Introduction

An endodontic revitalisation protocol involves disinfection of the infected root canal system followed by inducing bleeding into the canal through over-instrumentation (16, 396). This approach is technically simple, and does not required special equipment or preparation. Several case reports have demonstrated successful outcomes described as: increased in root length, thickening of canal walls and decreased size of the apex (16, 286, 397, 398). However, the nature of the healing tissue (whether it is odontogenic tissue or a repair tissue), as well as the long-term clinical outcome of the method, still need further investigation *in vivo*. Furthermore, in order to improve the quality and predictability of the healing tissue, additional development of the root canal cleaning protocols and optimisation of scaffolds is required before revitalisation can be confidently recommended as a routine clinical procedure.

Research of this type has used traditional laboratory animals such as rats and ferrets (318, 350). While these animals are preferred for reasons of familiarity and cost, the differences in teeth size and anatomy to humans, make them unsuitable models for long-term follow up revitalisation research.

Sheep have been used in many dental studies (367, 371, 399) but no previous studies have investigated their suitability for endodontic revitalisation research. In the previous Chapter 3, we showed that sheep dental anatomy is favourable for endodontic revitalisation research, with each dental age having advantages and disadvantages. For the study described in this chapter, sheep at "two-tooth" age were chosen as the most appropriate because of their ample supply, appropriate growth rate, and that the first incisors can easily be imaged without superimposition of other tissues or image distortion.

4.2 Aims

- Examine the response of immature sheep teeth to commonly used endodontic revitalisation protocol.
- Histologically examine of post-treatment revitalised tissues.

4.3 Materials and methods

4.3.1 Animals

Four healthy male Merino sheep at two-tooth age were used for this study. The animals were obtained from and cared for in the Large Animal Research and Imaging Facility (LARIF) maintained by South Australia Health and Medical Research Institute (SAMRI). Animal ethics for the research was granted by the University of Adelaide, South Australia/Animal Ethics Committee #M-2012-199 and by South Australia Pathology/Animal Ethics Committee # 71/13.

4.3.2 Surgical procedures

All surgical procedures were performed under general anaesthesia induced with thiopentone (10 - 15 mg/Kg) administered intravenously and maintained with 2.5% isoflurane in 4 L of O_2 through tracheal intubation during imaging and treatment.

4.3.2.1 Imaging

Directly after anaesthesia, orthodontic round bases were cemented with GIC (GC Fuji VII, USA) on the labial surface of both experimental and control teeth to act as fixed reference points for all radiographs taken (**Figure 4.1)**.

For each sheep, the right first incisor was the experimental tooth and the left first incisor tooth was left intact as a control.

Pre-operative radiographs were taken to confirm the presence of open apices (apical diameter > 1 mm), using a veterinary handheld intraoral x-ray machine (Nomad Pro, Aribex, USA) with exposure setting of 60 kV, 2.5 mA for 0.16 second using ultra-speed occlusal films (Insight Kodak, Rochester, NY). The film packet was placed on the occlusal surface of the teeth. A film holder designed especially for this study was used to ensure that the x-ray tube-head, film and teeth were at the same position throughout the study. The images were digitised using an optical scanner (Epson, Australia), with a resolution of 1200 dpi and 16 bit greyscale image type. These images were loaded to Image J software. TurboReg plug-in (Biomedical Imaging Group, Swiss Federal Institute of Technology, Switzerland) was used to mathematically reduce any dimensional changes in the preoperative and postoperative radiographs as a result of differences in angulation of the x-ray central beam at the time of image acquisition. Root length, root wall thickness and apical diameter were measured using IMAGE J measuring tools as shown in **Figure 4.2**. Root length was measured as a straight line from the cemento-enamel junction (CEJ) to the radiographic apex of the tooth. Dentinal wall thickness was measured at a level 3 mm from apex. Root width and pulp canal width were measured at the same level and the dentine thickness was calculated as following:

Dentine thickness = (root width - canal width) / 2

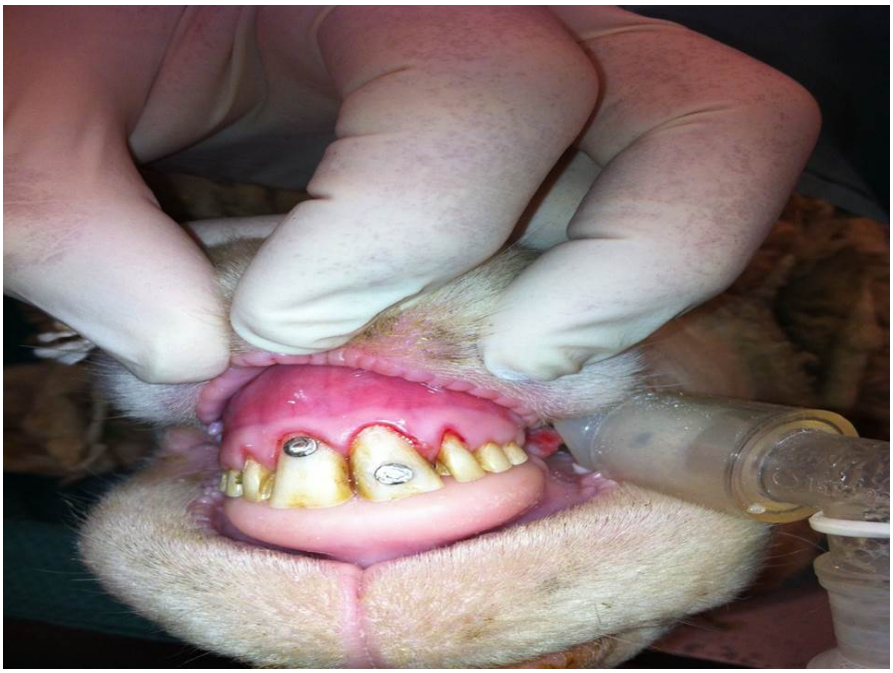


Figure 4.1 Orthodontic round bases on treatment and control teeth as reference points for radiographs.

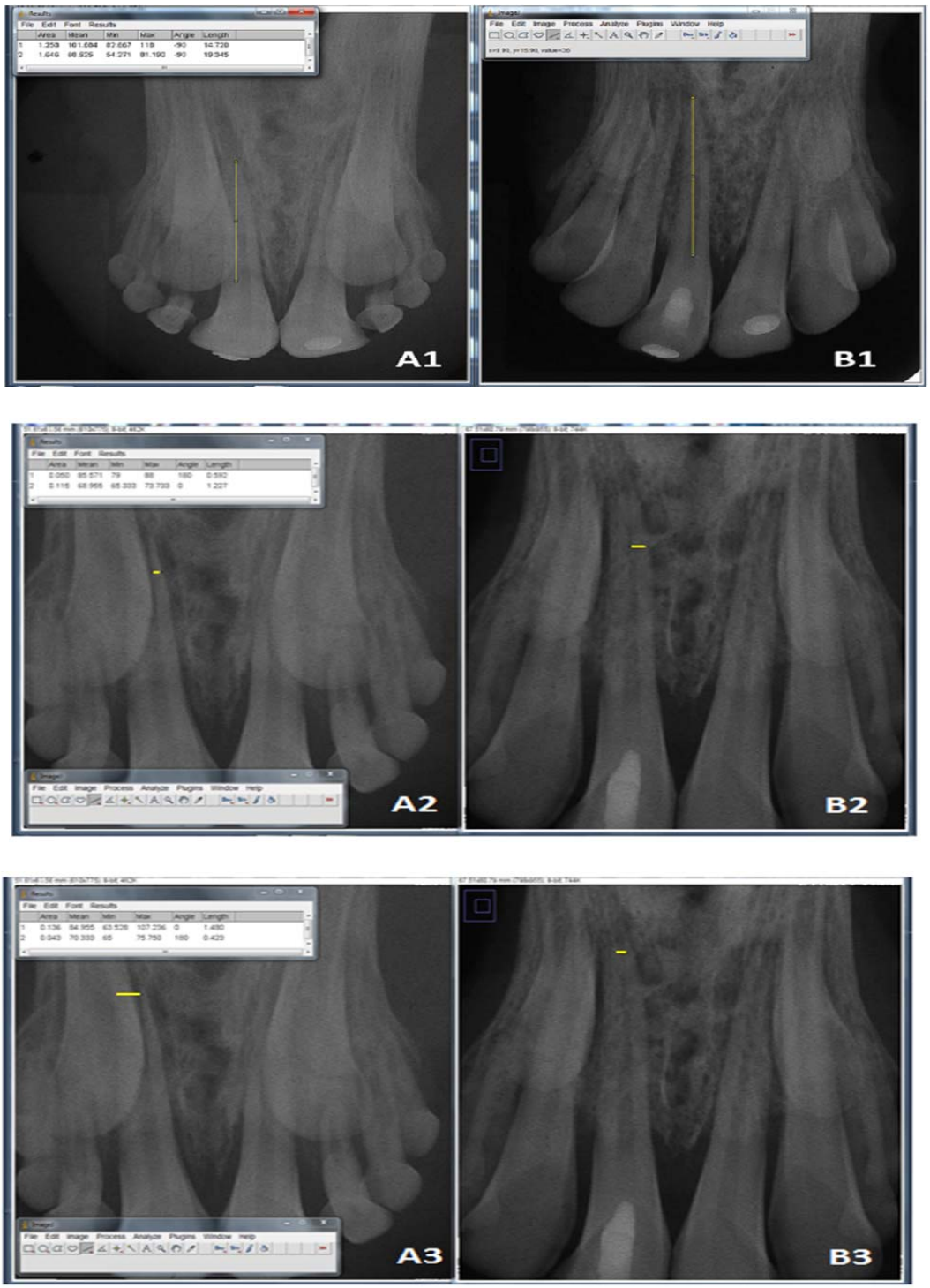


Figure 4.2 Measurement the root diameters using IMAGE J

A: Images before treatment.

B: Images after treatment.

A1 and B1: Measuring root length.

A2 and B2: Measuring wall thickness.

A3 and B3: Measuring apex diameter.

4.3.2.2 Treatment Protocol

Experimental animals were subjected to a treatment protocol comprising four sessions as illustrated in **Figure 4.3**. After each session, animals were given analgesics (50 mg/ml Rimadyl IM injection, Pfizer, Australia) and were visually monitored to check for any signs of distress. The sheep had free access to food and water throughout the study.

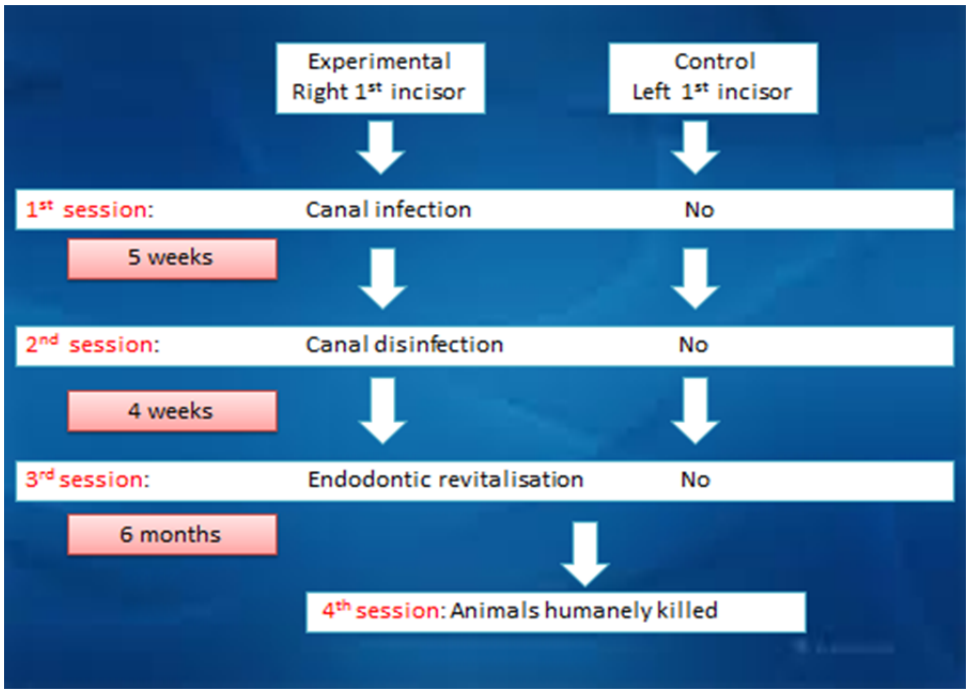


Figure 4.3 Treatment sessions

Session one:

The aim of the first treatment session was to infect the root canal of the experimental teeth. The pulp chamber of each tooth was mechanically exposed with round carbide burs (# 2) and the pulp tissue was disrupted to 12 mm depth with a sterile endodontic K-file (size15 SybronEndo, USA). Size 20 or 25 K-file was introduced to approximately 1 mm shorter than the root length on radiograph to estimate the working length. Supra-gingival plaque (approximately 15 mg) was scaled from sheep teeth and mixed with (2 ml) sterile saline. A sterile cotton pellet soaked in plaque suspension was sealed in the pulp chamber with a coronal seal (Cavit, 3M ESPE, USA).

Session two:

After five weeks, evidence of apical periodontitis was determined by presence of periapical radiolucency on radiograph (**Figure 4.7**) and/or canal infection was confirmed clinically by presence of intra-canal pus (**Figure 4.4**). The infected root canals were chemo-mechanically prepared to remove the smear layer and disinfected with a triple-antibiotic paste as following: after isolation of the experimental tooth with a rubber dam and disinfection of the operative field with 2% chlorhexidine gluconate as shown in **Figure 4.5**, the temporary restoration was removed and the canal were debrided with K-files (sizes 25 and 30). Files sized 35 and 40 were used subsequently with gentle filing movements. During preparation, 1 ml of 5.25% NaOCl (Scharlau, Spain) was used to irrigate the canal after each file size. Following instrumentation, the canal was irrigated with 10 ml 5.25% NaOCl followed by 10 ml saline and 5 ml of 17% EDTA (pH 7.4) (Scharlau, Spain) followed by 10 ml of normal saline prior to drying the canal with paper points as shown in **Figure 4.6**. Triple-antibiotic powder consisting of 20 mg each of metronidazole (400 mg Sanofi-Aventis, Australia), ciprofloxacin (500 mg Aspen Pharma, Australia) and amoxycillin (500 mg Sandoz, Australia) (prepared by Royal Adelaide Hospital, Pharmacy Department, South Australia) was mixed with 1 ml of sterile water and carefully introduce into the canal with a sterile lentulo spiral filler to 1mm below the working length (size 30, SybronEndo, USA). The coronal access was sealed with Cavit and GIC (GC Fuji IX, USA).

This session was repeated for Sheep-3 because there was evidence of persistent infection four weeks after treatment.

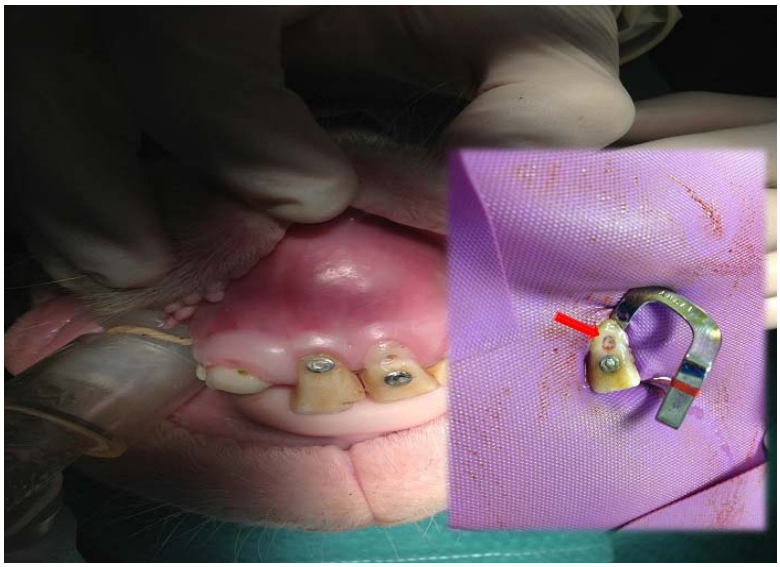


Figure 4.4 Buccal abscess associated with an infected tooth. Red arrow points to pus coming out of infected canal.

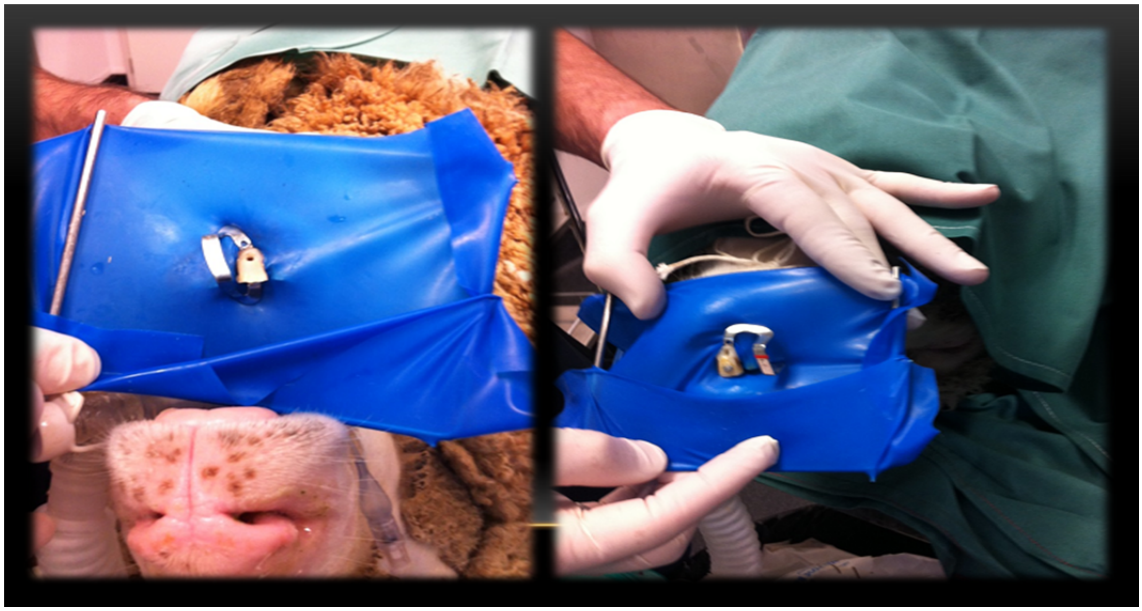


Figure 4.5 Isolation of infected tooth with a rubber dam. Composite ledge built at the crown cervically to prevent dislodgment of rubber dam clamp

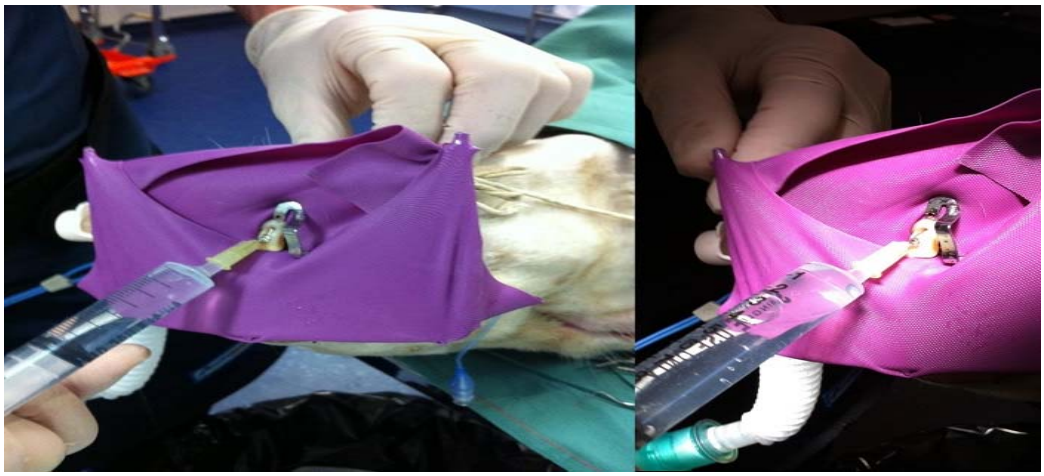


Figure 4.6 Canal cleaning with 5.25% NaOCl, 17% EDTA followed by saline.

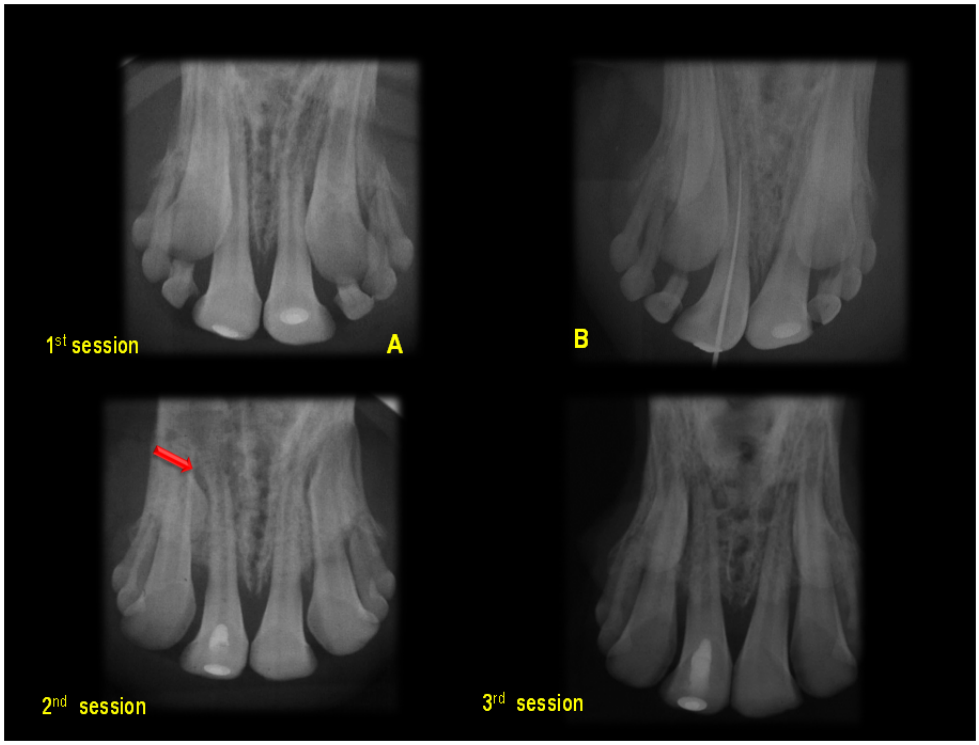


Figure 4.7 Radiographic images of treatment sessions.

First session: Upper row images (A) before treatment, (B) showing working length.

Second session: A periapical lesion associated with the infected tooth (Red arrow).

Third session: Revitalisation treatment.

Session three:

Four weeks after the second session all the infected teeth were re-accessed in an aseptic environment with rubber dam isolation and surface disinfection with 2% chlorhexidine gluconate. The temporary restoration was removed and each canal was irrigated with 10 ml sterile saline prior to drying the canal with paper points. A sterile endodontic file was used to irritate the apical tissue until bleeding occurred apically into the root canal space to create a biological scaffold for the healing process. Over a 15 minute time period, the blood was allowed to clot to a level approximately 3 mm below the CEJ. An absorbable collagen wound dressing (20 mg / canal, Geistlich Bio-Oss collagen, Switzerland) was condensed at the coronal third of the canal over the blood clot to prevent apical penetration of a barrier layer of MTA. (Dentsply, Germany). A moist cotton pellet was placed over the MTA for 15 minutes, before the access cavity was sealed with Cavit and GIC (**Figure 4.7)**.

After the third session all experimental teeth were checked for signs of swelling, mobility or the presence of a sinus tract. Teeth restorations were also checked. All teeth were checked twice a month for the first two months, which then decreased to once a month.

Session four:

Six months after the third session, all the sheep were humanely sacrificed using a lethal dose of 150 mg / kg sodium pentobarbital intravenously. The anterior segments of mandibles were removed from the animals.

4.3.3 Computer Tomography (CT) scanning

High resolution CT images of the specimens (anterior segments of the mandibles) were obtained using protocols already developed during the first phase of this project and

described in section (3.2.2.5). CT Analysis software tools were used to measure root length, root thickness and apex diameters of the experimental and the control teeth.

4.3.4 Tissue Processing

4.3.4.1 Fixation

Specimens were fixed in 10% neutral buffered formalin at 18° to 22°C following the protocol described in section (3.2.2.7).

4.3.4.2 Decalcification

All specimens were decalcified prior to further processing. Specimens were decalcified in laboratory decalcifying solution made of a combination of EDTA and hydrochloric acid (**7.2.1 Appendix**). Radiographs were taken to determine the completion of decalcification.

4.3.4.3 Histology

After decalcification, specimens were washed with water for ten days and the excess tissue was trimmed. Specimens were then processed in a Shandon Citadel 2000 (Industries, Pittsburgh) automatic tissue processor (**7.3 Appendix)** and embedded in Paraffin wax. Serial longitudinal sections, 7 μm thick, were cut in a mesio-distal direction including the apical 3 mm of the root and entire surrounding periapical region as shown in **Figure 4.8.** Sections were mounted on gelatine coated slides and stained with haematoxylin and eosin stain (**7.4 Appendix)**.

Stained slides were scanned and viewed using a slid scanner (NanoZoomer 2.0HT, Hamamatsu, Japan), or a stereomicroscope (Olympus, BX51; Leica, Germany).

The histology part focused on the interpretation of the tissue grown in the canal space after healing. The criteria for histology identification of dentine are the presence of regular or irregular dentinal tubules, cementum are the absence of dentinal tubules, adherence onto dentine and presence of cementocyte-like cells, bone the presence of Haversian canal within uniform distributed osteocyte-like cells, PDL: presence of Sharpey's fiber (21).

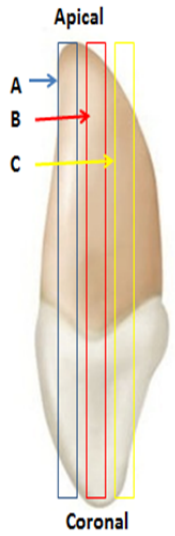


Figure 4.8 Schematic diagram showing regions of the tooth where planes of histological cross sections were taken.

A: anterior sections, B: middle sections and C: posterior sections.

4.4 Statistical Analysis

Statistical comparisons were performed using Student's (Paired) t-test between measurements (radiograph and CT) of experimental and control teeth. Also comparisons were performed for all teeth measurements before and after treatment. GraphPaD Prism was used, and a p value < 0.05 was considered significant.

4.5 Results

4.5.1 Radiographic results

Radiographic results showed significant increases in the root lengths of all the teeth except the experimental tooth in Sheep-4 **(Figure 4.9A)**. The results also showed no significant differences in root lengths between the experimental and the control groups ($p = 0.17$) (**Figure 4.9B)**. The differences in root length prior to and after treatment for the experimental group were not statistically significant ($p = 0.07$) **(Figure 4.9C),** probably because of the variation resulting from the inclusion of Sheep-4's data. The differences in root length at the beginning and the end of the experimental period for the control group were statistically significant (**Figure 4.9D)**. Before and after treatment radiographs of all sheep are shown in **Figure 4.10 - Figure 4.12**.

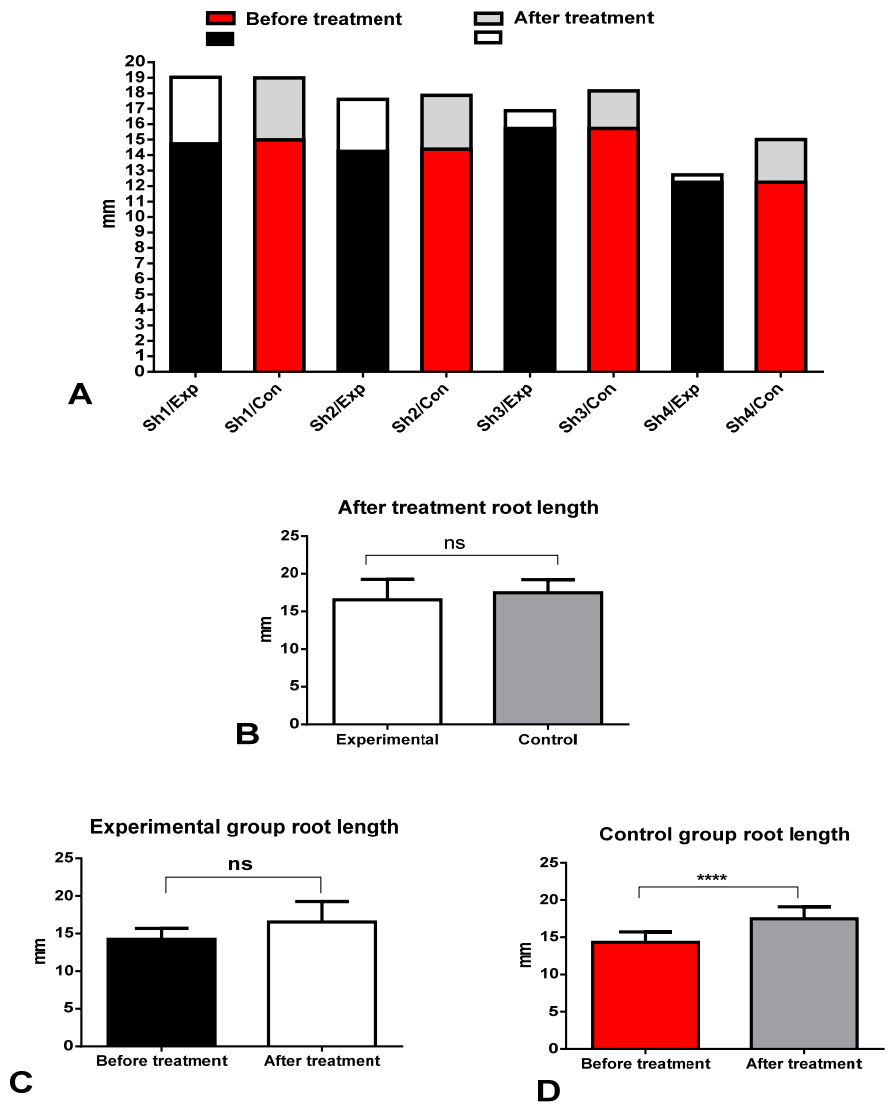


Figure 4.9 Root length measurements from radiograph

A: Root length of each sheep (experimental and control groups) before and after treatment.

B: Root length of experimental and control groups after treatment

C: Root length before and six months after treatment in the experimental group

D: Root length before and after six months in the control group.

Means ± SD of data collected from four animals are shown.

****$p < 0.00001$.

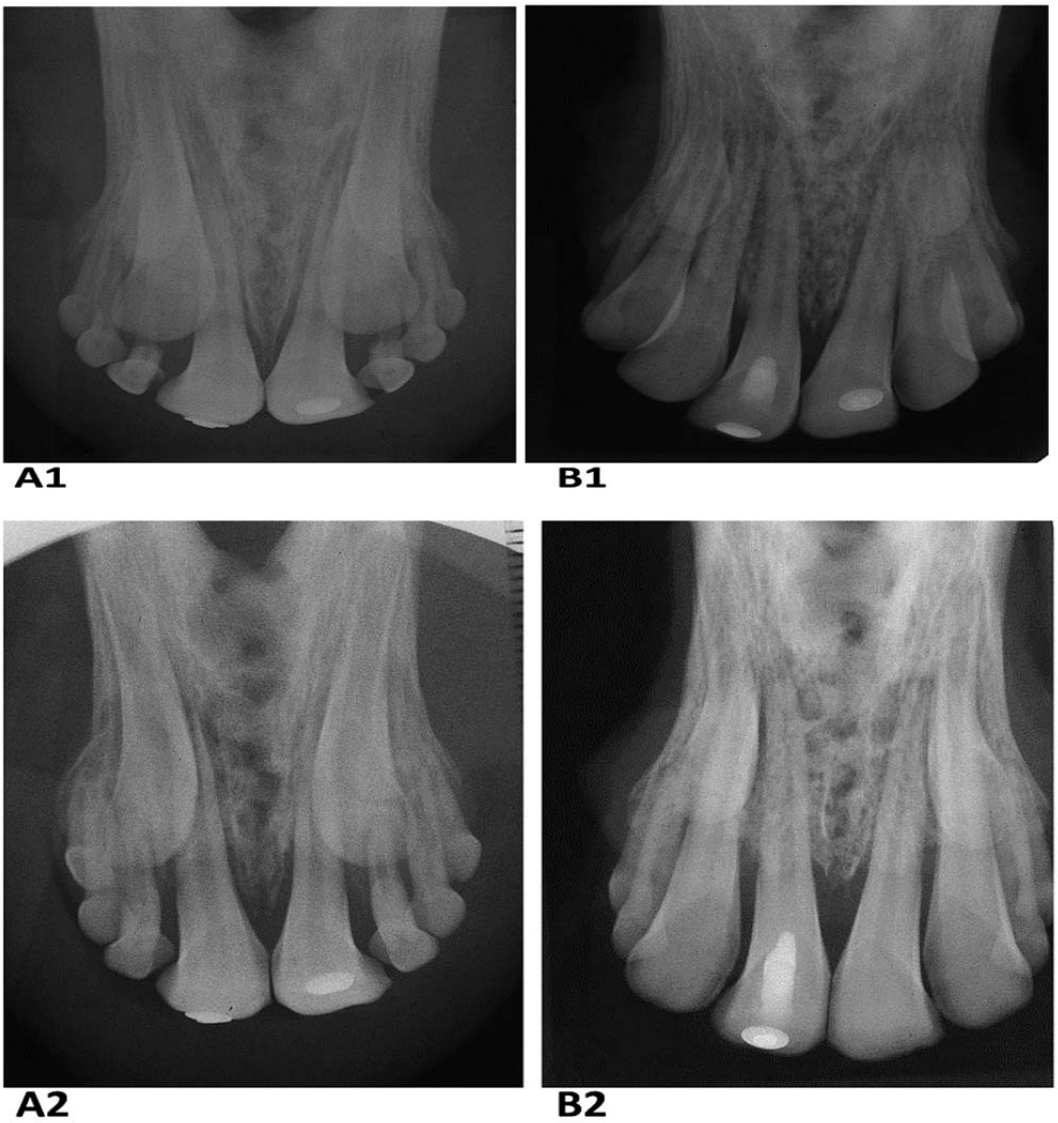


Figure 4.10 Sheep-1 (1) and Sheep-2 (2) radiograph images

A: Before treatment images showing the experimental and control teeth with short root, thin walls and open apices.

B: Fourth session (after treatment) images showing the experimental and control teeth with longer root, thicker walls and narrower apices.

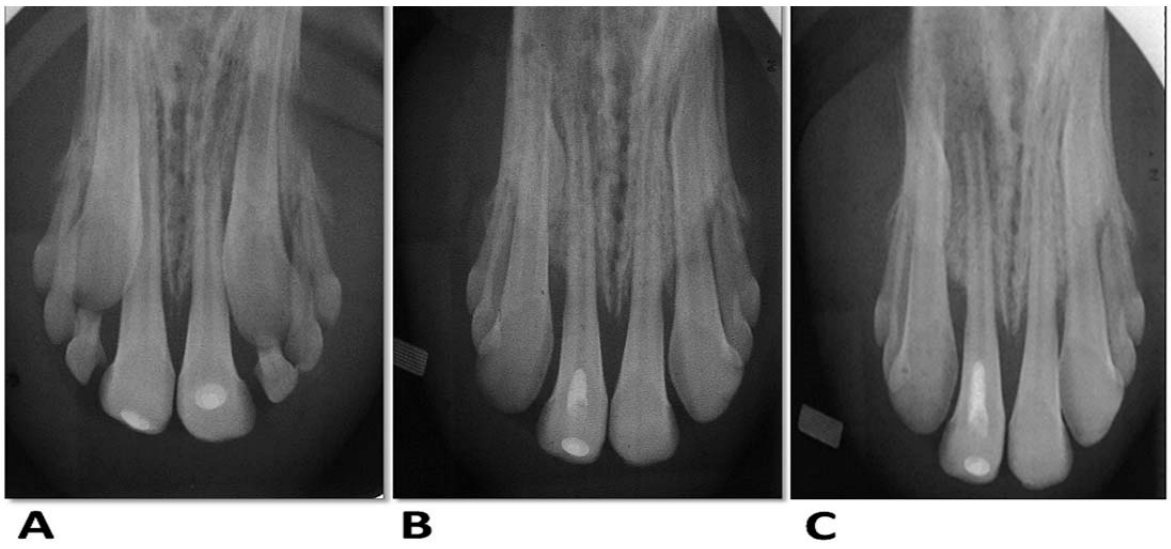


Figure 4.11 Sheep-3 radiograph images.

A: Before treatment images showing the experimental and control teeth with short root, thin walls and open apices.

B: Four weeks after the second disinfection session periapical radiolucency around the apex of the experimental tooth

C: Fourth session image showing the experimental tooth with thicker walls and narrower apex compare to before treatment image.

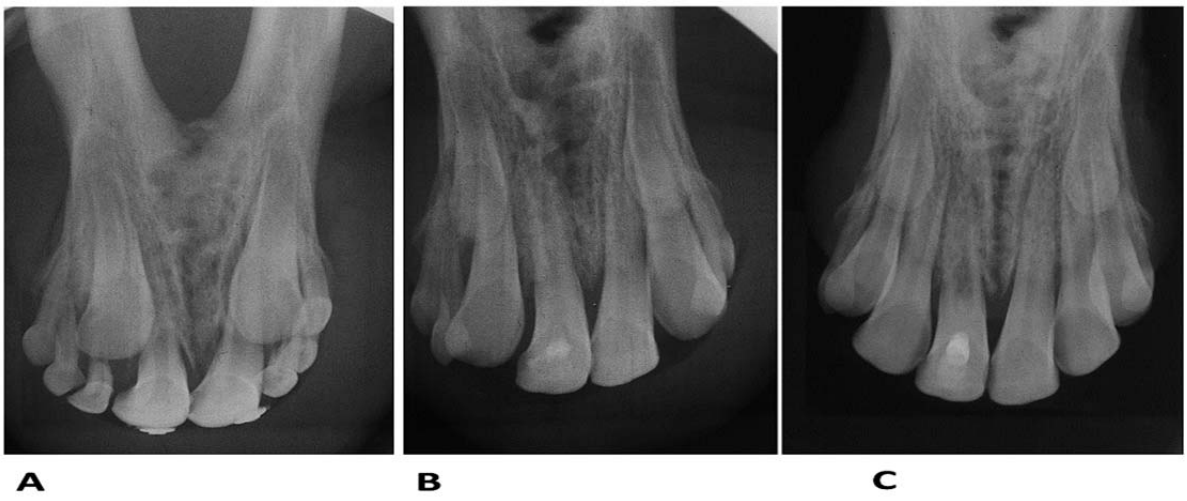


Figure 4.12 Sheep-4 radiograph images.

A: Before treatment images showing the experimental and control teeth with short root, thin walls and open apices

B: Four weeks after disinfection session images showing irregularities of the canal walls of the experimental tooth.

C: Fourth session images showing the experimental teeth with thicker walls with irregular inner border and narrower apex compare to before treatment.

