Physiological and molecular responses in the pulp associated with early inflammatory process

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Abstract

Therapies to promote pulp repair and regeneration after injury should be underpinned by a deep understanding of normal tissue behaviour, cellular cross-talk and regulation. The objectives of this study were to investigate the expression of cyclooxygenases (COX1 and COX2), prostanoid receptors (EP1 and EP2) and nitric oxide synthase 1 (NOS1) within the normal dental pulp. The effect of experimental inflammatory conditions on these elements at mRNA level (both normal and experimentally inflamed) were investigated to explore the possibility of a nitric oxide (NO)/prostaglandin (PG) signalling pathway interaction in the rat mandibular incisor (normal and experimentally inflamed).

Rodent mandibular incisors were utilised as a model throughout this thesis with structural and functional investigations on demineralised teeth, non-demineralised freshly extracted pulp tissues and tissue explants. The work described in this thesis used immunohistochemical, ELISA and quantitative reverse transcription polymerase chain reaction (q-RT-PCR) techniques.

Cellular heterogeneity was observed both in the odontoblasts population and in the interstitial cells forming the bulk of the pulp. Cellular processes were also observed in addition to the observation of cellular processes extending from interstitial cells in the cell-rich zone to odontoblasts. The presence and localisation of immunoreactivity to the above mentioned targets (COX1, COX2, NOS1 and prostanoid receptors) are novel and confirmed with q-RT-PCR. Isolated pulp tissues exposed to LPS were found to release an increased amount of PGE_2, which was found to be inhibited by other factors like the presence of NO and ATP.

Functional cross-talk between PG and NO was investigated by the ELISA technique, with experimentally-induced inflammation increasing PG release, whereas NO and ATP caused inhibition of PGE_2 release.

The effects of carefully selected pro-inflammatory agents (LPS, NO and ATP) on PG pathway were examined at the mRNA level by q-RT-PCR. Exposure to LPS was found to cause upregulation of all target genes, whereas variable reactions were observed in response to incubation with NO, ATP and PGE_2.

Certificate of approval

I confirm that, to the best of my knowledge, this thesis is from the student's own work and effort, and all other sources of information used have been acknowledged. This thesis has been submitted with my approval.

Professor
John M. Whitworth

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List of abbreviations

15-PGDH	15-hydroxyprostaglandin dehydrogenase.
AA	Arachidonic acid.
AGF	Angiogenic growth factor.
ATP	Adenosine triphosphate.
B0	Zero binding.
Barx	BarH-like homeobox.
BMP	Bone morphogenetic protein.
bp	Base pair.
BSP	Bone sialoprotein.
cAMP	Cyclic adenosine monophosphate.
CD	Calibrator diluent.
CD14	Cluster of differentiation 14.
cDNA	Complementary DNA.
CEJ	Cemento-enamel junction.
COX	Cyclooxygenase.
crz	Cell-rich zone.
DAPI	4’,6-diamino-2-phenylindole.
DC	Dendritic cell.
dcz	Diffuse cell zone.
DEJ	Dentino-enamel junction.
DMEM	Dulbecco’s modified Eagle’s medium.
DMP	Dentine matrix protein.
DNA	Deoxyribonucleic acid.
DPP	Dentine phosphoprotein.
DSP	Dentine sialoprotein.

DSPP	Dentine sialophospho protein.
EDA	Ectodysplasin A.
EDTA	Ethylenediaminetetraacetic acid.
EGF	Epidermal growth factor.
ELISA	Enzyme linked immunosorbent assay.
EP	Prostanoid receptor type E.
FGF	Fibroblast growth factor.
gDNA	Genomic DNA.
HERS	Hertwig's epithelial root sheath.
Hh	Hedgehog.
HIV	Human immune virus.
HRP	Horseradish peroxidase.
IGF	Insulin-like growth factor.
IL	Interleukin.
IR	Immunoreactivity.
Lhx	LIM homeobox protein.
LPS	Lipopolysaccharides.
MHC	Major histocompatibility complex.
MSX	Muscle segment homeobox.
NaKATPase	Sodium Potassium ATPase.
NLR	NOD-like receptor.
NO	Nitric oxide.
NOD	Nucleotide-binding oligomerization domain receptor.
NOS	Nitric oxide synthase.
NSAID	Non-steroidal anti-inflammatory drugs.
NSB	Non-specific binding.

OCB	Odontoblast cell body.
OCL	Odontoblast cell layer.
OCT	Optimal cutting temperature compound.
odp	Odontoblast process.
P2	Purinergic receptor type 2.
Par	Protease activated receptor.
Pax9	Paired box gene 9 transcription factor.
PBS	Phosphate buffer saline.
PCR	Polymerase chain reaction.
PDGF	Platelet-derived growth factor.
PFA	Paraformaldehyde.
PG	Prostaglandin.
PG F2 α	Prostaglandin F2 α.
PGD2	Prostaglandin D2.
PGE2	Prostaglandin E2.
PGG2	Prostaglandin G2.
PGH2	Prostaglandin H2.
PGI2	Prostaglandin I2.
PITX	Paired-like homeodomain.
PRR	Pattern recognition receptor.
RER	Rough endoplasmic reticulum.
RIG-I-like	Retinoic acid-inducible like receptor.
RNA	Ribonucleic acid.
RT	Reverse transcription.
SEM	Standard error of means.
Shh	Sonic hedgehog protein.

SOL	Sub-odontoblast cell layer.
TAE	Tris-acetate-EDTA.
TBS	Tris buffer saline.
TBS-T	Tris buffer saline with Tween.
TGF	Transforming growth factor.
Th	T helper cell.
TLR	Toll-like receptor.
TNF	Tumor necrosis factor.
TXA2	Thromboxane A2.
VEGF	Vesicular endothelial growth factor.

Chapter 1. Literature review

1.1. General introduction about the Pulp-dentine complex

Mammalian teeth are composed of rigid mineralized tissues (enamel, cementum and dentine) surrounding a specialized loose connective tissue called the pulp which resides in the central part of the tooth.

The dental pulp is a unique soft connective tissue, with many specific features including the presence of highly specialised odontoblasts, confinement within rigid mineralised tissues from all sides with the exception of a small apical foramen and the limited availability of a collateral blood supply. The intimate embryological, physical and functional relationships between dental pulp and the dentine make them regarded as a structural and functional unit, often referred to as the dentine-pulp complex (Goldberg and Lasfargues, 1995, Pashley, 2000, Nanci, 2007). From a clinical standpoint the concept of the dentine-pulp complex is highlighted by the fact that the odontoblast cell bodies (OCB) are embedded within the periphery of the pulp while their cytoplasmic processes extend peripherally within dentine and may be influenced by clinical manipulation of dentine. This positioning enables the odontoblasts to play a role in sensing aspects of the oral environment and playing a defensive role at the interface between dental hard and soft tissues, contributing to the development of the hard-tissue barriers (Turner et al., 1989, Bishop, 1991) and assisting in the maintenance of the hard tissue integrity in the face of injury (Frank and Nalbandian, 1989, Arana-Chavez and Massa, 2004). The dental pulp bears many resemblances to other connective tissues in the body, but its low compliance surroundings, relative incompressibility and limited expansion ability are unusual and of critical importance.

Histologically, the basic components of the dental pulp have much in common with other loose connective tissues, including vascular tissues, connective tissue fibres, ground substance, axons and cellular elements. Many cell types reside within the dental pulp including odontoblast, fibroblast, immunocompetent and undifferentiated mesenchymal cells.

1.2. Enamel

Enamel is the hardest mineralised tissue in mammalians, for example the contact hardness of enamel is about four to five times more than that of dentine, more than 8 times that of cortical bone and nearly ten times that of trabecular bone (Oyen, 2006, Chun et al., 2014).

This hardness allows the enamel-covered crown of the teeth to withstand masticatory forces and abrasive stresses throughout the animal life.

Enamel is formed by specialised cells called ameloblasts which are lost after tooth eruption making the enamel a non-renewable tissue (Cuy et al., 2002). The enamel hardness comes from its complex crystalline structure, and its low organic and water contents (approximately 4%). With hardness comes brittleness, and in order to function successfully, it is supported by an underlying layer of dentine. Enamel maximum stress is about 1:3 than that of dentine, and the maximum strain is about 1:2.5 than dentine (Chun et al., 2014). The inorganic component of the enamel is made up principally of crystalline calcium phosphate and carbonated apatite, so-called 'hydroxyapatite' though, any ions that may be present at the time of enamel matrix secretion and mineralisation could be incorporated in the hydroxyapatite crystals.

The basic structural units of enamel are called enamel prisms or rods which have a hexagonal cross section appearance and are densely packed together, cemented by interprismatic substance. The enamel rods may run throughout the entire thickness of enamel, with a wavy pattern as they are secreted by single ameloblasts. The term enamel rod is probably more accurate since these units have a cylindrical appearance in longitudinal section and usually have scales, an ovoid or keyhole appearance with their heads toward the occlusal or incisal enamel and their tails near and at right angles to the DEJ.

The organic protein and water-containing components are largely presented in the interprismatic substance between the hydroxyapatite crystals. These proteins are usually of two types: hydrophobic, low molecular weight amelogenins (90%) and high molecular weight nonamelogenins (10%).

1.3. Cementum

Cementum is an avascular mineralised connective tissue which covers the root of the tooth and is a key element of the periodontal attachment apparatus. It can be sub-classified according to the presence or absence of cells and fibres into three distinct types (Listgarten and Kamin, 1969): i. Acellular afibrillar, which contains no fibres and cells and is seen in the coronal cementum around the CEJ. Since it has no collagen, it has no role in periodontal attachment, at least in mature state. ii. Acellular extrinsic fibres (Primary Cementum) with fibres but no cells, is usually confined to the coronal two thirds

of molars and premolars, but covers the entire root of incisors and canines, and has a major role in tooth attachment. iii. Cellular intrinsic fibres (Secondary cementum) which may be absent in single rooted teeth, but seen in the apical part and inter-radicular areas of premolars and molars with an adaptive vital role in the tooth's response to wear and movement. This type of cementum forms after the teeth have reach a position of occlusal contact.

The biochemical composition of cementum is similar to that of bone with unique proteins. Inorganic hydroxyapatite forms about 45%-50%, the remaining are the organic (collagen and non-collagenous proteins). Approximately 90% of the collagen is Type I with lesser amounts of Type III. Non collagenous proteins include alkaline phosphatase, bone sialoprotein, fibronectin, osteocalcin, osteonectin, osteopontin, proteoglycans, proteolipids, vitronectin and several growth factors are also present in cementum (Nanci, 1999, Ikezawa et al., 1997, Yonemura et al., 1992).

Cementum contains many growth factors within its structure including FGFs, platelet derived growth factor (PDGF), TGF-β and BMPs (Cochran and Wozney, 1999, Grzesik and Narayanan, 2002, MacNeil and Somerman, 1999). Specific conditions might lead to the elaboration of these growth factors with reported relationship to cementoblast mitosis, differentiation and movement to the affected sites during the repair and healing of cementum following injury (Grzesik and Narayanan, 2002).

1.4. Dentine

Dentine is a collagen-based mineralised connective tissue forming the bulk of the tooth. It surrounds and encloses the pulp tissue from all directions, except for the apical and lateral foramina. It is covered by a cap made of enamel on the crown of the tooth and a coat of cementum on the root. This tissue is formed and mineralised by a highly specialised cell called the odontoblast (Linde and Goldberg, 1993) and has a very close relation with the dental pulp, with which it shares a similar embryological origin (Goldberg and Lasfargues, 1995).

1.4.1. Histology and morphology:

Dentine is a bone-like mineralised tissue with numerous densely packed tubules called dentinal tubules, usually representing about 20-30% of the tissue volume. These tubules usually cross the entire dentine thickness from the DEJ to the pulp in wavy, s-shaped lines and usually contain the cytoplasmic processes of the secretory odontoblasts, the so-called

odontoblast processes. The s-curvature is less prominent in dentine at the incisal edge and cusp tips, and results from the route of travel and packing of the odontoblasts as they retreat toward the centre of the tooth. Dentinal tubules are usually tapered with a wider portion near the pulp and narrower portion near the DEJ. In terms of density, tubules are almost twice as densely packed near the pulp than at the DEJ. This is due to the packing of odontoblast as the pulp space is reduced and odontoblasts are bunched on an ever diminishing pulp chamber circumference. Dentinal tubules are not simple tube-like structures. Many branches can be seen between the dentinal tubules usually at 45˚-90˚ to the main tubule (Schroeder and Frank, 1985), with a great degree of anastomoses which allows the dentine to have a high degree of permeability and facilitates fluid movement. These branches have been classified into three groups according to their size, location and direction from the primary tubule (Mjör and Nordahl, 1996): Firstly the major branches have a diameter of 0.5-1 µm, located about 250 µm from the edge of coronal and root dentine. Secondly, fine branches usually emerge at 45˚ angle from the primary tubule, ranging from 0.3-0.7 µm in diameter and usually limited to root dentine or crown dentine with low tubular density. Thirdly, microtubules are scattered throughout dentine with a very small diameter of 0.1 µm, emerging at right angles from the primary tubule. Some dentinal tubules end in tree-like branches near DEJ of coronal dentine or sometimes can breach the DEJ (Carda and Peydro, 2006).

Dentinal tubules are surrounded by a collar of highly mineralised dentine called peritubular dentine (Arana-Chavez and Massa, 2004, Carda and Peydro, 2006). This type of dentine measure about 1µm in thickness and is more mineralised than other types of dentine (40% more than intertubular dentine) with little or no collagen but rich in phosphoproteins (Gotliv and Veis, 2009, Gotliv et al., 2006, Xu and Wang, 2012). The thickness of this type of dentine varies greatly depending on the distance from the pulp, being very thin or completely absent near to the pulp and of greatest thickness near the DEJ. The formation and mineralisation of this type of dentine may result from the adsorption of unstructured non-collagenous proteins on the peripheries of the dentinal tubules. The deposition of this type of dentine leads to a continuous decrease in the tubule lumen, and it has been reported that the deposition of peritubular dentine may be accelerated by irritation and environmental stimulation (Nanci, 2012). Peritubular dentine has a porous nature (Gotliv and Veis, 2007, Gotliv and Veis, 2009), leading to the conclusion that the boundaries of dentinal tubules are fenestrated by numerous tiny pores,

which, together with the branches of the dentinal tubules, facilitate the movement of fluids and other matrix components across the peritubular dentine in both directions. Furthermore, peritubular dentine has an organic component called calcium-phospholipid-proteolipids (Gotliv and Veis, 2007). Similar proteins have been shown to have vital roles in brain neurological actions (Turner et al., 2005). Thus, peritubular dentine may have a potential role in active transport of ions and signalling (Irvine, 2003) between the odontoblast and the intertubular dentine or regulating the actions that help in maintaining the dentine as a live and vital tissue.

The main bulk of dentine is composed of intertubular dentine, which fills the spaces between the rings of peritubular dentine. About half of its composition is organic, mainly made up of collagen fibres that run circumferentially (Beniash et al., 2000) around each tubule. Because of this organic matrix, intertubular dentine is usually preserved after pathological or laboratory decalcification. Intertubular dentine is different from peritubular structurally and compositionally; it is highly mineralised but less than that of peritubular dentine (Xu and Wang, 2012) with a 1:3 minerals to matrix ratio, is less homogenous and has very different mechanical and physical properties including different elastic characteristics (Kinney et al., 1996), optical anisotropy (Iwamoto and Ruse, 2003) and fracture resistance (Wang, 2005). However, the main crystals in the inorganic phase are quite similar in alignment and size to those of peritubular dentine (Weiner et al., 1999).

1.4.2. Chemical properties

Generally, mature dentine is usually composed of organic and inorganic (mineral) phases. The inorganic phase represents about 70% of the dentine by weight and 45% by volume, while the organic phase comprises approximately 20% by weight and 33% by volume, water making up the remainder. Nevertheless, the distribution of water is not consistent throughout the dentine, because the water is largely located inside the dentinal tubules which have varying diameters in the dentine as previously described. This variation in the water contents can approach 20 fold (Pashley, 1996).

The inorganic contents of dentine is made up of hydroxyapatite crystals, while Type I collagen usually forms up to 90% of the organic component along with minor percentages of types III (Lukinmaa et al., 1993) and V (Bronckers et al., 1986, Lukinmaa and Waltimo, 1992). The remaining percentage is made up of ground substance which is composed of small fractions of non-collagenous proteins, proteoglycans, osteonectin,

osteopontin, decorin and biglycan, dentine sialoprotein (DSP), dentine phosphoprotein (DPP) and phospholipids (Goldberg and Smith, 2004). Moreover, growth factors can be found in the dentine matrix including TGFs (Cassidy et al., 1997), FGFs, BMPs, PDGF, insulin-like growth factor (IGF), epidermal growth factor (EGF), angiogenic growth factor (AGF) (Roberts-Clark and Smith, 2000) and vascular endothelial growth factor (VEGF). The composition of dentine shows considerable variation among the different areas of the tooth (Linde and Goldberg, 1993).

1.4.3. Physical properties

In young individuals, the dentine has a light yellowish colour but with age it will become darker in colour. Dentine is marginally harder than bone but remarkably softer than enamel, with viscoelastic properties and the ability to withstand mild deformation which helps in maintaining the full functionality of the tooth. This toughness or viscoelasticity assists in protecting the overlying brittle enamel coat from fracture.

There are slight variations in the mechanical properties of dentine in different types of teeth, in different areas in the same tooth, between the root and crown, in case of dehydration (Bajaj et al., 2006) and reported great variations with age (Arola and Reprogel, 2005, Fonseca et al., 2008). Dentine is relatively softer in the deciduous than in permanent teeth. Furthermore, the central portion of dentine in any tooth is harder than that near the pulp. Due to its relatively different organic contents, it appears more radiolucent than enamel and more radiopaque than the pulp, cementum and bone.

1.4.4. Types of dentine

Many classifications of dentine exist on the basis of position, function and origin. From a formative point of view, dentine can be classified into six distinct types: DEJ, mantle dentine, primary dentine, secondary dentine, tertiary dentine and predentine.