Statistical analysis of dentine thickness measurements showed no significant differences in dentine thickness between experimental and control groups ($p = 0.68$) after treatment. The experimental group showed significant increases in dentine thickness after treatment compared to before treatment. Similar changes were observed in the dentine thickness of control teeth (**Figure 4.13)**.

Both experimental and control groups showed significant reduction in apical diameters at the end of the experiment, compared to their diameters at the beginning of the experiment.

The changes after treatment in root length ranged (4-23%), dentine thickness ranged (26-53%) and apex diameters changes were (38-72%).

There were no significant differences between the groups in apical measurements at the end of the experiment ($p = 0.53$) (**Figure 4.14)**.

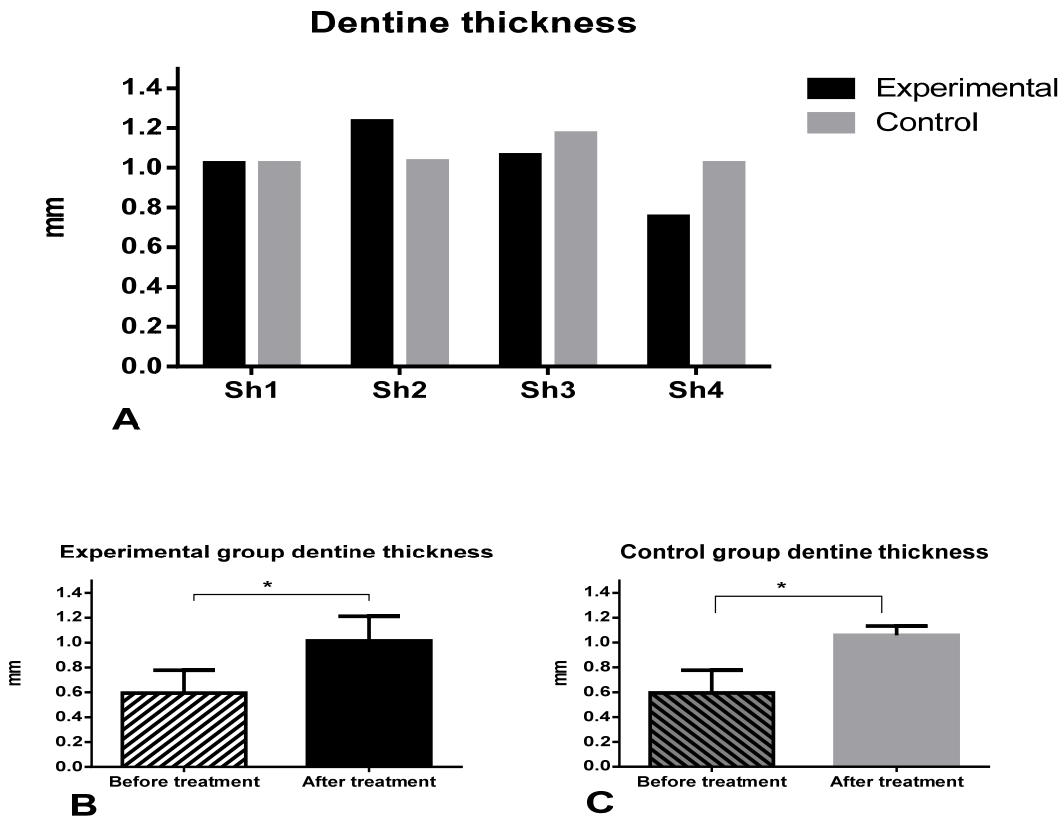


Figure 4.13 Measurements of dentine thickness

A: Dentine thickness of treatment and control groups (six months after treatment).

B: Dentine thickness before and after treatment in the experimental group.

C: Dentine thickness before and after treatment in the control group.

Means ± SD of data collected from four animals are shown. *$p < 0.05$.

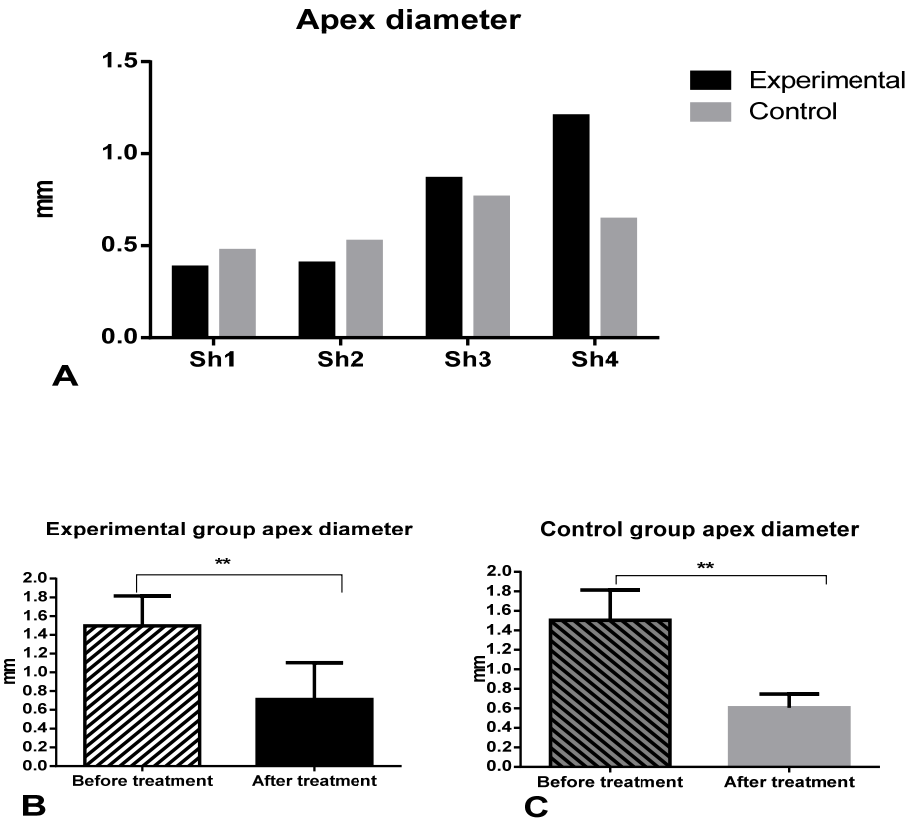


Figure 4.14 Measurements of apical diameters

A: Apical diameter of experimental and control groups six months after treatment.

B: Apical diameter before and after treatment in the experimental group.

C: Apical diameter before and after treatment in the control group.

Means ± SD of data collected from four animals are shown. **p < 0.009.

4.5.2 Computer Tomography scan analysis

CT measurements were generally larger than the radiographic measurements but these results confirmed the radiographic results that at the end of the experiment there were no significant differences between experimental and control groups in root lengths, dentine thicknesses or apex diameters (all $p > 0.26$), as shown in **Figure 5.15**. All experimental teeth after treatment had longer roots, thicker dentinal walls, and narrower apices, which were similar to the control teeth criteria (**Figure 4.16 - Figure 4.21)**. In Sheep-4 there were

also signs of internal resorption covered by a shadow of less hard tissue deposit on canal walls as shown in **Figure 4.22** and **Figure 4.23.**

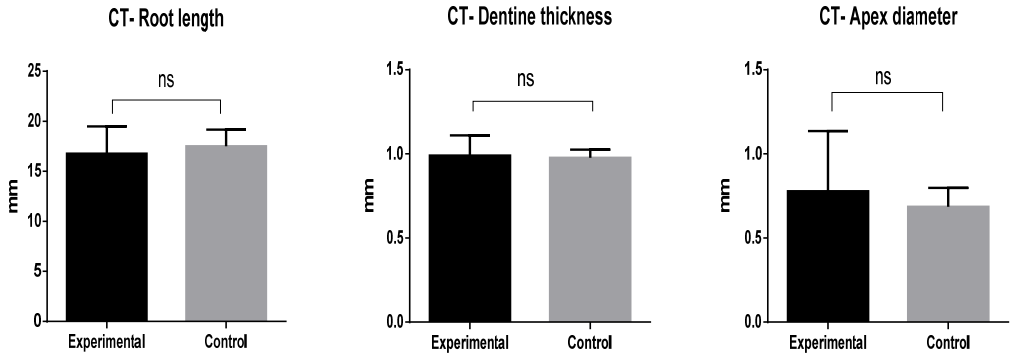


Figure 5.15 CT analysis of the differences between the experimental and the control group at the end of the experiment.

No significant difference was recorded in root length, dentine thickness or apex diameter. Results shown are Means ± SD of data collected from four animals.

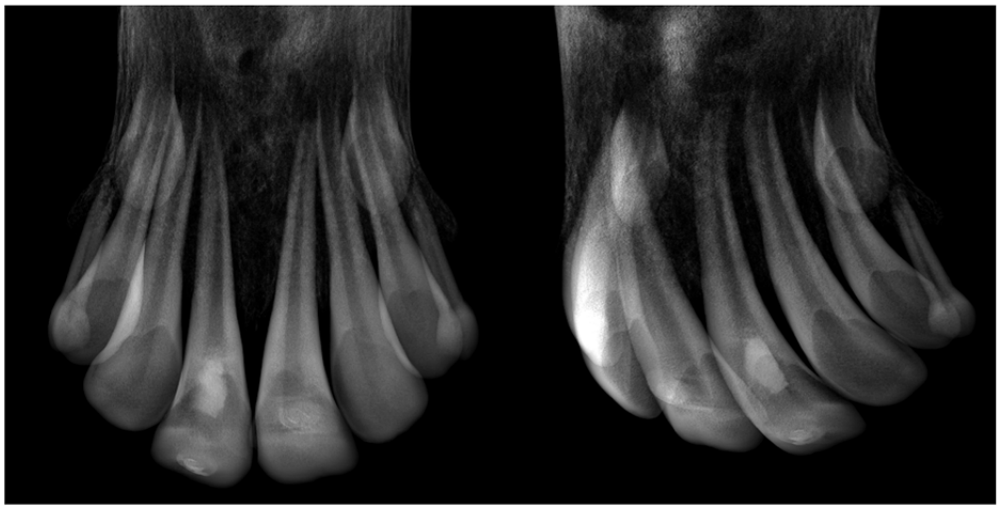


Figure 4.16 3D CT images of Sheep-1, showing coronal and lateral views of experimental and control teeth.

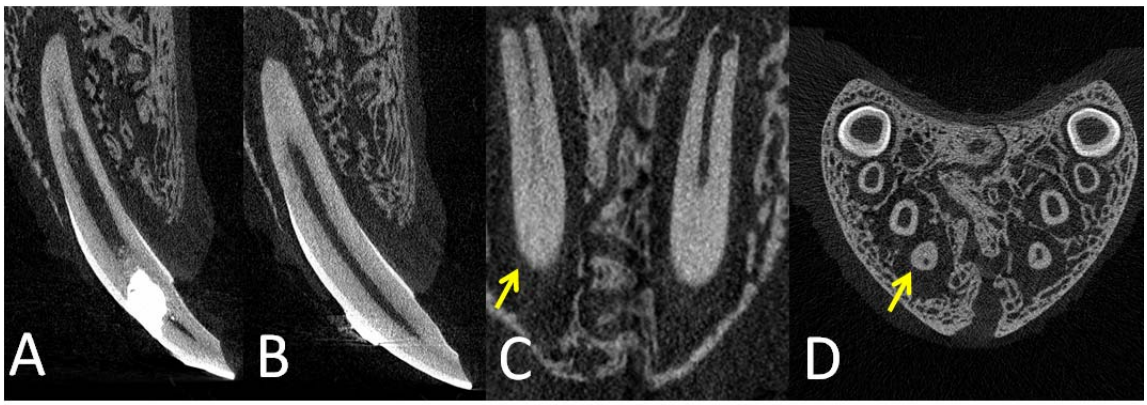


Figure 4.17 Sheep-1 CT image.

A: Sagittal section of control tooth.

B: Sagittal section of experimental tooth.

C: Coronal section.

D: Transaxial section showing teeth apices.

Yellow arrow indicate experimental tooth.

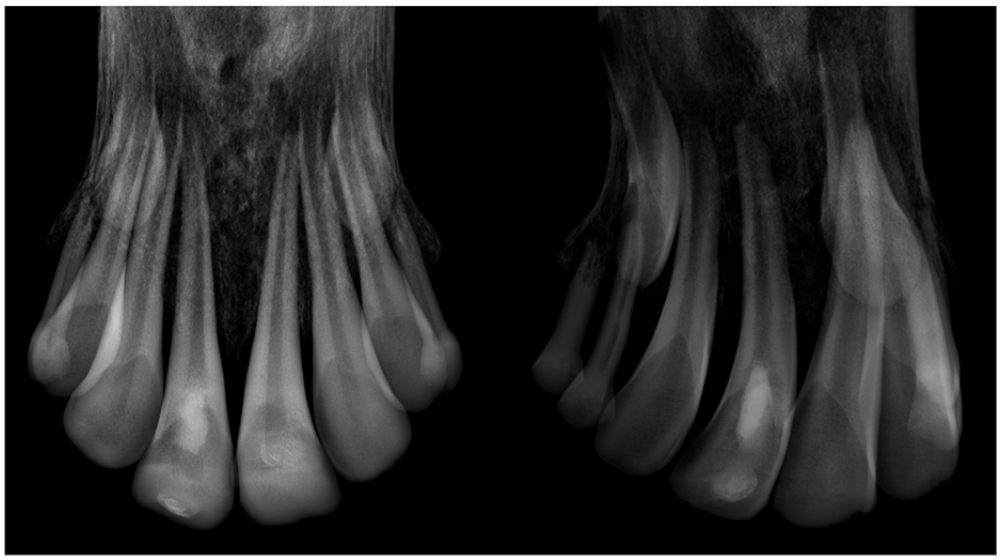


Figure 4.18 3D CT image of Sheep-2, showing coronal and lateral views of experimental and control teeth.

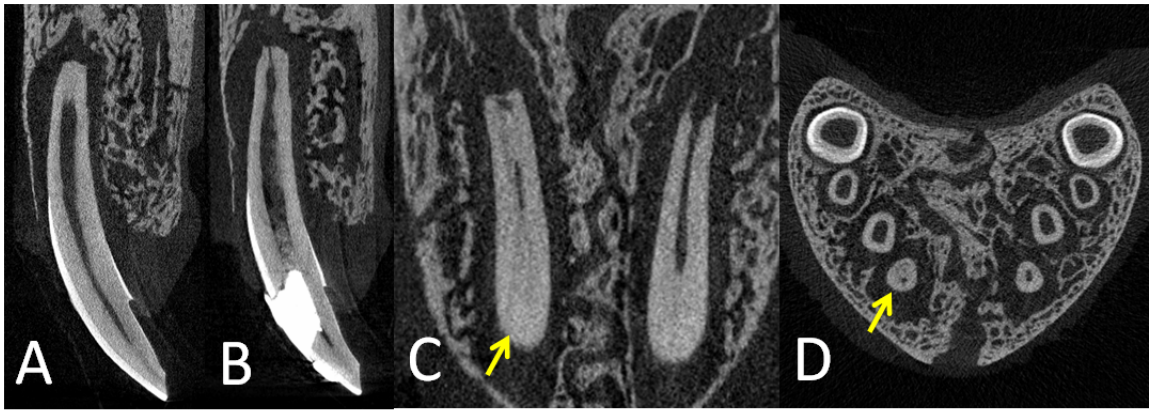


Figure 4.19 Sheep-2 CT images.

A: Sagittal section of control tooth.

B: Sagittal section of experimental tooth.

C: Coronal section.

D: Transaxial section showing teeth apices.

Yellow arrows indicate experimental tooth.

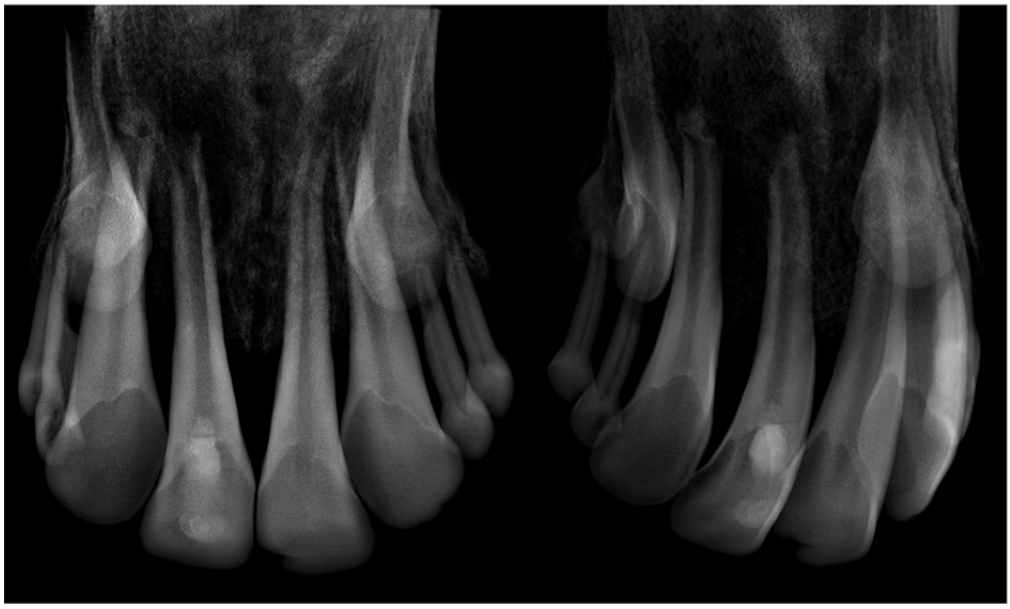


Figure 4.20 3D CT images of Sheep-3, showing coronal and lateral views of experimental and control teeth.

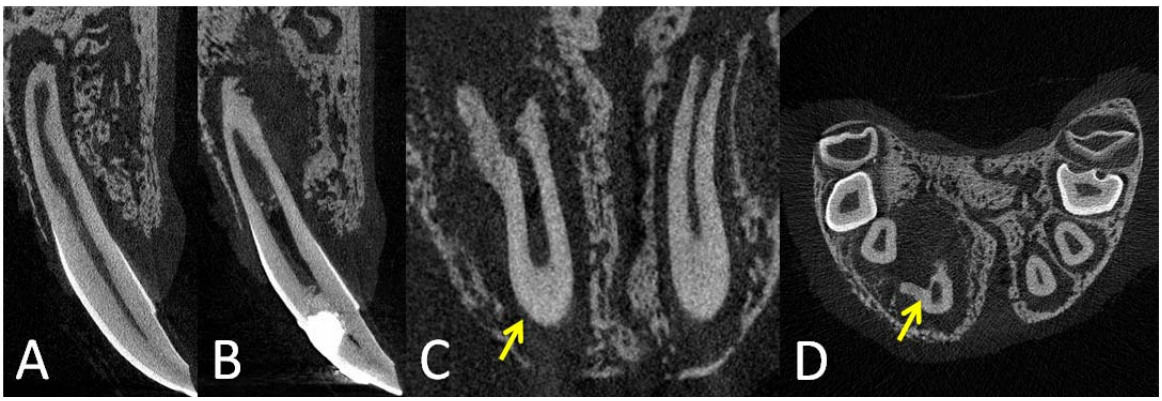


Figure 4.21 Sheep-3 CT images.

A: Sagittal section of control tooth.

B: Sagittal section of experimental tooth.

C: Coronal section.

D: Transaxial section showing teeth apices.

Yellow arrow indicate experimental tooth.

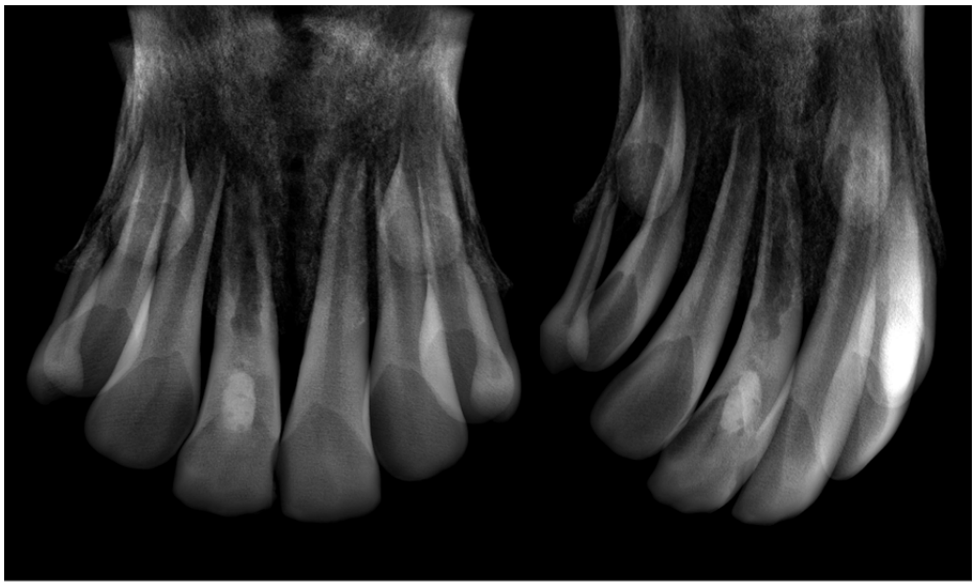


Figure 4.22 3D CT Sheep-4, showing coronal and lateral views of experimental and control teeth.

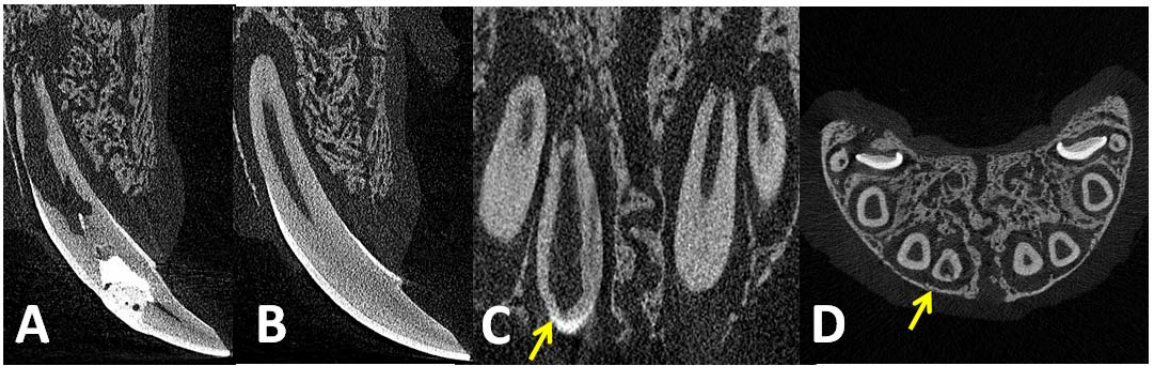


Figure 4.23 Sheep-4 CT images.

A: Sagittal section of control tooth.

B: Sagittal section of experimental tooth.

C: Coronal section.

D: Transaxial section showing teeth apices.

Yellow arrow indicates experimental tooth.

4.5.3 Histological observations

Histology sections of the experimental teeth six months after treatment showed that vital tissues developed in the canal cavities and hard tissues were deposited on the dentinal walls **(Figure 4.24)**.

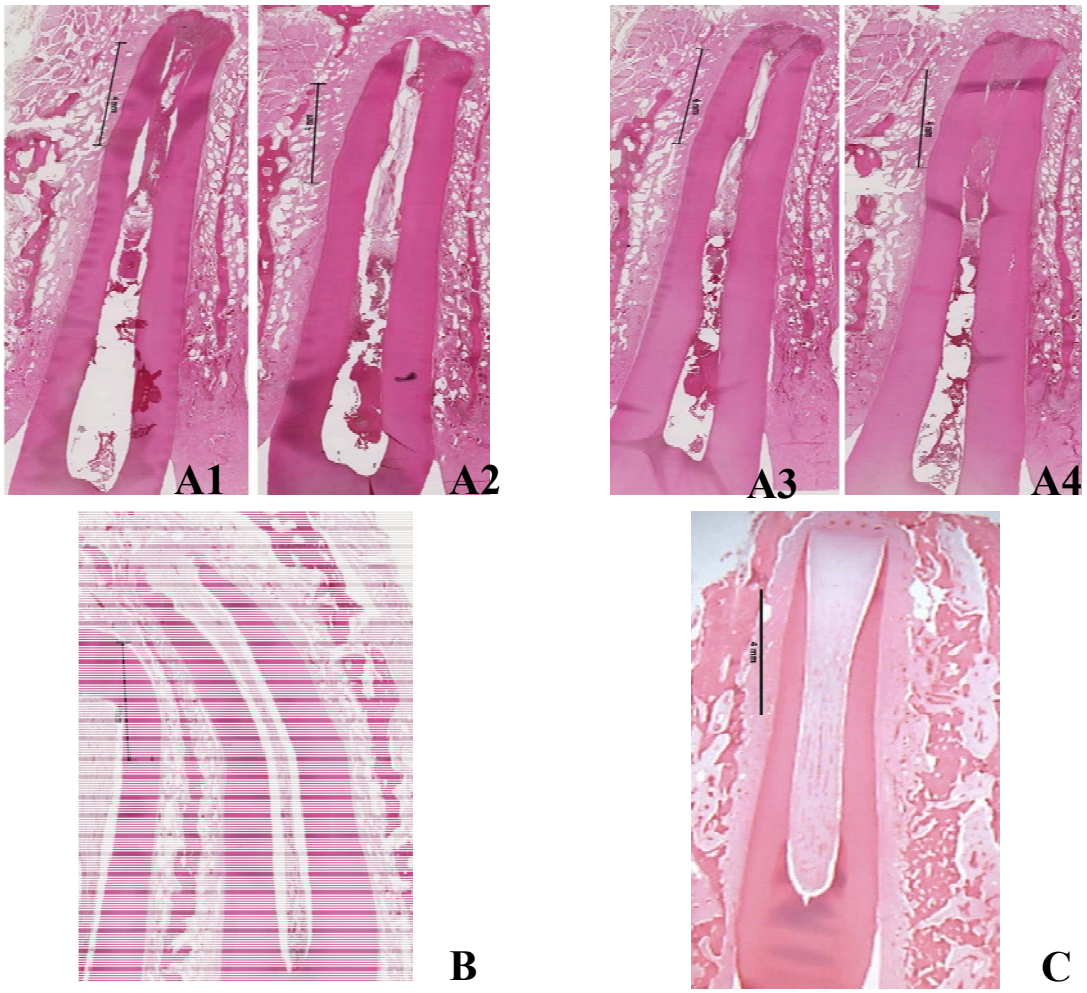


Figure 4.24 Experimental and control tooth sections.

A1: Anterior section of an experimental tooth showing tissues developed in the pulp canal.

A2: Middle section of an experimental tooth showing a narrow pulp cavity filled with loose fibrovascular connective tissues.

A3: Section approximately 350 µm posterior from (A2) showing a thick hard tissue deposit in the apical area.

A4: Posterior section of an experimental tooth showing hard tissue deposit on the root walls.

B: Section of a control tooth showing a normal pulp tissue, narrow pulp cavity and thick root walls.

C: For comparison reason a coronal section of a first incisor at two-tooth stage of dental development from a previous study, corresponding to the time of initiation of the experiment.

Based on the histologically structure and the differences of the healing tissues observed in the canal space and on the walls of the experimental teeth, it became apparently that there were three distinct regions in the healing response as shown in **Figure 4.25.** The root apices of the experiment teeth were analysed in a separate section.

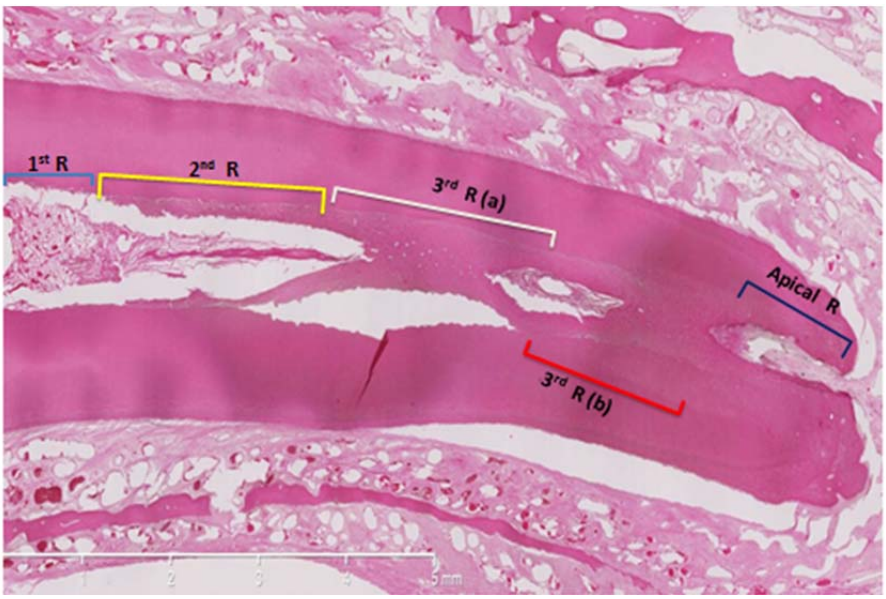


Figure 4.25 Histology section of the experimental tooth showing all regions.
R: region.

4.5.3.1.1 First healing region

The first healing region was seen in the coronal aspect of the experimental teeth next to the collagen-MTA plug (**Figure 4.26**). A row of cells lined the dentinal wall was visible in this region **(Figure 4.27)**. The tissues in the root canal consisted of stellate or spindle shape cells surrounded by a mesh-like matrix with hard tissue (cementum-like) islands and small blood vessels **(Figure 4.26 - Figure 4.28)**. The sizes of the hard tissue islands were larger at the apical part of the region (**Figure 4.28A**). **Figure 4.28C** shows an island with part of it has cementum-like structure and the rest still has a scaffold structure. There were variations between sheep in the first healing region **Figure 4.29**. In the experimental tooth of Sheep-3, this region extended into more than half the root (**Figure 4.29C**), while in

Sheep-4 this region was small, dense, rich in cells, and only a few small parts of the scaffold remained within the tissue (**Figure 4.29D**).

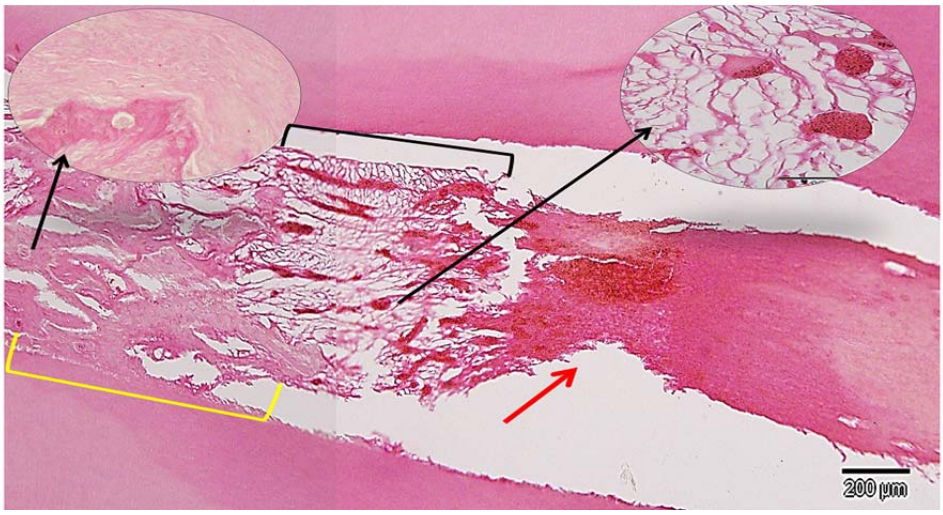


Figure 4.26 Experimental tooth healing regions

First region indicates by a black square bracket, second region indicates by a yellow square bracket, red arrow indicates collagen dressing.

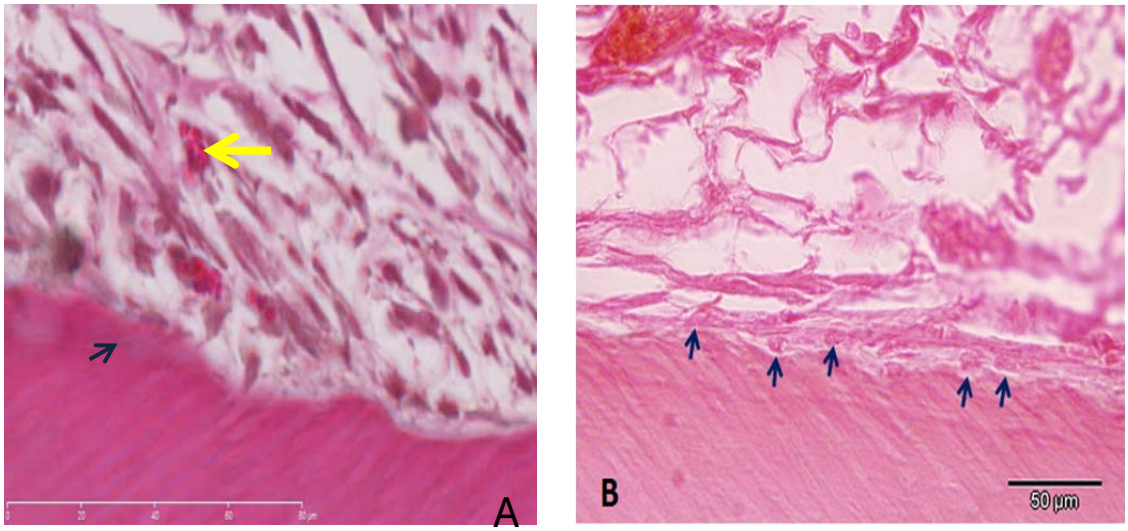


Figure 4.27 A and B High magnified sections of the first region.

Sections showing cells lined the dentine walls (black arrows).

Yellow arrow indicates a blood vessel.

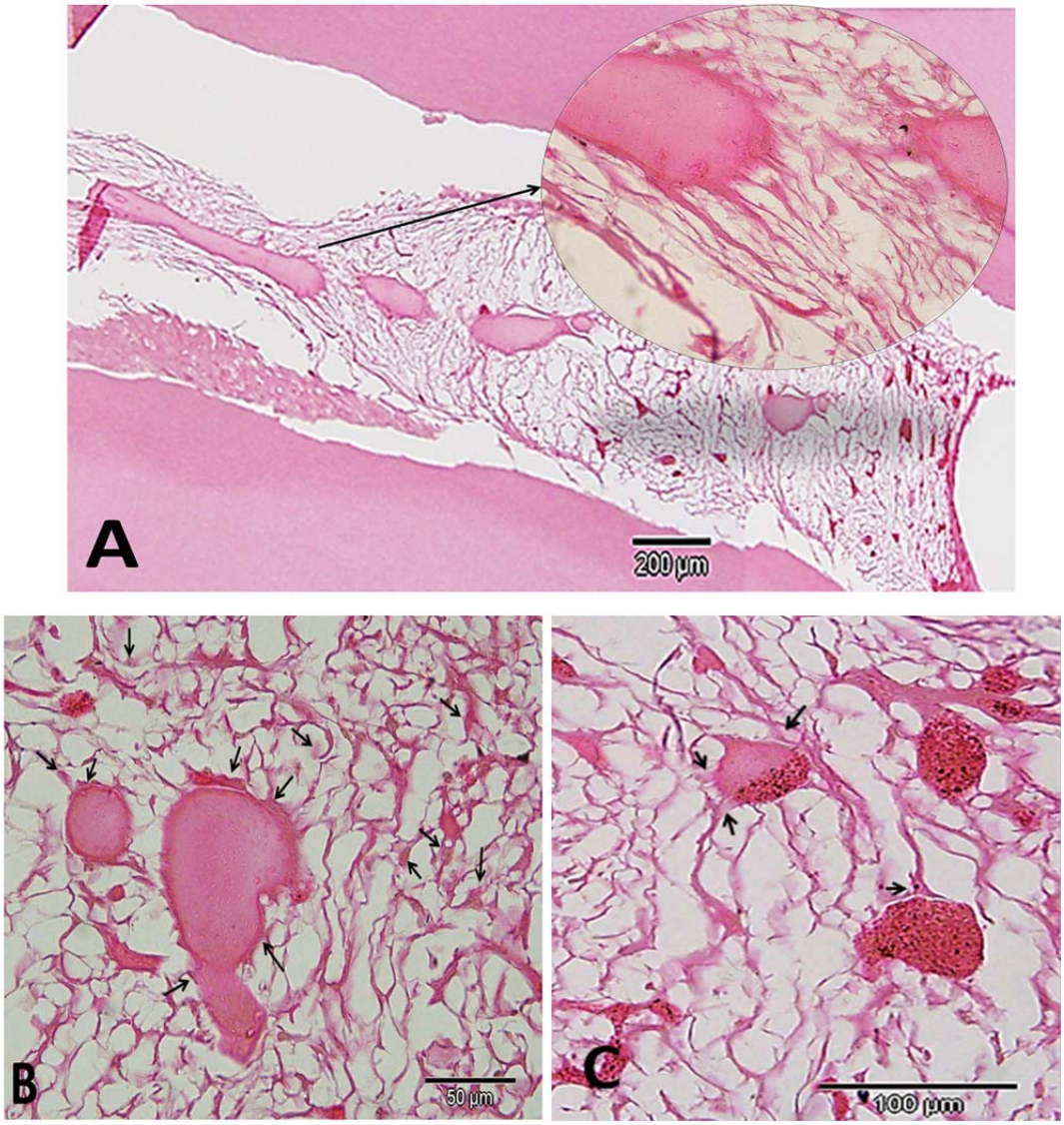


Figure 4.28 First region

A: A deep section showing the mesh-like soft tissue matrix with islands of hard tissue.

B and **C**: Magnified sections of the tissue matrix at the first region showing cells (black arrows) within a mesh-like matrix including some cells lined the surface of hard tissue islands.

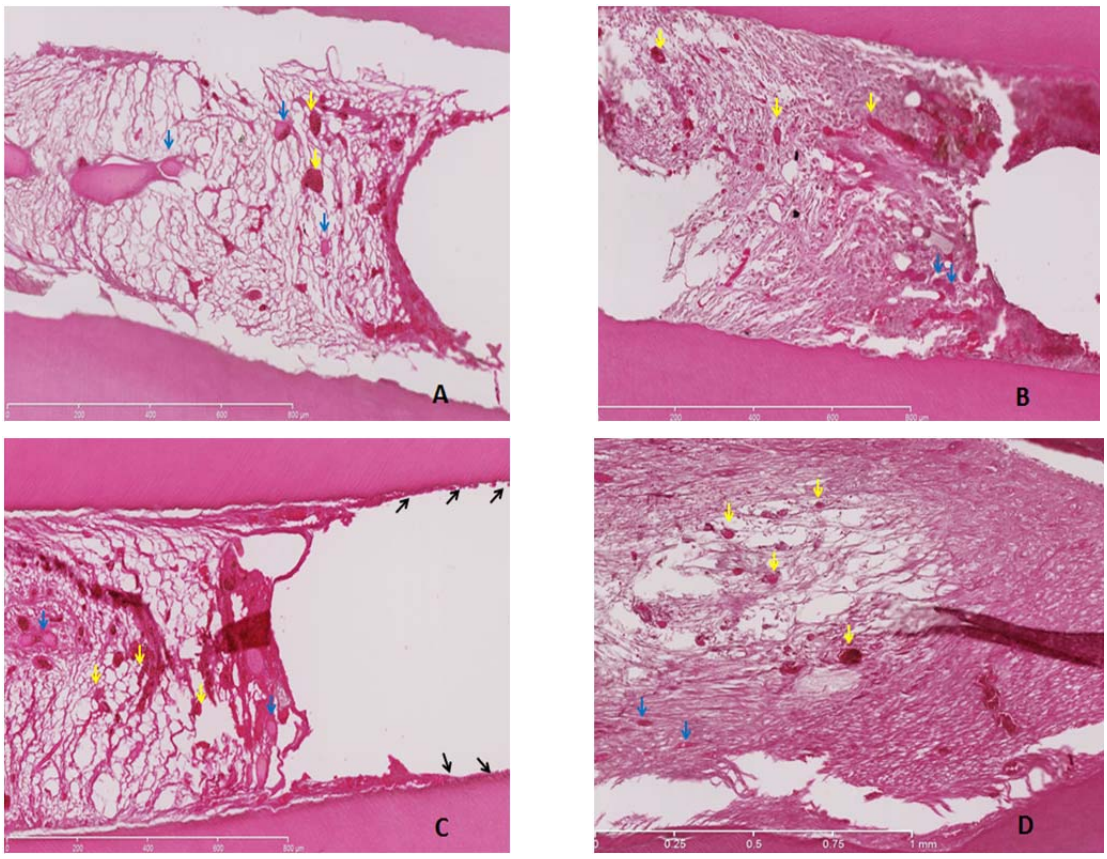


Figure 4.29 Experimental teeth sections showing the first region.

A: Sheep-1 first region with cell rich mesh-like tissue developed at the coronal third of the root.

B: Sheep-2 first region the same as Sheep-1 with a slightly condense structure of the tissue.

C: Sheep-3 first region with cell rich mesh-like tissue. The tissue shrank during histology processing; black arrows indicate the remnant of the tissue on the walls.

D: Sheep-4 first region with a more condense mesh-like structure rich in cells. Collagen scaffold did not exist, and only few islets remain.

Yellow arrows indicate scaffold islands and blue arrows indicate hard tissue (cementum-like) islands.

4.5.3.1.2 Second healing region

The second healing region showed more differentiated tissue with a mosaic-like matrix covering the canal walls. The matrix structure appeared to be at different stages of maturation. Some parts still had a mesh-like structure with big hard tissue (cementum-like) islands, while other areas looked similar to immature reparative dentine or immature

cementum **Figure 4.30.** The pulp cavity was filled with loose fibrovascular tissue (**Figure 4.30B)**. At the coronal margin of the region, there was a layer of cells with matrix deposit around them with an appearance similar to cellular dentine or cementum. Some cells were entrapped in lacunae (**Figure 4.31A)**. On the other hand, at the apical margin of the region the matrix was thicker and acellular (**Figure 4.31B)**. A cross-section of the region showed the different stages matrix mineralisation **(Figure 4.31C**). The second healing region was seen in the experimental teeth of Sheep-1, 2 and 3, and it was larger and more obvious in the experimental tooth of Sheep-1.

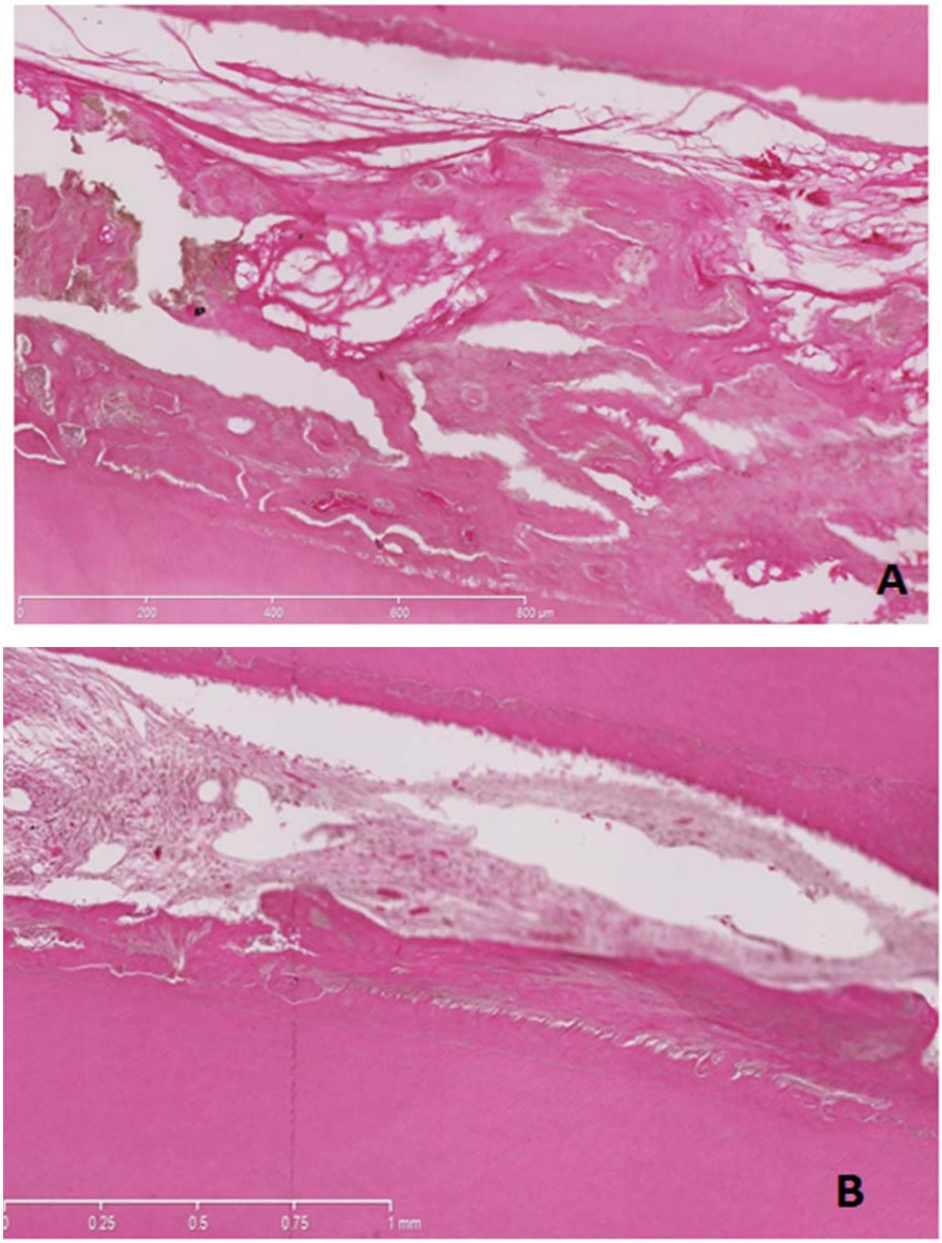


Figure 4.30 Section showing the second region with its hard tissue matrix structures.

A: An anterior section showing the mosaic-like structure of the matrix.

B: A middle section showing the hard tissue deposit on the wall and the cell rich fibrovascular tissue in the pulp cavity.

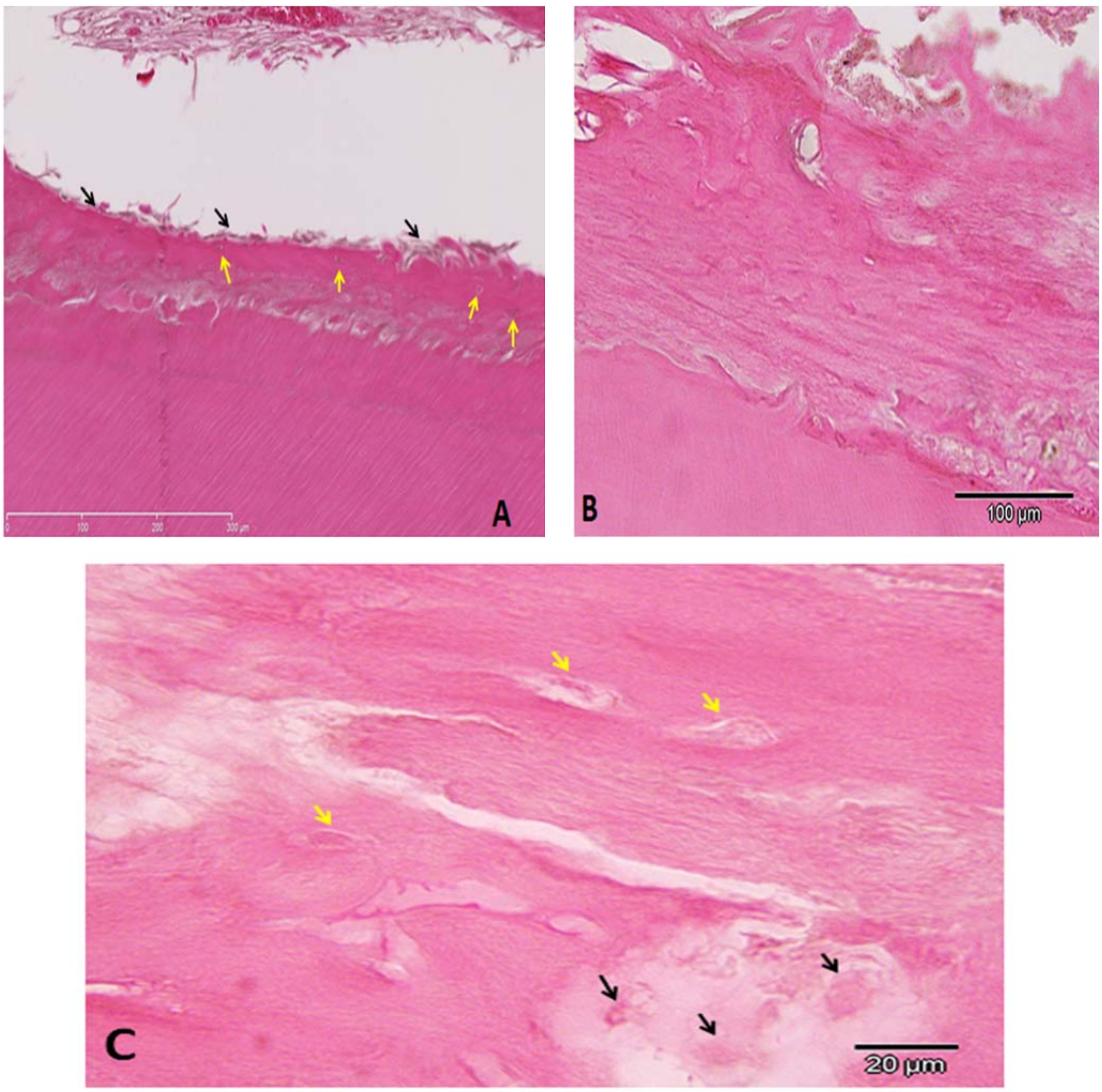


Figure 4.31 Enlargements of the second region.

A: The coronal margin of the region; a row of cells attached to the matrix with and some cells entrapped in lacunae.

B: The apical margin of the region with a more mature acellular matrix.

C: A cross-section of the region showing the hard tissue matrix at different stages of maturation and the cells forming the matrix. Some cells are in lacunae.

Black arrows indicate cells at the brim of the matrix, yellow arrows indicate a single cell in a lacuna.

4.5.3.1.3 Third healing region

The third region showed mature hard tissues covering the canal walls and loose fibrovascular connective tissues filling the pulp canal. The tissues on the walls were either

one type of hard tissue (cementum-like or atubular dentine-like) (region a), or two types of hard tissue (tubular dentine-like and cementum-like) (region b).

4.5.3.1.3.1 Third healing region (a)

The third region (b) in Sheep-1 and 2, showed one thick mature hard tissue layer covering the canal walls, as shown in **Figure 4.32**. The matrix was acellular and looked similar to irregular dentine or acellular cementum (**Figure 4.33**). Close to the pulp cavity, cells were seen lined the hard tissue matrix (**Figure 4.34)**. In a cross-section, the hard tissue layer covering the canal walls had bundle-like structures, which were perpendicular to the root surface (**Figure 4.34C)**. The third region (a) in Sheep-3 was smaller and less mature than in Sheep-1 and 2. It had a structure similar to cellular mixed cementum, where a deep layer of acellular cementum-like tissue was covered by superficial cellular cementum-like tissue (**Figure 4.35A**). The third region (a) in Sheep-4 was thick and similar to cellular cementum. In some sections this layer was a mixed cementum-like, but the cellular cementum-like tissue was thick and covered by a very thin acellular cementum-like tissue which had inserted fibers, similar to Sharpey's fibers (**Figure 4.35B** and **C**).

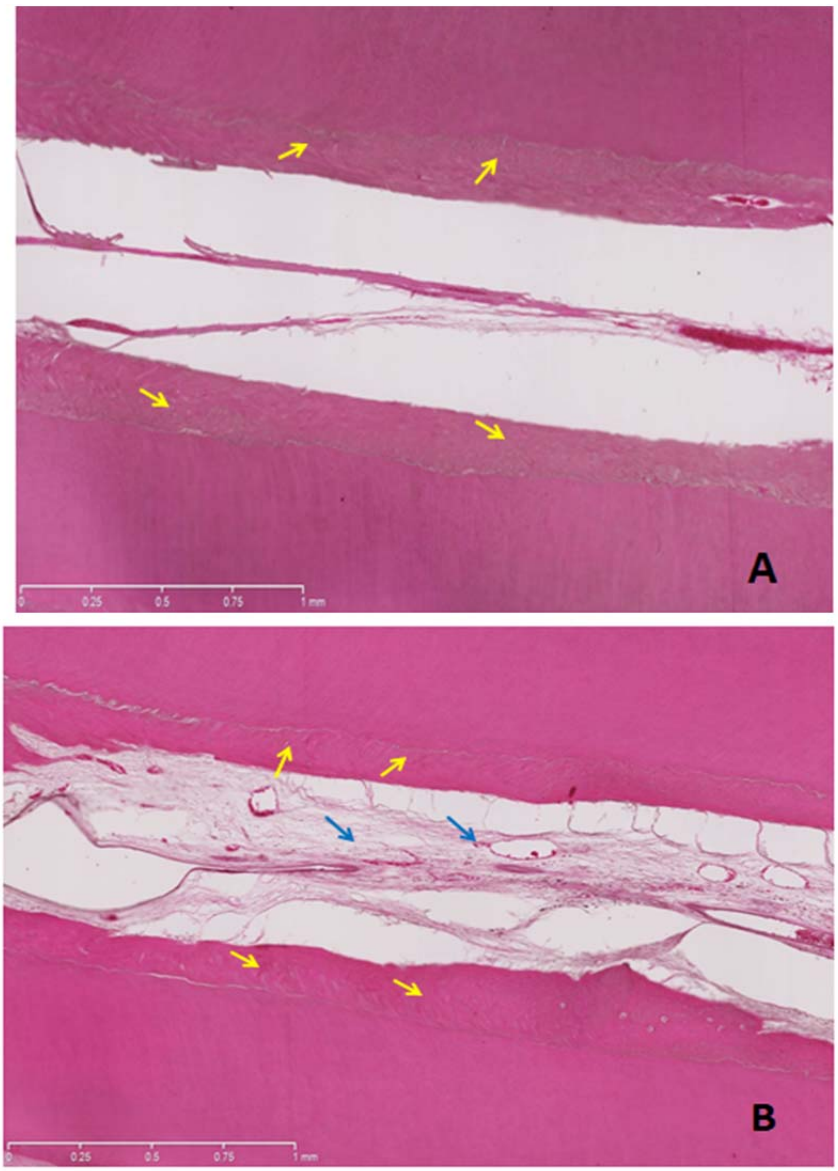


Figure 4.32 Section showing the third region (a).

A: From Sheep-1, mature hard tissue layers covering the root wall.

B: From Sheep-2, mature hard tissue layers covering the root wall with cell rich loose fibrovascular connective tissue in the pulp cavity.

Yellow arrows indicate the newly formed layers, histologically similar to reparative dentine or acellular cementum. Blue arrows indicate the tissue in the pulp cavity.

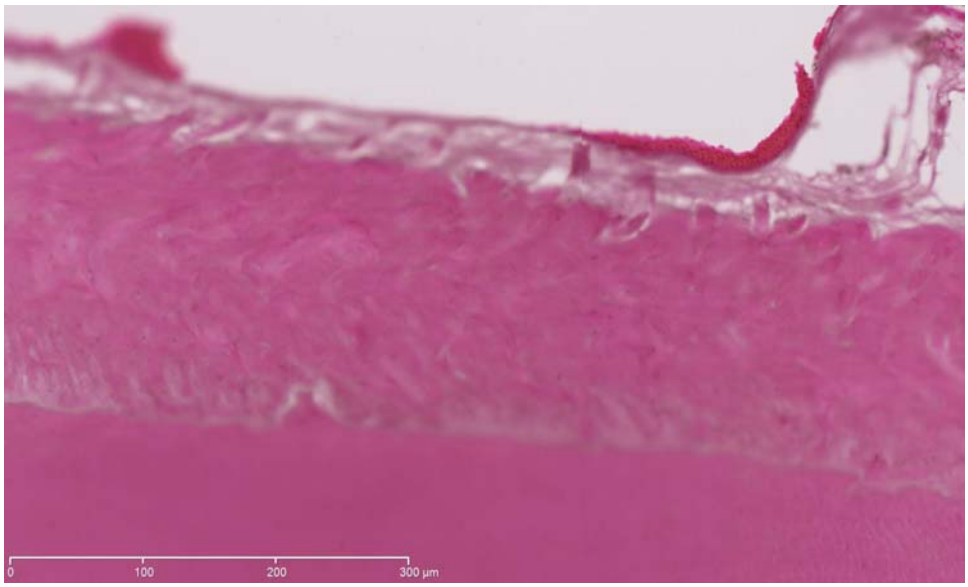


Figure 4.33 Enlarged section of the third region (a)

A section of the matrix structure of the hard tissue covering the primary dentin showing an acellular matrix similar to reparative dentine or acellular cementum.

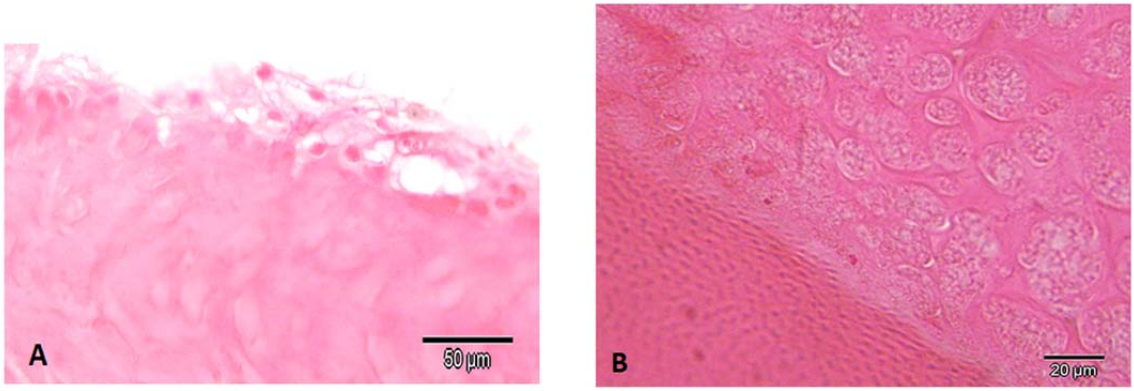


Figure 4.34 Magnified sections of the third region (a).

A: The hard tissue matrix close to the pulp cavity with a row of cells attached to the matrix.

B: Bundle-like structures in the hard tissue attached to the dentinal walls which run in the same direction as the dentinal tubules.

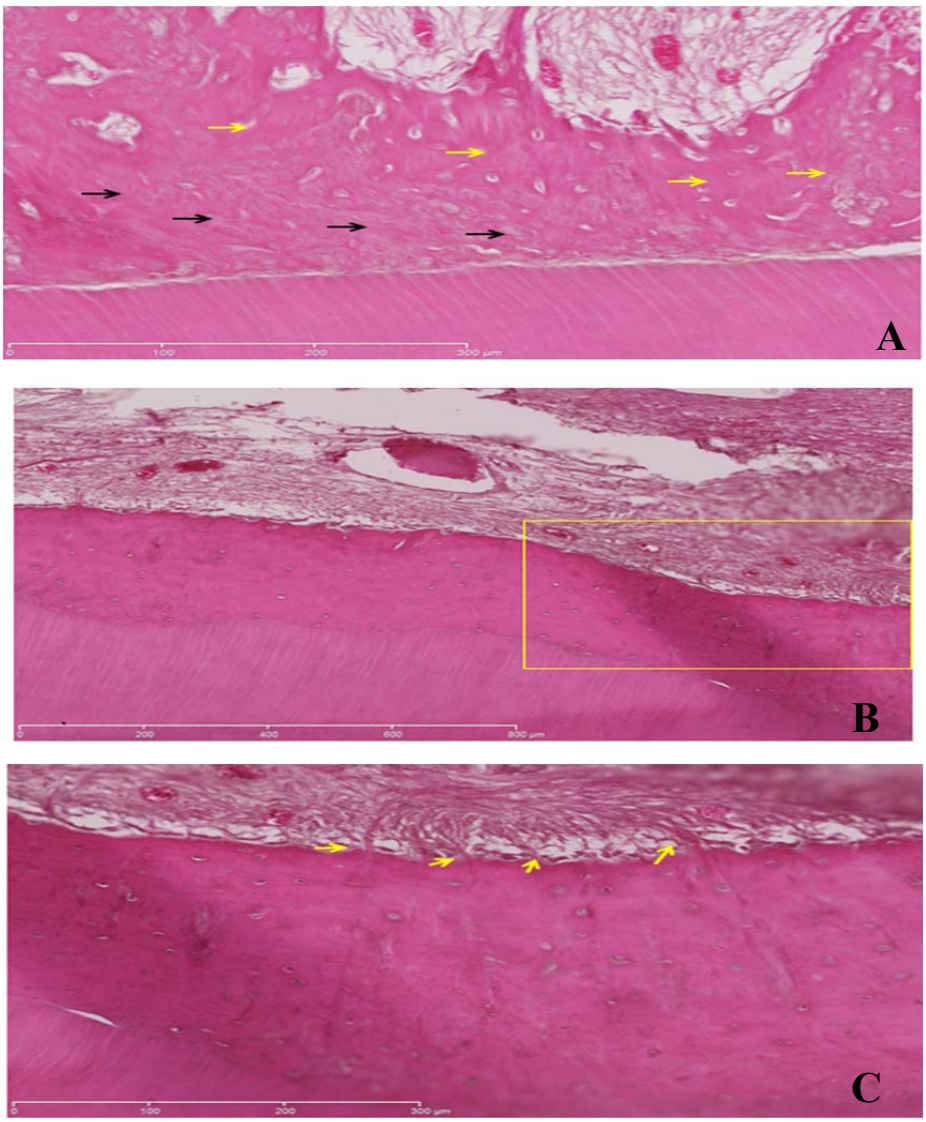


Figure 4.35 Third region (a).

A: The third region (a) of Sheep-3, deep layer of acellular cementum-like tissue (black arrows) covered by cellular cementum-like tissue (yellow arrow).

B: The third region (a) of Sheep-4, thick cellular cementum-like tissue covered by thin acellular cementum.

C: Magnification of the area indicated with the yellow rectangle in image (B) showing projections similar to Sharpey's fibers inserted into the cementum-like (yellow arrows).

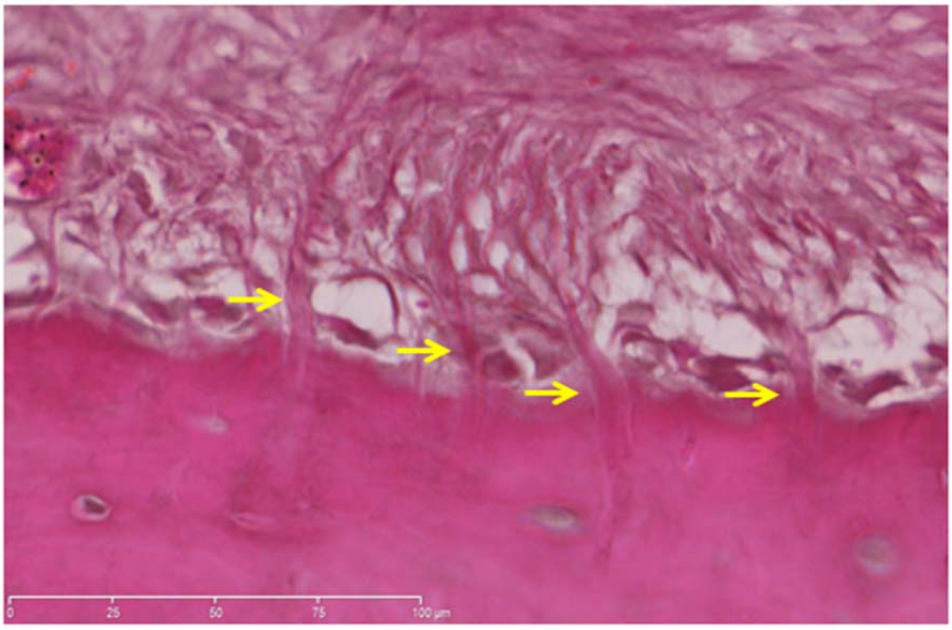


Figure 4.36 High magnified section of the third region (a) of Sheep-4.

Sharpey's fibers-like projections inserted into a cementum-like layer (yellow arrows).

4.5.3.1.3.2 Third healing region (b)

At the third region (b), the dentinal walls were covered by two thick layers of hard tissues (**Figure 4.37).** The first layer next to the primary dentine had a tubular-like matrix similar to secondary dentine or regular reparative dentine **(Figure 4.37A, Figure 4.39B and Figure 4.40A)**. The tubular structure of the matrix was better organised at some areas than other areas (**Figure 4.39B and Figure 4.40A)**. The second layer was similar to the third region (a), but showed a more mature histology (**Figure 4.37C, Figure 4.38 and Figure 4.40D**). The pulp cavity was filled with a loose fibrovascular connective tissue rich in cells. This region was seen in Sheep-1, 2 and 4 at the apical third of the root and also in the middle third of the root in Sheep-4.

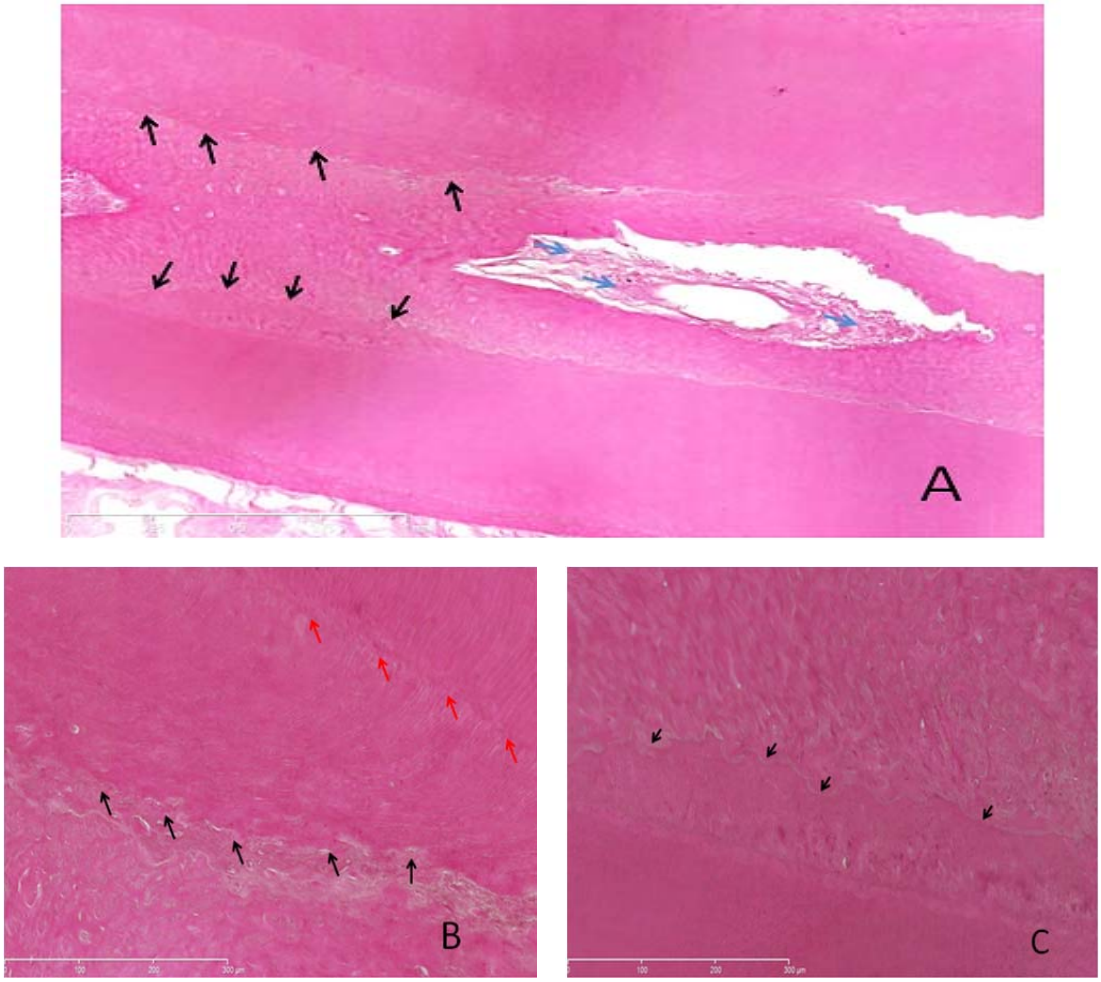


Figure 4.37 Sheep-1, third region (b).

A: A section showing two hard tissue layers covering the canal walls with fibrovascular connective tissue in the pulp cavity.

B: High magnification of the third region (b) showing the first layer with a tubular-like structure of the matrix.

C: High magnification of the second layer of the third region (b) with a similar appearance to the third region (a).

Red arrows indicate the line separating the primary dentine and the first layer. Black arrows indicate the line separating the first and the second layers. Blue arrows indicate the tissue in the pulp cavity.

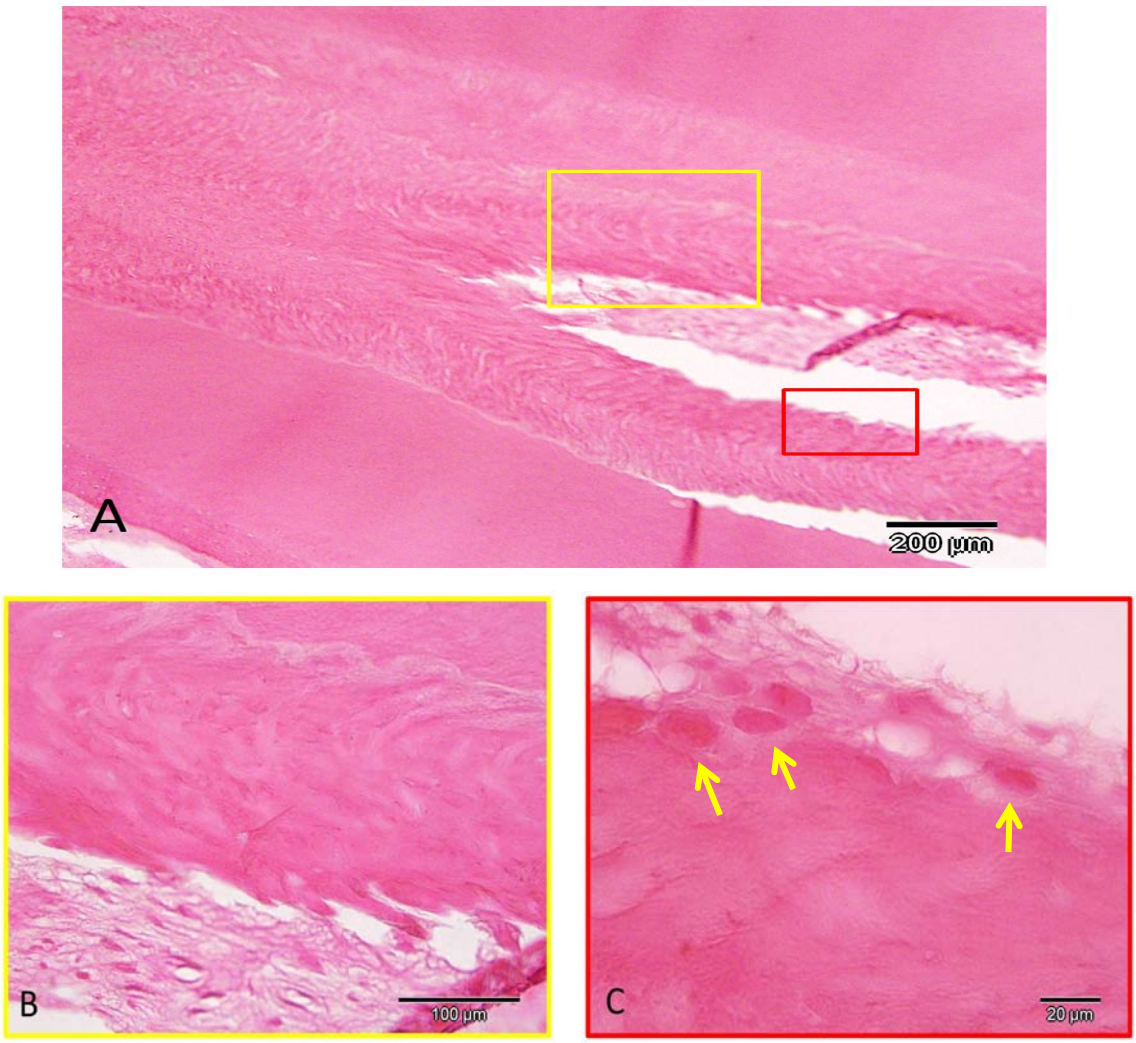


Figure 4.38 Third region (b).

A: A section showing two hard tissue layers covering the root walls and fibrovascular connective tissues occupying the pulp cavity.

B: High magnification of the area indicated with the yellow rectangle in image (A) showing the second layer matrix similar to third region (a).

C: High magnification of the area indicated with the red rectangle in image (A) showing the blast cells forming the second layer. Yellow arrows indicate the cells.

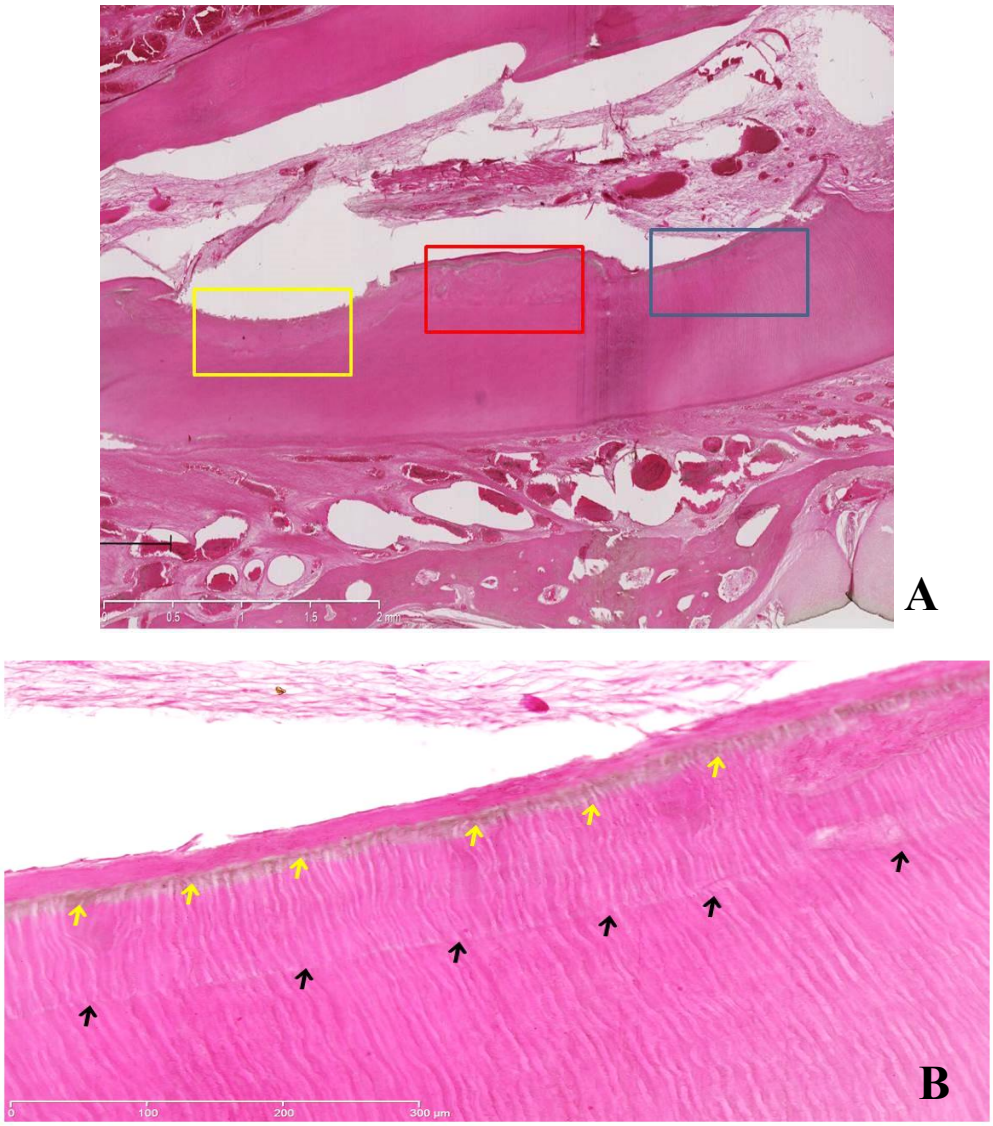


Figure 4.39 The experimental tooth of Sheep-4.

A: Third region (a) indicated with yellow rectangle. Third region (b) indicated with blue and red rectangles.

B: High magnification of the area indicated in the blue rectangle in image (A) showing third region (b). The first layer close to the primary dentine shows well organised tubular matrix, and the second layer is histologically similar to cellular cementum.