1.4.4.1. DEJ:

For a long time, this was considered simply as the biologically and anatomically inactive junction area between dentine and enamel, commonly seen as a wavy line measuring about 7-15µm in thickness (Gallagher et al., 2003). Nonetheless, there is an increasing belief that this junction is a more complex interface. One of the proposed complexities of this junction is that it is made of two delicate layers fused together, inner aprismatic enamel and mantle dentine (Goldberg et al., 2002) with a suggested cross-talk between dentine and enamel during the dentinogenesis and amelogenesis processes. The existence

of a specific enzymes (Goldberg et al., 2002, Boushell et al., 2008) and some growth factors like FGF-2 (Goldberg et al., 2002) may suggest biological activity in DEJ. The wavy nature of this interface (Radlanski and Renz, 2006, Marshall et al., 2003) works to facilitate the mechanical and structural engagement between enamel and dentine, being deeper and larger in incisal margins and cusp areas than in the cervical region (Radlanski and Renz, 2007), which explains the weaker mechanical engagement between enamel and dentine at the cervical area (Goel et al., 1991). In addition, there are two possible ways to enhance the interlocking between dentine and enamel: the continuity of the mineral inorganic crystals from dentine to enamel, and the engaging organic matrix (Jones and Boyde, 1984).

1.4.4.2. Mantle dentine:

This is the first type of dentine to be secreted, located adjacent to the enamel and cementum, measuring about 5-30µm in thickness, and with a more irregular organic matrix that differentiates it from the rest of the dentine (Linde and Goldberg, 1993). Mantle dentine usually contains bundled, coarse Type III collagen fibrils, with very little Type I collagen (Ohsaki and Nagata, 1994). The collagen fibrils are orientated parallel to the long axis of the odontoblast processes. This type of dentine is biochemically different from other types, with an absence of phosphoproteins and less inorganic contents than primary dentine, and with a gradual difference in the degree of mineralisation (Tesch et al., 2001). Mantle dentine is also devoid of dentinal tubules with the occasional existence of fine tubular branches (Mjör and Nordahl, 1996). Despite this fact, mantle dentine retains some permeability (Byers and Lin, 2003, Ikeda and Suda, 2006). The absence of dentinal tubules may indicate that the mantle dentine is secreted by differentiating or immature odontoblasts that lack the processes at the time of secretion. It is also worth mentioning that the fate of the basement membrane proteins degradation (which is located between the odontoblast and differentiating ameloblasts) is undiscovered yet, and it is logical to think that these degradation products or at least some of them might be integrated into the ground substance of mantle dentine before the mineralisation process begins (Tjäderhane et al., 2009).

It seems that the elastic characteristics of the teeth are mainly the responsibility of the mantle dentine, facilitating the teeth to withstand high occlusal loads during the routine daily function without fracturing.

1.4.4.3. Primary dentine:

The bulk of dentine is termed primary dentine, and is formed quickly during the process of dentinogenesis. Its organic matrix is secreted by odontoblasts and contains more dense and thin collagen fibres which are closely packed to each other forming a mesh around the dentinal tubules (Arana-Chavez and Massa, 2004). Primary dentine gives the tooth its final shape and size which is understood to be genetically predetermined. Structurally, it is composed of dentinal tubules, peritubular and intertubular dentine (Xu and Wang, 2012).

1.4.4.4. Secondary dentine:

After the complete formation of primary dentine, the odontoblasts retain the capacity to continue to lay down the dentine throughout the life of the tooth. Secondary dentine is laid down regularly, though at much slower rate (Arana-Chavez and Massa, 2004), and this process is believed to be physiological, rather than in response to injury. During secondary dentine deposition, odontoblasts retreat toward the pulp resulting in a reduction of the pulp volume and associated crowding of the odontoblast due to lack of space. From a structural point of view, secondary dentine has subtle differences from primary dentine, with slight differences in tubules curvature, and a slightly less regular tubular structure, though the tubules are continuous with those of the primary dentine.

The deposition of secondary dentine is not even throughout the pulp peripheries, with greater deposition on the roof and floor of the pulp chamber (Nanci, 2012).

The exact point where the odontoblast stops secreting primary dentine and starts laying down secondary dentine is poorly defined and controversial. It has been hypothesised from studies in rodent molars that the cessation of primary dentine formation occurs at the time of crown completion, where some cellular organelles were subject to programmed atrophy (Romagnoli et al., 1990). Others have reported the timing out of the primary odontogenesis to occur once the tooth comes into function (Linde and Goldberg, 1993) or after the completion of root formation (Nanci, 2012). The time between these events is considerable, highlighting the controversy that persists.

1.4.4.5. Tertiary dentine:

Almost any external noxious stimulus, including abrasion, erosion, attrition, caries, trauma and operative procedures will trigger the deposition of tertiary dentine in an effort

to protect the pulp by increasing the thickness of mineralised tissue. Throughout the literatures, many terms have been applied to describe this type of dentine including irregular dentine, irritation dentine and irregular secondary dentine (Cox et al., 1992). More recently, the terms tertiary reactionary and tertiary reparative dentine have been widely adopted (Smith et al., 1995). The molecular changes after each injury can be seen very early in the form of alteration in the enzymatic activities in the pulp-dentine interface, and even when the injury is limited to the overlaying enamel (Larmas, 1986), changes can be seen in dentine (Läikkö, 1984).

The morphology and uniformity of tertiary dentine depends on the severity and persistence of the external stimulus. This type of dentine has similar inorganic and organic phases to primary and secondary dentine.

In general, tertiary dentine is recognised in two forms: reactionary (produced by primary odontoblasts, which have persisted despite dental injury) and reparative dentine (produced by newly differentiated odontoblast-like cells where injury has led to the death of the primary odontoblast) (Smith and Lesot, 2001). These newly differentiated odontoblasts are believed to originate from cells within the cell-rich zone (crz) pulp cells that are de-differentiated into undifferentiated mesenchymal cells and then re-differentiate into odontoblast-like cells (Sloan and Smith, 2007) with a poorly defined degree of differentiation (Pääkkönen and Tjäderhane, 2010).

Dentinal tubules in reactionary dentine have some degree of continuity with that of the secondary dentine, while reparative dentine varies significantly from secondary dentine in terms of tubular absence, organization, structure and degree of mineralisation. The atubular nature of reparative dentine makes it a rather hermetic protective barrier between vascular pulp tissues and overlaying tubular dentine or caries (Mjör, 1985). However, complete isolation of the dental pulp tissue from the overlaying tubular dentine by a layer of atubular dentine is not always preferable, since the pulp contains sensory nerves that exhibit considerable changes when they sense danger and usually take part in recruitment of the immune and defensive cells to the traumatised pulp (Jontell et al., 1998). Thus, the formation of a substantial layer of reparative dentine may protect the pulp from microbial invasions but may slow the defensive actions of the pulp in the face of caries progression (Kamal et al., 1997).

The uniformity and extent of reparative dentine appears to be inversely related to the severity of the external noxious stimulus (Tziafas, 2007). Sometimes, reparative dentine formation is diffuse to the extent that it may block the entire pulp space leading to complete or partial loss of the root canal system which may further limit the pulp's ability to cope with injury and make the root canal treatment very difficult.

Pulp tissue preserves the ability to withstand significant dentinal loss by deposition of tertiary/reactionary dentine, even if the remaining dentine was as thin as 0.5 mm (Murray et al., 2000a, Murray et al., 2003) by increasing cellular proliferation and protein deposition. However, the ability of the pulp to heal is decreased by repeated injuries or trauma which further influence the pulp's ability to deposit reactionary dentine and cellular differentiation or recruitment of new odontoblasts together with significant reduction in cellular multiplication and deposition of proteins after the single injury (Johnson, 2004).

Tertiary odontogenesis is not limited to the dentine-pulp interface, but also can be distinguished as an increased thickness of peritubular dentine which might end with complete obliteration of the tubules (Smith et al., 1995).

1.4.4.6. Predentine:

Predentine occurs at the advancing front of primary, secondary and tertiary dentine, and is the non-mineralised organic dentine matrix, secreted by odontoblasts and measuring 10-50μm in thickness. The thickness of predentine is greatest during active dentinogenesis, and gradually decreases with age. The main component of predentine is collagen fibrils, mainly Type I and II, along with a group of non-collagenous proteins including phosphophoryn, proteoglycan and glycoproteins (Goldberg and Lasfargues, 1995). Phosphophoryn is a tissue specific and highly phosphorylated molecule believed to participate in tissue mineralisation via intercommunication with calcium (Goldberg and Smith, 2004).

Predentine successively mineralises to become normal dentine, however, its thickness remains the same because the mineralised predentine is continuously replaced by newly secreted non-mineralised matrix by odontoblasts.

1.5. Dental pulp

The pulp is derived from neural crest cells of ectomesenchymal origin. These cells proliferate and condense to form the dental papilla from which the connective tissue elements of the tooth develop. The fully mature pulp has a strong similarity with embryonic connective tissues in other parts of the body (Bletsa et al., 2006, Luukko et al., 2011).

The dental pulp can be defined as a loose connective tissue, with many features in common with other loose connective tissues of the body. It is composed mainly of gelatine-like material with proteoglycan and glycoproteins augmented by unevenly aligned and anastomosed collagen fibres (Yu and Abbott, 2007). Some of its unique features include its position within the non-compliant environment of the tooth; a rigid shell composed of dentine, which is covered with enamel on the crown and cementum on the root, and offers pulp tissue support and protection (Tziafas et al., 2000, Nör, 2006). In addition, the limited expandability, the limited collateral blood supply, high level of sensory innervation and rich microvascular elements, make the pulp a distinctive tissue and govern its inflammatory reactions (Berggreen and Heyeraas, 2000). If the pulp's protective barriers (enamel, dentine and cementum) are broken, it becomes vulnerable and threatened by insulting stimuli from the oral cavity. The breakdown of the protective barrier may result from caries, cracks, wear or defective restoration margins, which may provide a route for microbial invasion of dentine and pulp tissue (Yu and Abbott, 2007). Microbial invasion may in turn result in irritation and inflammation of the pulp tissue, with potential consequences including pulp necrosis (Smith et al., 2008, Bjørndal and Larsen, 2000).

A question that arises is whether the pulp is important after the full formation of the tooth or not? The overwhelming evidence from clinical practice is that teeth that have lost vital pulp functions and undergone root canal treatment may survive and function well for many years (Salehrabi and Rotstein, 2004, Ng et al., 2010). It is, however, recognised that teeth with vital pulp functions are significantly more resistant to bacterial invasion through dentinal tubules than pulpless teeth or teeth that have undergone root canal treatment (Nagaoka et al., 1995).

The primary role of the dental pulp is believed to be in tooth formation. However, even after dental development is complete, the pulp plays important roles in hydrating and maintaining the toughness of dentine, sensing dangers to the host due to occlusal overload

or breech of protective enamel and cementum, and orchestrating defensive hard and soft tissue defences (Luukko et al., 2011). Key to these roles are dense networks of blood vessels and nerves which enter through the apical foramen (Nakashima and Akamine, 2005) and complex cellular interactions. So important are the preservation of vital pulp functions that great interest is developing in the revascularisation and regeneration of pulp tissues damaged by disease, the mechanisms of which are again incompletely understood (Smith et al., 2008). The overall pulpal outcome in response to insult represents the balance between the stimulus, defence and repair processes.

1.5.1. Histological zones within the mature human pulp:

The dental pulp is usually considered to consist of four distinct histological zones as depicted in Figure 1-1 and as follows:

1. The pulp core: This is the central part of the pulp where the main nerve trunks and main blood vessels are found. These blood vessels are often venules and arterioles which enter through the apical foramen, extensively anastomosing, forming a rich and dynamic circulatory system. Several small branches of these vessels leave the pulp core and direct toward the peripheral zones where they form an extensive network of terminal capillaries in varying relation to odontoblasts depending on the odontoblast life cycle (Yoshida and Ohshima, 1996). These capillaries supply the odontoblasts with nutrients, minerals and ions which may be of particular importance during active tissue secretion. The overall pulp vascularity is comparable to the highly vascular areas of the tongue and the brain, making the pulp a highly vascular organ (Vongsavan and Matthews, 1992) with suggested protection from atherosclerosis (Krell et al., 1994). The vasculature of the pulp is responsible for the transport of oxygen, nutrients, minerals, gases, hormones, drainage of waste products and controlling the intra-pulpal pressure. It is conceivable that the control of the pulp microcirculatory system happens locally (Yu et al., 2002, Okabe and Todoki, 1999) but may also be modulated by physical stimuli, such as occlusal loading on the tooth (Shibutani et al., 2010). The regulation of local blood flow and pressure within the dental pulp is fundamental to the pulp survival, since inflammatory processes may lead to raised pressure within a low-compliance environment, with the potential for local or widespread ischaemic pulp necrosis (Heyeraas and Berggreen, 1999).

The pulp is a richly innervated organ in comparison to other tissues, where afferent nerves enter together with the blood vessels through the apical foramen and follow the same path, sending numerous branches peripherally. Principally, the pulp nerves are

sensory postganglionic nerves from the fifth cranial nerve (trigeminal), sympathetic branches from the superior cervical ganglia (Kerezoudis et al., 1993) and possibly some parasympathetic nerves (Luthman et al., 1992). The sensory innervation is responsible for transduction and sensation of pain. The observation of many thin nerves which come into close contact with the wall of the blood vessels of the pulp (Tabata et al., 1998) together with the hypothesis of two types of nerve endings: free and varicose (Okamura et al., 1995) suggest that some of these nerves are responsible for the regulation of blood flow. Generally, the nerves of the dental pulp, in addition to the regulation of the blood flow, may have roles in enhancing the wound healing, providing reflexes to protect and defend the pulp and promoting the dynamics of dentinal fluid (Byers and Narhi, 1999).

To date there has been consistent controversy on whether lymphatic vessels exist in the dental pulp or not (Martin et al., 2010).

2. Cell-rich zone (crz): This zone is positioned just peripheral to the pulp core and beneath the cell-free zone, is less prominent or absent in radicular pulp (Kumar, 2014) and contains highly packed cells. Usually, this layer is rich in undifferentiated mesenchymal cells and fibroblasts but also has immune cells, nerves and blood vessels. Cells of the crz vary in morphology from polygonal to star shaped with cytoplasmic extensions. A suggested role of these extensions is to enhance cellular communication, increase the surface area and active involvement in signalling pathways within the pulp system (Woodnutt et al., 2000, Yamaguchi et al., 2004).

Histologically, cells of the crz have poorly-developed endoplasmic reticulum and Golgi apparatus, making them unsuitable for protein synthesis (Gotjamanos, 1969a) and numerous well-developed mitochondria suggesting high energy requirement and high metabolic rates (Zhai et al., 2003). Cells of this zone share some histological and morphological features with the cells of transporting epithelia, like the cellular processes and highly-developed numerous mitochondria. This similarity suggests that cells of the crz may participate in active transport and reabsorption of fluids and ions between the pulp and odontoblasts (Gotjamanos, 1969a).

Some cells within this zone have cytoplasmic processes that extend into the odontoblast cell layer (Ohshima et al., 2003) with some of these cells displaying a dendritic shape.

It has been hypothesised that cells in this zone are able to differentiate into other cell types when they receive the correct stimulus or the proper command (Couble et al.,

2000). Some researchers have postulated that if odontoblasts are permanently damaged or degenerate in response to noxious stimuli, new odontoblast-like cells originating from the crz will replace the damaged odontoblasts (Tziafas et al., 2000, Ishikawa et al., 2010, Kenmotsu et al., 2010, Hosoya et al., 2012) after a reported increase in cellular division within this zone (Murray et al., 2002a, Murray et al., 2000a, Murray et al., 2000b). Furthermore, Notch pathway has been detected to have receptors in the cells of the crz and ligands in odontoblast cells (Mitsiadis et al., 1999, Mitsiadis et al., 2003). The importance of this pathway in regulating the formation and morphogenesis of organs (Artavanis-Tsakonas et al., 1999, Frisén and Lendahl, 2001), may suggest further communication between odontoblasts and crz to sense the injury of odontoblasts and signalling the need for replacement.

3. Cell-free zone of Weil: Immediately beneath the odontoblasts and is less prominent or completely absent in radicular pulp. This zone is occupied by a ramifying nerve plexus called the Plexus of Raschkow which is formed from the extensive branching of the main myelinated sensory nerve trunks in the pulp core and peripheral progression of these branches, the majority of which lose their myelin sheath within the crz. These unmyelinated nerves may be regarded as specific pain receptors, and may further progress to enter the odontoblasts layer, pass into the predentine and some may enter the dentinal tubules together with the odontoblasts processes. Up to half of the tubules under the cusp area contain nerves (Byers and Narhi, 1999), while only 5% of tubules in the root or cervical area contain nerves. These peripheral nerve endings are hypersensitive to fluid movements and any change in tissue pressure (Byers and Narhi, 1999). It is noteworthy that the cell-free zone and Plexus of Raschkow are predominant in the crown area, while in the root, every nerve gives rise to some branches which further supply their own localised area. Furthermore, this layer is completely absent in some species like the continuously growing teeth of rodents and in teeth with active dentine formation (Kumar, 2014).

4. Odontoblast cell layer (OCL): This represents the most peripheral zone of the dental pulp, where cells make direct contact with the mineralised tissue of the tooth. It is composed predominantly of odontoblasts, with the occasional presence of major histocompatibility complex II (MHC II) antigen presenting cells namely dendritic cells (DC) (Tsuruga et al., 1999). These cells may play a regulatory role in odontoblast function and differentiation (Ohshima et al., 1999). The cellular arrangement within this layer is variable depending on the stage or the degree of activity of odontoblasts, ranging

from a single cell row to 4-5 cell rows arranged in a pseudostratified pattern (Goldberg and Smith, 2004, Sasaki and Garant, 1996). Capillaries usually infiltrate this layer and their distribution and relation to odontoblasts is again variable depending on the stage of odontoblast maturation and degree of activity (Yoshida and Ohshima, 1996). In rodents, capillaries distribution differs in enamel-related dentine area from cementum-related dentine area (Ohshima and Yoshida, 1992, Yoshida and Ohshima, 1996).