Black arrows indicate the margin between primary dentine and the first layer. Yellow Arrows indicate the margin between the first and second layers. *The dark margin is due to EDTA irrigation effects on the pre-dentine.*

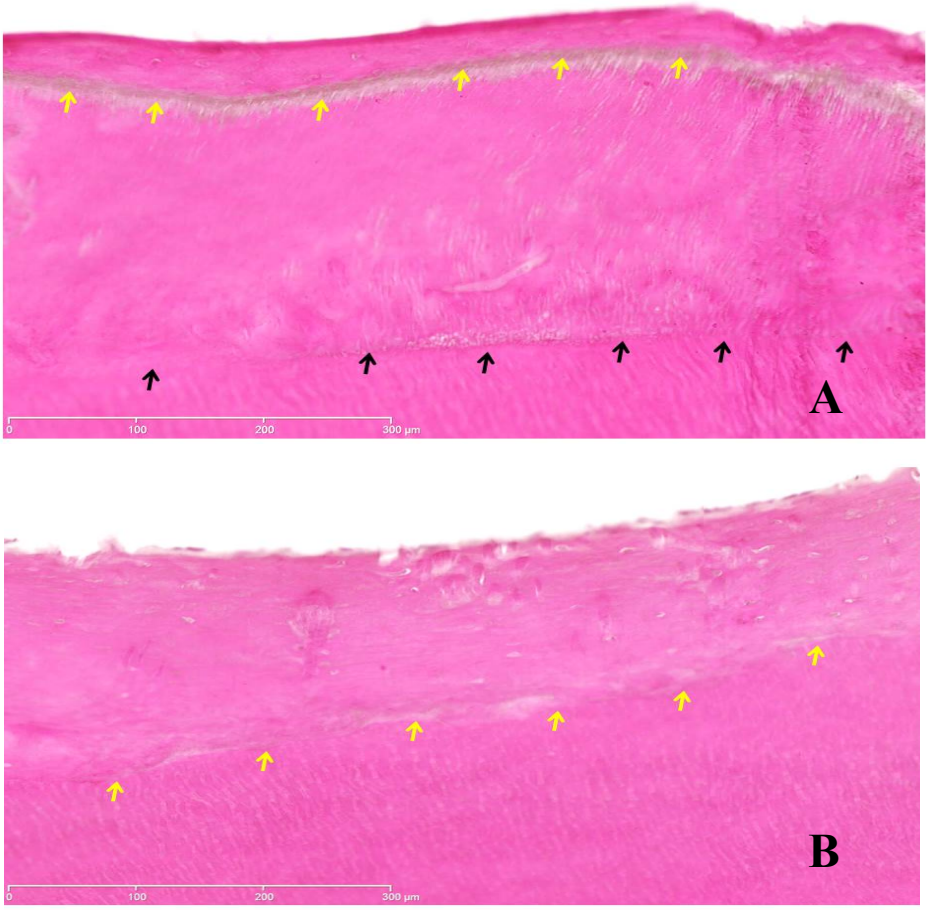


Figure 4.40 The experimental tooth of Sheep-4, third region (b).

A: High magnification of the area indicated by the red rectangle in Figure 4.39A showing third region (b). The first layer has a less organised tubular structure of the matrix than the previous region (Figure 4.39 B). Black arrows indicate the margin between the primary dentine and the first layer. Yellow arrows indicate the margin between the first and the second layers. *The dark margin is due to EDTA irrigation effects on pre-dentine.*

B: High magnification of the area indicated by the red rectangle in Figure 4.39A showing third region (a), with one hard tissue layer covering the primary dentine looking similar to cellular cementum tissue.

Yellow arrows indicate the margin between the primary dentine and the healing hard tissue.

4.5.3.2 Apical region

Histology sections of the experimental teeth showed well-developed apical regions with a narrow apical major foramen and canal walls covered by thick hard tissue. In Sheep-1 and 2, the layers were the same as the third region (b), and more similar to a cellular cementum

near the root tip (at the apical part of the intermediate dilatation and the minor foramen) (**Figure 4.41)**. In experimental teeth from Sheep-1 and Sheep-2, the apical part of the root (0.5 mm) consisted of cellular cementum (**Figure 4.42A)**, similar to the control tooth (**Figure 4.44).** In Sheep-3's experimental tooth, 1.3 mm of the apical length of the root consisted of cellular cementum-like tissue and a thick layer of cellular cementum-like tissue covered the canal, bridging it in some sections (**Figure 4.42B)**. In Sheep-4, cellular cementum-like tissue covered the canal wall and looked as a continuation of the external cementum. In the apical part of the root, islands of dentine were seen within the cellular cementum-like tissue, suggesting possible previous resorption of the primary dentine (**Figure 4.42C)** and (**Figure 4.43).** An intracanal bone-like tissue extending from the apex to a short distance in the apical third was seen in Sheep-4 experimental tooth.

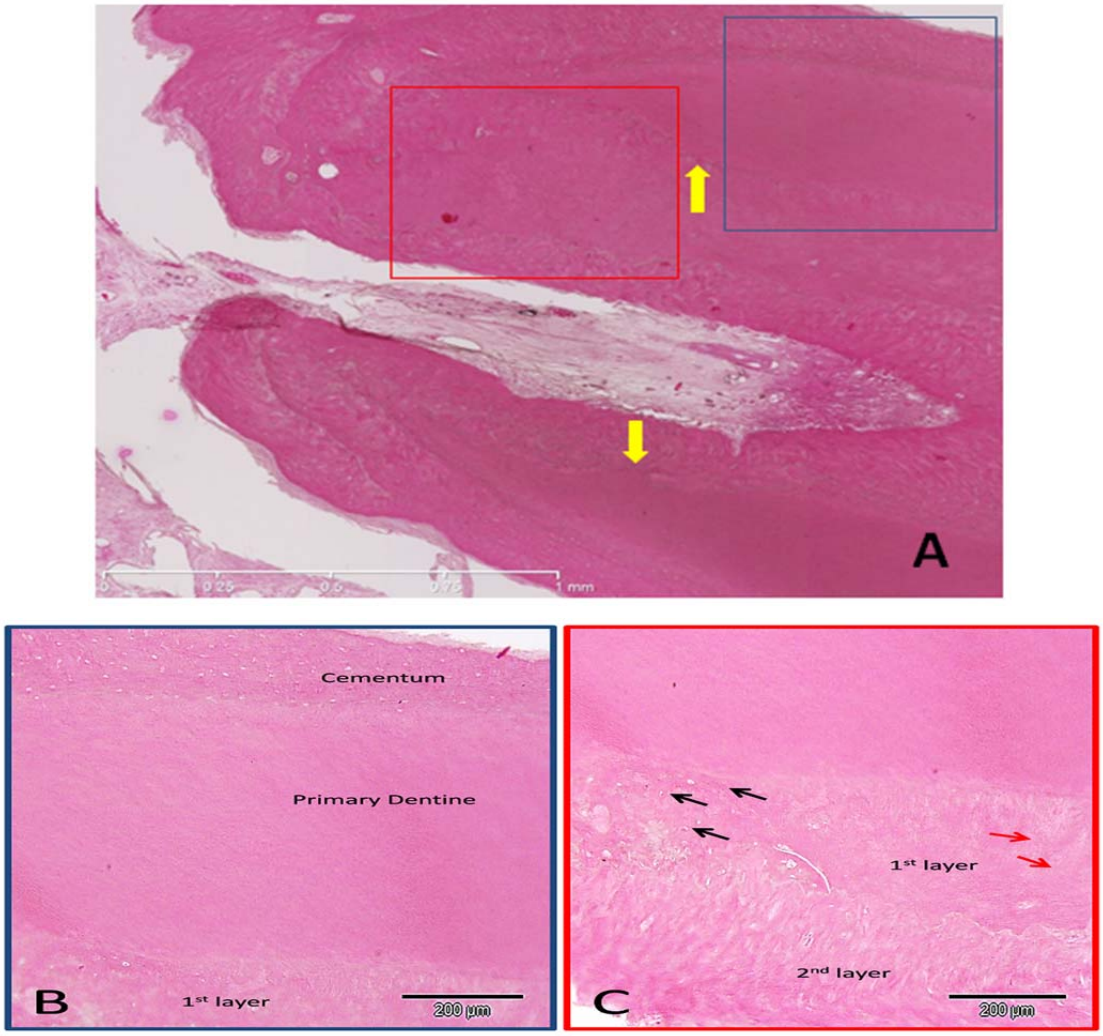


Figure 4.41 The apical area of Sheep-1 experimental tooth.

A: Section showing the hard tissue layers covering the pulp canal and the fibrovascular tissue in the pulp cavity. Yellow arrows indicate the position of the major foramen.

B: High magnification of the area indicated with the blue rectangle in image (A) showing cementum, primary dentin and first revitalised hard tissue layer.

C: High magnification of the area indicated with a red rectangle in image (A) showing the first layer covering the canal wall, and the second layer looking similar to irregular dentine or acellular cementum. Black arrows indicate the apical portion of the first layer looking similar to cellular cementum. Red arrows indicate the coronal portion of the layer.

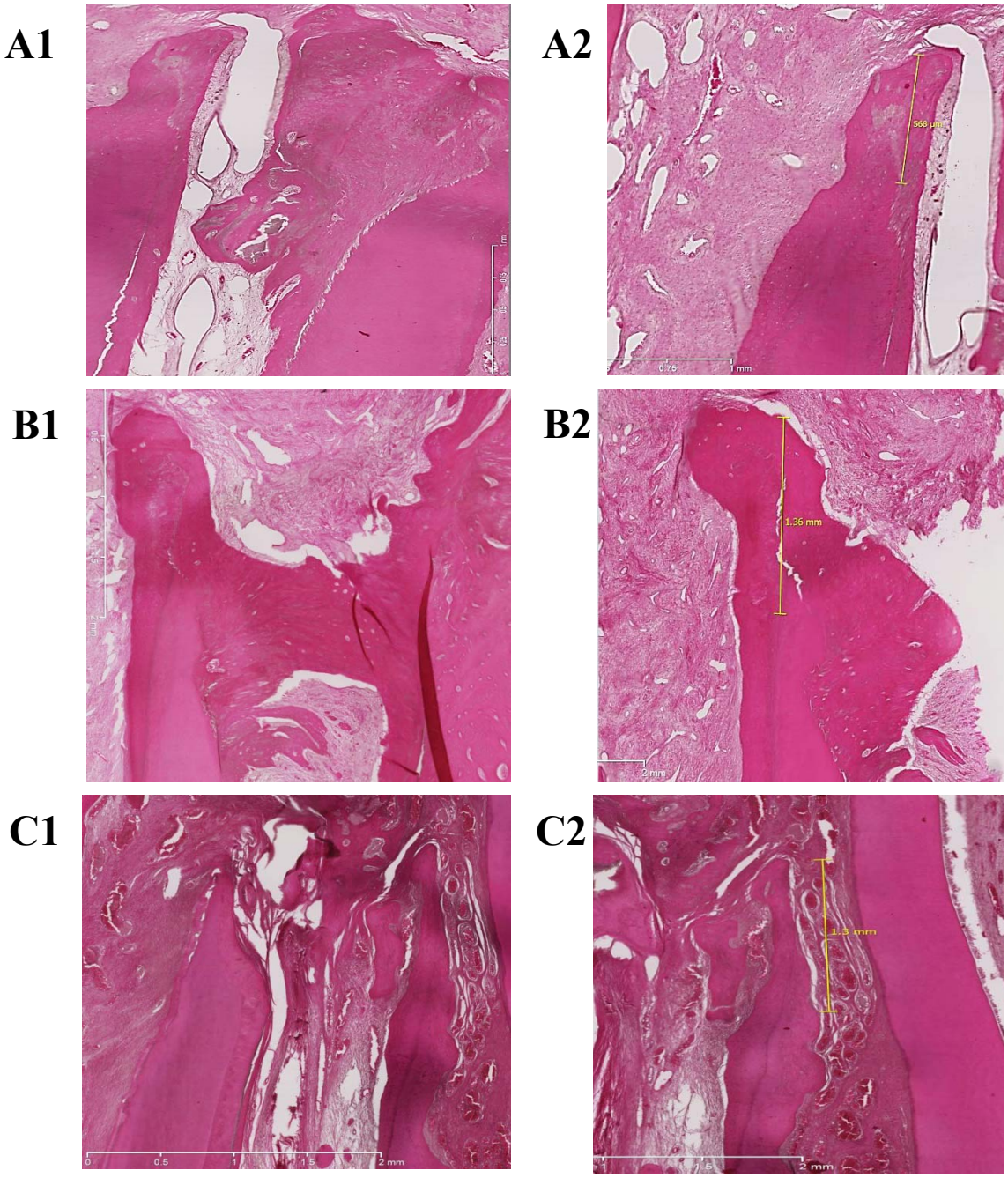


Figure 4.42 The apical regions of the experimental teeth

A: Apical region of Sheep-2

B: Apical region of Sheep-3

C: Apical region of Sheep-4

Left side images showing narrowed apices due to deposition of hard tissue (cementum-like) and fibrovascular tissue in the pulp cavities. Right side images showing the length of the root tips which has only cementum-like tissue.

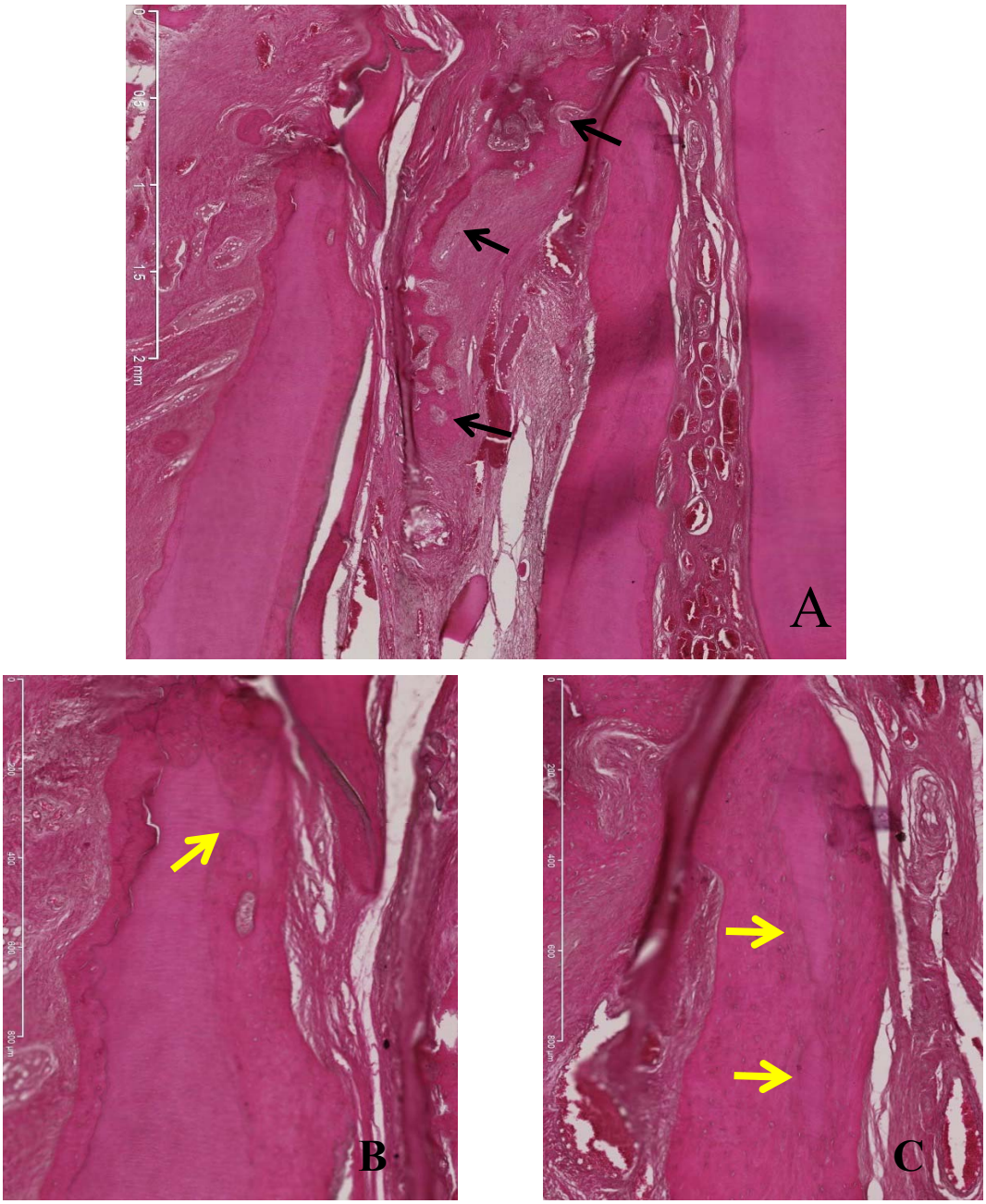


Figure 4.43 Sheep-4 apical region.

A: Cellular cementum-like layer covering the dentinal walls and bone-like island present from the apex to the apical part of the canal.

B: Magnification of the left wall.

C: Magnification of the right wall.

Yellow arrows indicate dentine tissue within a cellular cementum-like tissue.

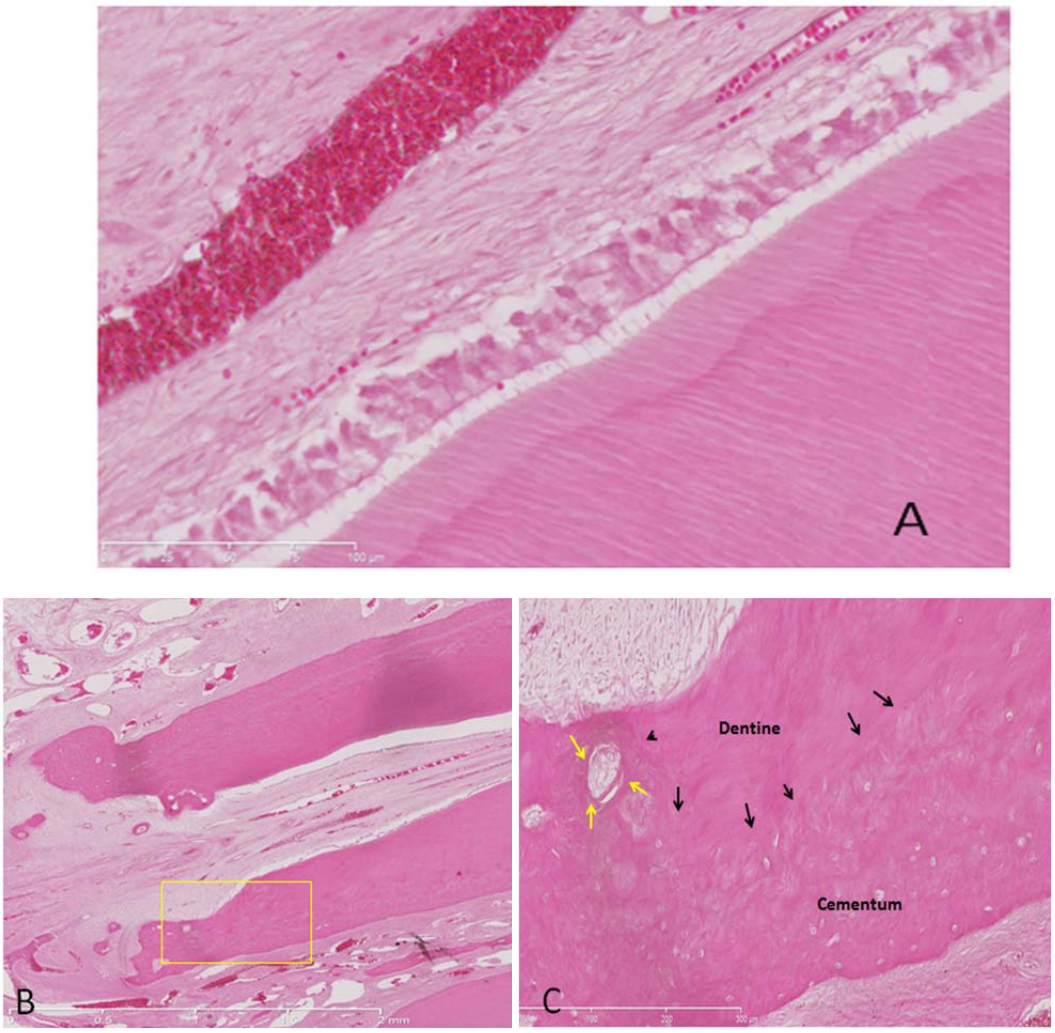


Figure 4.44 Control-tooth.

A: Coronal part of the pulp showing normal organised pulp tissue with odontoblasts at the dentine margin, connective tissue and blood vessels in the pulp cavity.

B: The apical part of the control-tooth showing a narrow apex, a canal filled with a normal pulp tissue and root walls cover by primary dentine and an outer layer of cementum.

C: Higher magnification of area indicated by the yellow rectangle in image (A), showing pulp tissues, primary dentine, and cementum. Black arrows indicate the margin between dentine and cementum. Yellow arrows indicate cells in a big lacuna within the cementum.

4.6 Discussion

Endodontic treatment for teeth with immature roots and infected canals is either by apexification or by revitalisation. Although apexification treatment, especially with MTA, has a good success rate (400, 401), the problem of thin root walls remain. Revitalisation as

a biologically based treatment has the advantages of further root development and canal thickening (16, 122, 123, 323). Case reports and animal research show controversial outcomes of this treatment (130, 289, 322, 335, 347, 350). Utilising revitalising revitalisation protocol, histology studies are unable to show that the revitalised tissue is dentine with associated odontoblast cells. Therefore more studies more studies are required to precisely identify the revitalised tissues and whether they are dentine derived from odontoblasts, cementum, bone or different type of tissue. Furthermore, in almost all previous research were done at very early stages of tissue maturation and remodelling. Maturation and remodelling of the wound healing occurs between 21 days and 1 year after injury (402). Insufficient information is therefore available about the healing tissue at a more mature stage (56, 124, 131).

In this *in vivo* study, the endodontic regeneration/ revitalisation protocol in immature infected sheep teeth generally showed positive outcomes with narrowing of the apices, thickening of the walls and increase in root length.

Histology sections showed three different healing or revitalisation regions six months after treatment.

In two sheep (Sheep-1 and 2), the infection of the experimental teeth was confirmed clinically at the time of disinfection session. However, changes in the periapical area were not visible on the radiographs suggesting a mild to moderate infection. The teeth were then treated with a revitalisation protocol, and six months after treatment, the radiographic results showed narrowing of the apices, an increase in the root lengths and root wall thickening. These measurements were confirmed by CT scans which showed no significant different between the experimental and the control teeth. Similar results have been reported in many cases that have been treated successfully with revitalisation protocol with continued root development (208, 288, 320, 403). In the present study, the histological

results showed three regions of healing. We suggest that the first region is likely to be an early stage of hard tissue development on canal walls in which the migrant stem cells has differentiated into hard tissue blast cells (cementoblast-like cells) and deposited a mesh-like matrix of collagen fibres, which mature, forming small hard tissue islands. These islands grow and merge forming a thin hard tissue layer on the walls with unmineralised fibrovascular tissue in the canal as seen in the second healing region. Further maturation of the layer results in development of layers in the third region (a and b). The third region (a) was characterised by the appearance of one type of well-developed hard tissue on the root walls. In Sheep-1 and Sheep-2, the appearance of this hard tissue was similar to acellular cementum or possibly irregular reparative dentine. The third region (b) was seen more apically, and characterised by the presence of two structurally different hard tissue layers on the dentinal walls. The first layer had a tubular matrix histologically similar to secondary or regular reparative dentine. This layer may have been deposited by local or differentiated odontoblasts as a reaction to trauma and bacterial assault to the pulp (56) at the first treatment session. However, the cells were not present at the end of the study, which might be due to them not surviving the bacterial infection or instrumentation trauma. Wang *et al*. (21) showed a partial survival of pulp odontoblasts in immature infected teeth treated by a revitalisation protocol using blood clot, but they did not use any instrumentation. The second layer may have been deposited by differentiated migrant stem cells after the third treatment session. In these animals, no periapical radiolucency was seen on the radiographs suggesting a moderate infection. This would make it possible for apical papilla stem cells to survive after the pulp infection. When a suitable environment was later available for these cells or periapical stem cells, they migrate into the pulp canal (116, 124, 312). Also, the epithelial root sheath may not have been damaged, thus the same continued development and increased root length as seen in the control teeth was possible.

As a result of root lengthening and root wall thickening, the apical area continued developing and the root apex became narrower.

In previous studies, MTA was condensed into the orifices of treated teeth without a collagen base and parts of the MTA pushed to the canal during condensation, which was associated with MIs near the orifices and scattered MIs inside the canal (21, 212). In the present study, there was no direct contact between MTA and the developing tissue, thus scattered calcification in the canal and apical bridge formation were avoided. This gave the opportunity for the tissue to progress from the apical to the coronal part of the root ending up with more mature regions close to the apex and less mature region (first region) in the coronal area. No intracanal hard tissue islands were seen apically in these teeth and the coronal centrally located hard tissue islands which were part of the first region, could have developed to replace the collagen scaffold. However, it is unknown if the deposition of hard tissue on the walls will be continuous leading to total calcification of the canal. A longer period for evaluating the maturation of the healing tissue and remodelling taking place is essential for a more complete understanding of the revitalisation or repair process.

In Sheep-3 the infected experimental tooth developed a periapical abscess which was confirmed radiographically. The infection was very severe and required an additional disinfection session. The inducement of bleeding during the revitalisation session in the experimental tooth was slightly difficult, probably due to the granulation tissue that had formed at the apical area. Similar experiences have been reported in previous research (1, 211, 212). Six months after treatment, the radiographic results showed changes in the teeth diameters but less so than in Sheep-1 and 2. Similar findings have been reported in cases which have pulp infections associated with periapical lesions, which were treated successful revitalisation treatments, but no increase in the root length after treatment (1, 17, 212, 289). The histology of the experimental tooth of Sheep-3 showed healing tissue

composed of cellular cementum-like and cellular mix cementum-like hard tissues. Similar histological results have been reported for human teeth with a similar history before revitalisation treatment (23, 347). Because of the severity of the infection of the Sheep-3 experimental tooth compared to Sheep-1 and 2, further damage to the pulp tissue and the epithelial root sheath would be expected, explaining the small increase in root length. In Sheep-3, the increase of the root length after treatment on determined radiographically was (1.54 mm) and in the histological images 1.3 mm of the apical part of the root consisted of a cellular cementum-like structure, suggesting that most of the length increase was achieved by cementum deposition.

The difference in the infection duration is a likely the cause for the difference in the healing tissue of Sheep-3 experimental tooth compared to Sheep-1 and 2. In Sheep-3, the infection persisted for two months which caused severe damage to the pulp and periapical tissues. Dental pulp tissues and apical papilla are unlikely to survive after such severe and long infection. A possible source of stem cells would be the periodontal tissue or periapical stem cells which could migrate into the root canal under the effect of a variety of signalling molecules from the blood clot scaffold and proteins at the exposed dentinal walls (404-407). Thus, the quality and the quantity of the available stem cells and the time factor may be the reason for observing a less mature healing region of Sheep-3 than in Sheep-1 and 2 experimental teeth. However, in spite of the severity of the infection, when a suitable environment was provided, the revitalisation process started and no signs of moderate or severe inflammation were seen in the pulp canal six months after treatment. All inflammation had resolved and only healthy fibrovascular tissue occupied the pulp cavity. The healing process seems to have started at the apical area and progressed coronal and if this tooth had been left for a longer time, further tissue maturation and remodelling could possibly have been seen.

In Sheep-4, the infected experimental tooth showed no apical radiolucency on radiograph after the first session, but the infection was confirmed clinically before the disinfection session. At the third session, radiographs showed irregularities on the intra canal wall. On clinical examination, there was no periapical swelling on the experimental tooth side. Intra canal examination did not show any signs of persistence of infection. However, there was a resistance to the irrigation needles and the endodontic file when introduced through the apex to mechanically irritate apical tissue to stimulate bleeding in the canal. It was difficult to stimulate bleeding in this tooth which was similar to Sheep-3. Six months later, radiographic results showed signs of internal resorption, narrower apex, and hardly any root lengthening, suggesting a substantial previous infection. These results were further confirmed by CT scans. A clinical case with resorption, treated with a revitalisation protocol showed hardly any further development of the root before 18 months postoperatively (408). Histology results of the Sheep-4 experimental tooth showed a small amount of the first healing region coronally and the third region (a) and (b) extended into most of the root. The reason for the appearance of stage three (a) and (b) in this case is possibly due to the assault of the pulp by infection and trauma at the infection session, leading to the stimulation of the local and differentiated odontoblasts to deposit reparative or secondary dentine (56). It has been reported that trauma or infection associated with the damage to the odontoblast layer and pre-dentine leads to the stimulation of resorption (409-412). The irrigation and disinfection protocols cleaned the canal dentinal walls from bacterial infection but also caused demineralisation of the superficial dentinal wall (413). The absence of infection and the presence of unmineralised dentine layer are an unfavourable environment for clastic cell attachment (410). On the other hand, the exposed signalling protein molecules in dentine could have attracted stem cells from periapical tissues (310, 407). The migrant stem cells could then have differentiated into hard tissue blast cells and deposited a layer of cementum-like hard tissue which filled resorption sites.

In Sheep-4 the histology sections showed signs of resorption at the root apex. The inflammatory resorption at the apical area possibly damaged the HERS and APSCs, which affected the development of the root. Thus, PDL stem cells and periapical cells were the only remaining source of stem cells to repair and support development of this tooth. This explains why the healing hard tissue was more cellular and mixed cementum-like in this animal compared to Sheep-1 and 2. Mixed cementum has been found at root resorption and fracture areas as a reparative cementum (58, 74). Intra canal bone-like island at the apical part of the root was observed in the histology section of Sheep-4 only. Similar histology findings were seen in ferret teeth which had had a problem with internal resorption after revitalisation treatment (350). In Sheep-4 there were no inflammatory cells seen in the pulp. All inflammation had resolved and only healthy fibrovascular tissue occupied the pulp cavity. Although, the inflammation in this animal was severe compared to Sheep-1 and 2, it was inside the root canal and for a shorter period than in Sheep-3. This could have accelerated the healing process and resulted in more mature regions after six months of treatment compared to the other experimental animals. The healing tissue structure after a longer evaluation period requires further investigations.

4.6.1 Research findings and future research direction

Endodontic revitalisation treatment in infected immature sheep teeth showed positive outcomes and provided valuable information about the revitalised healing tissues. However; whether these newly formed tissues can support the affected teeth and respond positively to various bacterial and traumatic assaults, needs further investigation. It is uncertain whether the long term prognosis of tissue maturation and remodelling will lead into total canal obliteration with calcified tissue or not. The type of hard tissue deposited, whether it is dentine, cementum, or a new kind of hard tissue, needs further investigation.

Various animal endodontic revitalisation or regeneration research models show development of hard tissues inside the root canal, similar to our findings, but with less organisation and maturation (131, 350, 414). Zhang *et al*. (414) Wang *et al.* (21) and Tawfik *et al*. (130) reported development of cementum-like tissues lining the canal walls after an endodontic revitalisation protocol in dog teeth, while other studies (131-133) claimed development of both cementum-like and bone-like tissues in the same model. Recently Torabinejad *et al.* (350) speculated that hard tissues developed on root canal walls in a ferret model were bone-like tissue (350). The differences in the outcomes of almost all previous research could be a result of using different animal models, teeth at different developmental stages, and the severity and length of pulpal and periapical tissue infection damage before treatment. A standardised model is needed. Furthermore, lack of specific antibodies for dentine, cementum and bone for each animal model make it hard to identify the nature of healing tissue using immunohistochemistry (17, 350). DSPP is a marker for odontoblasts which increases significantly during their differentiation and during the primary dentinogenesis processes (415-417). There are no sheep specific DSPP antibodies available on the market. We tried a polyclonal (anti-human, rat and mouse) antibody on sheep teeth (**7.1.2.2.2.4 Appendix**) but unfortunately it did not bind to the protein in sheep. Yamauchi *et al*. (109) studied the presence of DSSP and bone sialoprotein (BSP) in the mineralised healing tissue after revitalisation protocols in dogs' teeth. They reported that the newly formed hard tissue was different from dentine and bone, but had some similarities to cementum. The histology after treatment showed the presence of Sharpey's fibre like projections on the deposited hard tissues, similar to our observation in Sheep-4. Future immunohistochemical studies are essential for identifying the nature of the healing hard tissues after different stages of pulp and periapical tissues infection to establish whether the tissues are dentine, cementum, bone or a new type of hard tissue (350).

Sheep anterior teeth are single-rooted teeth with large canals making them a suitable model for various endodontic revitalisation investigations. The present small study showed promising outcomes for the use of sheep in revitalisation studies with similar outcomes as reported in human studies (23, 347). Further investigations at longer evaluation periods might improve our understanding of revitalisation treatment outcomes and could help develop clinical guideline.

4.7 Conclusion

Endodontic revitalisation protocol using a two-tooth age sheep model showed a positive outcome with narrowing of the apices, increased canal wall thickness, and root lengthening similar to the findings of human case reports. Histology analysis of the healing tissues showed development of three or four revitalised healing regions, which likely progressed from the apical to the coronal portion of the root.

Chapter 5. Simple Protocol for Platelet Rich Plasma Scaffold Preparation and Its Effect on Dental Pulp Cells.

5.1 Introduction

A three-dimensional scaffold is one of the essential elements that contribute to the success of a revitalisation protocol, as it supports cellular growth, migration and differentiation (12). Blood clots have been used as a scaffold for endodontic revitalisation (4, 123, 418), but there are some limitations. Firstly, the difficulty of inducing bleeding, a problem outlined in many case reports (1, 211, 212) and animal studies (350, 414). Secondly, the concentration of growth factors in the blood is limited and after clot formation, erythrocytes may undergo necrosis, which may affect the growth of the healing tissue (215).

In contrast, PRP is an autologous scaffold, alleviating the risks of an immunological reaction or disease transmission and is rich in growth factors (261, 419). It has the additional advantage of containing adhesive glycoproteins, which can react with cell receptors and facilitate cell attachment to the scaffold (420).

The effectiveness of PRP in improving the biological outcomes of regeneration and revitalisation protocols has been tested in many studies (23, 137, 333, 421), and has been used instead of blood clot for endodontic revitalisation cases (23, 265, 422), however there is considerable variations in the outcomes reported (23, 333, 349, 350). This may be due to either the variations in platelet product preparation methods like in preparation of lysate, glue, fibrin membrane, or scaffold, or differences in the platelet concentration in these preparations. Some research has shown that a high concentration of platelets could have an adverse effect on stem cell proliferation and differentiation (264, 423), and that PRP is only effective at specific concentrations (251, 261).

PRP preparation methods proposed by previous *in vitro* research and animal studies involve many steps of blood centrifugation and may also include freeze-thaw cycles to increase concentration of platelets and growth factors. These methods are currently too

complicated for effective clinical use. The protocol for a PRP preparation should be quick, easily applied in the dental clinic, and the scaffold needs to have appropriate concentration of platelets to be effective in improving revitalisation outcomes.

5.2 Aims

The aims of this *in vitro* study was to examine if a platelet rich plasma scaffold prepared using a simple protocol of blood centrifugation is effective in enhancing the proliferation, migration and differentiation activities of cultured ovine dental pulp cells (ODPCs).

5.3 Materials and Methods

5.3.1 Ovine dental pulp cells isolation

Immature incisors were collected from animals humanely sacrificed at the completion of other studies, following approved guidelines set by South Australia Pathology-Animal Ethics Committee (#ST25/12). The pulp tissue was extracted from each tooth through the open apex using a sterile stainless steel endodontic file (K-file size-20 SybronEndo, USA) under sterile conditions in a laminar flow hood. Using a surgical blade, the dental pulp was sectioned into small pieces. The minced pulp was digested in equal volumes of collagenase type I (3 mg/ml) and dispase type II (4 mg/ml) followed by incubation at 37 $^{\circ}$C between 1 and 1.5 hours in the tissue culture incubator, with rigorous shaking every 15 minutes to aid in digestion. Once the dental pulp appeared to be well digested, the tubes were removed from the incubator and the dental pulp suspensions were strained through a Falcon cell strainer (Becton Dickinsons Biosciences, San Jose, CA, USA) to remove the undigested tissues. The cell suspension was then centrifuged at 300 xg for five minutes and the supernatant was aspirated. The cells were resuspended in 7 ml of ODPCs media (10% v/v fetal calf serum, Life Technologies), 2 mM L-Glutamine (Life Technologies), 100 U/ml

penicillin and 100 μg streptomycin (Life Technologies), 1 mM L-ascorbate-2-phosphate (Novachem, Melbourne, Australia), in alpha modification of Eagle's medium (α-MEM) (Sigma-Aldrich, ST Louis, MO, USA) and seeded into a 25 cm^2 flask. The cultured pulp cells were incubated at 37 °C with 5% CO_2 for at least five days. Once ODPCs reached 50% confluence, half of the medium was aspirated and replaced with fresh medium. When ODPCs were 70 - 100% confluence, cells were harvested using 0.25% w/v trypsin and 0.0038% w/v EDTA in phosphate-buffered saline (PBS) (Life Technologies), washed with ODPC medium and seeded into a 75 cm^2 flask and incubated at 37 °C with 5% CO_2. The cells were stored in liquid nitrogen at -150°C (**Appendix 7.7**) and passaged two to five times before being used in experiments.

5.3.2 Sheep dentine discs preparation

Dentine discs were prepared from freshly extracted first incisors teeth (two-tooth age). After removal of the pulp tissue, commencing at the cement-enamel junction (CEJ) the root was sectioned into 1 mm thick discs using a low speed saw (Buehler, USA). The dentine discs were treated with 5.25% NaOCl (Scharlau, Spain) for 30 minutes, washed with sterilised Milli-Q water, washed with 17% EDTA (pH 7.4) (Scharlau, Spain) for 15 minutes. PBS (Sigma-Aldrich, USA) was used for several rinses and a two day soak to remove residual irrigants. The discs were subjected to ultraviolet (UV) light for at least six hours for additional sterilisation. The discs were then kept in α-MEM at 4 °C for up to seven days before commencing experiments.

5.3.3 Platelet rich and platelet poor plasma preparation

Venous blood was collected from healthy two tooth age sheep into 3.5 ml blood tubes containing 3.2% sodium citrate (Greiner Bio-One GmbH, Australia). PRP was prepared following a protocol provided by the Department of Haematology, Institute of Medical and Veterinary Science. The blood was centrifuged at 140 xg for 12 minutes to separate a PRP from the rest of the blood; resulting in PRP concentration of 150-270 K platelets/µl. The rest of the blood was centrifuged again at 2600 xg for 20 minutes to prepare platelet poor plasma (PPP) at a concentration of 3-5 K platelets/µl. To prevent infection, 100 U/ml penicillin and 100 µg/ml streptomycin was added to the prepared PRP and PPP and the plasma was subjected to UV light for 20 minutes for further sterilisation. To achieve clotting of PRP or PPP, 10 µl of $CaCl_2$ (100 mmol/L) (VWR International, Leuven, Belgium) was added to each 90 µl of PRP or PPP, and the scaffolds were then incubated for at 37 $^{\circ}$C for 20 minutes to allow clotting.

5.3.4 Ovine dental pulp cell proliferation assay

ODPC proliferation rate was evaluated by culturing ODPCs on PRP and PPP scaffolds (75 µl / well) or without a scaffold (control group) in 48-well plates. Each of the experimental and control groups included twelve wells, with six of them containing a single dentine disc each. For background correction, scaffolds (PRP or PPP) were added to two wells (no cells) and with the addition of dentine discs to another two wells (no cells) (**Figure 5.1**). ODPCs were seeded at a concentration 1 x 10^3 cells/well. ODPC medium (400 µl) was added to each well. The cells were cultured at 37 $^{\circ}$C with 5% CO_2 for one or five days without changing the medium.

Proliferation rates of the cultures were analysed after one and five days using a WST-1 cell Proliferation Assay Kit (Clontech Laboratories, CA, USA), as per the manufacturer's

instructions, 40 µl of premixed WST-1 cell proliferation reagent was added for each well. The plate was incubated for four hours at 37 °C in a humidified atmosphere with 5% CO_2. Medium (100 µl) was drawn from each well and transferred to 96-well plate. The absorbance level was measured at 490 nm using a multiwall plate reader spectrophotometer (Eppendorf, Hamburg, Germany).

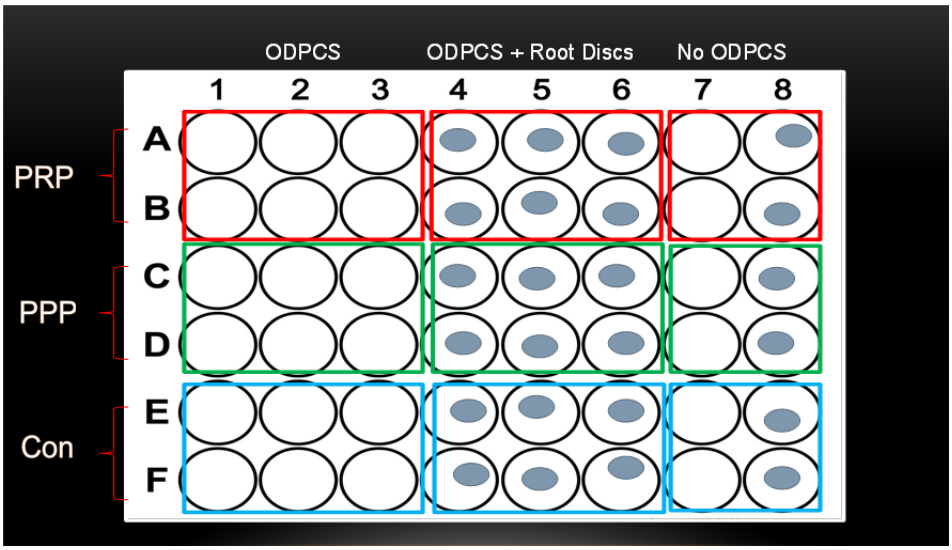


Figure 5.1 ODPC proliferation assay, experiment design.

5.3.5 Migration and distribution assays

5.3.5.1 Ovine dental pulp cell migration assay

The migration assay used for this study was based on the "Boyden Chamber Assay" (424) with some modifications. The time course to evaluate cell migration was chosen based on pilot studies which showed that minimal numbers of cells migrated through the external surface of the diaphragms to the scaffolds was after one day of seeding the cells into the transwell inserts.

Scaffolds PRP and PPP were placed in 12-well plates (150 µl / well). Collagen I (bovine protein, Life Technology, Australia) was used for the control group. Each group included six replicates; three of them with three dentine discs each. Transwell inserts were placed on the top of the wells, and ODPCs at a concentration 1.5 x 10^3 cells/well were seeded into

the transwell inserts (**Figure 5.2)**. After one day the cells in the transwell inserts were washed twice with PBS, fixed with 10% neutral buffered formalin (Australian Biostain) at room temperature for five minutes, and washed twice with Milli-Q water. In order to stain the cells, they were permeabilized using 100% methanol at room temperature for 20 minutes, washed twice with Milli-Q water, stained with haematoxylin for seven minutes, and washed with water for one minute, before the non-migrated cells inside the transwell inserts were scraped with a cotton swab. The transwells were dipped in 0.5% hydrochloric acid, washed twice in water, dipped in dilute alkaline (1% $NaHCO_3$), washed in running water for one minute then stained with eosin for 30 seconds. The diaphragms of the transwells were cut out and mounted on slides with aqueous mounting medium (chrome glycerine jelly). The number of migrant cells on the external surface of the diaphragm was counted using a stereomicroscope (Olympus BX51, Leica, Germany).

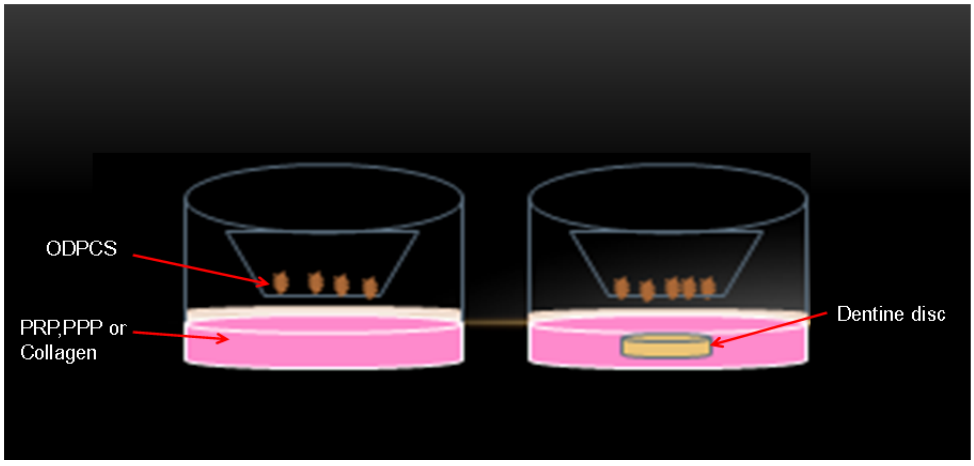


Figure 5.2 Migration assay, experiment design.

5.3.5.2 Ovine dental pulp cell distribution

Scaffolds (PPP, PRP, or collagen I (150 µl / well)), were added in transwell inserts (BD Falcon) (0.8 µm pore size). ODPCs were seed at a concentration 1 x 10^3 cells/well on the scaffolds. The transwells were placed in 12-well plates (**Figure 5.3**). Each group included six wells; three of them with three dentine discs each at the bottom of each well. After one

or five days of culturing, the scaffolds in the transwell inserts were rinsed three times with PBS and fixed in acetone and methanol (1:1 v/v) for five minute, washed with Milli-Q water five times before they were stained with nuclear staining 4',6-diamidino-2 phenlidole (ProLong® Gold Antifade Reagent with DAPI, Life Technologies, Australia). Cell distribution through the scaffold was observed using a spectral scanning confocal microscope (Leica TCS SP5, Wetzlar, Germany) (425). The numbers of ODPCs were measured at two randomly selected areas of each scaffold. At these areas, the measurement was taken every 50 µm until the diaphragm depth of 300 µm. For each 50 µm increment measurement, three scans were counted, the top, the middle and the bottom images, and the average cell number was recorded.

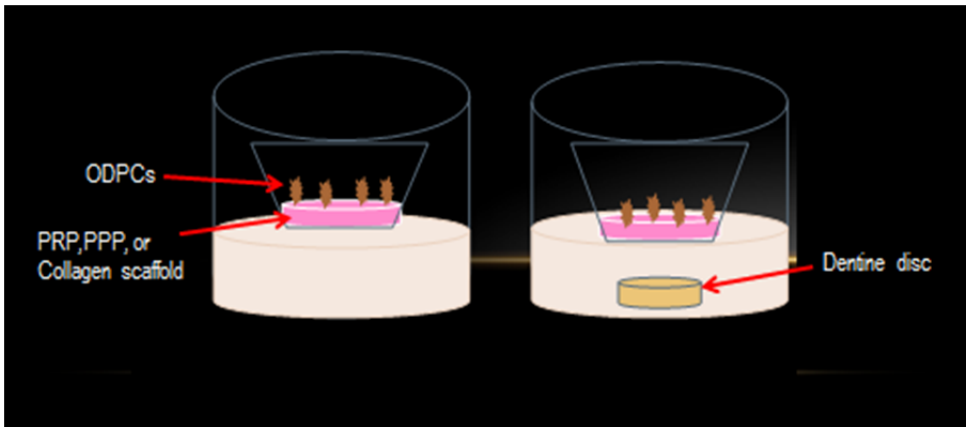


Figure 5.3 Cell distribution assay, experiment design

5.3.6 Ovine dental pulp cell differentiation

To assess *in vitro* differentiation of ODPCs, calcium deposits and alkaline phosphatase (ALP) activity of the ODPCs were evaluated.

5.3.6.1 Mineralisation detection and quantification

ODPCs at a concentration 3 x 10^3 cells/well were seeded on PRP, PPP scaffolds (200 µl / well), or control (without scaffold) (cells used for this assay were from passage three to

five). Each group included twelve wells, six of them with the addition of three dentine discs in transwell inserts (0.8 µm pore size) (Corning, Sigma-Aldrich, USA) (**Figure 5.4**).

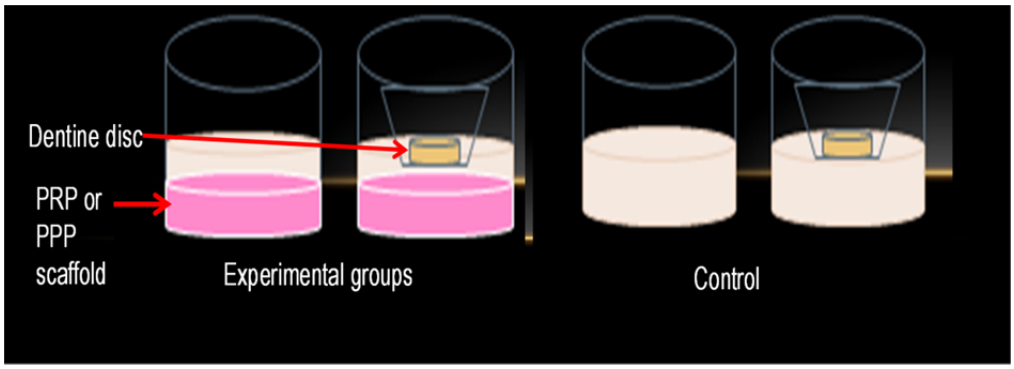


Figure 5.4. Differentiation assay, experiment design.

To enhance calcium deposits, mineralisation induction medium (MIM) was added to half of the replicates (six wells) in each experimental group and other half of the replicated remained in ODPC medium. MIM contained 100 mmol/L dexamethasone phosphate (Hospira, Australia), 2.64 mmol/L KH_2PO_4 (BDH Chemicals) and 10 mmol/L HEPES (4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid)) (Sigma-Aldrich), in addition to the ordinary ODPC medium. The medium was changed twice a week.

After 28 days of culturing, the wells were washed twice with PBS (pH 7.4, 1 ml/well), and fixed with 10% neutral buffered formalin at room temperature for 15 minutes. The fixative was then removed and the wells were rinsed three times for five minutes using Milli-Q water prior to the addition of 1 ml/well of 1% (w/v) Alizarin Red Stain solution (ARS, pH 4.1). The plates were incubated at room temperature for 15 minutes. After aspiration of the dye, the wells were rinsed gently four times with Milli-Q water for five minutes (427), before the wells were left to dry at room temperature for two days. The mineralisation nodules were observed under an inverted microscope (264). For quantitative analysis of mineralisation, ARS dye was extracted from the dry stained cellular matrix with 400 µl of 10% (w/v) cetylpyridinium chloride (CPC, pH 7) in 10 mmol/L sodium phosphate for 15

minutes at room temperature. Then ARS extracts were diluted 1/10 with 10% CPC, and ARS concentration was determined by absorbance measurement at 562 nm on a multiwall plate reader (Eppendorf, Hamburg, Germany) (427).

5.3.6.2 Alkaline phosphatase detection

ODPCs at a concentration 3 x 10^3 cells/well were seeded on PRP, PPP scaffolds (200 µl/well), or control (without scaffold). Each group included six wells, three of them with the addition of three dentine discs in transwell inserts (0.8 µm pore size) (Corning, Sigma-Aldrich, USA) (**Figure 5.4**).

ALP is a protein formed by the cells at the stage of mineralised tissue deposition. ALP activity was assayed in this study using an ALP assay kit (AnaSpec, CA, USA). Briefly, after 14 days of culturing the wells were washed twice with the assay buffer, and lysed with 0.1% v/v Triton X-100 (1 ml/well). The contents of each well were scraped and collected in a 1.5 ml microfuge tube, and sonicated using a Soniprobe (Dawe Instruments Ltd., London, UK) in an ice cold bath, three times for 10 seconds each. The tubes were then centrifuged at 10,000 xg for 20 minutes at 4 ˚C. The supernatant (50 µl) was added to 50 µl colorimetric ALP substrate, *p*-nitrophenylphosphate (*p*NPP), in a 96-well plate and incubated for 30 minutes at 37 ˚C. 50 µl of the stop solution was added into each well, and the absorbance was recorded at 405 nm using a multiwall plate reader spectrophotometer (Eppendorf, Hamburg, Germany) (264, 428).

5.3.7 Ovine dental pulp cell attachment to dentine

Root segments were prepared from scavenged sheep incisors (two-tooth age). The pulp tissue was removed. The apical 6 mm of the roots were cleaned following the same protocol as for preparing the dentine discs. ODPCs at a concentration of 5 x 10^3 cells were mixed with 50 µl of each scaffold (PRP or PPP) and then injected into each root tip. The scaffold in the root was allowed to clot in a 24-well plate at 37°C for 15 minutes. ODPCs medium (500 µl) was added to each well. The medium was changed every two days.

After 28 days, the roots were washed with PBS, fixed with 10% neutral buffered formalin for two days, followed by decalcified in 10% EDTA (Australian Biostain, VIC, Australia). The specimens were then embedded in paraffin wax (112), and sections (7 µm thick) were prepared and stained with hematoxylin and eosin (**7.3 and 7.4 Appendices).**

5.3.8 Statistical analysis

All experiments were performed at least in triplicate. Each value represents the mean ± standard deviation (SD). The data were analysed using a two-way ANOVA followed by Tukey's multiple comparisons test. Statistical significance was set at $p < 0.05$. All statistical analyses were performed using Prism 6 Statistics software (GraphPad Software Inc., San Diego, CA, USA).

5.4 Results

5.4.1 Ovine dental pulp cell proliferation assay

ODPC proliferation was evaluated with a WST-1 assay. The results showed that cell numbers increased significantly between day one and day five for all the groups (**Figure 5.5A and B**). There were no significant differences in cell proliferation rates between groups at day one. At day five, ODPCs cultured on PRP and PPP scaffolds showed

significantly higher proliferation rates compared to the control group (no scaffold) ($p < 0.0001$ and $p < 0.0017$, respectively). ODPC proliferation rate was also significantly higher on PRP scaffolds than on PPP scaffolds ($p < 0.0001$). Adding dentine discs to both PRP and PPP scaffolds significantly reduced the proliferation rate of the cells ($p < 0.0001$).

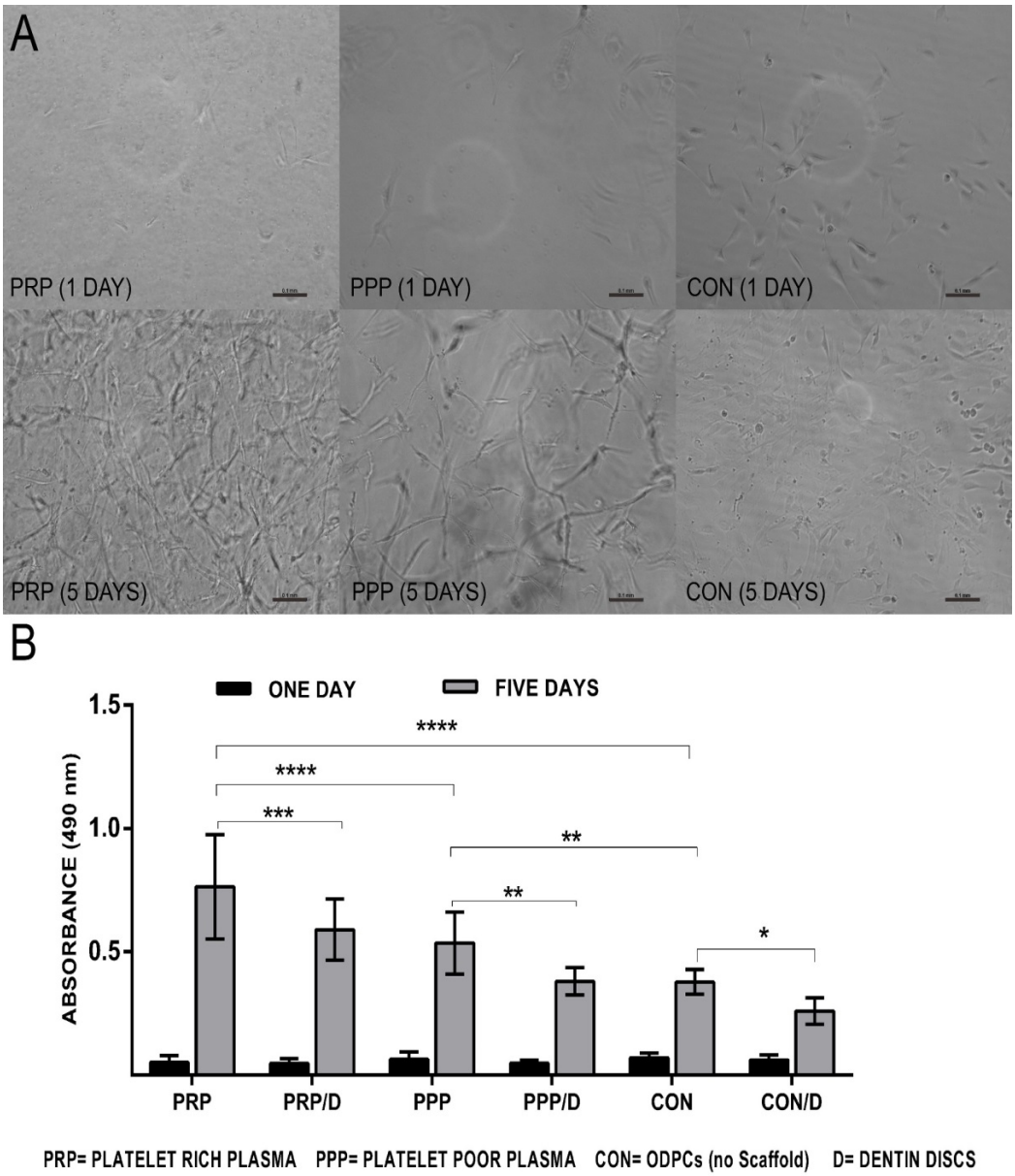


Figure 5.5 ODPC proliferation on PRP, PPP scaffolds and control (no scaffold).

A: Inverted light microscope images of ODPC proliferation is shown one day (top images), five days (bottom images) after seeding, scale bar=0.1mm.

B: Measurement of ODPC proliferation was measured using a WST-1 assay after one or five days of culturing.

The results are presented as means ± SD of three independent experiments.*$p < 0.05$, **$p < 0.01$, ***$p = 0.0004$, ****$p < 0.00001$.

5.4.2 Migration and distribution assays

5.4.2.1 Ovine dental pulp cell migration assay

Following one day of culturing in transwell inserts, the number of ODPCs that migrated toward PRP and PPP scaffolds was significantly higher than the control group (collagen scaffold ($p = 0.0001$, $p = 0.0002$). The number of cells migrating toward PRP was significantly higher than for PPP ($p = 0.02$) **(Figure 5.6)**. The effect of the addition of dentine discs on the migration activity of the experimental and control groups was not statistically significant ($p = 0.109$).

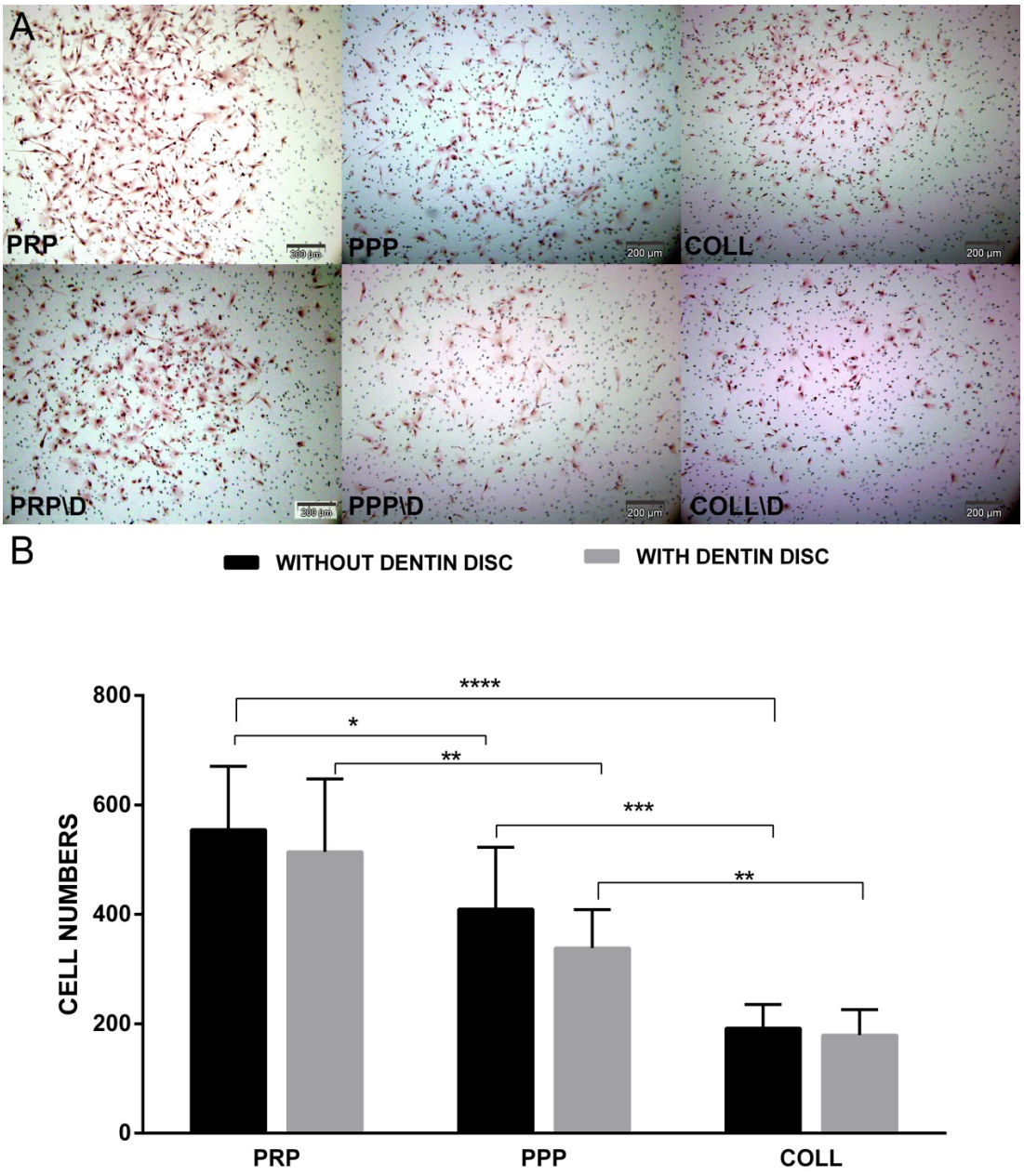


Figure 5.6. Scaffold type and root effect on ODPCs migration

A: ODPCs migration to each scaffold (top images) without root dentine discs, (bottom images) with root dentine discs. Scale bar =200µm.

B: Number of migrated ODPCs.

Results presented as means ± SD of three independent experiments. *p < 0.05, **p < 0.01, ***p = 0.0002, ****p < 0.00001.

5.4.2.2 Ovine dental pulp cell distribution assay

The results showed that at day five, more cells were distributed within all the scaffolds (**Figure 5.7)** compared to day one (**Figure 5.8)**. PRP and PPP scaffolds recruited more cells than the control (collagen scaffold). The presence of dentine discs showed no significant effect on cells distribution.

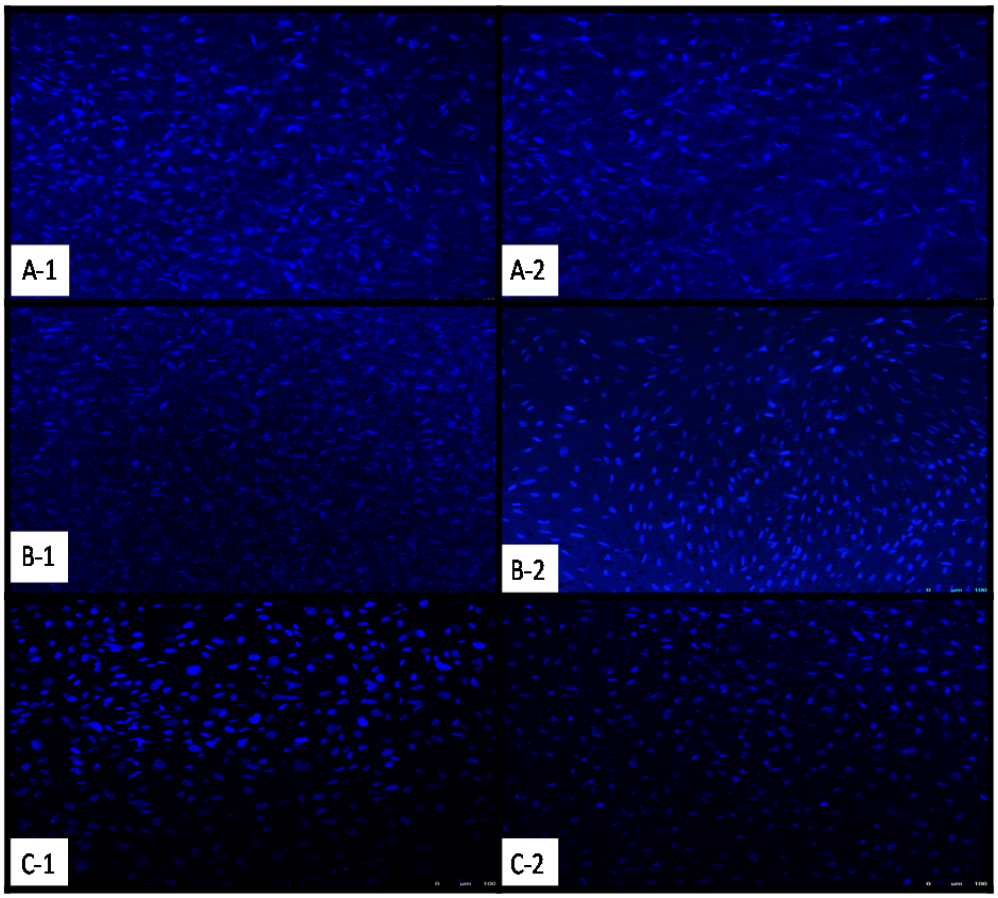


Figure 5.7 ODPCs distribution areas with the highest concentration of cells at day five.

A1: PRP scaffold, A2: PRP scaffold and dentine discs.

B1: PPP scaffold, B2: PPP scaffold and dentine discs.

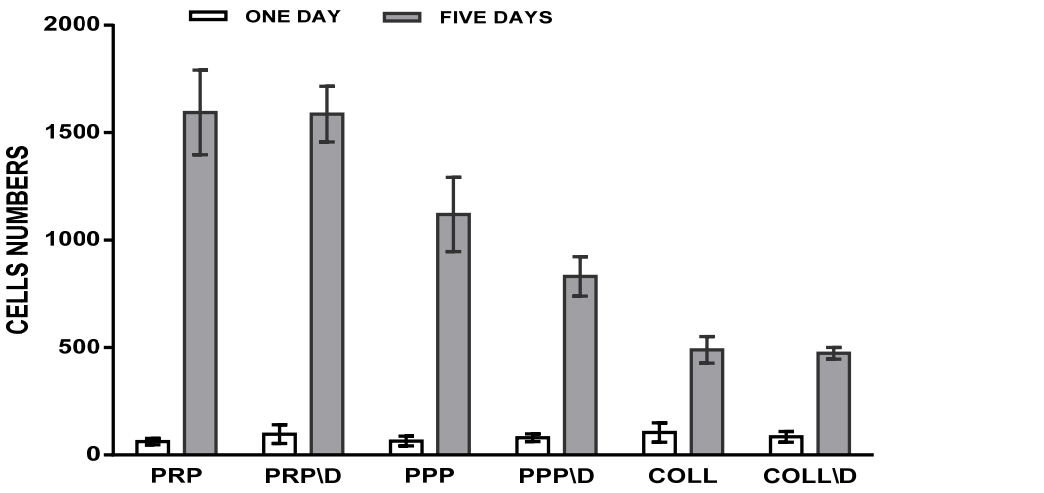
C1: Collagen scaffold, C2: Collagen and dentine discs.

-A-	PRP	PRP/D	PPP	PPP/D	COLL	COLL/D
50µm	*	*	*	*	*	*
100µm	*	**	*	**	**	*
150µm						
200µm						
250µm						
300µm						

-B-	PRP	PRP/D	PPP	PPP/D	COLL	COLL/D
50µm	*	*	*	*	*	*
100µm	***	***	***	**	******	*****
150µm	****	*****	***	**	*****	*****
200µm	*******	******	*****	***	*	*
250µm	*******	*******	******	****		
300µm	*******	*******	*******	*******		

CN:CELLS NUMBER **(50<CN≤100) ****(150<CN≤200) ******(250<CN≤300)

*(CN≤ 50) ***(100<CN≤150) *****(200<CN≤250) *******(CN> 300)



-C-

Figure 5.8 ODPCs distribution (300µm) for each scaffold observed by confocal microscope.

A: ODPCs distribution after one day.

B: ODPCs distribution after five days.

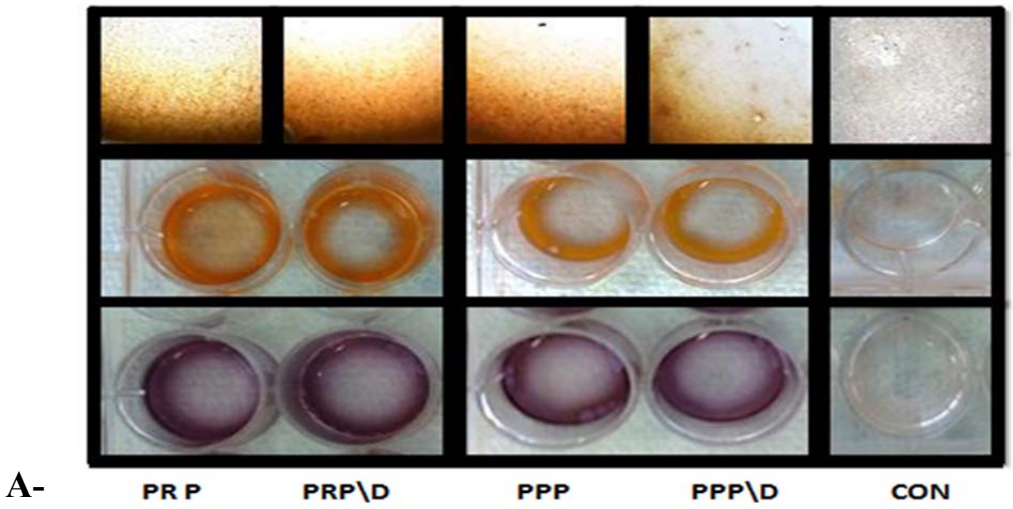
C: Average number of ODPCs in each scaffold.

Results presented as means ± SD of three independent experiments.

5.4.3 Ovine dental pulp cell differentiation

5.4.3.1 Mineralisation detection and quantification

ODPCs cultured on both PRP and PPP scaffold with and without MIM had a significant higher mineralising activity than the control group (without scaffold) ($p < 0.0001$). Cells cultured on PRP scaffolds showed significantly higher mineralisation activity than cells cultured on PPP scaffolds and the control group, with and without MIM (**Figure 5.9 and Figure 5.10**). The addition of MIM to the experimental and the control groups significantly increased the mineralisation activity of the cells. The addition of root dentine discs to PRP scaffold with and without MIM significantly reduced mineralised nodule formation of the cells.



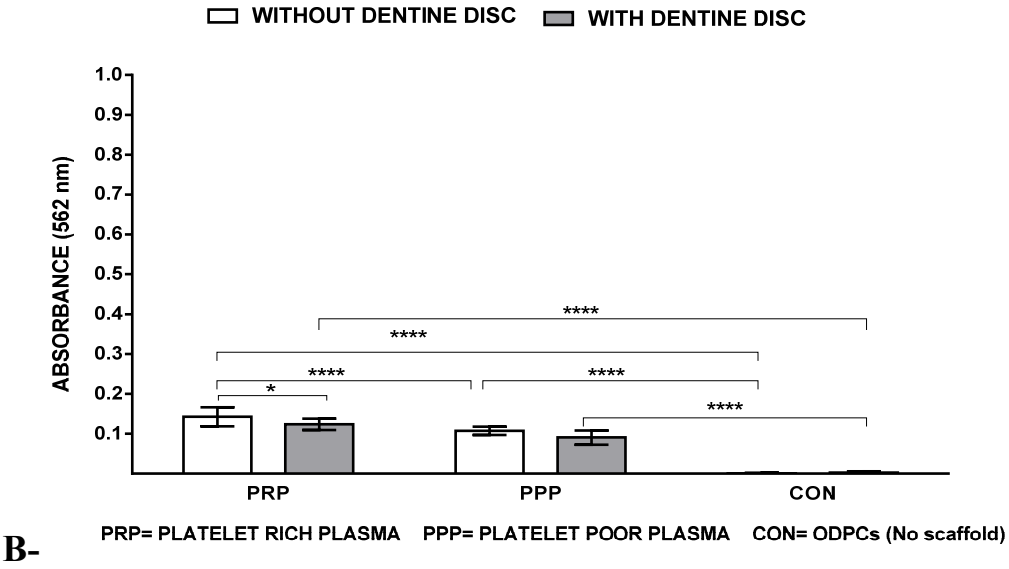
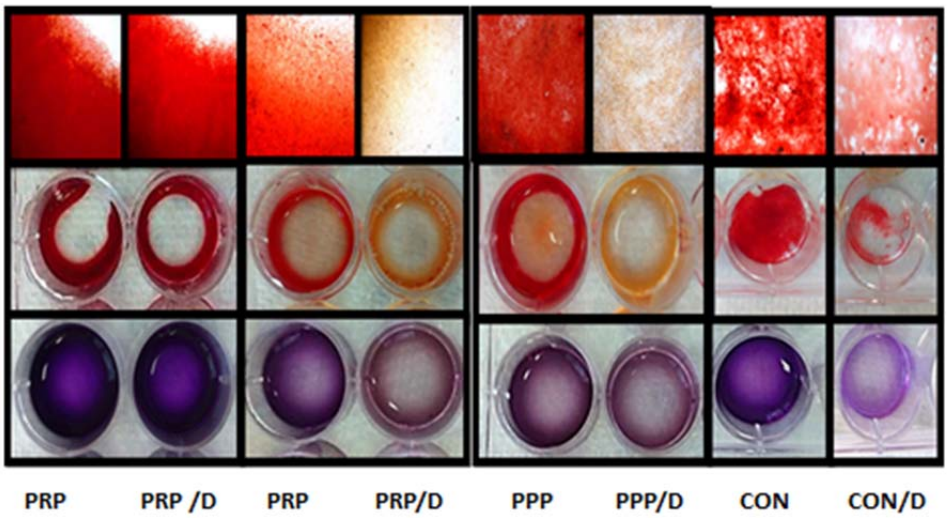


Figure 5.9 Qualitative and quantitative analysis of mineralised nodules formation by ODPCs cultured with and without scaffold.

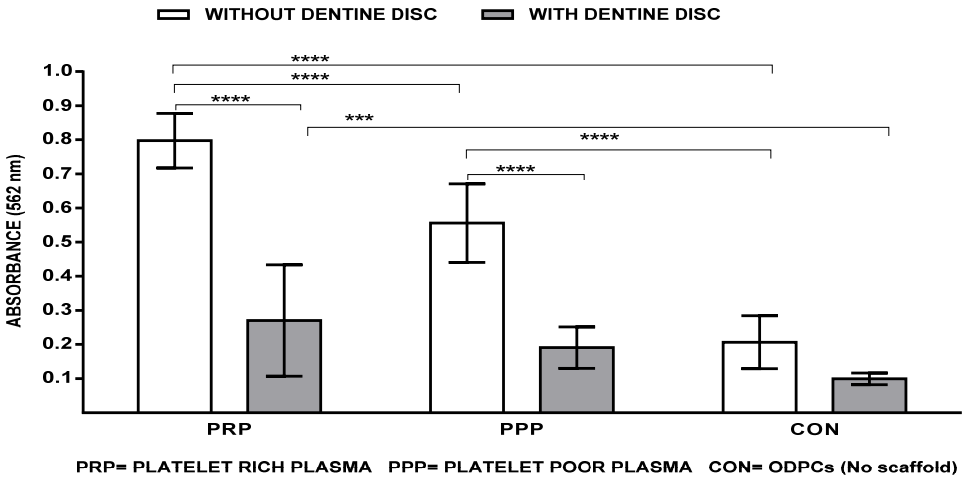
A: First and second rows are images of ARS stained matrix. The third row demonstrates the wells after extraction of the stain using the CPC method. Images shown are without the addition of MIM.

B: Results of staining quantification by measurement the absorbance at 562 nm, corresponding to amount of mineralisation nodules deposited in the experimental and control groups.

Results shown are without the addition of MIM. Means ± SD of four independent experiments are shown. (*p = 0.023, ****p < 0.0001).



A-



B-

Figure 5.10 Qualitative and quantitative analysis of mineralised matrix formation after adding of mineralisation induction medium to ODPCs.

A: First and second rows are images of ARS stained matrix. The third row demonstrates the wells after extraction of the stain using the CPC method. Images shown are with the addition of MIM.

B: Results of staining quantification by measurement the absorbance at 562 nm, corresponding to amount of mineralisation nodules deposited in the experimental and control groups.

Results shown are with the addition of MIM. Means ± SD of four independent experiments are shown.

***p = 0.0007, ****p < 0.0001.

5.4.3.2 Alkaline phosphatase activity

ODPCs cultured on PRP and PPP scaffolds showed significantly higher ALP activity than the control groups (without scaffold) ($p < 0.0001$). APL activity of the cells cultured on a PRP scaffold was also significantly higher than cells cultured on a PPP scaffold ($p = 0.018$). Addition of dentine discs to the experimental and control groups showed no statistically significant effect (**Figure 5.11)**.