Odontoblasts cells are connected to each other by many terminal web and gap junctions (Sasaki and Garant, 1996, Fried et al., 1996, Ikeda and Suda, 2006). These junctions allow the OCL to act as an epithelial tissue (Ushiyama, 1989), with gap junctions usually connecting this layer to the underlying pulp cells (Ikeda and Suda, 2006). Odontoblast-odontoblast junctions have been reported to be disrupted after operative dental interventions such as cavity preparation (Turner, 1991).

Sitting at the hard/soft tissue interface, the OCBs are located completely within the pulp and their cytoplasmic processes extended inside the dentine. The OCL is the first zone of pulp cells to confront fluid movements, microbial invaders and other chemical agents as they enter dentine following enamel, cementum or dentine loss. As such, the OCL may act as physical barrier to pulp invasion (Bishop and Yoshida, 1992) and serve important roles in immunological defenses, specifically sensing the presence of bacteria and their products (Veerayutthwilai et al., 2007).

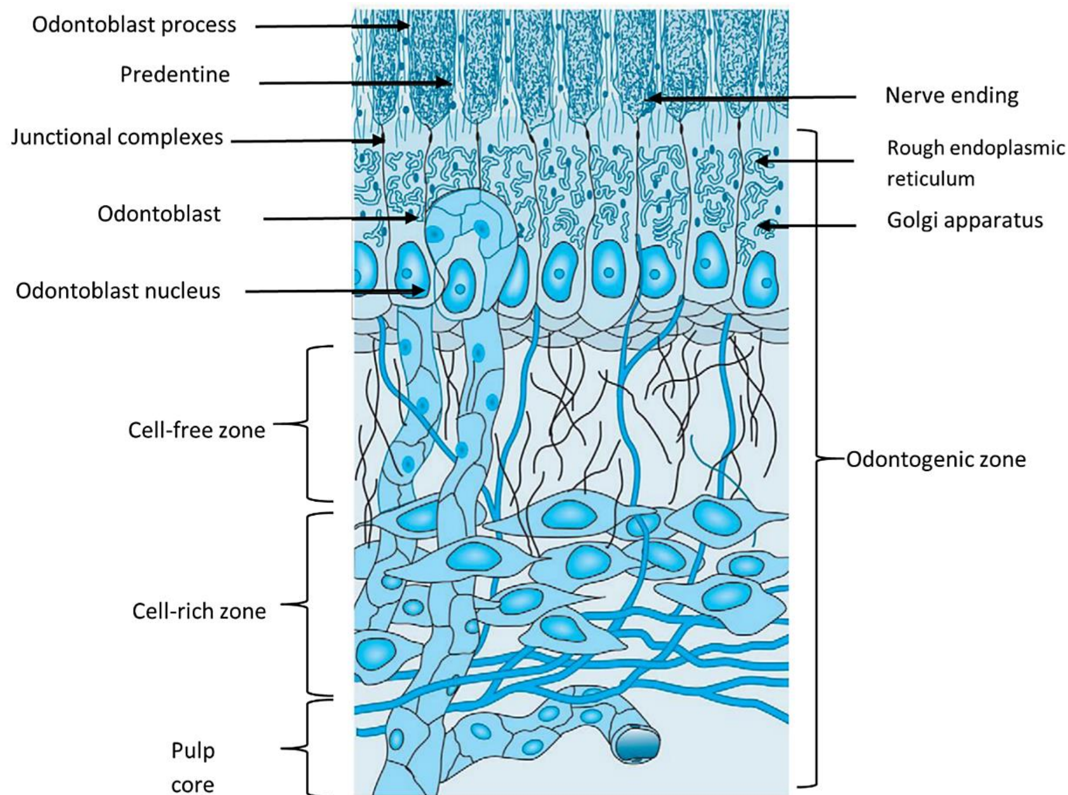


Figure 1-1: Zones of the dental pulp (Kumar, 2014.P. 126).

1.6. Cellular elements of the dental pulp

The dental pulp serves many functions, and in order to fulfil these functions, is pre-equipped with a heterogeneous population of cells. These cells include fibroblasts, undifferentiated mesenchymal cells, immunocompetent cells and finally the pulp's highly specialised cell, the odontoblast.

1.6.1. Fibroblasts:

Fibroblasts are the most common cells in the dental pulp with particular abundance in the coronal pulp and more specifically in the crz. Morphologically, the fibroblast is an elongated or spindle shape cell with many protracted cytoplasmic processes and an ovoid nucleus. It has a characteristic well-developed RER and Golgi apparatus in close proximity to the nucleus. The histological appearance of the pulp may be affected by cellular function, and in young, growing pulps where fibroblasts are actively elaborating matrix and fibres, the fibroblasts have abundant cytoplasm and organelles. Aged pulps, on the other hand, have a diminished need for the synthesis and secretion of matrix and fibre secretion, making the fibroblasts flattened with dense nuclei. They are also more abundant with age (Murray et al., 2002b).

The main function of pulp fibroblasts is the production and preservation of pulp matrix, more specifically collagen fibres and ground substance. Fibroblasts secrete mainly type I and III collagen fibres, together with a broad spectrum of non-collagenous extracellular matrix components like fibronectin and proteoglycans (Nanci, 2012). Moreover, fibroblasts are further involved in the degradation of components of the pulp matrix, thus playing a pivotal role in pulp repair and remodelling processes (Okiji, 2002). Pulp fibroblasts have been found to play a role in pulp defence and inflammation through the secretion of several molecules and chemicals including prostaglandin (PG)(Chang et al., 2006), tumor necrosis factor alpha (TNF-α) (Kokkas et al., 2007) and cytokines like interleukin (IL)1β, IL6 and IL8 (Yang et al., 2003, Zehnder et al., 2003). Furthermore, pulp fibroblasts may contribute in the neurogenic inflammatory process during pulp diseases through expression of the neuropeptide substance P (Killough et al., 2009). The ability of fibroblasts to secrete growth factors such as BMPs, TGF-β and VEGF (Sloan et al., 2000, Artese et al., 2002) makes them crucial in the stimulation and differentiation of pulp stem cells (About, 2011) and neoangiogenesis (Tran-Hung et al., 2006), which may be central to repair and regeneration processes.

Pulp fibroblasts have a limited life span and are often subjected to apoptosis (Vermelin et al., 1996) to keep the pulp volume constant and enhance the pulp functions. The degenerated cell bodies and contents are phagocytosed by macrophages.

1.6.2. Undifferentiated mesenchymal cells:

These cells represent the origin of many pulp cells, and can differentiate into numerous cell types when given the proper stimulus (Huang et al., 2009) for example they bear the ability to differentiate into osteoblast-like cells and produce osteoid-like tissue (Batouli et al., 2003, Carinci et al., 2008). They are located primarily within the crz, pulp core and in close association with the blood vessels (Téclès et al., 2005). These cells exhibit a polygonal appearance with large centrally located nuclei and have multiple cytoplasmic processes. They represent the progenitor cells that will replace the odontoblasts after severe injury (Hosoya et al., 2012). They will pass through proliferation and differentiation phases (Murray et al., 2002a) leading to the development of odontoblast-like cells which are capable of producing organic matrix and subsequently mineralising this matrix (Riccio et al., 2010, Zhang et al., 2011) after a programmed exodus to the pulp-injury interface especially when necrotic odontoblasts present as a triggering factor (Téclès et al., 2005).

1.6.3. Immunocompetent cells:

This group of cells includes DCs, macrophages, mast cells, T/B-lymphocytes and endothelial cells. Nearly all of them are MHC Class II antigen-presenting cells. They are abundant in the peripheral zones of the pulp, suggesting a critical role in the immune defence and pulp tissue reactions in the face of injury (Jontell et al., 1998, Okiji, 2002). Cells with MHC Class I expression are regarded as target or antigen-recognising cells and respond locally to cytokines and signalling molecules. The most intriguing cell within the dental pulp with such ability is the odontoblast. This cell has a suggested capability to nullify the incoming insults and call the other defence cells especially the MHC Class II cells including DCs, neutrophils and T-lymphocytes (Keller et al., 2010, Veerayutthwilai et al., 2007, Farges et al., 2011, Horst et al., 2011, Utreras et al., 2013).

1.6.4. Odontoblasts:

Odontoblasts are regarded as the most specialised, terminally differentiated and unique cells of the pulp with primary role in the formation and maintenance of dentine (Qin et al., 2004, Kuratate et al., 2008). Odontoblasts, in the phase of active dentine formation, exhibit a long columnar cell body with basely polarised nuclei, enormous RER, well-developed mitochondria and one cytoplasmic extension called the odontoblast process. This process extends into the dentinal tubules, contains endocytotic and exocytotic vesicles (Sasaki and Garant, 1996) and expresses bunches of cytoskeletal proteins including nestin, vimentin, actin microfilaments and microtubules (Yoshiba et al., 2002, Goldberg and Smith, 2004). Odontoblasts, after the completion of tooth formation, align themselves around the pulp in a pseudostratified layer forming the interface between the pulp and dentine. Odontoblasts express a variable morphology depending on the location within the pulp, being long columnar in the coronal pulp, cuboidal in the middle part and more flattened in the apical radicular pulp (Nanci, 2012). In addition, the functional activity of odontoblasts is reflected in their morphology. Elongated cells, basely situated nuclei, well-developed RER and Golgi, numerous mitochondria, prominent organelles and basophilic cytoplasm reflect a cellular profile of high activity. In contrast, a short cellular profile with little cytoplasm usually reflects resting cells (Lovschall et al., 2002).

Odontoblasts are held together by gap junction, tight junctions and desmosomes (Ikeda and Suda, 2006). These junctions play a key role in controlling cellular differentiation and polarization, in addition to regulating the permeability of this cell layer (Arana-Chavez and Massa, 2004, João and Arana- Chavez, 2004). Gap junctions are usually seen in the

lateral and the base boundaries of the OCB, connecting them to each other and to the subjacent pulp cells respectively (Ushiyama, 1989). At the distal end of odontoblast cell bodies, a junctional complex can be seen, made up of adherent junctions interconnected with tight junctions in the area (Couve, 1986).

Up to the time of completion of tooth formation, odontoblasts deposit different types of dentine, including mantle, intertubular, peritubular and circumpulpal dentine, all known as primary dentine. Odontoblasts continue to laydown secondary dentine throughout life which has a similar structure to primary dentine but at much slower rate. In addition, they rapidly lay down tertiary reactionary dentine in response to injury (Smith et al., 2001, Sangwan et al., 2013), a process which is initiated by a set of growth factors including TGF-β1, TGF-β3 and BMP-7 (Kalyva et al., 2010, Okabe and Matsushima, 2006, Cooper et al., 2010) but may be inhibited by intensive inflammatory processes (Cooper et al., 2010). To fulfil the dentine formation needs, odontoblasts secrete the organic matrix of the dentine which is mainly composed of collagens and proteoglycans, hand in hand with the production of a wide range of non-collagenous proteins including DSP, bone sialoprotein (BSP), DPP, dentine sialophosphoprotein (DSPP), dentine matrix protein-1 (DMP-1), osteocalcin, osteopontin, osteonectin and phosphoryn (About and Mitsiadis, 2001, Arana-Chavez and Massa, 2004, Sasaki and Garant, 1996).

Terminal differentiation and the post mitotic property of the odontoblast poses an intriguing problem. If a noxious stimulus or injury is applied to the tooth and if this stimulus is extensive enough to cause pulp exposure or loss of significant amounts of pulp-protecting hard tissues, the odontoblasts will be subjected to the harmful effects both directly and/or indirectly which may lead to odontoblast death and localised destruction of the integrity of the OCL (Bjørndal, 2008). With the urgent need to repair the dentine and following the death of odontoblasts, new odontoblast-like cells will be recruited, cytodifferentiated and moved to the affected area and start laying down a reparative dentine barrier (Smith and Lesot, 2001). This process is induced by specific signals (Nakashima et al., 2004), including the liberation of growth factors such as TGF-β1, FGF-2, BMP-2 and BMP-4 primarily from the destroyed dentine matrix. The repair process is enhanced by the rich vascularity and neuronal network within the pulp which plays a pivotal role (Kim et al., 2010).

All isoforms of voltage gated sodium channels are variably expressed by odontoblasts according to their position within the tooth (Byers and Westenbroek, 2011). These are

regarded as crucial tools for the cell to sense and actively respond to the environmental events (Catterall, 2010). In addition, odontoblasts express voltage-gated and voltage-insensitive calcium ion pathways within their cell membranes during active dentine formation (Lundgren and Linde, 1997). This further reflects the capability of odontoblasts to control the calcium ion influx to the odontoblast cell body and release to the dentine matrix. Furthermore, it has been suggested that odontoblasts have the ability to mechanically sense the stresses due to the presence of potassium and sodium channels and the generation of action potentials that can be conveyed to the nearby nerves, contributing to overall pain sensation (Allard et al., 2000, Allard et al., 2006, Magloire et al., 2003). A further suggestion is that the odontoblast may have the ability to transmit the pain themselves by mechanical sensing of the fluid movements (Magloire et al., 2003, Allard et al., 2000) or sensing the thermal changes (Okumura et al., 2005, Son et al., 2009).

The strategic location of odontoblasts makes them the frontline cells encountered by pathogens from a carious lesion. They are regarded as immunocompetent cells mediating the early defence against caries (Larmas, 2001) and play a vital role in the initiation, development and maintenance of pulp inflammatory and immune responses (Durand et al., 2006, Veerayutthwilai et al., 2007). Odontoblasts also contribute to a complex immunological defence mechanism with the class I MHC odontoblasts acting as antigen recognizing cells (Keller et al., 2010, Farges et al., 2011, Horst et al., 2011). The odontoblasts ability to respond rapidly to danger comes from the presence of specialised signalling receptors called Pattern Recognition Receptors (PRRs) including Toll-like receptors (TLRs) (Jiang et al., 2006, Mutoh et al., 2007), Retinoic acid-Inducible Gene-1-like (RIG-I-like) receptors and Nucleotide-binding Oligomerization Domain-like receptors (NLRs). The detection of pathogen toxins by PRRs elicits the secretion of pro-inflammatory cytokines and chemokines (Hahn and Liewehr, 2007, Bjørndal, 2008) which are of crucial importance in lymphocyte attraction and production of antibodies. IL1α, IL1β and TNF-α are regarded as the main controlling molecules in the pro-inflammatory process (Cooper et al., 2010). In addition, odontoblasts in situ can attract other immunocompetent cells like DCs, neutrophils and T-cells (Veerayutthwilai et al., 2007).

1.7. Functions of the dental pulp

The functions of the dental pulp are:

1. Formative: both primary tooth formation, and the maintenance and repair of tissues throughout the life of the tooth.
2. Nutritive: utilizing the rich microvasculature system within the pulp, the odontoblasts and their processes to provide nourishment to dentine.
3. Protective: Responses of the dental pulp to painful and noxious stimuli are very complex and somewhat different from those in other body organs. This is due to the unique characteristics of a limited collateral blood supply and a low-compliant environment of the dental pulp. The response comes from the interesting communication between odontoblasts and the nerves and capillary network in close proximity.
4. Reparative: dental pulp has an impressive regenerative and reparative ability to respond to mechanical, thermal, chemical and/or microbial stimuli through the deposition of protective layers of dentine namely reparative or reactionary dentine depending on the severity and persistence of the stimuli.

1.8. Pulp inflammation

Inflammation forms a critical element of host defenses in the face of injury. Bacterial infections, mechanical forces chemical and thermal challenges are the most common causes of injury to the pulp and mostly result from the dental caries, operative procedures and direct or indirect trauma. Injuries that expose the pulp to the oral environment may result in the loss of vital pulp functions and mixed microbial infections, dominated by Gram –negative anaerobes (Rôças et al., 2015).

Pulp inflammation triggers a wide spectrum of events at a molecular and cellular level. These aim to initiate the host immune response and to recruit immunocompetent cells from the local and systemic circulation and aid their migration across blood vessel walls to the affected site. This process helps to eliminate the pathogenic microorganisms, necrotic tissue debris and stimulate the resident pulp cells, aiming to limit the tissue damage and initiate reparative and regenerative processes. The nature of the inflammatory process after injury depends on the degree of tissue damage and the time elapsed since the injury (Kirkhorn et al., 1999). It is extremely important to control the inflammation, otherwise this may lead to a broad range of acute, chronic and systemic inflammatory disorders (Serhan and Petasis, 2011).

The presence of cariogenic microorganisms and their products is the most common cause of pulp inflammation worldwide (Love and Jenkinson, 2002). Its prevalence is closely related to socioeconomics, the absence of preventive measures and dietary factors (Bagramian et al., 2009). On the other hand, traumatic dental injuries remain common in both adult and child populations and may provide a route for the microbial colonization of previously healthy pulp tissue by their sudden exposure to a microbe-rich oral environment.

While microorganisms and their products may provoke inflammatory responses within the pulp, the liberation of dentine matrix components including cytokines, inflammatory mediators and growth factors may result from the demineralization of dentine by the caries process (Smith et al., 2012a, Goldberg and Smith, 2004). Some of these may have antibacterial properties (Smith et al., 2012b) and change the intensity and nature of bacterial threat to the dental pulp.

Following the pulp exposure to pathogenic organisms and/or their products, a complex series of molecular and cellular events will start which represent innate immunity. As the inflammatory process becomes chronic, the transition from an innate to an adaptive immune response will begin gradually. Ideally, the post injurious host defense will result in the elimination of infectious agents and promote an environment conducive to healing. However, host defenses are frequently incapable of creating such an environment, especially in the case of a deep and rapidly advancing carious lesion. The non-compliant environment of the dental pulp and its limited collateral circulation probably do not help.