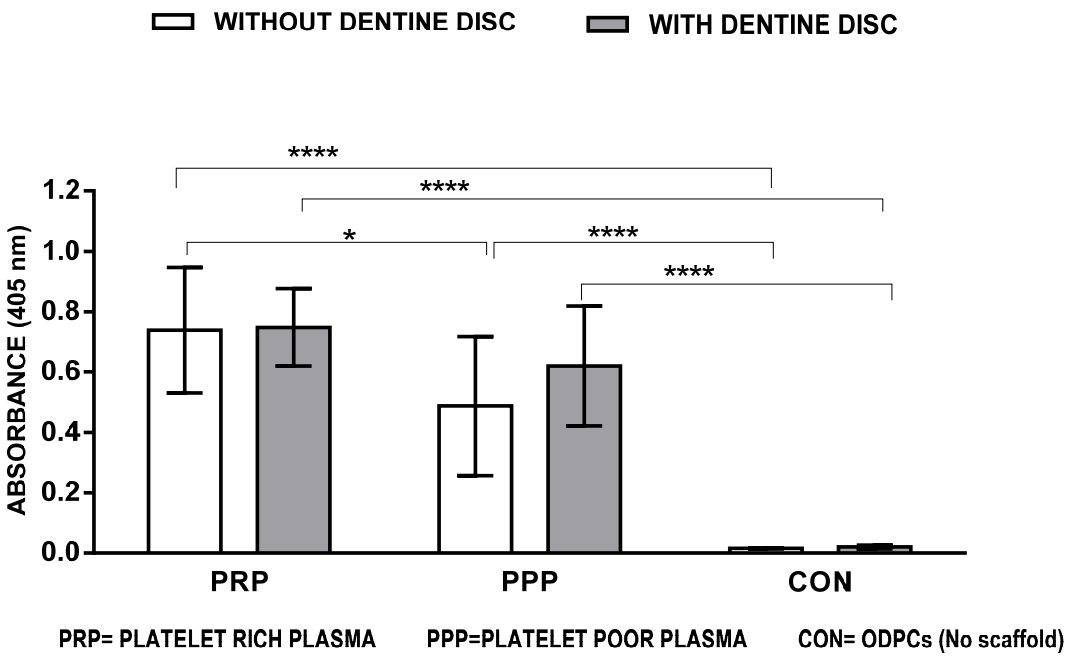


Figure 5.11. ALP activity of ODPCs cultured on PRP and PPP scaffolds with and without root dentine discs.

The chart shows the absorbance at 405 nm, corresponding to ALP activity for the experimental and control groups. Means ± SD of three independent experiments are shown.*$p = 0.018$, ****$p < 0.0001$.

5.4.4 Ovine dental pulp cell attachment to dentine

Culturing of ODPCs for 28 days on PRP and PPP scaffolds in root segments showed movement of the cells in both scaffold types, towards the dentine walls. It appeared from the histology that more cells attached to the apical part of the root than to the coronal part in both scaffolds. It also appeared that on PRP scaffold (**Figure 5.12).** There was a greater

number and better attachment of the cells to the dentinal walls compared with PPP (**Figure 5.13)**.

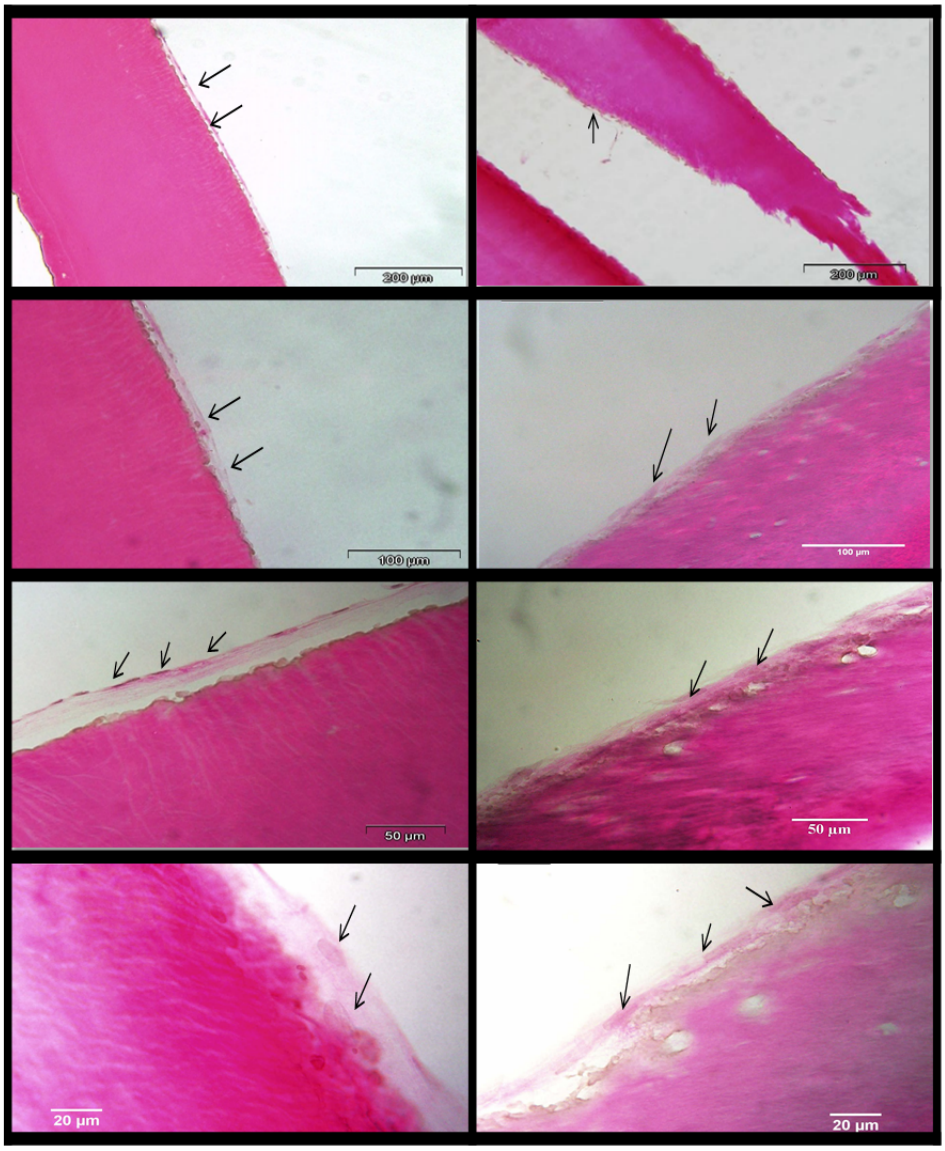


Figure 5.12 Histology sections of ODPCs cultured on PRP scaffold.

First row images were taken at magnification x 20, second row at x 40, third at x 60 and the fourth row at x 100. Images on the left side are from the coronal part of the root, on the right side from the apical part. Black arrows indicate ODPCs.

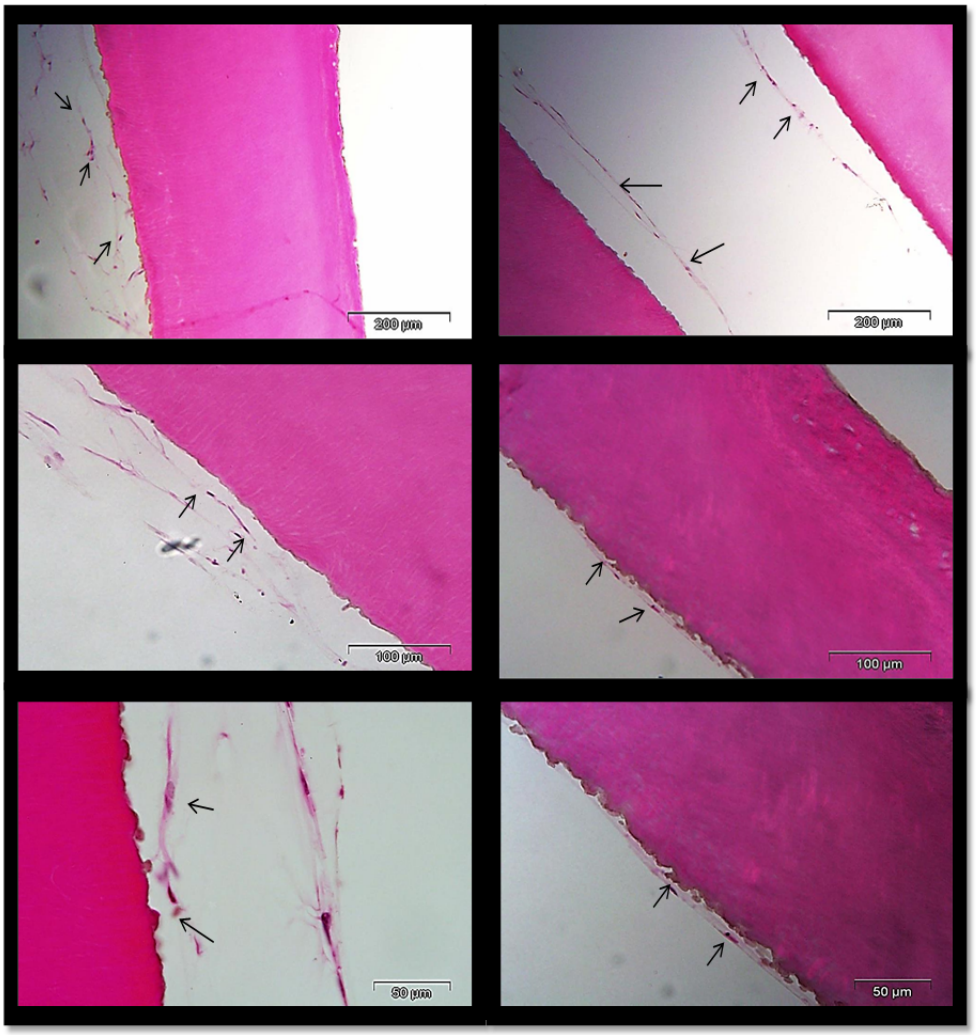


Figure 5.13 Histology sections of ODPCs cultured on PPP scaffold.

First row images were taken at magnification x 20, second row at x 40, and third row at x 60. Images on the left side are from the coronal part of the root, and images on the right side are from the apical part of the root. Black arrows indicate ODPCs.

5.5 Discussion

This study investigated if a platelet rich plasma scaffold prepared using a simple protocol of blood centrifugation is effective in enhancing the proliferation, migration and differentiation activities of cultured ovine dental pulp cells.

In the first experiments, the effectiveness of PRP and PPP scaffolds on proliferation of ODPCs was evaluated. Our data showed that both scaffolds significantly improved the ODPCs proliferation rate compared to cells cultured without a scaffold. It also showed that a PRP scaffold has a better stimulation effect on cells proliferation than a PPP scaffold and control (without scaffold). This is likely to be due to platelets releasing a variety of growth factors (261, 419), and this finding is in agreement with Anitua *et al*. (205) and Li *et al*. (256) who tested the effectiveness of PRP on a variety of MSCs (205, 256).

Appropriate concentration of platelet in the scaffold is essential for the scaffold to be effective and a high concentration of platelets can have adverse effects on stem cell proliferation and differentiation (264, 423). The results showed that the PRP scaffold prepared following the protocol presented at this study, had a suitable concentration of platelet to be effective in enhancing proliferation of cultured ODPCs.

To simulate the clinical environment, root dentine discs were added to each scaffold as well as to the control group. These discs were cleaned following a protocol similar to the clinical endodontic irrigation protocol using NaOCl and EDTA. The addition of dentine reduced the cell proliferation rate, which was also reported by Huang *et al.* (113). In this study, an extensive protocol of washing after the use of each irrigant was applied to reduce the chance for residual of the irrigant to remain in the dentine. Therefore, the adverse effect of the addition of dentine discs seen in this study is possibly related either to the type and concentration of the irrigation materials used to clean the dentine or the alteration of dentine structure after cleaning affected the ODPCs. Trevino *et al.* (298) evaluated the

effect of different combinations and concentrations of root canal irrigants on the survival of apical papilla stem cells on a PRP scaffold, and found that irrigation with 17% EDTA supported cell survival significantly more than irrigation with 6% NaOCl and 17% EDTA. The effect of intracanal irrigations and medicaments on the revitalisation outcomes requires further investigation *in vivo* and *in vitro*.

In the second part of this study, the effect of the scaffold on the stimulation of cell migration and distribution was evaluated. A collagen scaffold was selected for the control group, as it is the most commonly used scaffold for regeneration research (107, 429) and has previously been used in migration assays (425, 430). The present findings showed that PRP and PPP scaffolds could actively recruit more cells than a collagen scaffold, which could be due to the existence of a rich and diverse cocktail of signalling molecules like transforming growth factor-β, fibroblast growth factor, vascular endothelial growth factor and insulin-like growth factor I in the PRP scaffold (250, 251) that are known to significantly increase the cell migration rate (425, 431, 432). Similar findings were reported by Li *et al.* (256) who found that PR fibrin scaffolds induced migration of dental follicle progenitor cells more than PPP. The cell distribution assay also showed that PRP scaffold can actively recruit, support and permit the movement of the ODPCs. One drawback of using PRP and PPP scaffolds to evaluate the distribution of the cells is the expansion of these scaffolds that take place after setting, which is higher than seen with the collagen scaffolds. Therefore, statistical analysis of variance in cell distribution between these groups was not considered, but no cells migrated all the way down to the diaphragm in the collagen scaffold, while a large number of cells were seen at this level for both the PRP and PPP scaffold.

To investigate whether PRP and PPP scaffolds can enhance the mineralisation activity of ODPCs, we performed an ALP activity assay and used ARS to determine cellular matrix

mineralisation. Our results showed that PRP scaffold has a significant effect on the ALP activity of the cells when compared to the PPP and control groups (without MIM). Lee *et al.* (264) used PRP as a supplement with MIM added to the cell culture and showed that ALP activity of human dental pulp stem cells was significantly higher in the group treated with PRP (concentration 10630 platelets/µl) compared to the control group (without PRP). Kawase *et al*.(262) found PRP supplement failed to increase ALP activities of PDLSCs cultured with the addition of MIM that lacked dexamethasone, while, Li *et al*. (256) demonstrated that PR fibrin has a similar effect almost like MIM on increasing ALP activities of PDLSCs. The difference in the results of these studies could be related to the differences in PRP preparation protocol and/or ALP analysis methods. Mineralisation was also indicated by the increasing deposition of calcium. This study demonstrates that dental pulp cells cultured on PRP or PPP scaffolds significantly deposited more mineralised nodules compared to the control group. Cells cultured on PRP scaffolds deposited more mineralisation than cells on PPP scaffolds. The addition of MIM enhanced the mineralisation activity of the cells. Our results are supported by research by Li *et al.* (256) who found that dental follicle cells cultured on PR fibrin produced more mineralisation nodules than those cultured on PPP. They also are in agreement with Lee *et al.* (264) who reported that DPSCs cultured on PRP without MIM produced little calcium, while adding that MIM treatment of cells cultured on PRP caused a significant increase in the rate of calcium deposition. Similar result was also reported when 5% platelet lysate was added to DPCs cultured with the addition of MIM (433). These results suggested that a PRP scaffold can accelerate the mineralisation activity of dental pulp cells when a suitable mineralisation induction substance like dexamethasone is available. How to address this issue in the clinic requires further investigation *in vitro* and in *vivo*.

The addition of dentine discs to both scaffolds reduced cell mineralisation activity. This effect was more obvious in the groups with MIM than without MIM as mentioned. This

probably related to the effect of irrigants on the dentine matrix which in turn affect cells mineralisation activity. Changes of the irrigation protocol might prevent this effect.

Many case reports have shown that the addition of PRP as a supplement or as a scaffold improves the outcome of the treatment in terms of increasing root length, wall thickness and apical closure (333, 398, 422).

Culturing ODPCs on PRP or PPP scaffold in the pulp cavity of the root segments showed migration of cells toward the dentine wall, which is a characteristic behaviour of differentiated odontoblasts when reparative dentine is developed. Better attachment of the cells to the dentine wall was observed at the apical part of the root compared to coronal. PRP scaffold enhanced the attachment of the cells to the walls. The increase in the number of attached cells may lead to more calcified tissue deposit, which in turn can increase the thickness of root, which is important for treatment of immature teeth as the roots are thin, particularly apically.

Many studies have shown that PR products are only effective in stimulating cell proliferation and enhance differentiation at appropriate concentration (261, 264, 433). Furthermore, the use of only highly concentrated platelets or multiple growth factors without platelets or plasma has failed to stimulate cell proliferation and differentiation *in vitro* (423, 433, 434) or to improve treatment outcomes *in vivo* (137, 201). One reason for this could be that PRP contains other unidentified components which may play important roles in cell proliferation and differentiation (264).

5.6 Conclusion

The results of this study demonstrated that PRP scaffold prepared using a simple protocol of blood centrifugation protocol has similar characteristics to studies used complicated and long or many step protocols.

The response of ODPCs to the experiment conditions presented in this study is similar to the behaviour of human DPSCs subjected to similar experimental conditions in previous studies, which indicates that sheep model is a promising model for revitalisation research.

Chapter 6. Conclusions

6.1 Conclusions

From the results of the studies of the current project, it was concluded that:

1. Sheep mature incisor teeth are similar to human teeth in diameters and histological structure. Sheep teeth apices are different from human teeth by the presences of intermediate dilatation and minor foramen in addition to the major foramen.
2. Evaluation of sheep dental developmental stages from two-tooth to six-tooth ages, showed that:
    a. The use of sheep is a promising and reliable model for revitalisation research with each dental age having advantages and limitations.
    b. Two-tooth age sheep are readily available, small in size, and showed radiographical and histological favourable characters, which make it a more suitable model for endodontic revitalisation research than the other ages.
3. *In vivo* examine of endodontic revitalisation protocol in two-tooth age sheep model found that:
    a. The treatment showed a positive outcome with further development of the root, thickening of the dentine walls and narrowing of the wide open apex.
    b. Histological analysis of healing tissue showed development of three or four healing regions, with the more mature occurring apically and the less mature coronally, suggesting that these tissues developed from the apical area and progressed coronally.
    c. Sheep is a reliable model for endodontic revitalisation research
4. Evaluation of the effectiveness of PRP prepared using a simple protocol of blood centrifugation, as scaffold for revitalisation treatment showed that:

a. PRP scaffold significantly enhanced proliferation, migration, and differentiation activities of ODPCs similar to studies used complicated and long-step protocols.

b. Seeding ODPCs on chemically treated dentine discs with or without scaffold inhibited the activities of the cells.

Chapter 7. Appendices

7.1 Appendix: Pilot studies

7.1.1 Chapter 3 related pilot study

7.1.1.1 Development of a radiograph localizing frame

Using fixed sheep mandibles as a model, a radiograph localising frame was designed to standardise the angulation of the cone and film with respect to the lower anterior teeth.

The anterior segment of sheep mandible is flat and the mandibular floor is very shallow comparing to human mandible (**Figure 7.1**), therefore, it was very difficult to place an intra-oral periapical film in a parallel position to the anterior teeth. Extra-oral radiographs were hard to obtain and not practical because of the upper jaw restricting the position of the x-ray tube. As a result, it was decided to radiograph the anterior teeth using the bisecting technique with the occlusal film resting on the occlusal surface of the teeth.

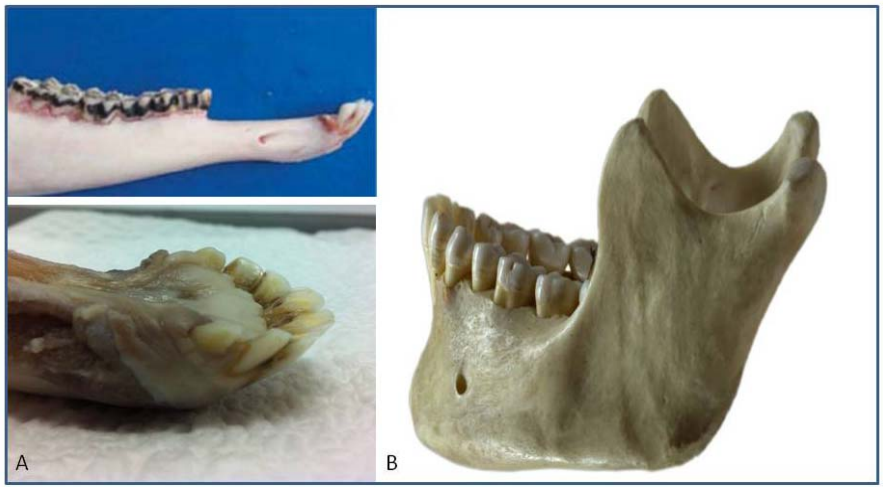


Figure 7.1 Sheep mandibles flat shape (A), human mandible (B), image added for a comparable reason.

A plastic cassette to hold the occlusal film was constructed, to which a holder arm was fixed at 90°. The holder arm (Super-Bite film holder arm) was modified by removing the backing plate. A Super-Bite localizing ring was fixed to the arm at 9.5 cm distance from the film and in a parallel position to the film cassette, as shown in (**Figure 7.2**). The focus to object distance was approximately 35 cm, and the focus to film distance was 39.5 cm.

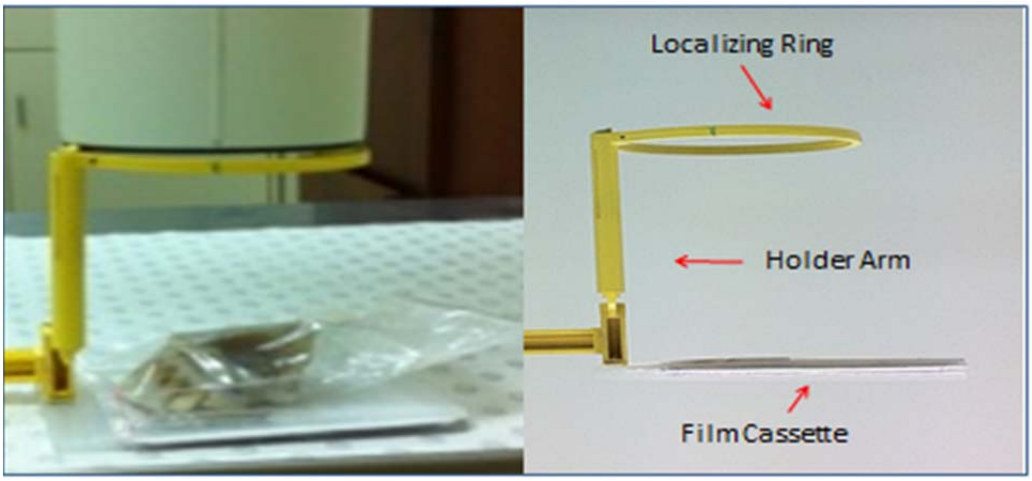


Figure 7.2 Radiograph localising frame.

It was not practical to construct a locating key on the anterior teeth as the sheep had not finishing developing. As a result, it was decided to use TurboReg plug-in within Image J software (Biomedical Imaging Group, Swiss Federal Institute of Technology, Lausanne, VD, Switzerland) to mathematically reduce any dimensional changes in the preoperative or postoperative radiographs as a result of differences in the angulation of the x-ray central beam at the time of image acquisition. This application required three fixed landmarks on the source image (preoperative) and the target image (postoperative) to mathematically align them, as described previously by Bose *et al*. (325). The landmarks previously used are: root apex; CEJ and a restoration (123, 325). A round orthodontic base, which was made by trimming the attached ring off an orthodontic eyelet, was cemented on the labial surface of the incisor tooth. The other reference points were the root apex and the CEJ, as illustrated in (**Figure 7.3**).

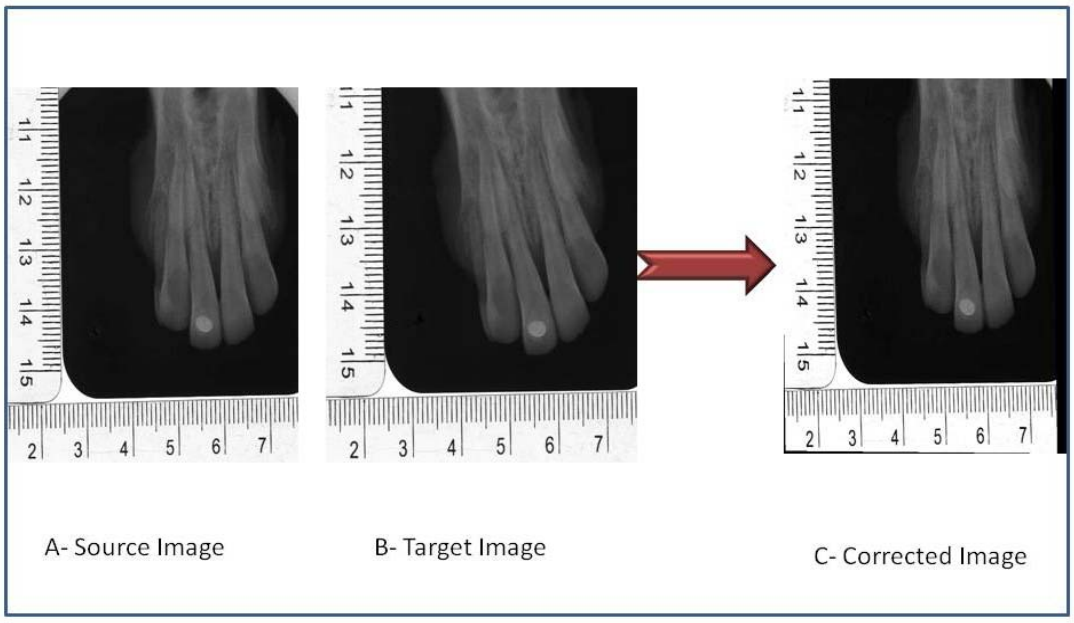


Figure 7.3 TurboReg plug-in used to correct errors in the image angulations

7.1.2 Chapter 4 related pilot study

7.1.2.1 Development of the experimental technique

Two sheep mandibles at the age of two-tooth were obtained from animals sacrificed at the completion of other studies. The two permanent incisors were used to examine the suitability of sheep immature teeth for trauma revitalisation endodontic research.

One sheep mandible was fixed in 10% neutral buffered formalin. Anterior teeth forceps (Upper Universal, DG, Henry Schein, USA) were used to extract the teeth. Despite the attempt to extract the teeth in a non-traumatic way, the extraction was difficult and one of the roots fractured during extraction. This most likely occurred as a consequence of fixation in formalin making them hard and rubbery. Therefore, the second attempt was to extract teeth from a fresh and non-fixed mandible. Teeth extraction from a fresh mandible was easy and uncomplicated. However, despite attempting a careful extraction, the left first incisor root tooth was empty when it was extracted, as all the pulp content had remained inside the socket. When the right first incisor was extracted, some of the periapical tissue remained attached to the tooth (**Figure 7.4**).

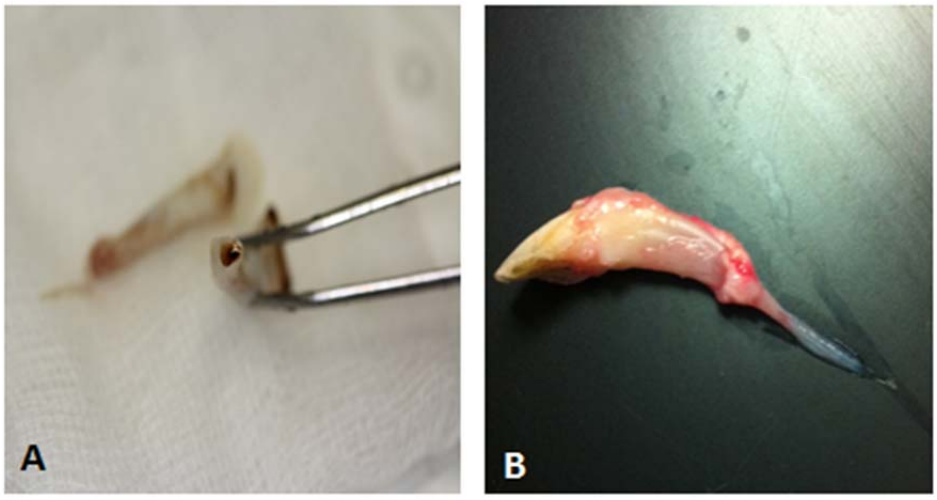


Figure 7.4 left incisor with a hollow cavity (A), Right first incisor with apical tissue (B).

Consequently, in light of the disadvantages of using immature sheep teeth for traumatic revitalisation endodontic research, it was decided to examine the suitability of immature sheep teeth for revitalisation subsequent to infection.

7.1.2.2 Immunohistochemistry (IHC) analysis of expression of DSPP in sheep teeth

7.1.2.2.1 Aims

This study aims to examine expression of DSPP by ovine odontoblasts and dentine. There is no available antibodies specific for ovine DSPP, therefore rabbit anti-DSPP polyclonal antibody was selected for this study.

7.1.2.2.2 Methods

7.1.2.2.2.1 Animals

Two sheep jaws and two rat jaws were scavenged from animals sacrificed at the completion of other studies following approved guidelines set by South Australia Pathology/Animal Ethics Committee (#ST25/12).

7.1.2.2.2.2 Fixation

The specimens were fixed in 10% neutral buffered formalin at 18 - 22°C.

7.1.2.2.2.3 Decalcification

The jaws were decalcified in 10% EDTA. X-ray analysis of each specimen was done each week to determine the completion of decalcification.

7.1.2.2.2.4 Tissue processing for immunohistochemistry stain

After decalcification, the specimens were washed in tap-water for approximately 7 days to eliminate the decalcifying agent. The tissues were placed in 10% neutral buffered formalin before automatic processing in a Shandon Citadel tissue processor (Shandon Industries, Pennsylvania) (**7.3 Appendix**). Throughout this process, the tissues were dehydrating in graded alcohols and cleared before impregnating in paraffin wax. The tissues were embed in Surgipath EM400 wax (Leica Microsystems, Richmond, UK) and 5 μm thick serial sections were cut and mounted on gelatine coated glass slides that were dried at 37°C for one day.

A detailed protocol for the IHC staining is found in (**7.5 Appendix)**. Briefly, tissue sections on gelatine coated slides were dewaxed, and then incubated with trypsin for 15 minutes at 37°C for antigen retrieval followed by washing with PBS. Heat antigen retrieval was also tried on sheep and rat tissues, but enzyme retrieval caused less damage to both tissues and gave better results

For inhibition of endogenous peroxidase activity, sections were incubated with 0.5% H_2O_2 in methanol at room temperature for 30 minutes followed by washing with PBS. Sections were then blocked for nonspecific antibody binding using 10% normal horse serum for 30

minutes. There are no sheep specific DSPP antibodies available on the market. Therefore, a rabbit polyclonal (anti-human, rat and mouse) was selected for this study. After further washing, sections were incubated with primary rabbit anti-DSPP polyclonal antibodies (Bioss Antibodies, USA) overnight at 4°C. After washing, the sections were incubated with a HRP conjugated anti-rabbi antibody (Dako, Denmark) for one hour. Following washing, sections were incubated with peroxidase substrate diaminobenzidine (DAB, Vector Laboratories Inc. CA, USA) for approximately 2 minutes. The sections were then washed and counter-stained with haematoxylin and mounted with Depex-mounting media.

7.1.2.2.3 Results

Rat teeth showed a positive reaction to anti-rat DSPP, while ovine teeth showed a negative reaction to anti-DSPP as shown in **(Figure 7.5).**

7.1.2.2.4 Discussion

The aim of this study was to demonstrate the expression of DSPP ovine odontoblast cells and dentine so it can be used as a method to identify dental pulp regenerative tissues. Unfortunately, there are no available anti-DSPP antibodies specific for ovine, and the rabbit-polyclonal anti-rat DSPP used in this study, did not demonstrate binding to ovine teeth.

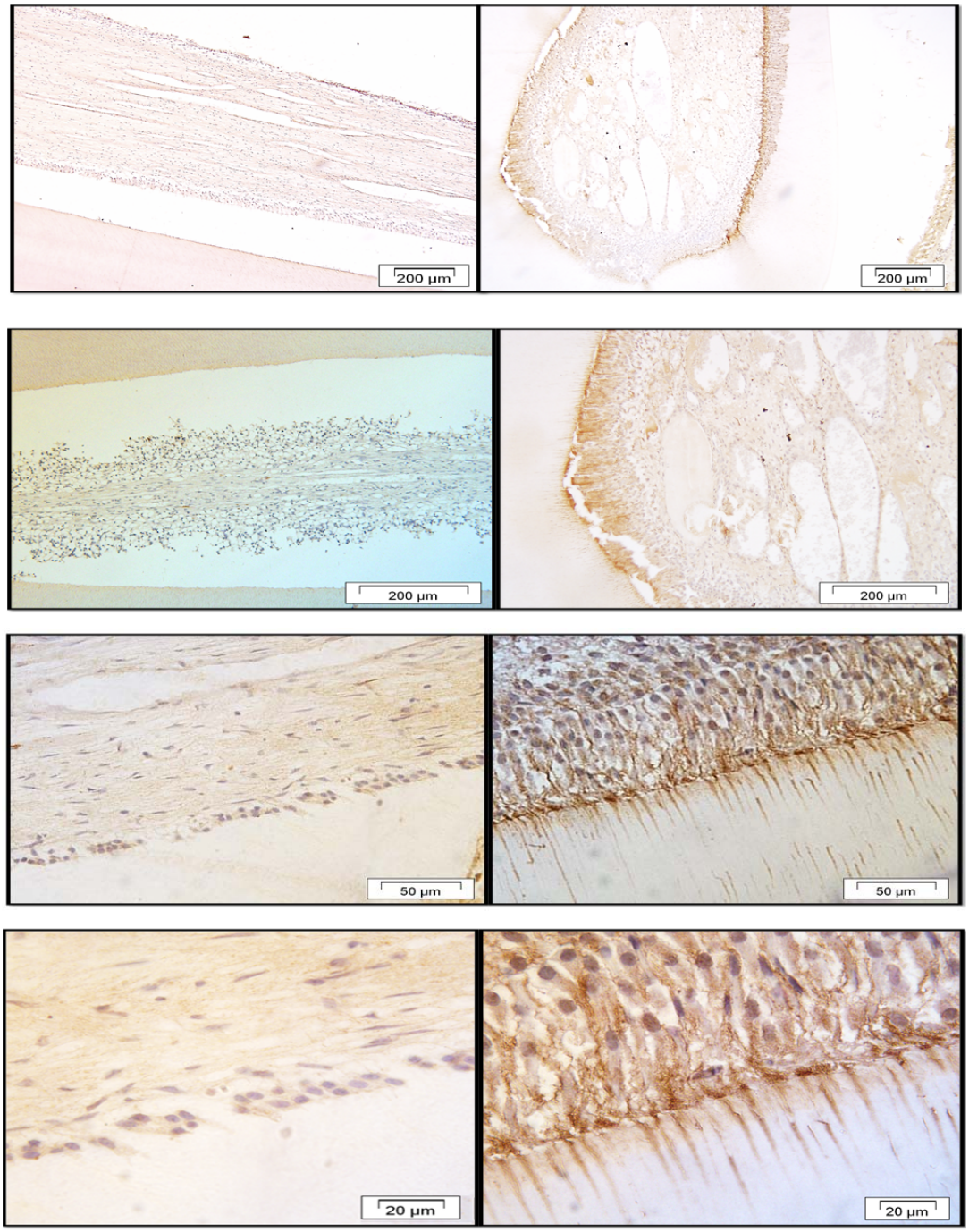


Figure 7.5 Negative expression of DSPP by ovine teeth (left side images) and positive expression of DSPP by rat teeth (right side images)

7.1.3 Chapter 5 related pilot studies

7.1.3.1 Analysis of the suitability of WST-1 colorimetric assay kit for measuring ODPCs proliferation rate

7.1.3.1.1 Introduction

Evaluation of the ODPCs proliferation rate on PRP and PPP is a vital part in the assessment of the suitability of the scaffolds for revitalisation treatment. Premixed WST-1 cell proliferation assay is based on the cleavage of tetrazolium salt WST-1 by cell mitochondrial enzymes to form formazan dye which can be detected by absorbance at 420-490 nm.

7.1.3.1.2 Aim

This study aims to evaluate the ability of Premixed WST-1 proliferation assay kit to detect the proliferation rate of ODPCs.

7.1.3.1.3 Methods

ODPCs were cultured on PRP and PPP scaffolds in 48-well plates following the protocol described in **Chapter 5, Section 5.3**. The cells were seeded on the scaffolds at concentrations (0, 1 x 10^3, 5 x 10^3 and 1 x 10^4 cells per well). Proliferation rate was analysed 24 hours after culturing the cells using WST-1 Proliferation Assay Kit at 460, as described in **Section 5.3.4**.

7.1.3.1.4 Results

The results showed that the absorbance rate increased significantly with the increases in the cell concentrations as seen in **Figure 7.6** and **Figure 7.7**.

7.1.3.1.5 Conclusion

The results showed that WST-1 kit can significantly detect the proliferation rate of the cells. We thus decided to use WST-1 proliferation kit for further studies of proliferation of ODPCs on PRP and PPP scaffold.

Date	1/08/2013	
Time	4:05:18 PM	
Reader Type:	Synergy Mx	
Reader Serial Number:	255460	
Reading Type	Reader	
Procedure Details		
Plate Type	96 WELL PLATE	
Read	Absorbance Endpoint	
	Full Plate	
	Wavelengths: 490, 630	
	Read Speed: Normal, Delay: 100 msec, Measurements/Data Point: 8	
Shake	Medium, 1:00 (MM:SS)	
Results		
Actual Temperature:	24.6	
Actual Temperature:	24.6	

	PR/no C	PR/10^3C	PR/50^3C	PR/10^4C									
	1	2	3	4	5	6	7	8	9	10	11	12	
A	0.079	0.254	0.49	0.78	0.045	0.045	0.045	0.045	0.045	0.045	0.045	0.044	490
	0.046	0.049	0.049	0.049	0.043	0.042	0.044	0.043	0.044	0.042	0.041	0.042	630
B	0.09	0.304	0.4	0.765	0.045	0.045	0.045	0.045	0.045	0.045	0.045	0.044	490
	0.047	0.046	0.048	0.048	0.041	0.041	0.042	0.043	0.043	0.042	0.042	0.042	630
C	0.095	0.271	0.417	1.011	0.046	0.045	0.045	0.045	0.044	0.045	0.045	0.045	490
	0.046	0.047	0.048	0.05	0.043	0.042	0.043	0.043	0.041	0.042	0.042	0.043	630
D	0.045	0.045	0.046	0.045	0.046	0.045	0.045	0.045	0.045	0.047	0.045	0.044	490
	0.043	0.044	0.044	0.043	0.044	0.042	0.042	0.042	0.042	0.043	0.042	0.042	630
E	0.044	0.045	0.044	0.045	0.044	0.045	0.045	0.045	0.046	0.045	0.045	0.045	490
	0.042	0.042	0.041	0.042	0.04	0.043	0.042	0.043	0.043	0.043	0.043	0.043	630
F	0.044	0.045	0.044	0.044	0.045	0.045	0.045	0.046	0.046	0.045	0.045	0.045	490
	0.042	0.042	0.041	0.041	0.042	0.042	0.043	0.044	0.044	0.044	0.043	0.042	630
G	0.044	0.045	0.044	0.045	0.046	0.045	0.045	0.045	0.045	0.045	0.046	0.045	490
	0.042	0.042	0.041	0.043	0.043	0.042	0.043	0.042	0.042	0.044	0.043	0.041	630
H	0.045	0.045	0.045	0.044	0.045	0.045	0.044	0.044	0.046	0.045	0.045	0.045	490
	0.042	0.041	0.042	0.043	0.043	0.043	0.041	0.042	0.042	0.043	0.043	0.043	630

Figure 7.6 Plate reading results of the absorbance rates of different concentrations of ODPCs on PRP scaffold.