The pulp inflammatory process is usually associated with a great increase in the production of pro-inflammatory cytokines including INF, TNF-α, IL-1β and IL-6, with particular upregulation of IL-10, which is responsible for limiting the immune response (Farges et al., 2011) and the anti-inflammatory radical nitric oxide (NO) which may often found at high concentrations (Connelly et al., 2001, Korkmaz et al., 2011).

Recently, it has been highlighted that inflammation is a crucial step to facilitate tissue repair and regeneration (Goldberg et al., 2008), so therapeutic steps to eliminate inflammation may not be helpful. In an age when the preservation of vital pulp functions and the regeneration of damaged tissues is coming to the fore, some of the challenges facing clinicians and scientists surround an appropriate level of inflammatory regulation in order to control infection and manage the removal of damaged tissues, while promoting

the complex cascades of cellular and molecular activity that are associated with healing and repair (Rutherford and Gu, 2000, Baumgardner and Sulfaro, 2001).

1.9. Role of PGE_2 in inflammation:

PGs are a group of small bioactive molecules derived from AA through a reaction controlled by enzymes referred to as COXs. COX exists in two forms: a constitutive form called COX-1 and an inducible form called COX-2 (Smith et al., 2000). COX-1 is found in almost all cells and is regarded as the major source of PGs under normal physiological conditions, where they are responsible for a range of housekeeping actions such as homeostasis and cytoprotection (Dubois et al., 1998). The PG release from this enzyme is usually in small amounts and for short periods of time. On the other hand, COX-2 induction occurs in response to inflammatory stimuli, growth factors and hormones. It is regarded as the dominant source of PGs in the case of inflammation and cancer (Dubois et al., 1998). PG production in these cases is high and continues for the duration of the stimulus during episodes of inflammation and other pathological conditions. PGs have been identified as some of the most common local inflammatory mediators in almost every tissue (Waterhouse et al., 1999) and most importantly, control vasodilation and vascular permeability.

Different PGs (collectively called prostanoids) have been identified, including: Prostaglandin E_2 (PGE_2), Prostaglandin I_2 (PGI_2), Prostaglandin D_2 (PGD_2), Prostaglandin $F_2\alpha$ ($PGF_2\alpha$) and Thromboxane A_2 (TXA_2) (Calder, 2001).

1.9.1. PGE_2 Synthesis

PGE_2 is regarded as the most abundant prostanoid, produced by virtually all cell types. PGE_2 synthesis involves the mobilization of AA from the cell membrane by the effect of phospholipase A_2 enzyme (Lambeau and Lazdunski, 1999), conversion of AA into PGH_2 by the action of COX enzymes (COX-1 and COX-2), followed by the final formulation to PGE_2 by PGE synthase enzyme (Park et al., 2006). Although, this synthesis process seems to be controlled by other elements such as AA availability, the main governing factor that dictates the rate of synthesis is the local expression and activity of COXs.

The synthesis of PGs takes place in the lumen of the endoplasmic reticulum (Morita et al., 1995) from which they are immediately released to the extracellular spaces either by active transport, through ATP-dependent transporters, or by diffusion across the cell membranes (Park et al., 2006) to exert their wide ranging effects by acting on special cell

membrane receptors in an autocrine or paracrine manner (Narumiya et al., 1999). Despite the stability of PGE_2 *in vitro*, it has very a high degradation rate *in vivo* and is eliminated rapidly from the tissues as well as from the circulation (Förstermann and Neufang, 1983). This fast turnover is controlled by the enzyme 15-hydroxyprostaglandin dehydrogenase (15-PGDH) (Tai et al., 2002, Myung et al., 2006).

1.9.2. PGE_2 receptors

After release, PGE_2 acts either at or nearby the release site, by binding to EP receptors. The diversity of PGE_2 functions may be partly attributed to the diversity of the targeted receptors, with four main types: EP1, EP2, EP3 and EP4, in addition to at least eight splice variants of EP3 in humans (Hata and Breyer, 2004). Each of these receptors has a distinct function, location within the tissue and affinity to bind to PGs. The diversity of prostanoid receptors reflects the ubiquitous effects of PGE_2. EP3 and EP4 are the most abundant and highest affinity receptor types for PGE_2 binding (Abramovitz et al., 2000). EP3 has the highest affinity, and thus requires the least PGE_2 concentration for signalling. EP2 is the least expressed receptor type with low affinity for PGE_2 binding (Sugimoto and Narumiya, 2007). Distribution of EP1 receptors is restricted to some organs including the kidney, stomach and lung tissues, with the least affinity to bind to PGE_2.The affinity to bind to PGE_2 is species-dependant, for example, EP2 affinity to PGE_2 in rats is significantly higher than that in human or mouse (lest affinity) (Dey et al., 2006). In addition to site specific functions, the main signalling pathway for EP1 leads to an increase in intracellular calcium ion levels (Funk et al., 1993), while EP2 and EP4 pathways are linked to the elevation of intracellular cyclic adenosine monophosphate (cAMP) (Regan, 2003). By contrast, stimulation of EP3 receptors tends to reduce the cAMP concentration inside the cell (Boie et al., 1997).

1.9.3. PGE_2 effects

Under normal physiological conditions, PGE_2 is a key mediator of diverse biological activities including: nociception, neuronal signalling, vascular tone regulation, blood pressure, renal filtration, fertility, regulation of gastrointestinal mucosa integrity, control of vascular permeability and smooth muscle functions (Yang and Chen, 2008, Durand and Zon, 2010, Nagamatsu and Schust, 2010, Rivest, 2010, Yuhki et al., 2011).

However, under pathological conditions, down or upregulation of PGE_2 has been observed (Legler et al., 2010), suggesting further diversity and involvements, which makes PGE_2 of great importance as it participates in virtually all processes leading to

redness, fever, swelling and hyperalgesia, the classic signs and symptoms of inflammation (Funk, 2001). This contribution in inflammation occurs mainly through arterial vasodilation and increasing microvascular permeability, and/or acting directly on peripheral and central sensory neurons (Funk, 2001, Moriyama et al., 2005, Minami et al., 2001). PGE_2, by acting on different EP receptors, plays a key role in regulating the function of numerous types of cells, including DCs, macrophages, and both B and T lymphocytes, thus exerting PGE_2-associated pro-inflammatory and anti-inflammatory actions.

The pro-inflammatory role of PGE_2 is achieved through a local vasodilatory effect, neutrophil attraction (Yu and Chadee, 1998), mast cell chemotaxis (Weller et al., 2007) and macrophage attraction and activation (Nakayama et al., 2006, Wang and Lau, 2006), regulation of the expression of cytokines of the DC, together with an evident control of T cell differentiation to either T helper (Th) 1 or Th2 cells (Egan et al., 2004, Yao et al., 2009). In addition, PGE_2 plays a crucial role in inducing the migration of DC and directing them to drain lymph nodes through a selective effect on EP receptors (Legler et al., 2006). Concurrently, the presence of PGE_2 during the maturation of DC plays an inductive effect on specific molecules on DC to enhance T cell proliferation and activation (Krause et al., 2009).

PGE_2 anti-inflammatory actions can be observed in innate immune cells, with suppression of natural killer cells (Goto et al., 1983), neutrophil and monocyte inhibition (Harris et al., 2002), limitation of the pathogen eradicating ability of macrophages (Aronoff et al., 2004, Serezani et al., 2007) and degranulation of mast cells (Gomi et al., 2000). Furthermore, during neuro-inflammation, the contrasting effect of PGE_2 appears clearly. It has been reported that PGE_2 exerts a suppressive effect on Th1 differentiation and B cell function associated with allergy (Harris et al., 2002), with a reported limitation to the nonspecific immune response related to cancer and chronic inflammation through a direct suppressive effect on the production of pro-inflammatory cytokines (Wang et al., 2007). PGE_2 released in response to microbial LPS has harmful effects ranging from enhanced pain transmission to neuronal lesions (Lehnardt et al., 2003, Montine et al., 2002, Reinold et al., 2005).

Although PGE_2 has a supportive effect on the acute local inflammatory process and phagocyte-mediated immune response at the site of injury or pathogen entry, it has a controlling effect on the cell-mediated cytotoxic immune response, more specifically at

the late stage. Despite the fact that such a suppressive effect of PGE_2 directs the immune response to be less harmful (a potentially crucial step in the preservation of affected tissues), it is detrimental in the event of infections with intracellular organisms (e.g. HIV infection) and in cancer since both rely on increasing PGE_2 synthesis or inhibiting its turnover to establish a suppressive immune response and disease propagation (Kalinski, 2012).

1.9.4. PGE_2 in the dental pulp

The factors contributing to inflammation and pain are of great importance in the dental pulp, as dental pulp tissue may be subjected to a wide spectrum of injuries including chemical, mechanical and microbial, with a cumulative effect that may ultimately cause varying degrees of reversible or irreversible damage. The ability of the dental pulp to withstand these insults and recover is highly dependent on the adequacy of its blood supply. This is of critical importance since pulp tissue resides in a noncompliant hard tissue environment with little if any collateral blood supply.

PGE_2 is regarded as one of the main mediators implicated in pulpal inflammation, nociception and regulation of pulp cell function (Goodis et al., 2000). PGE_2 was found to alter vascular permeability in the pulp (Funahashi et al., 2009), both after the application of mechanical stimulation (dentine removal) or following chemical irritation (Chidiac et al., 2001). Some authors regard this as involvement of PGE_2 in the regenerative processes (Ohnishi et al., 2000). Furthermore, painful pulps are associated with higher PGE_2 levels than healthy pulps, and within painful pulps, PGE_2 levels were higher in reversibly pulpitic than irreversibly pulpitic pulps (Petrini et al., 2012). This may reflect the damage of cellular components releasing the PGE_2 or a simple termination of the signal as a result of tissue necrosis. Interestingly, in a study on the clinical outcome of pulpotomy procedures in human primary molars, high levels of PGE_2 within the dental pulp were associated with poorer clinical outcomes (Waterhouse et al., 2002) and directly correlated to the extent of pulpitis determined histologically (Petrini et al., 2012). It is therefore desirable to control the production of these mediators, to limit their unnecessary detrimental effects which may lead to complete pulp necrosis, and to promote the events of pulp regeneration (Nakashima et al., 2009).

The anti-inflammatory effect of PGE_2 within the dental pulp start immediately after microbial contact with the tissue, or the introduction of microbial toxins. PGE_2 plays a major role in characterising pulp pathology and orchestrating the events of pulpitis.

In addition to previously mentioned PGE_2 effects, more sophisticated roles for PGE_2 have been found within the dental pulp. For example, it was reported that PGE_2 acts to boost the ability of pulp cells to mobilise calcium ions, possibly through EP1 and/or EP3 signalling. This may regulate various cellular activities such as changing cAMP levels, altering the roles of glycosaminoglycan and alkaline phosphatase (Chang et al., 2006) and possibly modulating dentine matrix mineralisation and the various complex events associated with it (Couble et al., 2000).

In terms of pulpal pain, PGE_2 has been identified to have key modulatory effects on numerous events within pain transmission pathways, specifically in two fashions. Firstly, hyperalgesic actions by acting directly on nerve endings (Moriyama et al., 2005), and secondly by acting to enhance neuronal sensitivity to other mediators of pain, such as bradykinin (Cui and Nicol, 1995).

For decades, the PG pathway has been targeted to minimise pain, nociception and inflammation in clinical (including dental) practice, with the discovery of a range of medications targeting essential events within this pathway both selectively and non-selectively like NSAIDs, which may non-selectively inhibit both COX enzymes or selectively inhibit COX-1 or COX-2. These drugs have proven efficiency in reducing pain, fever and inflammation (if used in high concentration) even in single doses (Clarke et al., 2009).

1.10. Animal models

The host response to an infective agent sustains a high degree of complexity which makes its in vitro reproduction impossible. In addition, the inflammation and healing processes are multifactorial, and in order to study these processes from all perspectives, and to allow studies to confirm both short-term and long-term consequences of inflammation, a suitable experimental model is required. However, it is important to address the fact that there is no universal animal model that is ideal for all research needs. Therefore, clinicians and researchers must be aware of the relative strengths and weaknesses of the diverse animal models available.

Another important factor is the ability to induce inflammation or other required conditions to mimic human disease conditions. However, other factors involved in the disease process include the dose of causative agent, the mode of entry of the agent and the

related physiological changes which may have an important role in the outcome. All must be taken into consideration, and few ideal experimental models exist.

Nevertheless, animal models assist scientists in obtaining new knowledge and better understanding of various physiological and pathological conditions.

Rodents have featured strongly among the research models for studying the dental pulp (Saghiri et al., 2015), with much accrued knowledge about the structure and physiology of their tissues. Rodents are additionally easy to handle and house for long periods, and are relatively low cost which enhances the possibility of large sample sizes. They are readily adaptable to the lab environment, are associated with lower social and ethical concerns than other animals such as primates and finally, a diverse array of commercially available antibodies for cellular and molecular techniques are available for rats; perhaps more so than any other laboratory animal.

The rat incisor represents a particularly well-established model for the study of pulp tissue. Its continuous growth makes it a valuable model to investigate the lifecycle of tissues from formation to maturation and repair in the face of injury (Harada et al., 1999, Harada et al., 2002b, Harada and Ohshima, 2004). Rodent studies have included investigations on the effects of therapeutic agents (Murray et al., 2002a), bioactive molecules (Sloan and Smith, 1999), responses following injury (D'Souza et al., 1995), surgical procedures and the application of filling materials (Murray et al., 2000b, Murray et al., 2002b), orthodontic tooth movement (Ren et al., 2004), tooth bleaching (Cintra et al., 2016) and pulp capping (Dammaschke et al., 2006, Lovschall et al., 2002).

In addition, rat incisors provide the researcher with appreciable amounts of pulp tissue suitable for tissue culture (Sloan et al., 1998) and RNA-based investigations, even from a single tooth (McLachlan et al., 2003).

It should always be recognised, however, that animal models cannot flawlessly simulate the human *in vivo* situation, and it is recognised that caution must be exercised in the interpretation of results from animal studies, including those included in this thesis. The insight developed do, however, contribute usefully to the body of knowledge and understanding on complex physiological systems.

1.11. Aims of this study

Overall, this research aimed to investigate the presence and exact localisation of the PG and NO pathways elements within the dental pulp, and to examine the changes of these elements in response to certain laboratory environment. In details the aims were:

1. To re-examine cellular components and interrelations in the rodent incisor pulp using contemporary immunohistochemical techniques.
2. To investigate the presence and localisation of physiological markers, notably those of the PG and NO pathways in the rat incisor pulp.
3. To determine the amount of PG released from isolated rat pulp tissue in stimulated and unstimulated conditions.
4. To assess the expression of genes relating to basic elements of the PG and NO pathways, and other key anti and pro-inflammatory cytokines in stimulated and unstimulated isolated rat pulp tissue.

These aims were explored in the following investigations:

1. Immunohistochemical investigation of rat mandibular incisor dental pulp using specific structural antibodies and fluorescent microscopy with confocal abilities (Chapter 3).

2. Immunohistochemical analysis of rat mandibular incisor using specific antibodies against key elements of PG and NO pathways utilizing the fluorescent microscope with confocal abilities (Chapter 4).

3. Analysis of the quantity of PGE_2 released from the rat dental pulp both unstimulated and stimulated for short and longer time-periods with (lipopolysaccharides (LPS), NO, adenosine tri-phosphate (ATP) and combined ATP and NO), utilizing enzyme linked immune serpent assay (ELISA) (Chapter 5).

4. Utilizing q-RT-PCR to determine the gene expression of basic elements of PG and NO pathways in addition to some crucial pro and anti-inflammatory elements (IL1, IL1R, IL6, IL6 receptor (R) and TLR-4) in the unstimulated rat dental pulp and determining the effect of different stimulants (LPS, NO, ATP and PGE_2) on the RNA level of target genes (Chapter 6).

This general literature review is supplemented by shorter and more focussed introductory literature reviews linked to each of the experimental chapters (Chapters 3-6).

Chapter 2. Materials and methods

General materials and methods used in this thesis will be described in this chapter. Some details that were specific to each of the investigative chapters will be included in smaller materials and methods sections specific to those chapters. Detailed samples collection is described in the corresponding result chapter.

A. Structural Investigations

All work was performed in line with the standard guidelines for studies with experimental animals set by the UK Home Office. All the animals were housed in Comparative Biology Centre- Newcastle University and all the research work was carried out in compliance with the animals (scientific procedures) Act 1986 and its associated codes of practice.

2.1. Immunohistochemistry (IHC)

2.1.1. Sample collection

A detailed description of sample collection was included in the methods section of each results chapters.

2.1.2. Fixation

Fixation is a mandatory step in the preparation of a biological sample for immunohistochemistry. The fixation process helps in maintaining the cellular architecture and components including proteins, carbohydrates and other bioactive cellular elements (Fox et al., 1985). Two techniques were used to fix the samples: a). Intravenous infusion of the animal prior to sample collection, and b). Fixing the samples after sectioning or extraction of tissues. The fixative used in both techniques was freshly prepared 4% paraformaldehyde (PFA) in phosphate buffer saline (PBS) (Dapson et al., 2005, Leong, 2004, Grube, 2004). The procedures were as follows:

a) Whole animal perfusion: whole animals were perfused with 4% PFA according to the protocol of Gage (2012), the entire process being conducted in the specialised animal facility, housed in the Newcastle General Hospital Campus for Ageing and Vitality, by trained and licenced individuals. After complete fixation and animal death, the teeth and mandibles were obtained as described previously.

b) Animals were not perfused. After cervical dislocation, the extracted teeth and sectioned mandibles were immersed in 4% PFA in PBS at 4˚C for 24 hours (Boushell et al., 2008, Sigal et al., 1985).

After complete fixation, all samples were washed in Tris-Buffered Saline (TBS), then Tris-Buffered Saline with Tween 20 (TBS-T), then TBS for 20 minutes each on a 3D rocking platform STR9 (Stewart Scientific, UK), to remove any excess fixative. Samples for examination without demineralisation were transferred into graded sucrose solutions (10%, 20% and 30 %) for 24 hours in each concentration for cryopreservation (Rumsey et al., 1992, Santos et al., 2006).