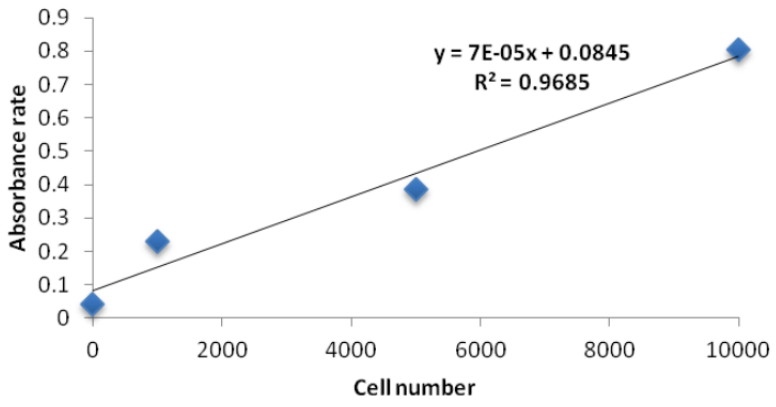


Figure 7.7 Chart showing almost linear relation between cell concentration and absorbance rate.

7.1.3.2 Development of the proliferation assay protocol

7.1.3.2.1 Introduction

The proliferation study was designed to measure the proliferation rate after one and seven days. For the seven day group, it was planned to change the medium every two days. But we noticed that after adding the medium to PRP and PPP scaffolds, the scaffolds absorbed some of the medium, which resulted in variations in unabsorbed medium volume for the experimental compared to the control groups. Less free supernatants were available for the experimental groups than the control group, and when replaced with 400 ml medium in total, there was more medium available in the experimental group (absorbed volume + the new medium volume) than in the control group. This variation affected the final volume of the WST-1 reagent, which was supposed to be added at equal volume to all groups and thus caused variations in the results. For the purpose of standardisation, we decided not to change the medium during the proliferation period and follow the protocol used by Anitua *et al.* (251).

7.1.3.2.2 Aims

The aims of this study were to:

- Evaluate the proliferation rates of ODPCs after, 1, 5 and 7 days without changing the medium.
- Find the longest period that ODPCs can proliferate actively without changing the medium.

7.1.3.2.3 Method

ODPCs were cultured at a concentration 1 x 10^3 per well on PRP, PPP and without scaffold (control) in 48-well plates following the same protocol described in **Chapter 5, section 5.3.4.** The proliferation rate was evaluated using WST-1 assay kit.

7.1.3.2.4 Statistical analysis

The data were analysed for significant difference between proliferation rates after one, five, and seven days for each group using a two-way ANOVA followed by Tukey's multiple comparisons test. Each value represents the mean ± standard deviation (SD). All statistical analyses were performed using Prism 6 Statistics software (GraphPad Software Inc.). Statistical significance was set at $p < 0.05$.

7.1.3.2.5 Results

The results showed that ODPCs numbers increased significantly between day one and day five for all the groups ($p < 0.05$) (**Figure 7.8)**. There were significant reductions in cell proliferation rates at day seven compared to day five in all the experimental groups, except for the control with dentine discs ($p = 0.93$).

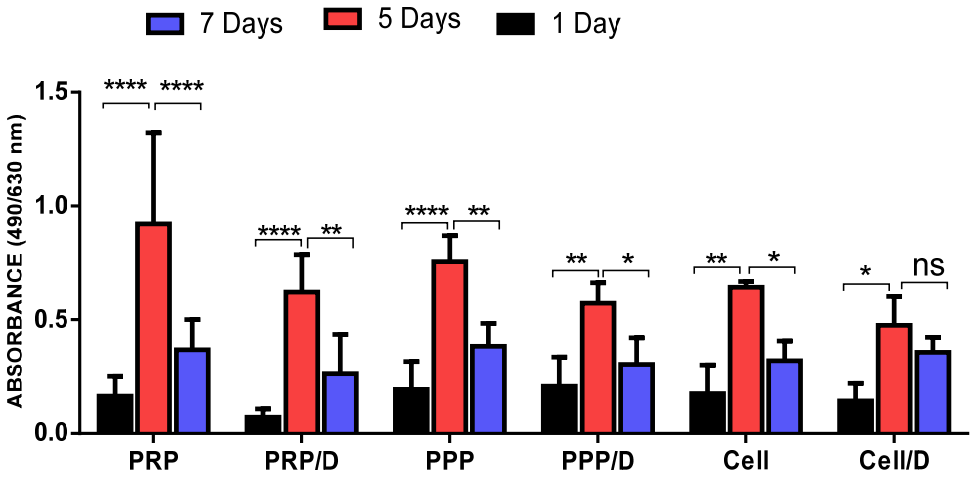


Figure 7.8 ODPCs proliferation after one, five, and seven days of culturing with and without scaffolds.

PRP: platelet rich plasma scaffold, PPP: platelet poor plasma scaffold, Cell: ODPCs, D: dentine discs.

Means ± SD.*p < 0.05, **p < 0.01, ****p < 0.00001.

7.1.3.2.6 Conclusion

Proliferation rate of ODPCs increased significantly between one and five days of seeding, with and without scaffolds. 400 ml medium per well supplement can support the proliferation of ODPCs at a concentration 1 x 10^3 per well for five days without changing the medium.

7.1.3.3 Evaluate the attachment of ODPCs to dentinal walls using SEM

7.1.3.3.1 Aims

Evaluate migration, attachment, proliferation and differentiation of ODPCs on dentine surfaces in the presence of PRP or PPP scaffold using Scanning Electron Microscope.

7.1.3.3.2 Methods

Dentine discs, prepared following the protocol described in Chapter 5, with a notch made on the side of each disc to mark the upper and the lower surface was used. The discs (two discs per well) were inserted in 48-well plates. The scaffolds were prepared by adding 68 µl from PRP or PPP to each well plus 8 µl (100 mmol/L) $CaCl_2$, and added to the dentine discs. Four replicas were used for each scaffold. ODPCs at a concentration 5 x 10^3 per well were seeded on the scaffolds for ten days.

After ten days, the specimens were prepared for SEM analysis following the protocol used by Huang *et al*. (112). Briefly, the wells were washed in PBS. Dentine discs retrieved, fixed, dehydrated in a series of alcohol concentrations, mounted on SEM specimen stubs, as shown in (**Figure 7.9),** and left to dry at room temperature for one day. After gold sputter coating, the discs were observed with a Phillips XL30 Scanning Electron Microscope (Eindhoven, Netherlands).

Figure 7.9 image of specimens prepared for SEM

7.1.3.3.3 Results

SEM images showed some cells on the surfaces of the dentinal walls. It also, showed dentinal walls covered by the scaffolds in both groups and it was hard to detect the cells, especially in the PRP group due to the scaffold. Only a few cells were able to be detected near the surface as shown in (**Figure 7.10)**.

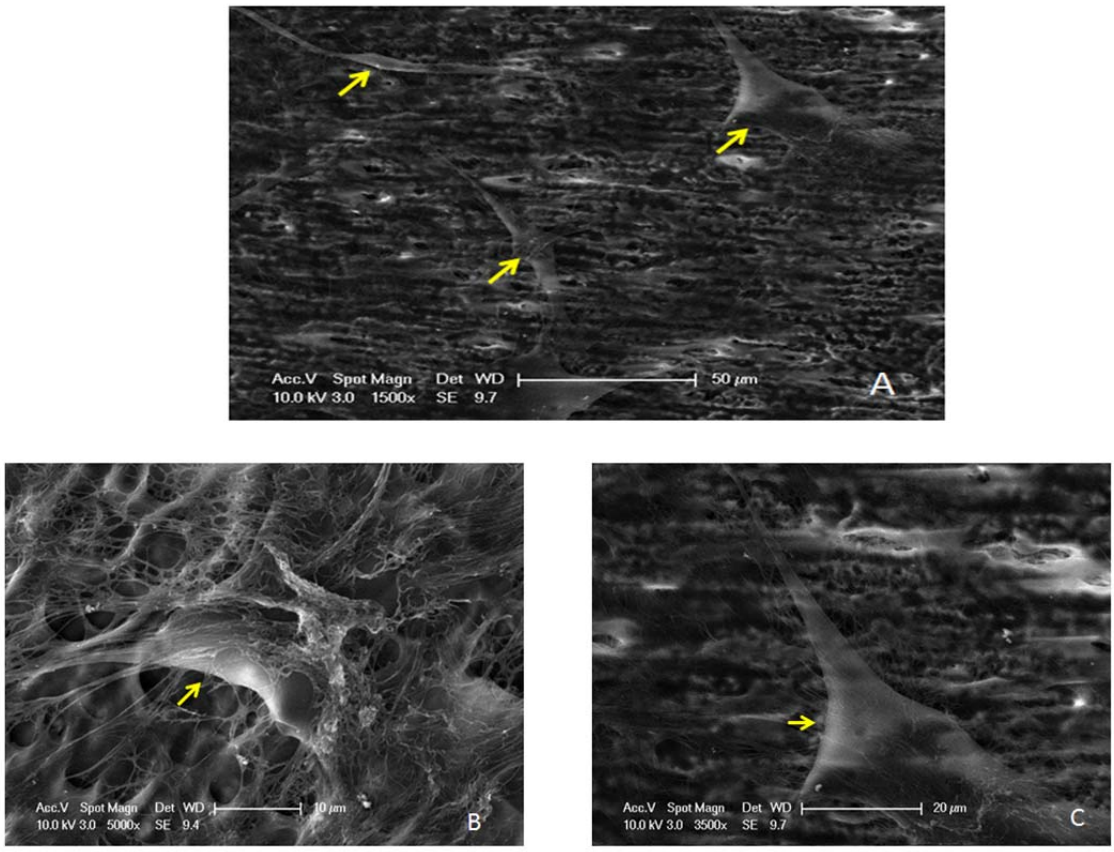


Figure 7.10 SEM images of ODPCs attach to dentine walls after ten days of culturing on PRP and PPP scaffolds.

A: A group of cells on dentine surface and covered by PPP scaffold.

B: Magnified image from PRP group showing single cells covers by scaffold.

C: Magnified image from PPP group showing single cells covers by scaffold.

Yellow arrows indicate cells.

7.1.3.3.4 Discussion

This study aimed to visualise the attachment, proliferation and differentiation of ODPCs on dentine surfaces by seeding the cells on PRP and PPP scaffolds. After ten days, the attached cells in both scaffolds migrated deep towards the dentine, resulting in a layer of the scaffold covering the cells, making it difficult to detect the cells especially in the PRP group. Only a few cells near the surface were detected by SEM as seen in (**Figure 7.10).**

Therefore, alternative methods like histological preparation of the dentine discs or even root segments could provide better information about the effect of both PRP and PPP scaffolds on proliferation and attachment of the ODPCs.

7.1.3.3.5 Conclusion

Using SEM methods to evaluate migration, attachment, proliferation and differentiation of ODPCs to dentine surfaces was not effective in the presence of the PRP and PPP scaffolds.

7.2 Appendix: Decalcifying solutions

7.2.1 Laboratory decal solution

7.2.1.1 Reagents:

- 950 ml hydrochloric acid (HCl 36% concentration).
- 100 g sodium acetate MW 82.04 dissolved in 1 L of distilled water.
- 100 g EDTA (ethylenediaminetetra-acetic acid) dissolved in 1L of distilled water.
- $[CH_2N(CH_3COOH)\ CHC_2OONa]_2\ 2H_2O$ in 1L of distilled water.
- 7050 ml of distilled water.

7.2.1.2 Method for making 10 L.

1. Weigh sodium acetate and dissolve in 1 L of warm distilled water.
2. Add to a large container.
3. Weigh 100 g of EDTA and dissolve in 1 L of warm distilled water.
4. Add to container.
5. Add 7050 ml of distilled water to container.
6. Add with care 950 ml of hydrochloric acid.

7.2.2 EDTA 10% pH 7.4

7.2.2.1 Preparation

1- Weigh 100g of EDTA.

2- Dissolve in 1 L of warm distilled water.

3- Adjust the pH using 5 M sodium hydroxide and 1 M hydrochloric acid.

7.3 Appendix: Tissue dehydration and paraffin embedding protocol

Shandon Citadel (Shandon Citadel ^{TM} Tissue processor), Shandon Industries, Pittsburgh, Pennsylvania).

The following automatic program was used for impregnation of tissue with paraffin wax prior to embedding:

Program steps	Time
1. Fixative or Buffer	time as needs
2. 70% Ethyl Alcohol	4h Cold vacuum to remove any trapped air
3. 80% Ethyl Alcohol	4h
Return to the machine	
4. 90% Ethyl Alcohol	4h
5. 100% Ethyl Alcohol	4h
6. 100% Ethyl Alcohol	4h
7. 100% Ethyl Alcohol	4h
8. 100% Histolene	4h Transfer to cold vacuum until bubbling stops, then resume the processing schedule.
9. 100% Histolene	4h
10. Paraffin wax	4h
11. Paraffin wax	4h
12. Paraffin wax	4h
14. Paraffin wax (in a hot vacuum until bubbles cease)	

The tissues were then embedded in Surgipath EM400 wax.

7.4 Appendix: Stains for light microscopy protocol

Mayer's haematoxylin-eosin staining protocol:

Remove wax	
Xylol	2 x 2 min
Absolute Alcohol	2 x 2 min
Rehydrate stepwise in lower concentrations of alcohol	
Check for incomplete removal of wax and xylol	
Dip in Tap Water	
Staining	
Stain in Haematoxylin	10 min
Wash in running tap water	1 min
Differentiation	
0.5% HCl Hydrochloric Acid	one dip
Blue	
Wash in running water	10 min
Blue in Dilute Alkali (NH_4OH)	2 dips
Wash in running water	1 minute
Eosin	
Counterstain in eosin	30 s
Differentiation	
70% alcohol	few dips
Dehydration and Clearing	
Absolute Alcohol	2 x 2 min
Xylol or Clearene or Histoclear	2 x 2 min
Mounting with coverslips	
Mount in DePex	

7.5 Appendix: Immunohistochemistry stain protocols

Trypsin (Enzyme digestion):

Trypsin solution preparation protocol supplied by abcam (www.abcam.com/technical)

Trypsin stock solution (0.5%):

Trypsin II	50 mg
Distilled water	10 ml

Mix to dissolve and store at - 20 ˚C

Calcium Chloride stock solution (1%)

Calcium Chloride	0.1g
Distilled water	10 ml

Mixed and store at 4 ˚C

Trypsin working solution (0.05%)

Trypsin stock solution (0.5%)	1 ml
Calcium Chloride stock solution (1%)	1 ml
Distilled water	8 ml

Adjust pH to 7.8 using 1N NaOH. Store at 4 ˚C for one month

Staining protocol:

Add a small quantity of water to the bottom of the humid container. Two containers: 1st at 4C and 2nd at room temperature

Note: Do not allow the slides to 'dry out' at any time during in this procedure. Slides may be rested in PBS if staining is halted or not begun immediately.

Sections dewaxing

1. Xylene: 2 x 3 minutes
2. 100% ethanol: 2 x 3 minutes
3. 90% ethanol: 3 minutes
4. 70% ethanol: 3 minutes
5. 50% ethanol: 3 minutes
6. Running cold tap water to rinse

Antigen Retrieval:

Trypsin (Enzyme digestion):

1-Preheat trypsin working solution (0.05%) to 37ºC. Remove excess water from section and then pipet the enzyme solution, approximately 200 µl per section.

2-Incubate slides in a humidified container at 37ºC for 10-15 minutes.

3-Rinse slides in phosphate buffered saline**:** 2 pots, 1 minute each

Endogenous Peroxidase Block: 0.5% hydrogen peroxide in methanol (30% w/v solution) for **30 min**

Phosphate Buffered Saline Rinse: 2 pots, 3 minutes each. Sections can be held here if catch-up period is required.

Non-specific antibody Block: (3% Normal Horse Serum in PBS) Pipette 2-3 drops onto slides. Incubate for **30 min** at room temperature.

Drain well: Tap off excess:

Phosphate Buffered Saline Rinse: Pipette PBS over slides several times. Tap and wipe the excess from around the section.

Primary antibody: (1:200) diluted in PBS (5 µl for 995 PBS)

Pipette approximately 200 µl per section. Incubate overnight at 4^0C in the cold room.

Phosphate Buffered Saline Rinse: Pipette PBS over slides several times. Tap and wipe off the excess.

Secondary antibody: (200 µl per section) Incubate for one hour on covered racks.

Drain well: Tap off excess.

Phosphate Buffered Saline rinse: Pipette PBS over slides several times. Tap and wipe off the excess around each section.

DAB Staining:

DAB working solution: In a separate vial add1 drop (approximately 0.5ml) of DAB for every 1 ml of substrate buffer. Add onto sections. Incubate for approximately 2 minutes,

Drain waste DAB into the waste beaker: Tap the slide on a funnel. Allow DAB to drain into a beaker below. At the end of the staining denature the DAB according to the DAB Preparation SOP.

Rinse slide well: Pipette distilled water into slide surface to gently rinse. Collect waste into the DAB waste beaker.

Counter stain nuclear cells: Stain in haematoxylin for 1 minute

Wash in running water: 10 minutes gently running tap water

Blue in alkali solution: 2 minutes

1. Running cold tap water to rinse for 1 minute
2. 50% ethanol: 3 minutes
3. 70% ethanol: 3 minutes
4. 90% ethanol: 3 minutes
5. 100% ethanol: 2 x 3 minutes
6. Xylene: 2 x 3 minutes
7. Mounting with coverslips (Mount in DePex)

7.6 Appendix: Cell counting protocol

- Aspirate media from cell culture and wash the culture with 1 x PBS
- Detach the cells using trypsin/EDTA mix and incubate at 37 °C for approximately 5 minutes until cells detach.
- Add an equal volume of media to neutralise the trypsin.
- Spritz cells up and down using a pipette and pellet the cells via centrifugation at 300 g for 5 minutes
- Aspirate medium from the cell pellet and resuspend the pellet in 1 ml new medium.
- Take 20 µl from the suspension and add it to 180 µl trypan blue dye (Sigma - Aldrich) to get 200 µl mixtures.
- Take volume from cell suspension and trypan blue dye mixture and count the number of cells using a haemocytometer.
- Count the average number of the cells per each square of haemocytometer.
- The dilution factor = $\frac{Mixture\ volume}{cell\ suspension\ volume} = \frac{200\mu l}{20\mu l} = 10$
- Cell concentration in 1 ml = Average number of cells (from haemocytometer) x dilution factor x 10^4.

7.7 Appendix: ODPC cryopreservation and thawing protocols:

7.7.1 ODPC cryopreservation and thawing protocols

- Perform a cell count following the previously mentioned counting protocol to determine the concentration of the cells of cells in suspension. The required concentration for cryopreservation of the cells is 1x 10^7 cells.
- Dilute cell suspension to desirable concentration in a 10% DMSO/90% FCS mixture, and immediately aliquot into cryotubes (Greiner Bio - one, Germany) of 500 or 1000 µl.
- Place the cryotubes in freezing container "Mr frosty" at room temperature, and then place them in (-80°C) freezer for 24 hours.
- Transfer to liquid nitrogen vapour for an extended storage at (-150 °C).

7.7.2 Thawing of cryopreserved ODPCs

- Remove the tubes from the liquid nitrogen (-150 °C), and place them in - 80°C freezer for at least 4 hours.
- Remove the tubes from the freezer and thaw them at 37 °C in a water bath.
- Once thawed, add slowly 1 ml of ODPCs medium, aspirate the cells and medium, and transfer into a 15 ml Falcon tube contains 8 ml ODPCs medium.
- Centrifuge the tube at 300 g for 5 minutes to pellet the cells, aspirate the medium, and repeat the washing step again.
- Suspend the cell pellet according to the required concentration.
- Add 1 ml of cell suspension into a T75 flask containing 15 ml ODPCs medium and incubate at 37 °C.

7.8 Appendix: Results related tables

7.8.1 Chapter 3 related results

7.8.1.1 Mature age measurement (mm)

Root length				Root wall thickness/BL				Root wall thickness/MD				Apex diameters			
1st incisor	2nd incisor	3rd incisor	4th incisor	1st incisor	2nd incisor	3rd incisor	4th incisor	1st incisor	2nd incisor	3rd incisor	4th incisor	1st incisor	2nd incisor	3rd incisor	4th incisor
14.8	14.7	14.0	13.3	0.75	0.71	0.74	0.76	0.85	0.85	0.62	0.81	0.35	0.3	0.2	0.25
15.2	14.1	14.0	13.2	1.3	1.1	1	1	0.9	0.74	0.86	0.8	0.3	0.35	0.4	0.3
14.3	12.9	13.3	12.7	1.36	1.19	1.35	1.2	1.13	1	1.04	0.88	0.2	0.35	0.5	0.5
13.3	13.9	13.7	12.3	1.5	1.3	1.24	1.3	1.14	1.01	1	1.03	0.3	0.35	0.2	0.2
14.5	13.8	12.7	11.2	1.39	0.9	0.85	1.23	1.18	0.83	0.78	0.8	0.3	0.35	0.25	0.2
15.4	14.3	13.2	13.2	1.23	1.22	1.09	1	0.89	0.92	0.97	0.89	0.3	0.3	0.25	0.2
16	15.26	13.6	11.07	1.11	1.1	1.01	0.71	1.11	1	1.01	0.56	0.4	0.3	0.35	0.55
15.9	15.2	13.4	11.4	1.15	1.1	1.09	0.75	1.15	0.96	1.09	0.59	0.4	0.3	0.4	0.6
17.5	16.3	14.9	12.5	1.61	1.3	1.08	0.86	1.61	1	1.08	0.75	0.3	0.35	0.35	0.65
17.8	16.5	14.5	12.6	1.5	1.4	1.1	0.93	1.5	0.95	1.1	0.79	0.35	0.35	0.4	0.6
16.8	16.2	13.3	11.6	1.75	1.1	1.01	0.85	1.75	1.54	1.01	0.74	0.35	0.2	0.4	0.4
16.7	16.2	13.5	11.6	1.67	1.2	1.05	0.89	1.67	1.45	1.05	0.92	0.3	0.25	0.4	0.45
16.5	16.8	14.6	11.8	1.36	1.28	0.94	0.99	1.36	1.02	0.94	0.95	0.35	0.35	0.3	0.4
15.9	15.5	15	11.6	1.31	1.16	0.95	0.92	1.31	0.97	0.95	0.65	0.35	0.35	0.35	0.4
15.3	15.5	14.8	10.5	1.56	1.57	1.11	0.99	1.56	1.14	1.11	0.63	0.35	0.3	0.4	0.6
15.8	15.2	14	10.3	1.63	1.4	1	0.89	1.63	1.12	1	0.81	0.3	0.25	0.4	0.55

Table 7-1 Direct measurements of incisor teeth of the mature age sheep (mm).

Root length				Root wall thickness				Apex diameters			
1st incisor	2nd incisor	3rd incisor	4th incisor	1st incisor	2nd incisor	3rd incisor	4th incisor	1st incisor	2nd incisor	3rd incisor	4th incisor
15.54	14.63	13.17	9.64	1.27	0.97	1.08	0.94	0.38	0.47	0.55	0.42
15.56	14.89	13.22	10.19	1.06	1.19	1.19	1.03	0.38	0.55	0.6	0.65
17.68	15.86	14.32	11	1.32	1.15	0.91	0.82	0.51	0.48	0.49	0.5
17.07	15.89	14	11.33	1.37	1.15	0.91	0.82	0.59	0.48	0.49	0.51
14.67	13.78	12.79	10.73	1.3	0.85	0.95	0.82	0.44	0.51	0.49	0.38
14.48	14.3	12.84	11.17	1.41	0.93	0.82	0.87	0.44	0.51	0.49	0.38
14.14	12.89	12.49	8.6	1.02	1.06	0.82	0.82	0.45	0.31	0.78	0.4
14.36	12.66	12.4	8.33	1.19	1.06	0.93	0.82	0.37	0.31	0.78	0.4
14.45	14.4	13.97	10.67	1.02	0.98	0.93	0.81	0.33	0.4	0.35	0.33
14.52	14.1	12.9	9.4	1.4	0.98	0.93	0.81	0.33	0.4	0.35	0.33
16.24	16.1	15.4	13.11	1.39	1.2	1.06	0.89	0.42	0.4	0.43	0.35
16.6	16.77	15.11	13.69	1.31	1.02	1.25	0.98	0.42	0.42	0.44	0.38
16.34	15.03	14.18	12.08	1.16	1.04	1.27	0.79	0.37	0.34	0.49	0.32
15.89	15.42	14.15	11.16	1.04	1.06	0.86	0.86	0.34	0.32	0.44	0.44
17.55	16.77	15.1	12.56	1.16	0.95	0.95	0.92	0.42	0.48	0.51	0.51
17.14	16.92	14.56	11.45	1.19	0.95	0.95	0.92	0.42	0.48	0.51	0.51
17.27	16.12	14.51	10.07	0.96	0.95	0.94	0.99	0.4	0.45	0.68	0.93
17.91	16.23	14.45	9.86	0.96	0.95	0.94	0.99	0.48	0.45	0.68	0.96
15.49	15.28	15.12	10.84	1.19	1.06	0.98	0.79	0.38	0.4	0.4	0.65
16.3	15.83	14.87	10.46	1	1.07	0.98	0.79	0.42	0.45	0.45	0.62
17.09	16.02	16.81	11.71	1.4	1.01	1.1	0.85	0.38	0.45	0.5	0.7
17.1	16.56	16.81	11.22	1.5	1.01	1.1	0.83	0.32	0.42	0.53	0.7
16.48	16.11	14.54	10.24	1.5	1.02	0.85	0.73	0.3	0.52	0.48	0.63
16.42	16.71	14.15	10.3	1.32	1.02	0.85	0.73	0.55	0.52	0.48	0.63
17.61	16.7	14.28	9.7	1.4	0.93	0.89	0.75	0.45	0.54	0.5	0.72
17.32	16.39	14.81	9.03	1.4	0.93	0.89	0.75	0.45	0.5	0.5	0.61
16.05	16.67	13.85	9.53	1.1	1.27	1.1	0.93	0.37	0.31	0.37	0.63
16.15	16.67	13.85	9.53	1.1	1.27	1.1	0.93	0.37	0.31	0.37	0.63

Table 7-2 Radiographic measurements of incisor teeth of the mature age sheep (mm)

Root length				Root wall thickness								Apex diameters							
1st incisor	2nd incisor	3rd incisor	4th incisor	1st incisor		2nd incisor		3rd incisor		4th incisor		1st incisor		2nd incisor		3rd incisor		4th incisor	
				BL	MD	BL	MD	BL	MD	BL	MD	BL	MD	BL	MD	BL	MD	BL	MD
15.15	14.67	12.59	10.82	1.34	1.3	1.25	1.25	1.13	1.13	1.07	1.05	0.8	0.41	0.76	0.43	0.8	0.51	0.64	0.69
14.81	14.74	12.6	10.72	1.49	1.35	1.2	1.26	1.14	1.19	0.94	1.03	0.97	0.45	0.7	0.41	0.86	0.65	0.55	0.58
17.48	16.5	15.8	11.49	1.52	1.35	1.32	1.35	1.54	1.7	1.16	1.19	0.75	0.35	0.98	0.34	0.93	0.4	1.2	0.44
17.44	16.5	15.53	12.18	1.6	1.56	1.64	1.6	1.63	1.78	1.28	1.63	0.9	0.3	0.95	0.4	0.92	0.42	1.29	0.5
16.42	15.84	12.69	10.035	1.6	0.95	1.07	0.87	0.95	0.84	0.88	0.94	0.8	0.48	1.02	0.47	0.95	0.47	0.84	0.49
16.4	15.19	12.38	10.51	1.56	0.95	1.1	0.87	0.95	0.78	0.78	0.82	0.75	0.47	0.9	0.45	0.9	0.48	0.87	0.56
14.29	14.08	12	9.62	1.05	1.04	0.98	0.91	0.97	0.92	0.92	0.92	0.55	0.35	0.6	0.35	0.5	0.37	0.5	0.48
14.46	14.08	12.3	9.8	1.06	1.03	0.98	1.1	0.93	0.97	0.97	0.92	0.55	0.3	0.6	0.3	0.53	0.39	0.46	0.5
15	14.33	14.73	12.11	1.14	1.2	1.24	0.96	1.11	0.98	0.93	0.91	0.75	0.35	0.75	0.4	0.7	0.4	0.4	0.35
15.08	15.37	14.73	11.96	1.04	0.97	1.04	0.94	1.05	0.85	0.93	0.86	0.7	0.35	0.6	0.3	0.7	0.4	0.45	0.35
18.89	17.17	16.7	13.7	1.07	0.95	1.15	0.97	0.86	0.88	1.03	0.86	0.62	0.4	0.65	0.35	0.51	0.4	0.68	0.5
18.3	17.3	16.44	13.36	1.2	0.94	1.12	0.95	0.94	0.88	0.96	0.94	0.6	0.4	0.62	0.25	0.5	0.44	0.7	0.5
17.75	15.19	14.5	12.14	1.04	1.05	1.14	0.94	1.06	0.96	0.97	0.97	0.64	0.25	0.53	0.53	0.64	0.37	0.53	0.31
16.54	15.5	14.3	11.51	0.99	0.93	1.12	0.95	1.04	1.18	1.09	0.75	0.69	0.25	0.4	0.44	0.6	0.35	0.58	0.35
18.41	17.23	15.44	12.08	1.19	1.07	1.04	0.86	1.07	0.74	0.88	0.68	0.97	0.62	1	0.64	1.2	0.7	1.79	1.14
18.34	17.16	15.85	12.04	1.03	0.96	1.105	0.93	1	0.75	0.88	0.69	0.83	0.67	0.92	0.57	1.3	0.7	1.6	1
18.55	17.74	15.37	10.73	1.06	0.98	0.98	0.84	0.92	0.69	0.65	0.67	0.81	0.48	0.91	0.47	1.21	0.44	2.07	1.19
18.55	17.86	15.65	10.53	1.07	0.94	0.95	0.99	0.92	0.69	0.75	0.58	0.85	0.44	0.92	0.62	1.35	0.46	1.96	1.03
16.03	15.41	13.7	11.3	1.25	1.15	1.23	1.19	1.09	1.2	0.65	0.54	0.85	0.45	0.81	0.4	1.05	0.38	1.48	0.63
16.2	15.12	13.4	11.2	1.21	1.16	1.2	1.15	1.1	1.25	0.65	0.56	0.87	0.47	0.82	0.42	1.04	0.34	1.5	0.58
17.79	17.31	16.4	11.69	2	1.7	1	0.81	0.9	0.91	0.62	0.62	0.85	0.35	0.94	0.4	0.88	0.48	1.09	0.64
17.95	17.3	15.09	11.11	1.9	1.3	0.96	0.81	0.83	0.98	0.68	0.61	0.83	0.38	1	0.38	1.2	0.42	1.1	0.69
16.05	15.06	13.35	12.03	1.57	1.51	1.22	1.02	0.82	1.06	0.95	0.85	0.85	0.35	0.7	0.4	0.99	0.44	1.04	0.63
16.64	15.12	13.42	12	1.16	1.68	1.19	1.06	1.04	1.02	0.86	0.85	0.82	0.35	0.75	0.45	0.9	0.43	0.93	0.53
18.69	17.27	15.44	11.45	1.4	1.3	1.08	0.91	0.93	0.87	0.74	0.93	0.85	0.47	0.9	0.53	1.2	0.35	1.96	0.5
18.34	17.06	15.26	11.59	1.19	1.2	1.06	0.94	0.93	0.84	0.83	0.66	0.89	0.43	0.95	0.6	1.36	0.36	1.96	0.54
16.37	15	12.07	10.19	1.105	1.61	1.22	1.05	1.28	1.04	1.034	0.63	0.74	0.25	0.6	0.58	0.94	0.49	0.77	0.41
16.16	15.33	12.77	11.24	1.5	1.4	1.13	1.03	1.104	0.98	1.096	0.71	0.77	0.3	0.8	0.6	0.86	0.44	0.56	0.3

Table 7-3 CT measurements of incisor teeth of the mature age sheep (mm).

7.8.1.2 Two-tooth age measurements (mm)

Root length	Root wall thickness		Apex diameters
	BL	MD	MD
16.05	0.64	0.62	2.7
16.39	0.64	0.55	2.5
16.66	0.73	0.72	2.25
14.07	0.63	0.48	2.25
14	0.5	0.4	2.4
15.96	0.63	0.61	1.95
15.87	0.65	0.59	1.94
15.56	0.66	0.62	1.97
16.03	0.62	0.55	2

Table 7-4 Direct measurements of incisor teeth of two-tooth age sheep (mm).

Root length	Root wall thickness	Apex diameters
15.11	0.63	2.62
14.73	0.63	2.8
14.99	0.34	2.41
14.8	0.34	2.16
14.35	0.55	2.98
15.81	0.55	2.1
15.11	0.68	1.78
15.46	0.65	1.31
16.13	0.59	2.14
15.87	0.55	2.09
14.77	0.51	1.61
14.64	0.55	1.4
14.35	0.43	1.83
14.3	0.51	2.01
13.88	0.51	1.75
14.05	0.51	2
15.05	0.68	1.52
15.17	0.68	1.56
15.95	0.68	1.67
15.98	0.68	1.87
16.52	0.72	2.08
16.78	0.72	1.95
13.65	0.52	2.02
13.9	0.56	2.1
14.06	0.59	2
14.79	0.55	1.98

Table 7-5 Radiographic measurements of incisor teeth of two-tooth age sheep (mm).

Root length	Root wall thickness		Apex diameters	
	BL	MD	BL	MD
15.58	0.6	0.62	2.96	2.43
14.88	0.68	0.67	2.96	2.49
15.3	0.68	0.63	2.18	1.81
15.26	0.74	0.68	2.3	1.76
16.1	0.67	0.6	3.06	2.3
15.73	0.8	0.6	2.85	2.5
14.47	0.62	0.66	3.12	2.23
14.19	0.62	0.64	3.05	2.27
14.05	0.53	0.52	2.96	2.07
14.23	0.55	0.57	2.94	2.17
16.7	0.51	0.46	2.7	2.2
16.13	0.55	0.48	2.6	2.3
16.29	0.52	0.55	2.4	2.2
16.29	0.52	0.51	2.5	2.1
16.82	0.6	0.71	2.82	2.53
16.85	0.66	0.71	2.9	2.4
14	0.67	0.67	3.19	2.4
13.7	0.64	0.59	3.13	2.3
15.65	0.55	0.58	2.13	1.91
15.64	0.69	0.52	2.18	2.04
15.39	0.62	0.60	2.75	2.22
1.01	0.08	0.07	0.35	0.22

Table 7-6 CT measurements of incisor teeth of two-tooth age sheep (mm).

7.8.1.3 Four-tooth age measurements (mm)

Root length		Root wall thickness		Apex diamerters	
1st incisor	2nd incisor	1st incisor	2nd incisor	1st incisor	2nd incisor
14.68	12.99	1.07	0.67	0.6	1.8
14.78	14.78	1.09	0.67	0.65	1.75
18.16	12.53	0.69	0.4	1	2.14
18.06	12.74	0.75	0.38	1.08	2.3
15.63	13.17	0.93	0.41	0.4	1.53
15.18	12.9	0.92	0.45	0.45	1.52
17.14	13.64	0.6	0.48	1.4	2.3
17.34	13.22	0.65	0.47	1.7	2.3

Table 7-7 Direct measurements of incisor teeth of four-tooth age sheep (mm).

Root length		Root wall thickness		Apex diameters	
1st incisor	2nd incisor	1st incisor	2nd incisor	1st incisor	2nd incisor
16.83	12.51	0.97	0.59	0.76	1.07
16.38	12.19	0.97	0.5	0.79	1.16
16.58	12.52	0.92	0.39	0.59	0.95
17.1	12.44	0.95	0.52	0.63	1.7
16.49	13.99	0.95	0.76	0.72	1.95
16.03	13.92	1.02	0.93	0.78	1.52
15.78	13.94	1.06	0.56	0.48	1.33
15.82	13.61	1.03	0.59	0.48	1.33
15.63	13.46	0.93	0.55	0.7	1
16	13.48	0.97	0.55	0.75	1.14
15.75	13.45	1.06	0.59	0.73	1.69
15.8	13.97	1.05	0.59	0.68	1.66
15.74	11.11	1.02	0.51	0.6	1.8
15	11.11	1.02	0.51	0.6	1.8
17.94	13.52	0.76	0.43	1.07	1.79
17.39	13.08	0.79	0.43	0.98	2.24
14.11	12.39	0.93	0.39	0.45	1.23
14.15	12.39	0.96	0.49	0.45	1.59
17.06	13.2	0.72	0.5	1.27	2.13
16.94	12.05	0.69	0.51	1.19	2.3

Table 7-8 Radiographic measurements of incisor teeth of four-tooth age sheep (mm).

Root length		Root wall thickness				Apex diameter			
1st incisor	2nd incisor	1st incisor		2nd incisor		1st incisor		2nd incisor	
		BL	MD	BL	MD	BL	MD	BL	MD
16.68	12.87	0.95	0.82	0.77	0.47	1.1	0.92	2.76	1.82
16.61	13.29	0.95	0.86	0.85	0.32	1.21	0.92	2.36	2.01
16.89	14.32	1.015	0.97	0.52	0.5	1.33	0.7	2.5	1.83
16.71	14.05	0.9	0.94	0.55	0.41	1.3	0.67	2.4	1.86
16.75	13.88	1.18	1.14	0.66	0.66	1.07	0.71	2.16	1.49
16.82	13.81	1.075	1.035	0.71	0.66	1.29	0.7	2.32	1.45
16.6	12.8	0.93	0.94	0.54	0.6	1.09	0.48	2.77	1.89
15.57	12.87	0.99	1.04	0.37	0.47	1.34	0.45	2.65	2.19
15.98	13.5	1.1	0.88	0.46	0.43	1.3	0.76	1.26	1.016
15.98	13.9	1.05	0.99	0.44	0.44	1.01	0.74	1.6	1.04
14.29	12.67	1.08	1.04	1.39	0.66	1.39	0.66	2.58	1.56
14.7	12.48	1.01	1.06	0.67	0.72	1.49	0.69	2.37	1.53
15.45	11.54	1.08	0.93	0.48	0.49	1.38	0.79	2.48	1.95
15.33	12.97	1.02	0.92	0.49	0.48	1.28	0.87	2.38	1.87
18.55	12.8	0.75	0.65	0.48	0.48	1.16	1.03	3.11	2.27
18.1	12.32	0.7	0.65	0.44	0.36	1.38	1.02	3.22	2.21
15.33	14.43	1.07	0.94	0.5	0.35	1.16	0.42	2.66	1.43
15.33	12.46	1	0.93	0.46	0.46	0.98	0.55	3.02	1.59
17.26	15.08	0.69	0.63	0.58	0.52	2.25	1.78	2.95	2.29
17.46	14.43	0.7	0.68	0.52	0.48	2.41	1.77	3.18	2.44

Table 7-9 CT measurements of incisor teeth of four-tooth age sheep (mm).

7.8.1.4 Six-tooth age measurements (mm)

Root length			Root wall thickness			Apex diameter		
1st incisor	2nd incisor	3rd incisor	1st incisor	2nd incisor	3rd incisor	1st incisor	2nd incisor	3rd incisor
16.92	15.76	12.42	0.95	0.62	0.35	0.55	1.28	1.95
16.92	15.37	12.15	1.05	0.66	0.37	0.6	1.3	1.7
19.14	16.21	15.46	1.01	0.72	0.78	0.45	1.1	1.45
19.17	16.68	15.36	1.03	0.7	0.66	0.45	1.06	1.38
18.63	16.82	13.73	0.99	0.94	0.67	0.35	0.45	1.1
18.61	16.39	12.68	0.98	0.93	0.66	0.35	0.45	1.17
16.93	13.69	12.84	1.05	0.68	0.45	0.96	1.18	1.9
16.69	13.77	12.38	1.09	0.77	0.62	0.96	1.2	1.7

Table 7-10 Direct measurements of incisor teeth of six-tooth age sheep (mm).