2.1.3. Demineralisation

The principle of demineralisation is that the demineralising agents (chelating agents) can bind to calcium and other minerals in the tissue and remove them gently. As teeth and jaw samples require extensive demineralisation, immediately after washing from fixative solution, the samples to be demineralised were immersed in freshly prepared 0.1M 17% ethylenediaminetetraacetic acid (EDTA), pH 7.4 at 37˚C for 4-6 weeks (Cho et al., 2010) with constant agitation on a temperature controlled shaker (Environ-shaker, Lab-line, Jencons Scientific Ltd). The demineralising solution was changed every two days. After four weeks, the samples were examined by x-ray to check the degree of demineralisation. If successful demineralisation was confirmed by a loss of radioopacity, the samples were checked again with a scalpel to confirm that they were easily cut. It has been confirmed that this technique can result in good demineralisation in a relatively short period of time, without harmful effects on the tissue (Cho et al., 2010).

2.1.4. Freezing and sectioning

Demineralised (after confirmation of demineralisation) and non-demineralised samples were washed again with TBS, TBS-T and TBS for 20 minutes each on a 3D rocking platform. Samples were then gently dried on tissue paper and immediately immersed in a cryo-embedding material (Optimal Cutting Temperature compound (OCT), Tissue Tek, Netherlands) using pieces of laboratory paraffin thin film (Parafilm M, Bemis flexible packaging, USA) as a base. By preference, samples were no larger than 10 mm in diameter and the amount of OCT used was the least possible to cover the whole sample. Samples with OCT were snap frozen using isopentane cooled in liquid nitrogen. After that, samples were removed from the isopentane and either sectioned immediately, or stored in a -80˚C freezer for later use. The frozen blocks to be sectioned were moved to a

cryotome (SHANDON Cryotome FSE, ThermoFisher Scientific, USA), mounted on serrated discs using OCT and cut using a new blade (MX35 Premier +Microtome Blade, Thermo Scientific, USA).

8-10 µm thick sections were prepared, mounted on polysine slides (Thermo Scientific, USA), numbered, dated and allowed to bench dry for 24 hours. Resulting slides were either to be used immediately after drying, or were wrapped with cling film, avoiding any touch or damage to the tissues, and stored in a -80˚C freezer for later use.

2.1.5. Staining procedure

Slides were removed from the -80˚C freezer, allowed to equilibrate to room temperature for one hour and carefully unwrapped. A hydrophobic barrier pen (PAP pen) was then used to encircle the tissue pieces on the slides, taking care to ensure that a complete circle was made around each of the tissue pieces, then allowed to bench dry for 5 minutes. The slides were then stored back to back in pairs in a Coplin jar and washed in TBS, TBS-T and TBS for three consecutive periods of 5 minutes each on a 3D rocking platform.

Antibody	Abbr.	Conc.	Cat#	Company
Mouse monoclonal anti-vimentin	vim	1:5000	MU074-UC	Biogenex
Rabbit monoclonal anti-α smooth muscle actin	actin	1:200	Ab32575	Abcam
Rabbit polyclonal anti-α tubulin	tub	1:1000	GTX102078	Gene Tex
Rabbit monoclonal anti-NaK-ATPase	NaK-ATPase	1:500	Ab76020	Abcam
Goat polyclonal anti cyclooxygenase-1	COX-1	1:100	sc-1752	Santa Cruz Biotech.
Rabbit monoclonal anti cyclooxygenase-2	COX-2	1:100	M3214	Spring Bioscience
Rabbit polyclonal anti nitric oxide synthase 1	NOS1	1:500	sc-648	Santa Cruz Biotech.
Rabbit polyclonal anti EP1	EP1	1:500	sc-20674 sc-98388 sc-22648	Santa Cruz Biotech.
Rabbit polyclonal anti EP2	EP2	1:500	sc-20675	Santa Cruz Biotech.
Goat polyclonal anti TLR4	TLR-4	1:100	sc-16240	Santa Cruz Biotech.
Rabbit polyclonal anti IL1β	IL1β	1:100	bs-6319R	BIOSS.
Rabbit polyclonal anti IL1RI	IL1RI	1:100	sc-689	Santa Cruz Biotech.
Goat polyclonal anti IL6	IL6	1:100	sc-1265	Santa Cruz Biotech.
Rabbit polyclonal anti IL6Rα	IL6Rα	1:100	sc-13947	Santa Cruz Biotech.

Table 2-1: **List of primary antibodies employed in this study.**

After washing, slides were removed from the jar, carefully dried around the PAP pen circle with tissue paper, and transferred to a humidified chamber. Primary antibodies (Table 2-1) were then applied using 100-200 μL of stock solution for each slide depending on the size and number of tissue sections mounted on the slide. The humidified chamber containing the slides was then stored in a 4°C refrigerator overnight.

The slides were then transferred back to a Coplin Jar and washed once again in TBS, TBS-T, and TBS for three periods of 20 minutes each.

After removal from rinsing solutions, slides were carefully dried around the PAP pen circle with tissue paper, and first secondary antibodies (Table 2-2) applied, before incubating the slides for 1 hour in a dark, humid environment. The slides were then washed in TBS, TBS-T, and TBS for three periods of 20 minutes each before applying the second secondary antibodies and storage once again in dark humid environment for

one hour. Slides were then taken out of the humidified chamber, and washed again in TBS, TBS-T, and TBS for three periods of 20 minutes each.

Antibody	Type	Concentration	CAT No.	company
Alexa Fluor	Anti-goat 488	1:500	A11055	Invitrogen
Alexa Fluor	Anti-goat 594	1:500	A11058	Invitrogen
Alexa Fluor	Anti-rabbit 488	1:500	A21206	Invitrogen
Alexa Fluor	Anti-rabbit 594	1:500	A21207	Invitrogen
Alexa Fluor	Anti-mouse 488	1:500	A21202	Life Technologies

Table 2-2: **List of secondary antibodies employed in this study.**

Finally, all slides were removed from the rinsing solution, dried carefully around the PAP pen circle with tissue paper and covered with one drop of Vectashield hard set mounting medium with DAPI (4',6-diamidino-2-phenylindole) (Vector laboratories Inc., Burlingame CA 94010 U.S.A). DAPI has the ability to fluoresce when bound to DNA and is usually used as a chromosomal or nuclear stain. After one minute, a drop of glycerol in PBS was applied, followed by covering the tissues on the slide with a glass cover slip (Menzel-Gläser, Thermo Scientific, USA) and sealing the margins with nail varnish.

2.1.6. Examination and analysis

Prepared slides were then examined at various levels of magnification powers with an Olympus BX-61 microscope. Representative images were captured using a microscope-mounted Olympus XM10 monochrome camera.

In an attempt to gain the quality of confocal images, z-stack images were captured with the BX-61 microscope and XM10 camera and transmitted into a computer with AutoQuant x software (Media Cybernetics Inc) which had 2D and 3D deconvolution algorithms available.

Basic manipulation of the images in addition to creating composite images was achieved using Image J software (Java- based image processing program, National Institute of Health (USA).

2.1.7. Controls:

Positive and negative control samples were included according to protocols reported by Sigal et al. (1985) and Sigal et al. (1984). In the negative controls for the primary antibodies, the slides were incubated with PBS instead of the primary antibodies, before staining with

the secondary antibodies only. In the negative controls, the slides were incubated with PBS only.

In addition, the specificity of the key antibodies used in this work were previously confirmed with blocking peptides including COX-1 (De Jongh et al., 2007, Fornai et al., 2005), EP1 and EP2 (Rahnama'i et al., 2010) and NOS1 (Ling et al., 2012).

Some representative images for the external positives and negative controls for the key antibodies are shown in the Appendix.

2.2. Quantitative determination of prostaglandin E_2 (PGE_2)

2.2.1. Principle

PGE_2 quantitative determination assay is designed to measure PGE_2 in cell culture supernatants, serum, plasma and urine. The basic principle of this assay is a forward sequential competitive binding technique, where a competition occurs between endogenous PGE_2 in a given sample and PGE_2 labelled with horseradish peroxidase (HRP) for a limited number of binding sites on a mouse monoclonal antibody. The first binding allowance is offered to PGE_2 of the sample in the first incubation period, while the second incubation period allows the HRP-PGE_2 to bind to the remaining binding sites on the antibody. A wash is subsequently required to remove the remaining unbound materials, followed by the addition of a substrate material which helps to determine the bound enzyme activity. A colour change will result, which can be fixed by the addition of a chemical to arrest the reaction at a given stage before reading the absorbance at 450nm. The PGE_2 concentration in the sample is inversely proportional to the intensity of the colour change.

2.2.2. Materials

Parameter^{TM} Prostaglandin E_2 assay kits were employed (Catalogue Number SKGE004B, R&D systems), with the component materials explained in Table 2-3. Each kit contains enough materials to run ELISA on six 96 well plates. It is important to mention that this kit is temperature sensitive and should be stored at -20 ˚C in a manual defrost freezer before reconstitution and equilibrated to room temperature (18-23˚C) prior to use.

Part	Part No.	Description	Storage
Goat anti-mouse microplate	892575	96 well polystyrene microplate (12 strips of 8 wells) coated with a goat anti-mouse polyclonal antibody.	Return unused wells to the storage bag. May be stored at 2-8 °C for up to 1 month.
PGE_2 Standard	893377	25,000 pg/vial of PGE_2 in buffer with preservatives; lyophilized.	Aliquot and store at ≤ -20 °C for up to 1 month in a manual defrost freezer
PGE_2 Conjugate	893375	6 mL/vial of PGE_2 conjugated to horseradish peroxidase with red dye and preservatives.	May be stored for up to 1 month at 2-8 °C
Primary Antibody Solution	893376	6 mL/vial of a mouse monoclonal antibody to PGE_2 in buffer with blue dye and preservatives.	
Calibrator Diluent RD5-56	895612	21 mL/vial of a buffered protein base with preservatives	
Wash Buffer Concentrate	895003	21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservative	
Colour Reagent A	895000	12.5 mL/vial of stabilized hydrogen peroxide	
Colour Reagent B	895001	12.5 mL/vial of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895926	11 mL/vial of 2 N sulphuric acid.	
Plate Sealers	N/A	Adhesive strips.	

Table 2-3: Details and contents of Parameter™ Prostaglandin E_2 assay kit

2.2.3. Reagent preparation

- Any DMEM solution mentioned throughout this thesis contained 1% FBS and 50 (µg/ml, IU/ml) penicillin-streptomycin.
- All the assay reagents must be left on the bench to equilibrate to room temperature before use.
- Wash buffer: it was mixed gently until a homogenous clear solution was formed, then 20 mL of wash buffer concentrate was added to 480 mL distilled water to prepare 500ml of working concentration wash buffer.

- PGE_2 standard: was supplied as a powder and dissolved in 1 mL of distilled water with mixing to ensure complete reconstitution of the powder and forming a stock solution of 25000 pg/ml PGE_2, which was left on the bench for 15 minutes and gently shaken before use. The solution was divided into 10 aliquots of 100 µL each, nine being stored in a -20˚C and one only being used in each experiment. Seven eppendorf tubes were prepared and a dilution series commenced as follows (see Figure 2-1):
- Tube 1: 900 µL of calibrator diluent (CD) was added to the first tube together with 100 µL of PGE_2 standard solution and mixed well by pipetting, resulting in a 2500 pg/mL PGE_2 standard solution.
- Tube 2: 500 µL from tube 1 was pipetted and added to tube 2, before adding 500 µL of CD and mixing well, resulting in 1250 pg/mL PGE_2 standard solution.
- Tube 3: 500 µL from tube 2 was pipetted and added to tube 3, before adding 500 µL of CD and mixing well, resulting in 625 pg/mL PGE_2 standard solution.
- Tube 4: 500 µL from tube 3 was pipetted and added to tube 4, before adding 500 µL of CD and mixing well, resulting in 313 pg/mL PGE_2 standard solution.
- Tube 5: 500 µL from tube 4 was pipetted and added to tube 5, before adding 500 µL of CD and mixing well, resulting in 156 pg/mL PGE_2 standard solution.
- Tube 6: 500 µL from tube 5 was pipetted and added to tube 6, before adding 500 µL of CD and mixing well, resulting in 78 pg/mL PGE_2 standard solution.

Tube 7: 500 µL from tube 6 was pipetted and added to tube 7, before adding 500 µL of CD and mixing well, resulting in 39 pg/mL PGE_2 standard solution.

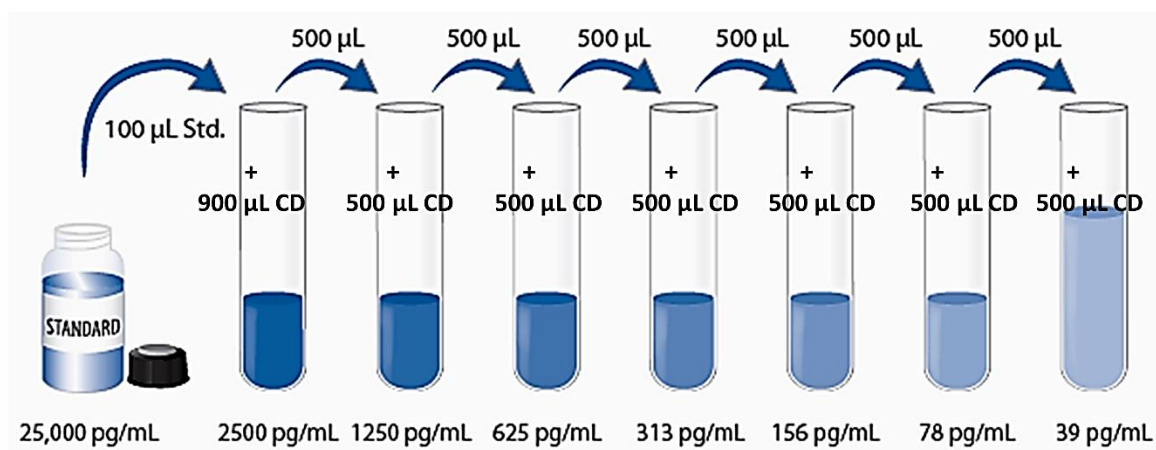


Figure 2-1: PGE_2 standard dilution series (R&D systems).

The purpose of this serial dilution was to provide the investigator with measurements that help to produce a PGE_2 standard curve. The solution in tube 1 will serve as the high standard and the other tubes serve as diluted concentrations in sequence ending with zero PGE_2 concentration obtained from CD.

- Substrate solution: equal volumes of colour reagents A and B were mixed together within 15 minutes of use, and protected from light. 200 µL of the resultant solution was required for each well.

2.2.4. Assay procedure

- All reagents were allowed to equilibrate to room temperature (18-25˚C) before use. The plate frame was prepared to have the desired number of microplate strips, and the extra strips were stored back in the foil pouch with a desiccant pack and sealed.
- All the samples, controls and standards were assayed in duplicates.
- In every reaction, internal assay positive and negative controls were included.
- 200 µL of CD was added to nonspecific binding (NSB) wells, 150 µL of CD was added to the zero standard (B_0) wells and 150 µL of standard, control or sample was added to the remaining wells. It was important to dilute the sample by three folds with CD before use.
- 50 µL of the primary antibody was added to all wells except the NSB wells. All wells (except NSB) were changed to blue in colour by this addition. The plate was covered securely with the provided plate sealer and incubated for 60 minutes at room temperature on a horizontal orbital plate shaker (IKA Schuttler MTS 4, IKA Labortechnik) set at 500 ± 50 rpm.
- Without washing, 50 µL of PGE_2 conjugate was added to all wells. A violet colour resulted in all wells except NSB. The plate was covered with new plate sealer and incubated for 120 minutes at room temperature on the orbital shaker.
- The plate sealer was removed and the plate transferred to an autowasher (ELx 50, Bioteck Instruments Ltd, UK) for four washes in wash buffer. After completion of the washing process, the plate was taken out of the autowasher, inverted and blotted against clean paper towels.
- 200 µL of substrate solution (prepared within 15 minutes of use and kept protected from light) was added to each well, covered with a plate sealer and

incubated for 30 minutes at room temperature and protected from light on the bench top.

- 100 µL of stop solution was added to each well, resulting in a colour change to yellow. Sometimes gentle tapping was required to achieve good mixing of the well contents when the colour changes were not uniform.
- Within 30 minutes, the plate was placed in the microplate reader (Synergy HT, BioTek Instruments Ltd, UK). The optical density was determined at 450 nm with wavelength correction set to 540 or 570. Reading calculation, data correction and analysis was achieved with microplate data collection and analysis software (Gen5, BioTek Instruments Ltd, UK).

2.2.5. Quality control, analysis and statistics:

For quality control within each run, all samples were tested in duplicate with the inclusion of double standard curve samples, internal and external positives, internal negatives and absorbance correction.

The raw measurements of PGE_2 concentration were in pg/ml. These values were then divided by the mean pulp weight for each group (11 mg for LPS groups and 15 mg for all other groups) resulting in measurements in pg/mg tissue.

At the end of each experiment and reading the plate, the system will provide an excel sheet with all the readings including a graphical standard curve. The data were then assembled from all experiments, categorised and initially analysed using excel in which mean, standard deviation and standard error values were calculated. To determine the level of significance, a student *t* test was accomplished using online version of GraphPad software.

2.2.6. Statistics

The data are presented as the mean ± standard error of means (SEM) throughout. A simple paired *t* test was used in all cases to determine significant differences between measurements before treatment (basal) and after treatment (stimulated), at the $P<0.05$ level.

2.2.7. Precautions:

- Foaming should be always avoided when mixing or reconstituting protein solutions.

- The assay procedure is sensitive to temperature changes, and should always be conducted between 18-23 ˚C.
- Contamination from other reagents of the same kit should always be avoided.
- Repeated cycles of freezing and thawing of the PGE_2 standard should be avoided.

2.3. Polymerase chain reaction (PCR)

2.3.1. Sample collection:

Detailed samples collection is described in the corresponding result chapter.