Root length			Root wall diameter			Apex diameter		
1st incisor	2nd incisor	3rd incisor	1st incisor	2nd incisor	3rd incisor	1st incisor	2nd incisor	3ed incisor
15.24	14.92	11.71	0.97	0.87	0.59	0.55	0.79	1.12
15.2	13.66	10.88	0.98	0.82	0.53	0.52	0.72	1.26
16.05	14.48	11.9	1.27	0.85	0.39	0.46	1.01	1.38
16.42	14.09	11.8	1.18	0.92	0.59	0.56	0.91	1.4
16.34	15.62	14.44	1.02	0.94	0.35	0.38	0.81	1.27
17.06	16.64	14.54	0.99	0.81	0.35	0.43	0.77	1.35
15	14.19	12.86	0.9	0.88	0.77	0.59	0.82	1.16
15.58	14.55	12.64	0.89	0.79	0.69	0.62	0.8	1.28
15.82	15.48	12.24	1.35	0.93	0.79	0.65	0.95	1.48
15.81	15.58	12.98	1.3	0.72	0.69	0.68	0.95	1.6
16.44	14.12	13.09	0.9	0.59	0.34	0.64	1.33	1.5
16.71	14.4	13.03	0.89	0.59	0.38	0.64	1.14	1.63
18.03	15.55	14.4	0.96	0.63	0.59	0.58	0.99	1.24
18.98	15.55	14.4	0.93	0.63	0.59	0.6	0.99	1.24
18.49	16.42	12.91	1.06	0.76	0.45	0.6	0.89	1.73
18	16.89	12.91	0.79	0.69	0.45	0.65	0.85	1.73
18.3	17.71	12.92	1.02	0.72	0.51	0.63	0.89	1.46
18.98	17.71	12.02	1.06	0.72	0.59	0.68	0.89	1.64
17.05	14.29	12.68	1.1	0.6	0.65	0.71	1.02	1.51
17.13	14.08	12.53	1.01	0.51	0.36	0.78	1.26	1.63

Table 7-11 Radiographic measurements of incisor teeth of six-tooth age sheep (mm).

Root length			Root wall thickness						Apex diameter					
1st incisor	2nd incisor	3rd incisor	1st incisor		2nd incisor		3rd incisor		1st incisor		2nd incisor		3rd incisor	
			BL	MD	BL	MD	BL	MD	BL	MD	BL	MD	BL	MD
15	15.55	12.55	0.97	0.92	0.88	0.62	0.49	0.55	0.59	0.42	1.3	0.67	1.93	1.43
15.64	14.5	11.56	0.97	0.84	0.85	0.78	0.49	0.53	0.59	0.45	1.23	0.68	2.24	1.61
16.95	15.71	11.67	1.04	0.98	0.75	0.76	0.43	0.45	1.38	0.78	1.6	1.38	2.28	1.55
16.86	15.47	11.67	1.01	0.95	0.79	0.69	0.48	0.45	1.5	0.88	1.85	1.3	2.35	1.54
17.71	16.82	15.97	1.03	0.91	0.75	0.69	0.68	0.59	0.7	0.74	0.97	0.84	1.16	1.31
17.69	16.71	15.91	1.06	0.86	0.75	0.65	0.61	0.46	0.7	0.65	0.85	0.84	1.26	1.23
14.18	14.3	12.01	0.92	0.95	0.7	0.71	0.72	0.64	0.7	0.54	2.03	0.97	1.88	1.14
14.44	14.09	12	1.03	0.94	0.74	0.71	0.68	0.66	0.85	0.57	2.03	0.96	2.04	1.21
16.02	15.47	12.56	1.1	0.95	1.1	0.85	0.5	0.54	0.95	0.57	1.4	0.77	2.29	1.67
15.42	15.58	12.08	1.14	1.09	1.05	0.87	0.45	0.58	0.95	0.62	1.26	0.87	2.51	1.82
16.43	15.54	12.04	1.09	0.97	0.55	0.6	0.3	0.41	1.55	0.67	2.3	1.02	2.69	1.98
16.43	15.44	12.81	1.09	0.97	0.54	0.58	0.32	0.35	1.2	0.61	1.22	1.07	2.77	1.76
19.27	18.9	16.09	0.93	0.88	0.75	0.66	0.58	0.51	0.91	0.53	1.58	1.17	1.72	1.73
19.27	18.44	16.3	0.98	0.87	0.7	0.67	0.51	0.43	0.94	0.48	1.45	1.19	1.83	1.64
17.3	15.85	13.77	1.07	1.03	0.93	0.93	0.58	0.63	0.98	0.38	1.21	0.51	1.74	1.05
18.03	16.41	13.42	1.05	0.93	0.94	0.94	0.57	0.51	0.98	0.31	1.22	0.52	1.59	0.93
18.41	16.09	12.91	1.09	0.9	0.63	0.65	0.45	0.48	1.32	0.76	2.05	1.4	2.4	1.71
17.65	16.54	13.02	1.078	1.04	0.62	0.67	0.38	0.45	1.36	0.88	2.12	1.13	2.47	1.4
16.82	13.94	11.25	1.018	0.85	0.63	0.54	0.52	0.33	1.28	0.65	2.4	1.02	3.13	1.9
16.44	13.82	10.9	0.97	1	0.58	0.62	0.68	0.53	1.5	0.74	2.34	1.04	2.7	1.82
16.80	15.76	13.02	1.03	0.94	0.76	0.71	0.52	0.50	1.05	0.61	1.62	0.97	2.15	1.52
1.43	1.34	1.71	0.06	0.07	0.16	0.11	0.12	0.09	0.32	0.16	0.48	0.26	0.51	0.30

Table 7-12 CT measurements of incisor teeth of six-tooth age sheep (mm).

7.8.2 Chapter 4 related results

Sheep-1	Tooth		Root length	Root wall thickness	Apex diameter
Before treatment	RI1	Radio	14.72	0.75	1.14
	LI1	Radio	14.98	0.75	1.17
6 months after treatment	RI1	CT	18.95	1.07	0.49
		Radio	19.03	1.02	0.38
	LI1	CT	18.87	0.95	0.58
		Radio	18.98	1.02	0.47

Sheep-2	Tooth		Root length	Root wall thickness	Apex diameter
Before treatment	RI1	Radio	14.24	0.59	1.48
	LI1	Radio	14.38	0.59	1.55
6 months after treatment	RI1	CT	18.07	1.05	0.49
		Radio	17.6	1.23	0.4
	LI1	CT	18.09	0.95	0.63
		Radio	17.86	1.03	0.52

Sheep-3	Tooth		Root length	Root wall thickness	Apex diameter
Before treatment	RI1	Radio	15.72	0.65	1.44
	LI1	Radio	15.72	0.65	1.45
6 months after treatment	RI1	CT	17.26	1.03	0.9
		Radio	16.87	1.06	0.86
	LI1	CT	18.02	0.96	0.84
		Radio	18.15	1.17	0.76

Sheep-4	Tooth		Root length	Root wall thickness	Apex diameter
Before treatment	RI1	Radio	12.24	0.35	1.92
	LI1	Radio	12.24	0.35	1.92
6 months after treatment	RI1	CT	12.88	0.81	1.23
		Radio	12.72	0.75	1.2
	LI1	CT	15.07	1.05	0.69
		Radio	15.003	1.02	0.64

Table 7-13 Radiographic (mm) and CT measurements (mm) of the experimental and control teeth before treatment and six months after treatment, (*in vivo* revitalisation research protocol in a sheep model).

7.8.3 Chapter 5 related results

PR/C	RP/C/D	PP/C	PP/C/D	CELL	CELL/D
0.048	0.0360	0.0240	0.032	0.0850	0.049
0.035	0.0350	0.0500	0.039	0.0770	0.051
0.042	0.0350	0.0250	0.028	0.0650	0.054
0.038	0.0290	0.0500	0.043	0.0720	0.079
0.040	0.0350	0.0440	0.045	0.0890	0.062
0.046	0.0320	0.0690	0.038	0.0670	0.041
0.101	0.0895	0.0929	0.056	0.0709	0.053
0.099	0.0655	0.1189	0.064	0.0789	0.049
0.089	0.0725	0.1079	0.054	0.0839	0.051
0.069	0.0505	0.1009	0.056	0.0809	0.047
0.082	0.0575	0.0829	0.065	0.0869	0.044
0.073	0.0755	0.0849	0.067	0.0619	0.037
0.018	0.0260	0.0350	0.030	0.0310	0.038
0.032	0.0520	0.0370	0.032	0.0330	0.093
0.034	0.0280	0.0590	0.054	0.0290	0.080
0.022	0.0580	0.0730	0.068	0.0490	0.097
0.037	0.0540	0.0520	0.047	0.0890	0.099
0.015	0.0200	0.0320	0.039	0.0910	0.073

Table 7-14 ODPCs proliferation after one day of culturing with and without scaffold.

PR/C	RP/C/D	PP/C	PP/C/D	CELL	CELL/D
0.7700	**0.7040**	**0.5310**	**0.3820**	**0.469000**	**0.2830**
0.7820	**0.6370**	**0.4980**	**0.3910**	**0.343000**	**0.2200**
0.8320	**0.5820**	**0.5080**	**0.3840**	**0.339000**	**0.2900**
0.8110	**0.6560**	**0.4550**	**0.4030**	**0.462000**	**0.2770**
0.9110	**0.6800**	**0.5220**	**0.3350**	**0.416000**	**0.2990**
0.7870	**0.6190**	**0.6210**	**0.3960**	**0.304000**	**0.2990**
1.0035	**0.7910**	**0.7565**	**0.3465**	**0.357000**	**0.2520**
0.9785	**0.7300**	**0.6945**	**0.4535**	**0.401000**	**0.3240**
0.8645	**0.6630**	**0.7355**	**0.4765**	**0.422000**	**0.2880**
1.1975	**0.6300**	**0.6675**	**0.3515**	**0.412000**	**0.2870**
0.8515	**0.6790**	**0.5005**	**0.4445**	**0.390000**	**0.2200**
0.8935	**0.6260**	**0.6665**	**0.4765**	**0.343000**	**0.2960**
0.5177	**0.4420**	**0.4100**	**0.3670**	**0.310000**	**0.2980**
0.4767	**0.4550**	**0.3480**	**0.3340**	**0.408000**	**0.2800**
0.5097	**0.3670**	**0.4110**	**0.3170**	**0.340000**	**0.2060**
0.5547	**0.4840**	**0.3640**	**0.3670**	**0.299000**	**0.1300**
0.5747	**0.4720**	**0.4630**	**0.2750**	**0.381000**	**0.1500**
0.4256	**0.3880**	**0.4780**	**0.3330**	**0.397000**	**0.2800**

Table 7-15 ODPCs proliferation after five days of culturing with and without scaffold.

PR	PR/D	PP	PP/D	COLL	COLL/D
516.	453.	341.	282.	163.	183.
570.	470.	402.	267.	169.	190.
520.	501.	412.	305.	157.	127.
467.	420.	315.	406.	150.	201.
510.	489.	392.	260.	156.	106.
456.	360.	411.	310.	211.	191.
627.	701.	483.	360.	270.	245.
459.	771.	270.	395.	239.	139.
769.	462.	661.	460.	215.	231.

Table 7-16 ODPCs migration to each scaffold without root dentine addition.

PR/C/	PR/C	PP/C/	PP/C	PR/C/M/	PR/C/	PP/C/M/	PP/C/	C/M	C/M/	C	
0.757	0.202	0.542	0.113	0.442	0.084	0.286	0.053	0.35	0.128	0.00	0.001
0.772	0.136	0.4475	0.100	0.5475	0.110	0.275	0.093	0.28	0.117	0.00	0.002
0.917	0.152	0.453	0.093	0.594	0.120	0.215	0.084	0.20	0.102	0.00	0.001
0.887	0.120	0.508	0.103	0.2045	0.118	0.207	0.091	0.11	0.115	0.00	0.001
0.942	0.125	0.725	0.122	0.205	0.133	0.210	0.096	0.14	0.116	0.00	0.001
0.698	0.145	0.550	0.103	0.2115	0.133	0.202	0.076	0.13	0.076	0.00	0.001
0.747	0.122	0.513	0.120	0.185	0.111	0.101	0.083	0.26	0.084	0.00	0.001
0.732	0.124	0.421	0.108	0.106	0.111	0.108	0.119	0.25	0.103	0.00	0.002
0.752	0.137	0.480	0.10	0.1095	0.077	0.101	0.105	0.27	0.089	0.00	0.001
0.841	0.142	0.758	0.126	0.206	0.091	0.204	0.079	0.15	0.091	0.00	0.009
0.796	0.133	0.716	0.102	0.219	0.081	0.190	0.075	0.14	0.096	0.00	0.009
0.729	0.172	0.560	0.102	0.212	0.074	0.1915	0.066	0.15	0.076	0.00	0.004

Table 7-17 Quantitative analysis of mineralised nodules formation by ODPCs using ARS assay.

PR/C	PR/C/D	PP/C	PP/C/D	C	C/D
1.066	0.738	0.417	0.466	0.017	0.010
1.097	1.062	1.073	1.057	0.017	0.020
0.766	0.669	0.321	0.338	0.014	0.034
0.606	0.726	0.443	0.581	0.018	0.019
0.589	0.671	0.566	0.660	0.012	0.024
0.699	0.810	0.400	0.615	0.014	0.018
0.518	0.728	0.341	0.684	0.015	0.022
0.610	0.690	0.411	0.662	0.018	0.016
0.695	0.635	0.409	0.515	0.015	0.016

Table 7-18 Quantitative analysis of ALP activity of ODPCs cultured on PRP and PPP scaffolds with and without root dentine discs.

7.9 Appendix: Results related figures

7.9.1 Chapter 3 related figures

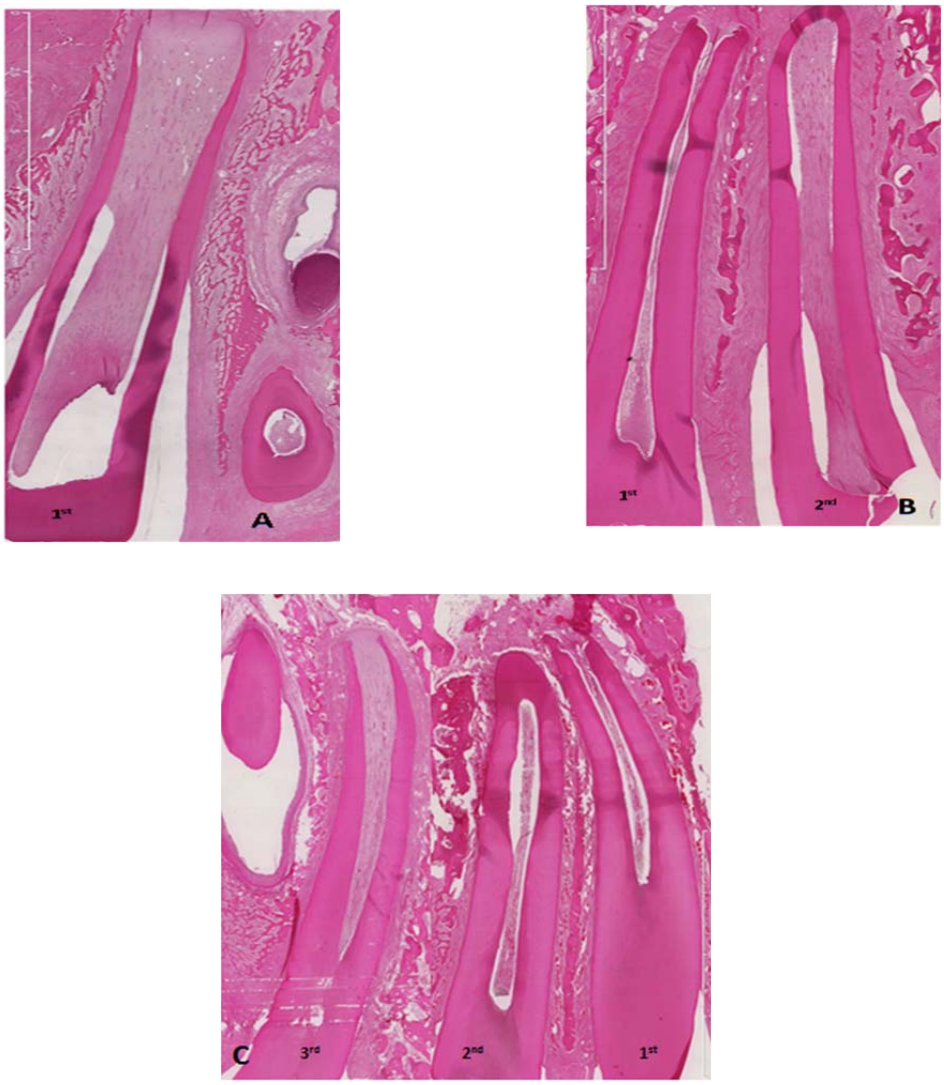


Figure 7.11 Histology sections of sheep incisor teeth.

A: Two-tooth age, permanent first incisor tooth has thin dentine walls apically and wide apex.

B: Four-tooth age, permanent first incisor has thick dentine wall and narrow apex. Second incisor tooth has thin dentine walls apically and wide apex.

C: Six -tooth age, first and second incisors have narrow canal and thick dentinal walls. Second incisor tooth has thin dentine walls apically and wide apex.

7.9.2 Chapter 4 related figures

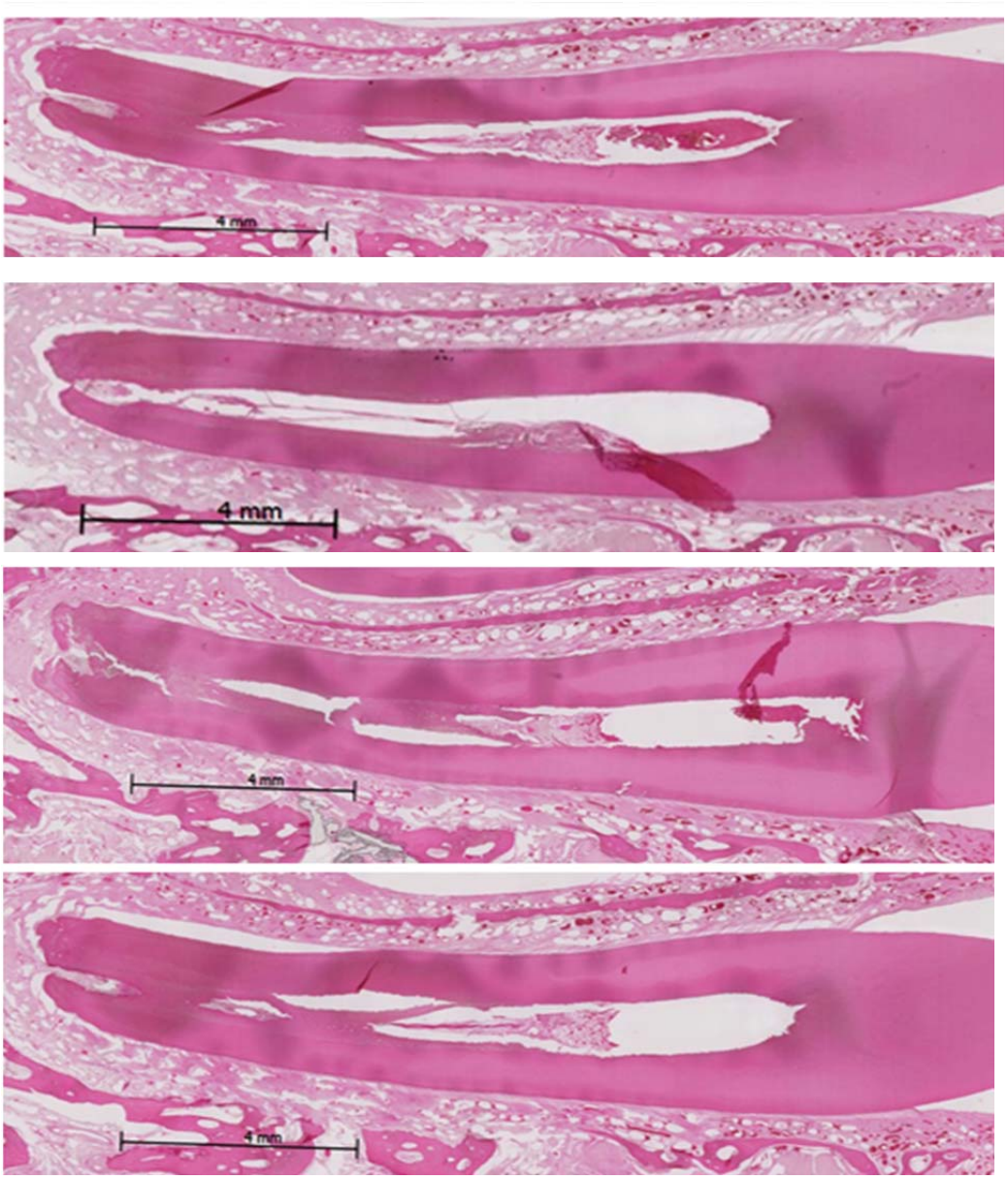


Figure 7.12 Histology sections from Sheep-1 experimental tooth.

A: Anterior section of an experimental tooth showing tissues developed in the pulp canal,

B: Middle section of an experimental tooth showing a narrow pulp cavity filled with loose fibrovascular connective tissues.

C: Section approximately 400 µm posterior from (B) showing a thick hard tissue deposit in the apical area,

D: Posterior section of an experimental tooth showing hard tissue deposit on the root walls.

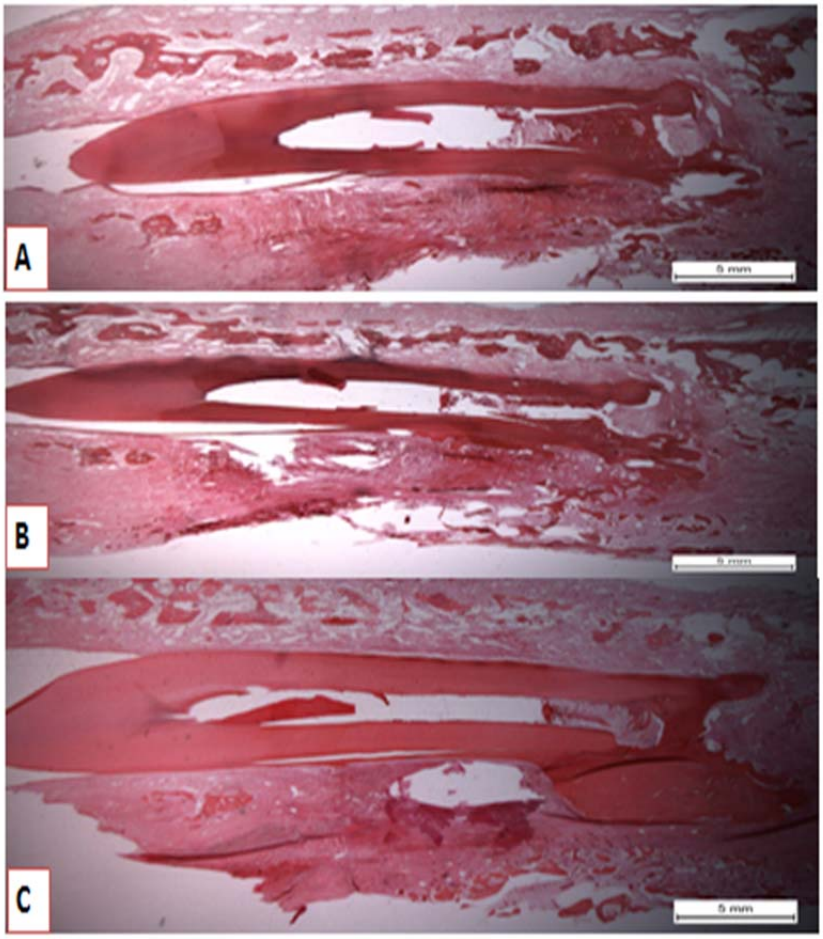


Figure 7.13 Histology sections from Sheep-3 experimental tooth.

A: Anterior section of an experimental tooth showing hard tissues developed on dentinal walls and soft tissue in the pulp canal.

B: Middle section of an experimental tooth showing narrow pulp cavity filled with loose fibrovascular connective tissues and narrow apex.

C: Posterior section of an experimental tooth showing hard tissue deposit on dentine walls and bridging the apex.

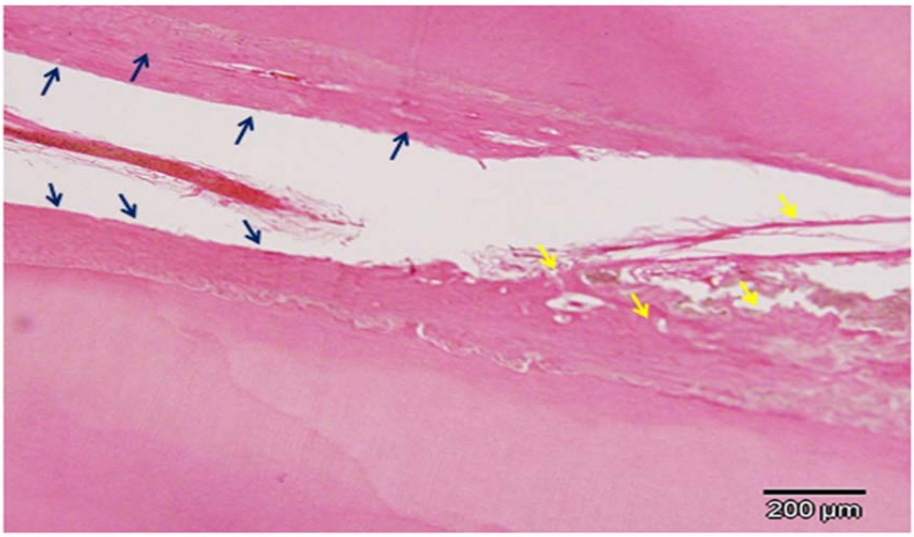


Figure 7.14 Histology section showing the second and third healing regions.

Yellow arrow indicate the second healing region, black arrows indicate the third healing region (a).

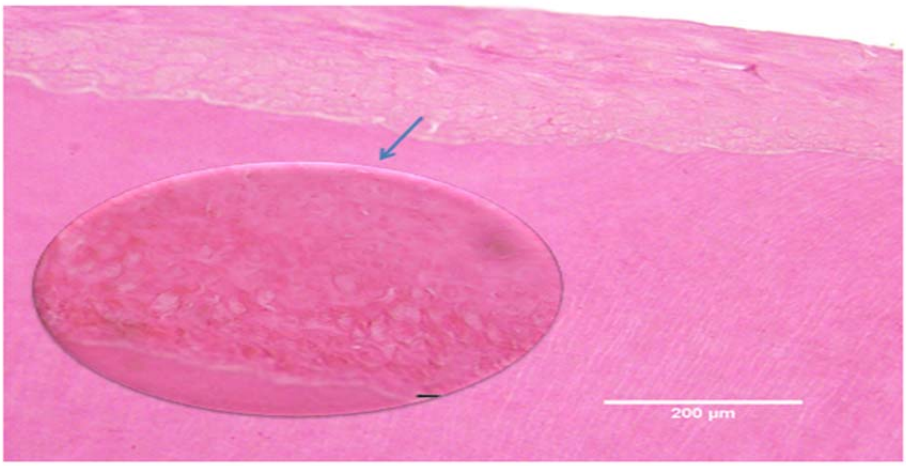


Figure 7.15 Third region (a).

Blue arrow indicates magnified section of the deep layer of the third region showing the structure of the matrix similar to bundle-like structure

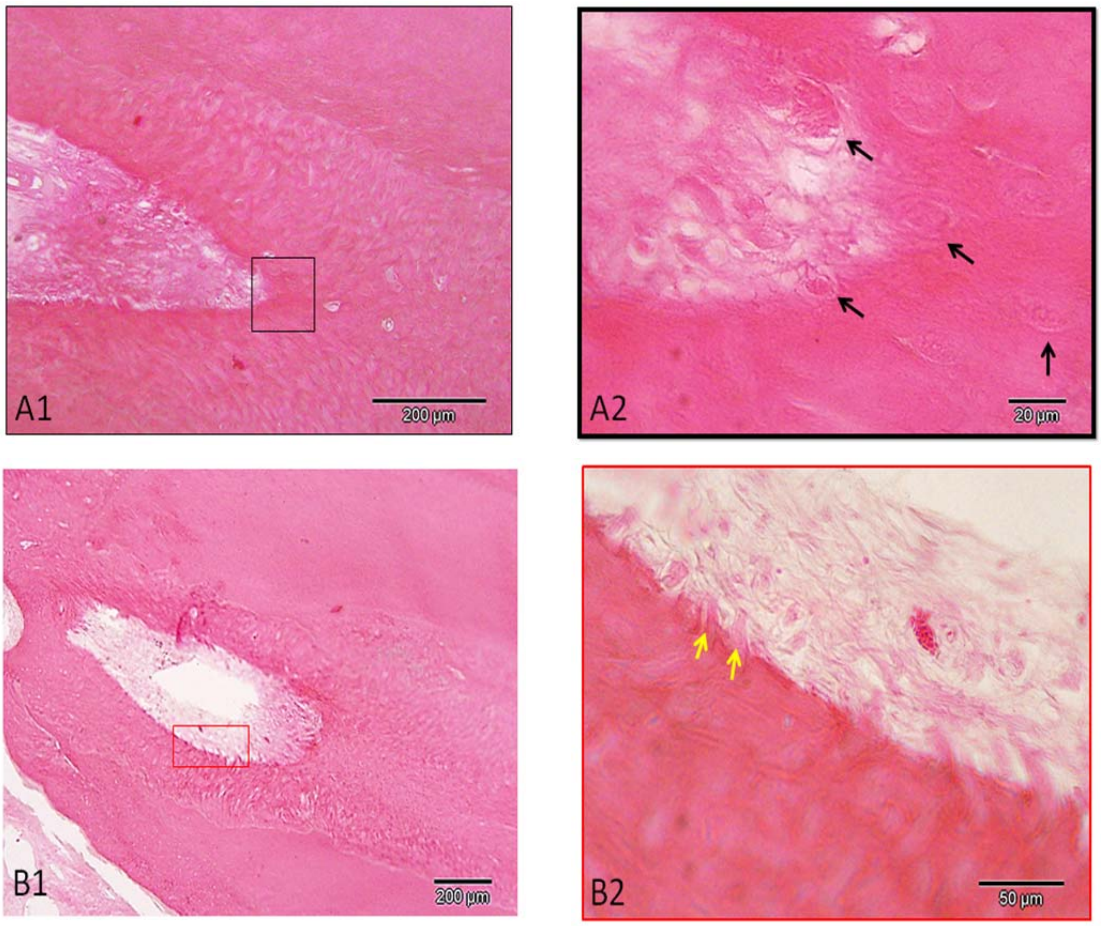


Figure 7.16 Histology sections at the apex of the experimental tooth.

A1: apical region with hard tissue on the walls and fibrovascular tissue in the canal.

A2: high magnification of the area indicates by Black Square in image (A1), showing cells attached to the hard tissue and some cells embedded in the hard tissue matrix.

B1: Section in the apical region.

B2: high magnification of the area indicates by the red rectangle, showing projections from the fibrous tissue in the canal inserted in the walls hard tissue.

Black arrows indicate cells; yellow arrows indicate fibres projection inserted in the walls hard tissue.

7.9.3 Chapter 5 related figures

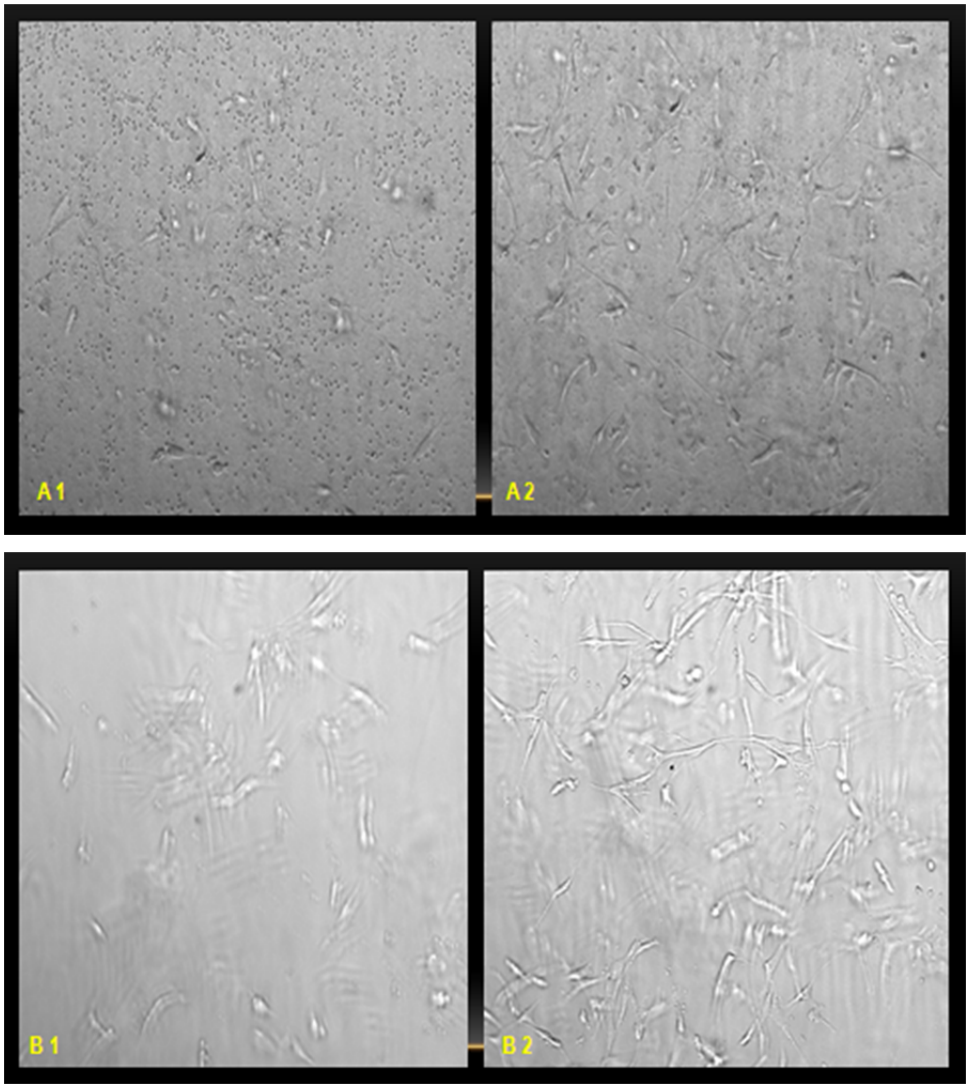


Figure 7.17 ODPCs proliferation on PRP and PPP scaffolds.

A1: Proliferation of ODPCs on PRP scaffold after one hour of culturing.

A2: Proliferation of ODPCs on PRP scaffold after one day of culturing.

B1: Proliferation of ODPCs on PPP scaffold after one hour of culturing.

B2: Proliferation of ODPCs on PPP scaffold after one day of culturing.

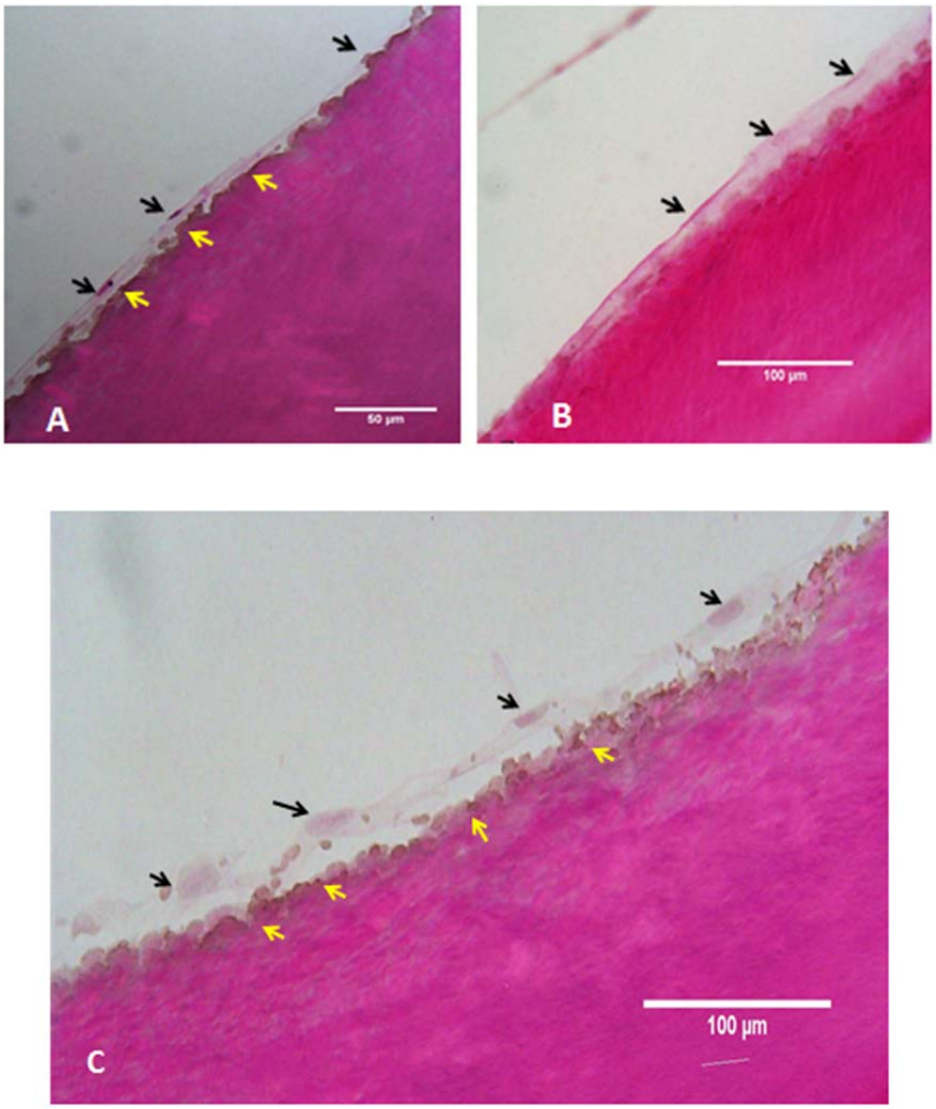


Figure 7.18 ODPCs cultured on PRP and PPP scaffolds in sheep roots.

A: ODPCs cultured on PRP scaffold attached to dentine walls.

B and **C**: ODPCs cultured on PRP scaffold attached to dentine walls.

Black arrows indicate cell, yellow arrows indicate a brown margin owing to EDTA effect.

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