2.3.2. RNA extraction:

Extraction of total RNA from the pulp tissues was accomplished using RNeasy mini kit (Cat. No. 74104, Qiagen, Germany) and PureLink RNA mini kit (Cat. No. 12183018A, Ambion, Life Technologies, UK). The detailed protocol for each kit was as follows:

2.3.2.1. RNeasy mini kit: the contents of the kit are shown in Table 2-4. Before using the kit, four volumes of ethanol (96-100 %) were added to Buffer RPE and 10 µL β-mercaptoethanol (M7154, Sigma, Germany) was added to every 1 mL of the Buffer RLT to prepare a working solution according to the manufacturer’s instructions.

Material	Supplied
Buffer RLT	45 mL
Buffer RW1	45 mL
Buffer RPE (Concentrate)	11 mL
RNase-free water	10 mL
RNeasy mini spin column	50
Collection tubes (1.5 ml)	50
Collection tubes (2 ml)	50

Table 2-4: The contents of RNeasy mini kit

The protocol was as follows:

- Pulp tissues were gently taken from the RNAlater solution with forceps, dried gently and cut into small pieces using a new scalpel for each sample, then immersed in a micro centrifuge tube containing 600 µL of the Buffer RLT with β-mercaptoethanol according to manufacturer’s instructions. Additionally, the tissue was disrupted more while being immersed in the Buffer RLT using Pellet Pestle motor and disposable autoclaved tips.

- Homogenisation was achieved using TissueLyser LT (Qiagen, Germany) at maximum speed for 5 minutes. The supernatant was then pipetted and transferred to a gDNA eliminator spin column placed in a 2 ml collection tube and centrifuged (SLS 4600, Scientific Laboratory Supply, UK) for 30 seconds at ≥8000 x g. The columns were then discarded and the flow-throw saved. This step is very important because it helps to remove the insoluble materials and genomic DNA (gDNA) that may interfere with DNA removal.
- The flow-through was transferred to a new micro centrifuge tube before the addition of 600 µL of 70% ethanol and the contents of the tube were thoroughly mixed by pipetting.
- 600 µL of the mix was then transferred to an RNeasy mini spin column, placed in a 2 mL collection tube supplied by the manufacturer. The lid of the collection tube was closed tightly and the tube centrifuged for 15 seconds at ≥8000 x g. The flow-through was discarded. This step was repeated until all the initial mix was fully used.
- 700 µL of Buffer RW1 was added to the RNeasy spin column, lid closed, centrifuged for 15 seconds at ≥8000 x g and the flow-through was discarded.
- 500 µL of Buffer RPE was added to the RNeasy mini spin column, lid closed, centrifuged for 15 seconds at ≥8000 x g and the flow-through was discarded.
- 500 µL of Buffer RPE was added to the RNeasy mini spin column, lid closed, centrifuged for 2 minutes at ≥8000 x g, the flow-through discarded.
- The RNeasy mini spin column was placed in a new collection tube supplied by the manufacturer and centrifuged for 1 minute at full speed to further dry the membrane, and the collection tube with the flow-through were discarded.
- The RNeasy mini spin column was placed in a new 1.5 mL collection tube supplied by the manufacturer, before the addition of 50 µL RNase-free water directly to the spin column membrane, lid closure and centrifuged for 1 minute at ≥8000 x g to elute the RNA.
- The RNA was quantitatively analysed and then either stored on ice for immediate use or stored in -80 ˚C freezer for later use.

2.3.2.2. PureLink RNA mini kit: the contents of this kit are shown in Table 2-5. Before using the kit, 16 mL of ethanol (96-100 %) was added to Buffer RPE and

10 µL β-mercaptoethanol was added to every 1 mL of the Lysis Buffer to prepare a working solution according to the manufacturer instructions.

Material	Supplied volume/ quantity
Lysis Buffer	125 mL
Wash Buffer I	50 mL
Wash Buffer II	15 mL
RNase-free water	15.5 mL
Spin cartridge	50
Collection tubes	50
Recovery tubes	50

Table 2-5: Contents of PureLink RNA mini kit.

RNA was isolated and purified according to the following protocol:

- Pulp tissues were taken from the RNAlater solution with forceps, disrupted and cut into small pieces using a new scalpel, then immersed in 600 µL of the Lysis Buffer with β-mercaptoethanol in a micro centrifuge tube according to manufacturer's instructions.
- The tissue was disrupted while being immersed in the Lysis buffer using Pellet Pestle motor and disposable autoclaved tips. Additional disruption was achieved using TissueLyser LT at maximum speed for 5 minutes, followed by the addition of 600 µL of 70% ethanol and thoroughly mixing using a vortex (SA2 Autovortex mixer, Stuart Scientific, UK).
- 600 µL of the mix was transferred to a spin cartridge with collection tube (supplied by the manufacturer), centrifuged for 15 seconds at 12000 x g, followed by discarding the flow-through. This step was repeated until the entire mix was used.
- 700 µL of Wash Buffer I was added to the spin cartridge with collection tube, centrifuged for 15 seconds at 12000 x g, followed by discarding the flow-through.
- 500 µL of Wash Buffer II with ethanol was then added to the spin cartridge, centrifuged for 15 seconds at 12000 x g and the flow-through was discarded. This step was repeated one more time.
- The spin cartridge was then centrifuged for 2 minutes at 12000 x g to further dry the membrane, followed by the discard of the flow-through and the collection tube.

- The spin cartridge was inserted in a new recovery tube, followed by the addition of 60 µL of RNase-free water to the centre of the spin cartridge and centrifuged for 2 minutes at 12000 x g to elute the RNA from the membrane into the recovery tube.
- The RNA was quantitatively analysed and then either stored on ice for immediate use or stored in a -80 ˚C freezer for later use.

2.3.3. RNA quantitation:

The total RNA products yielded from both RNA isolation protocols were tested with NanoDrop ND-1000 spectrophotometer (ThermoFischer Scientific, USA) with the NanoDrop 1000 specific software (NanoDrop 1000 Version 3.8.1). The protocol was as follows:

- The software initialised, the sampling arm opened and the measuring pedestal cleaned using a soft laboratory wipe.
- 1 µL of autoclaved distilled water or elution buffer was used to create the blank measurement, pipetted carefully into the pedestal, the arm closed and the blank measurement made.
- Upon completion, the sampling arm was opened, the pedestal cleaned thoroughly, 1 µL of the sample RNA (according to the manufacturer's instructions) was pipetted onto the pedestal and the spectral measurements made for each sample.
- The pedestal was cleaned thoroughly following every measurement and a blank measurement was made before each sample measurement.
- For every sample quantified by NanoDrop, the purity of the nucleic acid was estimated by calculating the ratio between the spectrophotometric readings at 260 nm and 280 nm. The purity of all samples were ranging from (2.06-2.16).

Individual samples have been measured separated from other samples (not pooled) and the RNA extraction procedure yielded 156-648 ng/mg RNA per pulp tissue.

Due to inherent inaccuracy in quantifying total RNA using absorbance with NanoDrop (Aranda et al., 2009), the amount of RNA added to a PCR from each sample was more accurately determined by normalising against reference gene.

2.3.4. Reverse transcription (RT) and complementary DNA quantitation:

1 µg of total RNA was used in the synthesis of complementary DNA (cDNA) using High-Capacity cDNA Reverse transcription kit (Cat No. 4368814, ThermoFischer Scientific, USA). The contents of the kit are listed in Table 2-6.

Component	Quantity
10X RT Buffer	1 tube x 1mL
10X Random Primers	1 tube x 1mL
25X dNTP Mix (100 nM)	1 tube x 0.2 mL
MultiScripe Reverse Transcriptase (50U/µl)	2 tubes x 1 mL
RNase Inhibitor	2 tubes x 200µL

Table 2-6: The components of High-Capacity cDNA kit.

To synthesize a single-stranded cDNA from total RNA using this kit, the following protocol was employed:

- All the components of the kit were allowed to thaw on ice, then 2X RT master mix was prepared according to manufacturer's instruction as illustrated in Table 2-7, always kept on ice and mixed gently. This master mix was for 20 µl yield reaction.

Component	Volume
10X RT Buffer	2 µL
10X Random Primers	2 µL
25X dNTP Mix (100 nM)	0.8 µL
MultiScripe Reverse Transcriptase (50U/µl)	1 µL
RNase Inhibitor	1 µL
Nuclease-free water	3.2 µL
Total volume per reaction	10 µL

Table 2-7: Volume components needed to prepare RT master mix.

- 10 µL of the 2X master mix was pipetted into a PCR tube followed by the addition of 10 µL of sample RNA of 1 µg concentration, pipetting up and down two or three times to mix the contents.
- PCR tubes were labelled, dated and the lid closed tightly. Tubes were then centrifuged for 15 seconds at 8000 x g to spin down the contents and eliminate any air bubbles and always kept on ice.

- To create negative controls (RT –ve), we included RT reaction tubes containing the same components of Table 2-7 excluding the MultiScripe enzyme. To create no template –ve control (NTC), all the components in Table 2-7 were included without the addition of RNA, but added the same volume of nuclease free water instead.
- The tubes were then transferred to the thermocycler (T100 Thermal cycler, BIO-RAD, USA) using a special program suggested by the manufacturer as shown in Table 2-8.

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature (˚C)	25	37	85	4
Time	10 min	120 min	5 Sec	∞

Table 2-8: The program used with thermal cycler.

- The cDNA products were tested with NanoDrop ND-1000 following the same protocol as previously described for RNA quantitation in section 2.3.3.
- The synthesised cDNA product was either kept on ice for immediate use or stored in a -20˚C freezer for later use.

2.3.5. Primers

Primer design was performed using Primer 3 input http://primer3.ut.ee/ (Rozen and Skaletsky, 1999), before checking all primers for specificity using UCSC In-Silico PCR https://genome.ucsc.edu/cgi-bin/hgPcr . The primers were designed to create amplicons of up to 200 base pairs (bp), have GC content of 40-60% and primers with long runs of a single base were avoided as they can mis-prime. Then, primers were synthesised by Metabion (Metabion International AG, Germany), purified by desalting and with an annealing temperature of 58˚C. A full list of primers used in this work and their product size are listed in Table 2-9.

Primer name	Sequence	Product bp length
COX2 -FP	CTTCCTCCTGTGGCTGATGA	115
COX2 -RP	CCGGGATGAACTCTCTCCTC	
B- Actin-FP	TACTGCCCTGGCTCCTAGC	81
B- Actin-RP	AGAGCCACCAATCCACACA	
COX1-FP	TCATGAGTCGTGAGTCCGAC	132
COX1-RP	GGAAACTGGAGCGAGAGACT	
IL6-FP	CTCATTCTGTCTCGAGCCCA	146
IL6-RP	TGAAGTAGGGAAGGCAGTGG	
IL1-FP	CACCTCTCAAGCAGAGCACAG	79
IL1-RP	GGGTTCCATGGTGAAGTCAAC	
TLR4-FP	GATTGCTCAGACATGGCAGTTTC	135
TLR4-RP	CACTCGAGGTAGGTGTTTCTGCTAA	
EP1-FP	CATCATGGTGGTTTCGTGCA	198
EP1-RP	GGGTAGGAGGCGAAGAAGTT	
EP2-FP	CTTTAACGAGGTGGTTGGGG	115
EP2-RP	TAGATGCAGCTGGTTGTCCC	
NOS1-FP	AGGACCAGCTCTTCCCTCTA	145
NOS1-RP	GTGTCCTTGAGCTGGTAGGT	
IL6R-FP	GGCTCAAAGTCAGTTGGCAA	139
IL6R-RP	ATGACACATTGATGCTGGGC	
IL1R-FP	GACGTGGAGGATAGTGGGAG	115
IL1R-RP	TTCCTCCAGACCCAACTTCC	

Table 2-9: List of primer pairs used in this study and their product size.

2.3.6. End point (qualitative) Polymerase Chain Reaction (PCR):

PCR reaction was accomplished using GoTaq Green Master Mix (Cat. No. M7122, Promega, USA). The protocol used was as follows:

- GoTaq master mix was thawed at room temperature, vortexed briefly and centrifuged for 15 seconds.
- The reaction mix was prepared in PCR tubes on ice as shown in Table 2-10.

Component	Volume	Final
GoTaq green master mix, 2X	12.5 µL	1X
Forward primer (10 µM)	1 µL	0.4 µM
Reverse primer (10 µM)	1 µL	0.4 µM
Sample cDNA	3 µL	NA
Nuclease-free water	7.5 µL	NA

Table 2-10: End point PCR reaction mix/ 25 µl.

- The PCR tubes were transferred to the thermal cycler using a specifically designed program according to the manufacturer's instructions as shown in Table 2-11.
- The yielded PCR products were immediately detected by gel electrophoresis.

Cycles	Temp	Time	Notes
1	95˚C	2 min	Polymerase activation
40	95˚C 58˚C 72˚C	30 sec 30 sec 30 sec	Denaturation Annealing Extension
1	72˚C	5 min	Final extension
	4˚	∞	Holding until usage

Table 2-11: Detailed thermal cycler program used for end point PCR.

2.3.7. Post PCR detection and gel documentation system:

2% agarose gel was prepared by dissolving 2 gm of agarose (MB1200, Melford laboratories, UK) in 100 µL of 1X Tris-acetate-EDTA (TAE) buffer and completely dissolved by heating the mix with microwave oven for 5 minutes. The mixture was then allowed to cool down for few minutes but not solidify, followed by the addition of 10 µL of GelRed Nucleic Acid Gel Stain 10000x (Cat 41003, Biotium, UK) to allow

visualisation of nucleic bands. The solution was poured in a suitable gel tray, with the associated comb in place. After complete setting of the gel (around 30 minutes), the comb was removed, the tray transferred to the electrophoresis tank and more 1X TAE buffer added until it reached a specified level marked on the side of the tank. 3 µL Ladder (Hyper Ladder IV 100bp, Bioline, UK) was mixed with 5 µl gel loading buffer (5X DNA loading buffer blue, Cat. Bio-37045, Bioline, UK) followed by the addition of 3µL autoclaved distilled water, and loaded in the first well to the left. The samples did not need to be mixed with that stain, because the GoTaq master mix already contained two dyes (blue and yellow) that allowed monitoring progress during electrophoresis. 10 µL of each sample was loaded in the specified well. Negative controls for each gene were prepared following the same technique as with the samples but without cDNA and loaded in other wells.

Gel electrophoresis was subsequently performed by running TAE gels at 85 volts and 400 mAmp for 1-2 hours, until separation of DNA fragments were achieved using BIO-RAD Power Pac 300 device (BIO-RAD, UK).

After completion of the electrophoresis process, the gel was transferred to the Electrophoresis Documentation Analysis System (G:Box, Syngene, UK) for exposure of the gel to ultra violet light. Relevant images were captured with GeneSnap software (Version 7.08, Syngene, UK), saved and finally printed with a digital monochrome printer (P93D, Mitsubishi, Japan). The size of PCR products was subsequently determined by correlating the size and location of each sample band with the known size bands of the ladder. In order to achieve a quantitative value of the product bands of amplified genes, a specific software was used (Gene Tools software version 4.01, Syngene, UK). By drawing a rectangular shape of even sizes around each product band, the software provided a value representing the density of the respective bands.

2.3.8. Quantitative Reverse Transcriptase Polymerase Chain Reaction (q-RT-PCR):

q-RT-PCR was achieved using 2X SensiFAST SYBR No-ROX kit (BIO-98005, Bioline, UK), where all PCRs were prepared in 96 well optical reaction plates (RT-PL96-op, Eurogenetics, UK). All the reagents were taken out of the freezer and allowed to thaw on ice. All the work was accomplished on ice, using disposable filtered tips (StarLab, UK) and special pipettes with the SensiFAST kept protected from light. The contents of each well are shown in Table 2-12.

Reagent	Volume	Final Concentration
2X sensiFAST	10 µL	1X
10 µM Forward primer	0.8 µL	400 nM
10 µM Reverse primer	0.8 µL	400 nM
cDNA (Template)	1 µL	NA
Nuclease free water	7.4 µL	NA
Final volume	**20 µL**	

Table 2-12: Contents of each individual well for q-RT-PCR reaction.

In all the reactions, 50 ng of cDNA was used in each well and a house keeping gene was included (β-actin) as a positive control and to ensure that all data were expressed in close relation to an internal reference. All samples were tested in triplicates plus a negative control (NTC and RT-ve). Adhesive seals (Microseal B, cat. No. MSB 1001, BIO-RAD, UK) were fitted firmly over every reaction well plate, to avoid samples evaporation during the thermal cycling. Subsequently, the prepared and sealed 96 wells reaction plates were loaded into the DNA Engine Opticon 2 system (BIO-RAD, UK) operated by Opticon Monitor software (Version 3.1, BIO-RAD, UK). A plate layout was required for every reaction plate then the amplification conditions and protocol were determined. A three-step protocol was used with all reactions, with details of the protocol shown in Table 2-13 according to the manufacturer's instructions and the annealing temperature of the primers used in this study.

Cycles	Temperature	Time	Notes
1	95˚C	2 minutes	Polymerase activation
40	95 ˚C	5 seconds	Denaturation
	58 ˚C	10 seconds	Annealing
	72 ˚C	10 seconds	Extension

Table 2-13: Details of protocol used in q-RT-PCR.

At the end of each reaction, the data were collected by the system and graphically displayed. The files were saved containing all the specific details of the reaction including a melting curve, amplification curve and *Ct* values. The threshold was set at the linear part of the amplification curve and the number of cycles needed to reach it was calculated.

2.3.9. Results, analysis (real time) and relative quantification

Relative mRNA levels for each gene were determined by using a standard curve and by further normalisation to the reference gene to adjust for uncontrolled variabilities between the samples.

Calibration of the q-RT-PCR system was accomplished by including a standard curve prepared from gradual dilution of the cDNA template in each reaction. This can also serve as an internal positive control for primer function.

The melting curve analyses were performed by the system upon the completion of the cycles and was used to determine the specificity of each primer set. Melting curve allows the confirmation of specific PCR products and the absence of non-specific products.

Following each reaction, the system will present the data in numerical and graphical manner. All the data were then moved to excel sheet where they were assembled from all experiments. The data was then categorised and initially analysed using excel in which mean, standard deviation and standard error values were calculated. To determine the level of significance, a student *t* test was accomplished using online version of GraphPad software.

Chapter 3. Complexity of Odontoblast and Subodontoblast Cell Layers in Rat Incisor

3.1. Introduction:

The odontoblasts represent a specialized cell population with primary roles in the secretion and mineralization of dentine matrix (Qin et al., 2004). Further roles are also recognized in forming and regulating dentinal fluid and dentine permeability (Turner et al., 1989, Bishop, 1991), hard and soft tissue induced immune responses (Veerayutthwilai et al., 2007, Horst et al., 2009), enzyme production (Engström et al., 1976, Karim et al., 1979), signalling and mechano-transduction (Magloire et al., 2009, Allard et al., 2000) through the generation of action potentials (Allard et al., 2006), thermosensitivity (Son et al., 2009), and additional sensory roles (Okumura et al., 2005, Byers and Westenbroek, 2011). Odontoblasts are described as being arranged in columnar or pseudo stratified formation with one cytoplasmic process extending from each cell into the tubular structure of the overlying dentine (Luukko et al., 2011). After the tooth has fully formed, odontoblasts align along the periphery of the pulp, with their cytoplasmic processes embedded within the dentine and their cell bodies located within the soft tissues of the pulp. Their location makes them well-positioned to regulate the interaction between the oral environment and the dental pulp and engage in sensory and defence function playing important roles in regulating the pulp-dentine barrier (Turner et al., 1989, Bishop, 1991) and preserving the tooth integrity in response to noxious stimuli (Frank and Nalbandian, 1989, Arana-Chavez and Massa, 2004). Odontoblasts are unlikely to fulfil their various roles without the support of adjacent and subjacent cells, and it is for this reason that understanding of the sub-odontoblast cell layer (SOL) and its possible interaction with the odontoblast cell layer (OCL) is important.

The rat incisor is a well-established model for the study of pulp tissue. Its continuous growth allows tissues at all stages of development and maturation to be investigated within a single specimen (Smith and Warshawsky, 1975, Smith and Warshawsky, 1976, Ohshima and Yoshida, 1992).

These features make the rat pulp a valuable model to investigate tissue structure and function within a single organ that may represent the whole life cycle of cellular activity from formation to maturation and repair in the face of injury (Harada et al., 1999, Harada et al., 2002a, Tummers, 2003, Harada and Ohshima, 2004). Studies involving rat teeth

have included investigations on the effects of therapeutic agents (Murray et al., 2002a), bioactive molecules (Sloan and Smith, 1999), responses following injury (D'Souza et al., 1995), surgical procedures and the application of filling materials (Murray et al., 2000b, Murray et al., 2002a).

A deeper understanding of fundamental pulp physiology may be critical to the development of vital pulp therapies for the preservation of vital pulp functions, and for the development of tissue engineering approaches for the revascularization and regeneration of pulp tissues damaged by disease or trauma (Smith et al., 2008).

Based on that, this chapter was an attempt to explore the dental pulp histologically to investigate the structural and cellular complexity in more details.

3.2. Aim of study

The aim of this study was to use the rat mandibular incisor to explore the structural arrangements of cells of the OCL and SOL and within different zones of the dental pulp using contemporary immunohistochemical techniques.

3.3. Materials and methods

Every immunoreactivity image for every antibody presented in this work was considered representative if the observation were repeated in sections from seven different animals.

3.3.1. Sample collection

Twenty healthy male Wistar rats (300-600 gm weight) were killed by cervical dislocation, before collecting mandibular incisor teeth in three ways:

a) Whole mandibles were dissected as one piece with all teeth intact, then the mandible was divided into three separate pieces as shown in Figure 3-1. Piece one contained the incisal half of the incisor, piece two contained the rat molars and the apical half of the incisor and piece three contained the apical bud of the incisor.
b) Mandibular incisors were surgically removed from the mandible by dissecting away the overlying bone with a new scalpel to loosen them. When the incisors became mobile, they were removed with artery forceps, then divided into two groups:

1. Teeth were cut into two halves, incisal and apical, using a diamond coated disc mounted in a slow-speed straight handpiece under constant water cooling.

2. Teeth were grooved longitudinally on their mesial and distal surfaces with high speed diamond burs under constant water cooling, taking care to avoid pulp tissue exposure. Teeth were then readily split with a scalpel before carefully lifting the pulp tissue free with tweezers.

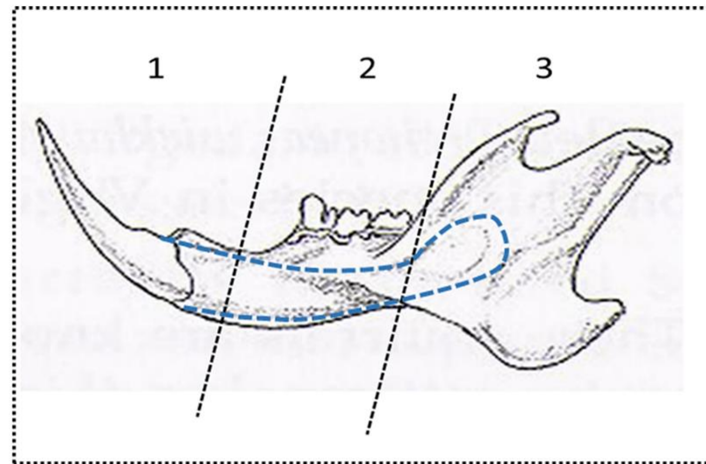


Figure 3-1: Schematic illustration showing the division of the whole rat mandible into three separate pieces.

In methods (a) and (b1), pulp tissue was investigated in situ following tooth demineralisation to preserve hard and soft tissue relationships. In method (b2), free pulp tissues were removed and examined without reference to hard tissue, to compare the results with demineralised specimens, and to exclude any possible harmful effects of the demineralisation process.

Specific structural antibodies against Vimentin, α-smooth muscle actin and NaKATPase were used in this study to provide an overview of the cellular arrangements histological zones within the dental pulp. For a detailed information about the positive and negative controls used in this study see chapter two section 2.1.7.

3.4. Results

In order to provide a better understanding and to help in orientation, an overview image for the cellular and morphological zones within the dentine-pulp complex were prepared (Figure 3-2) utilizing haematoxylin and eosin (H&E) and immunohistochemistry staining. Similar morphological zones were shown in both labial (A and C) and lingual (B and D), namely the OCL, cell-rich zone (crz) and the central part of the pulp. In immunohistochemistry images (C and D), vim IR was seen in the distal regions of the odontoblasts and in the odontoblast processes, with the nuclei located in the proximal regions of the cells. These images appear to show that the odontoblasts are arranged in a

stratified or pseudo stratified pattern, and usually these odontoblasts have a globular rather than tall columnar shape, with the nuclei arranged in two or more lines. No cell-free zone was observed in any section, which is in agreement with the literature in continuously growing teeth. The cells of the crz were different from that of OCL and dcz in terms of morphology, size, density of the cells and the immunoreactivity. These cells have small size, small round nuclei and present in very high density. NaKATPase IR appeared to be very intense in the crz and show much lower intensity in the odontoblast and dcz. The sections also illustrate differences between labial and lingual sides of the pulp, with apparent differences in average OCL thickness (thicker labially (72 µm) than lingually (66 µm). The thickness of crz which was NaKATPase IR was again thicker labially (53 µm) than lingually (37.5 µm).

Further structural details within the OCL were investigated utilizing IHC and high magnification of a sections from the apical half of a demineralized mandibular rat incisor Figure 3-3. The most striking observation to emerge from this set of figures was the presence of actin IR cells scattered within the OCL. These cells had no or very much reduced vim IR compared with the surrounding odontoblasts (see individual black and white panels), and appeared to be found only in the distal half (close to dentine) of the OCL, with appearances suggestive of some sort of connection between the actin IR cells. Observations from the representative black and white images provide an evidence that these population of cells within OCL have different morphology and IR.

Observations of vascular complexity were clearly visible and quite common in almost all sections with quite repetitive globular shape of the odontoblasts and wavy lines of nuclei Figure 3-4. The sections show many small blood vessels in different patterns, including tubes close to the mineralizing front (panels A and E), a single blood vessel running parallel to the OCL before bifurcating to form Y shaped vessels (panel B), a single blood vessel running parallel to the odontoblasts and crossing the OCL to reach the mineralizing front (panel C) and relatively wide blood vessels in the SOL, narrowing as they enter the OCL and crossing its thickness before bifurcating at the mineralizing front (panel D). This section suggests that most of the blood vessels within the OCL are located near to the mineralizing front where the minerals and nutrients are needed for the mineralisation process.

Investigating the crz in more details illustrates the presence of cellular processes extending from cells within the crz toward the odontoblast cell layer Figure 3-5, some of

which infiltrated the OCL and ran between the odontoblasts (arrows), while others sent short horizontal processes that remained within that zone (arrows with star *). There was no evidence of cellular processes extending from the cells of the crz downwards toward the dcz.

Further evidence of these cellular processes is illustrated in the higher power images of tissues from a demineralized section in Figure 3-6. In this figure, a cell whose cell body and nucleus is located completely in the crz (arrow), sends a cellular extension toward the OCL, which runs between the odontoblasts, extending to about half of the thickness of the OCL with arrows referring to the cell body and the process. The globular shape of odontoblasts and the wavy nuclei lines were again noted.

Another fascinating finding that emerged while investigating the pulp tissues at low power was the distribution of immunoreactivity to α-smooth muscle actin in the whole pulp with variable intensities Figure 3-7. Variation in the staining distribution and intensity was observed between labial and lingual sides, with higher intensity in the lingual side as shown in the individual black and white panel.

One of the most important observations was the heterogeneous nature of the cellular components of the dental pulp. Clear evidences were shown for the presence of cellular heterogeneity (multiple cell types) within the SOL and the centre of the pulp (Figure 3-8). Panel A shows cells with positive cyclooxygenase-1 IR scattered throughout these cell layers, while B show cells with tubulin IR^{+ve} within the vimentin IR^{+ve} cells appearing as single spindle like cells. Panel C shows tubulin IR^{+ve} cells or aggregates of cells forming specific shapes and patterns like tubes, lobes or star shapes arranged and sometimes this aggregation of cells surround a lumen. All cells in panels A, B and C showed very little or no vimentin IR.

Histological observations obtained from this study are summarised in the cartoon shown in Figure 3-9.

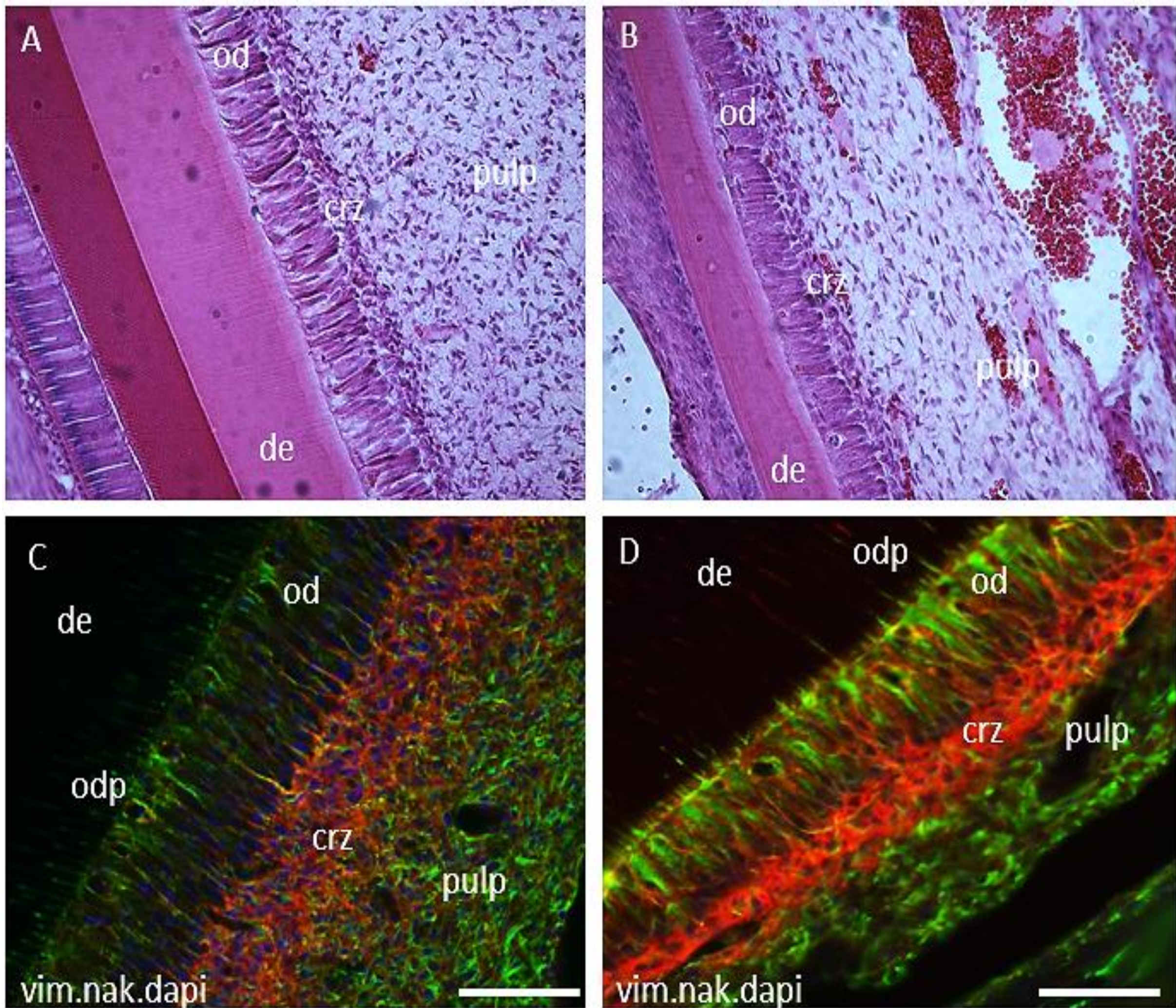


Figure 3-2: Overview of the cellular components and dentine of the apical half of demineralized rat incisor root. A (labial) and B (lingual) shows haematoxylin and eosin stained sections. C and D shows sections stained with antibodies to vimentin (green), NaKATPase (red) and nuclear stain (blue). In all images, the dentine (de) is clearly identified, containing several odontoblast processes (odp), and just beneath is the pulp which contains a layer of odontoblast cells (od), a cell rich zone (crz) and a diffuse cell zone (dcz). Calibration bars of 100 µm are shown in all images.

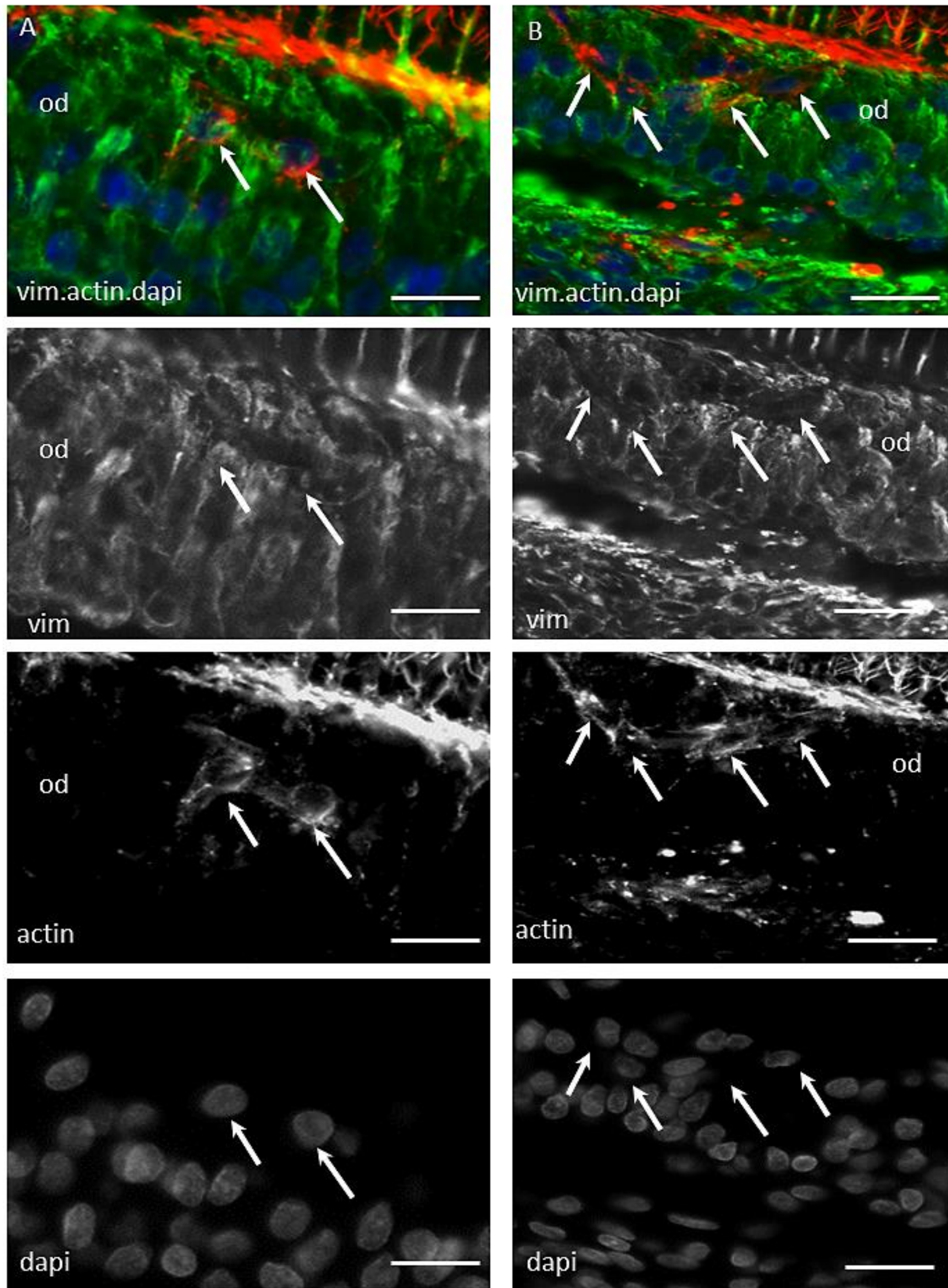


Figure 3-3: Demineralized sections of the apical half of mandibular rat incisor showing the odontoblast cell layer. Sections stained with antibodies to vimentin (green), α-smooth muscle actin (red) and nuclear stain (blue). A and B are high magnification images showing the odontoblasts, with the presence of other cells (arrowed) within the OCL, at the distal part of OCL close to the pulp/predentine junction. These cells show α-actin-IR but were negative to vim as shown in the images beneath for individual stains. Calibration bars of 50μm are shown in all images.

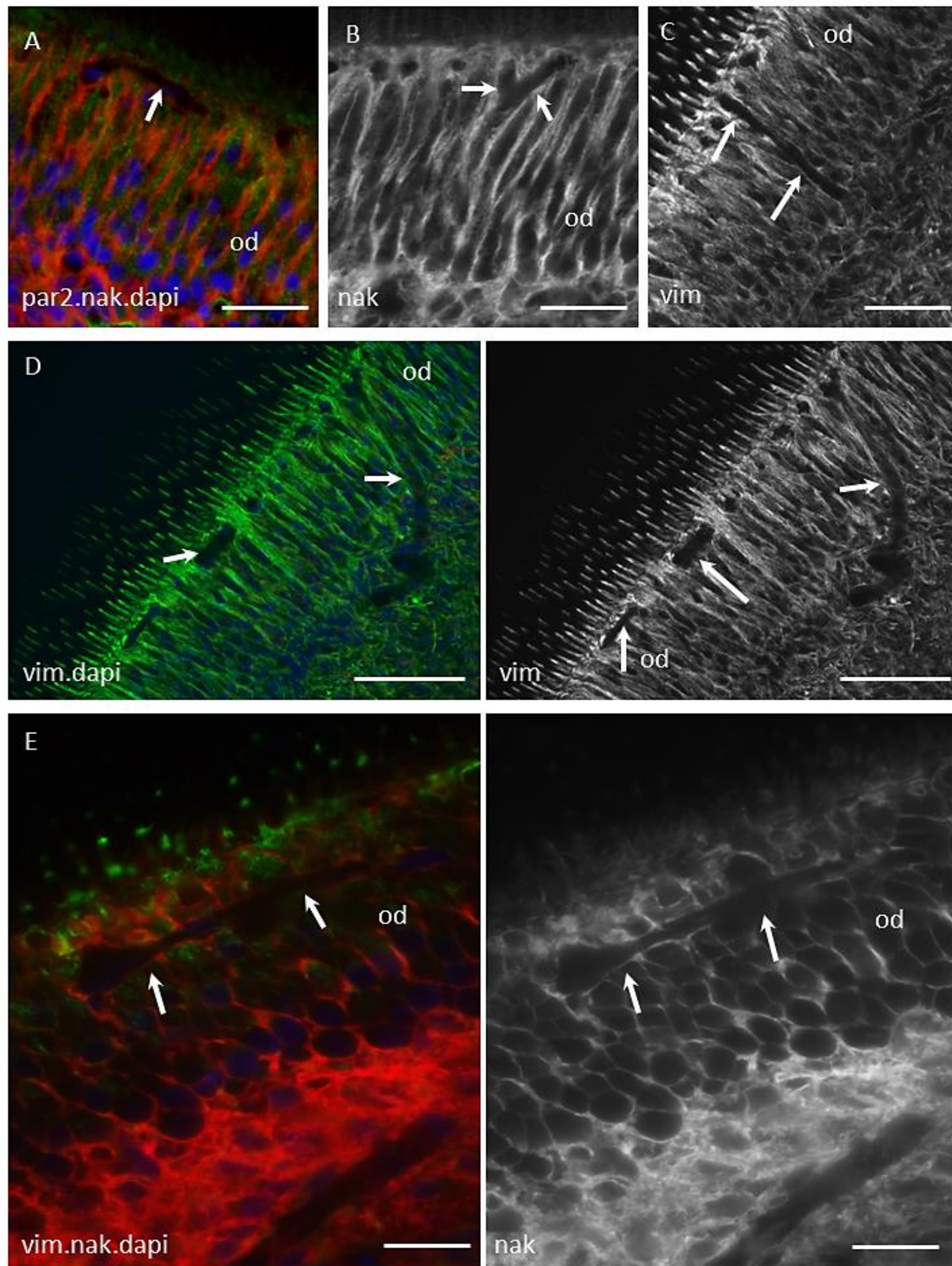


Figure 3-4: Demineralized sections from the mid part of the mandibular rat incisor showing vascularity in the odontoblast cell layer. Section A stained with antibodies to PAR2 (green), NaKATPase (red) and dapi (blue), B shows a section from a similar area, stained with NaKATPase, C and D stained with vimentin. D section stained with antibody to vimentin (green) and dapi (blue), E shows a section stained with antibodies to vimentin (green), NaKATPase (red) and dapi in blue. Calibration bars are 50 µm in A, B and C and 100µm in D and E.

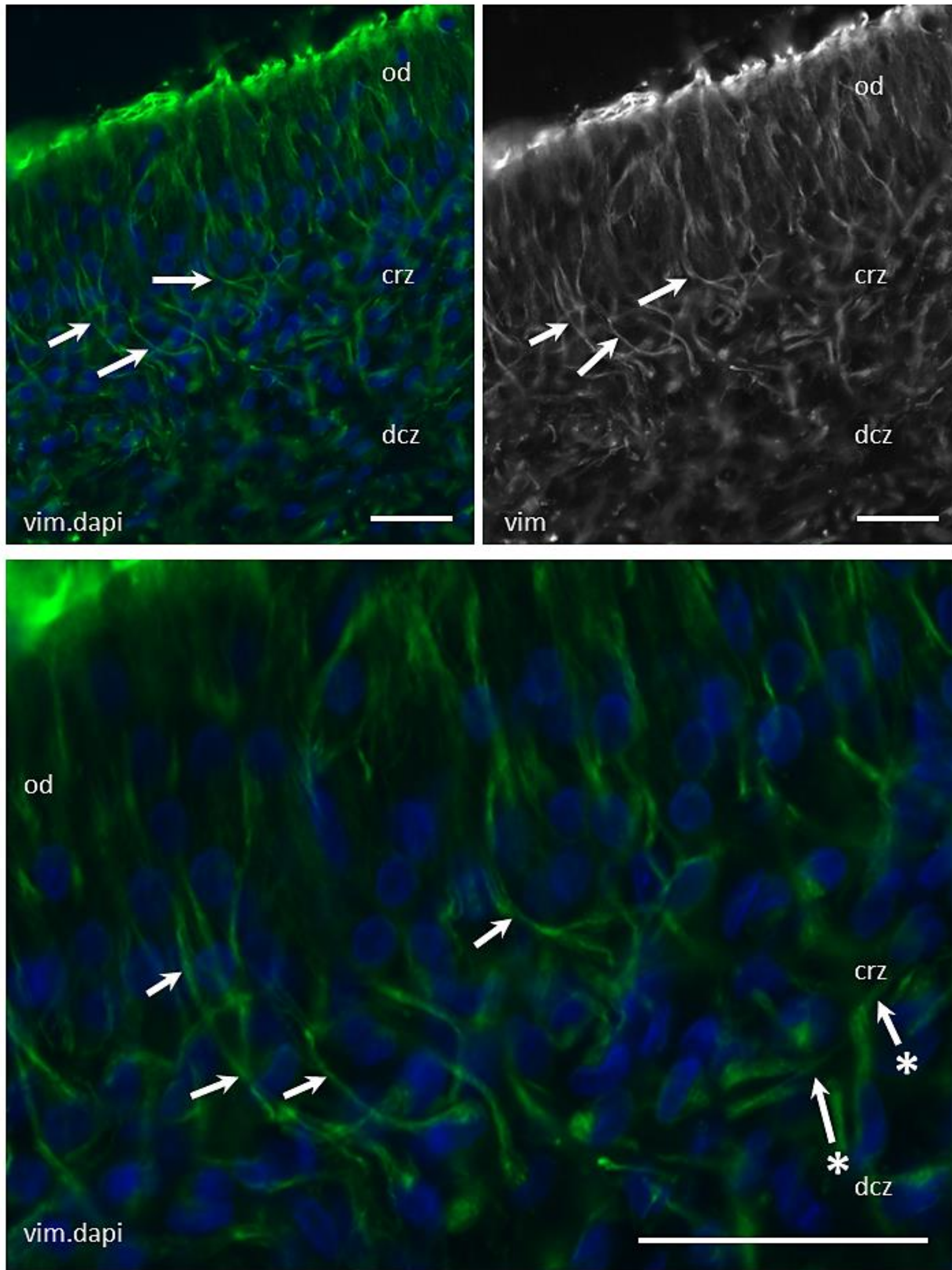


Figure 3-5: Isolated rat incisor pulp tissue (apical half) showing the odontoblast cell layer and subodontoblast area. Sections stained with antibodies to vimentin (green) and dapi (blue). The odontoblast (od), crz and dcz can be identified. Arrows refers to the long cellular processes extending from crz towards the OCL, while arrows with * refers to the cellular processes from crz cells that remain in crz. No cellular processes were seen to extend from the crz towards the dcz. Calibration bars of 100 μm shown in all images.

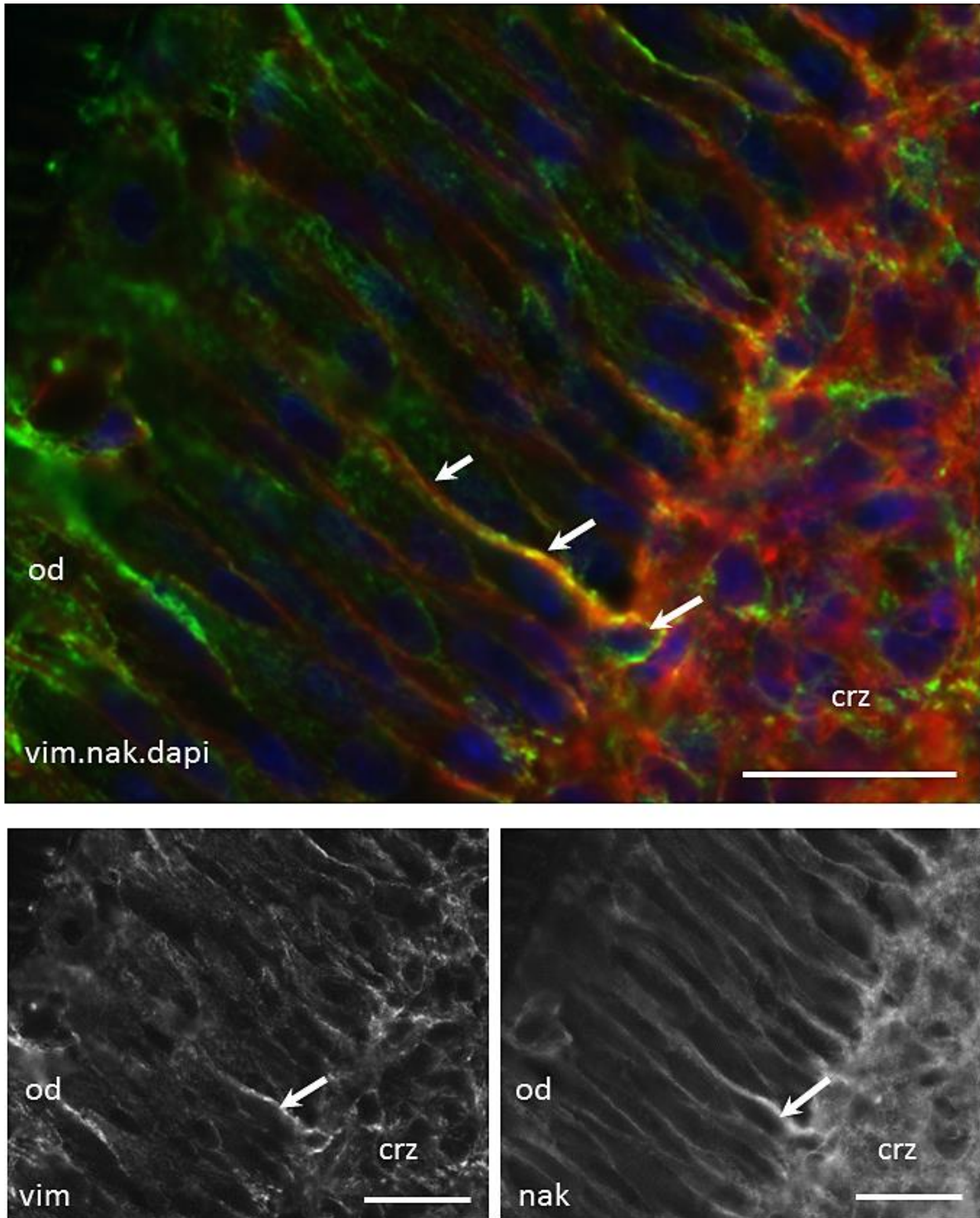


Figure 3-6: Demineralized sections of rat mandibular incisor showing the odontoblast and subodontoblast cell layers. Higher magnification images from a section stained with antibodies to vimentin (green), NaKATPase (red) and dapi (blue). The arrows in the colour image refer to a cell body and its process that appear to pass from the crz to the odontoblasts. The panels below are the individual images for vimentin and NaKATPase. Calibration bars are 50 µm in all images.

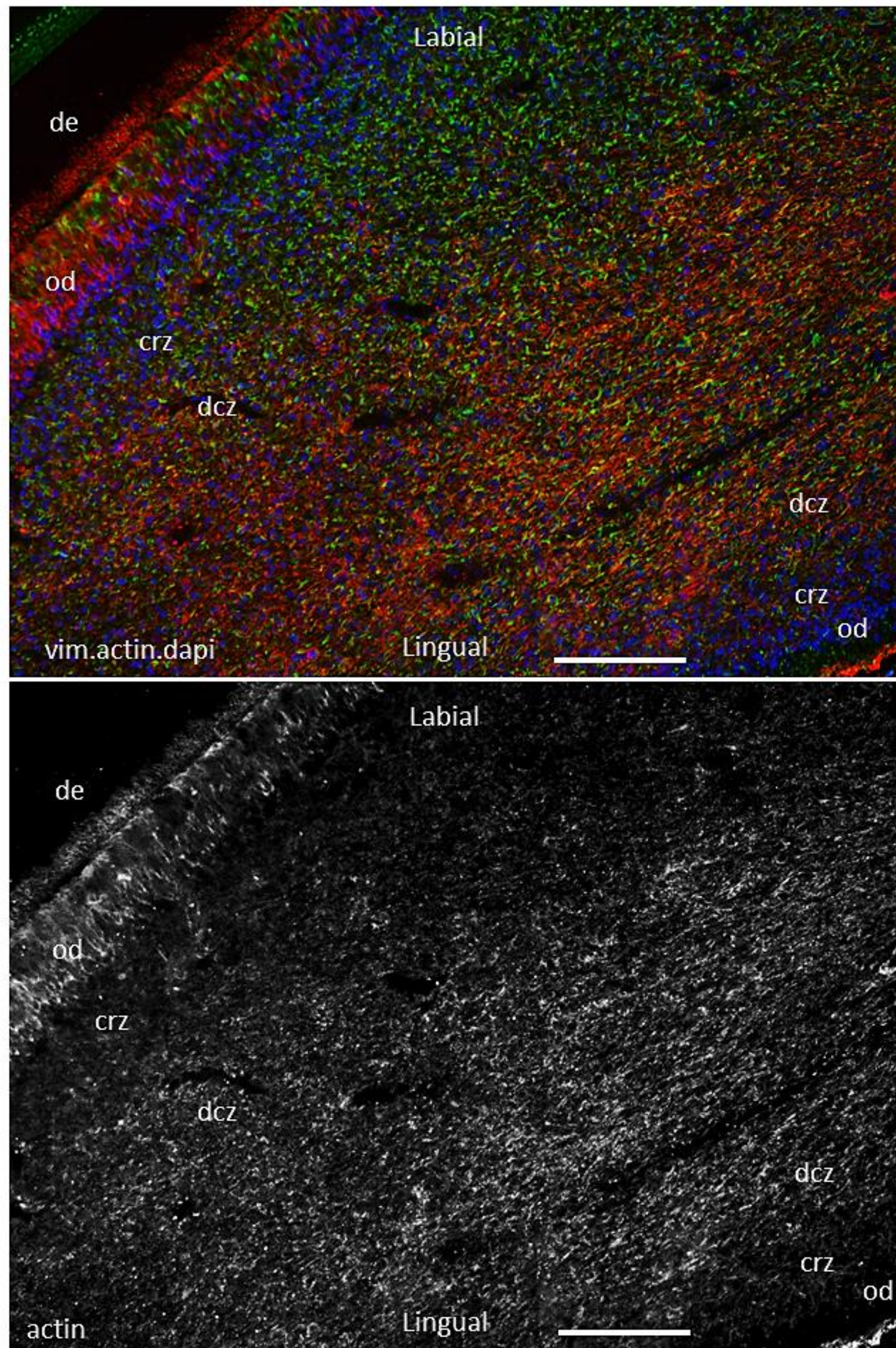


Figure 3-7: Demineralized section of rat mandibular incisor showing an overview of the whole thickness of the pulp. A shows section stained with antibodies to vimentin (green), α smooth muscle actin (red) and nuclear stain with dapi (blue). In this section both labial and lingual compartments of odontoblast (od), crz and dcz are clear with the labial side to the top and lingual to the bottom. Note the intense α smooth muscle actin IR on the lingual side compare to very weak IR on the labial side. Calibration bars is 150 µm in both images.

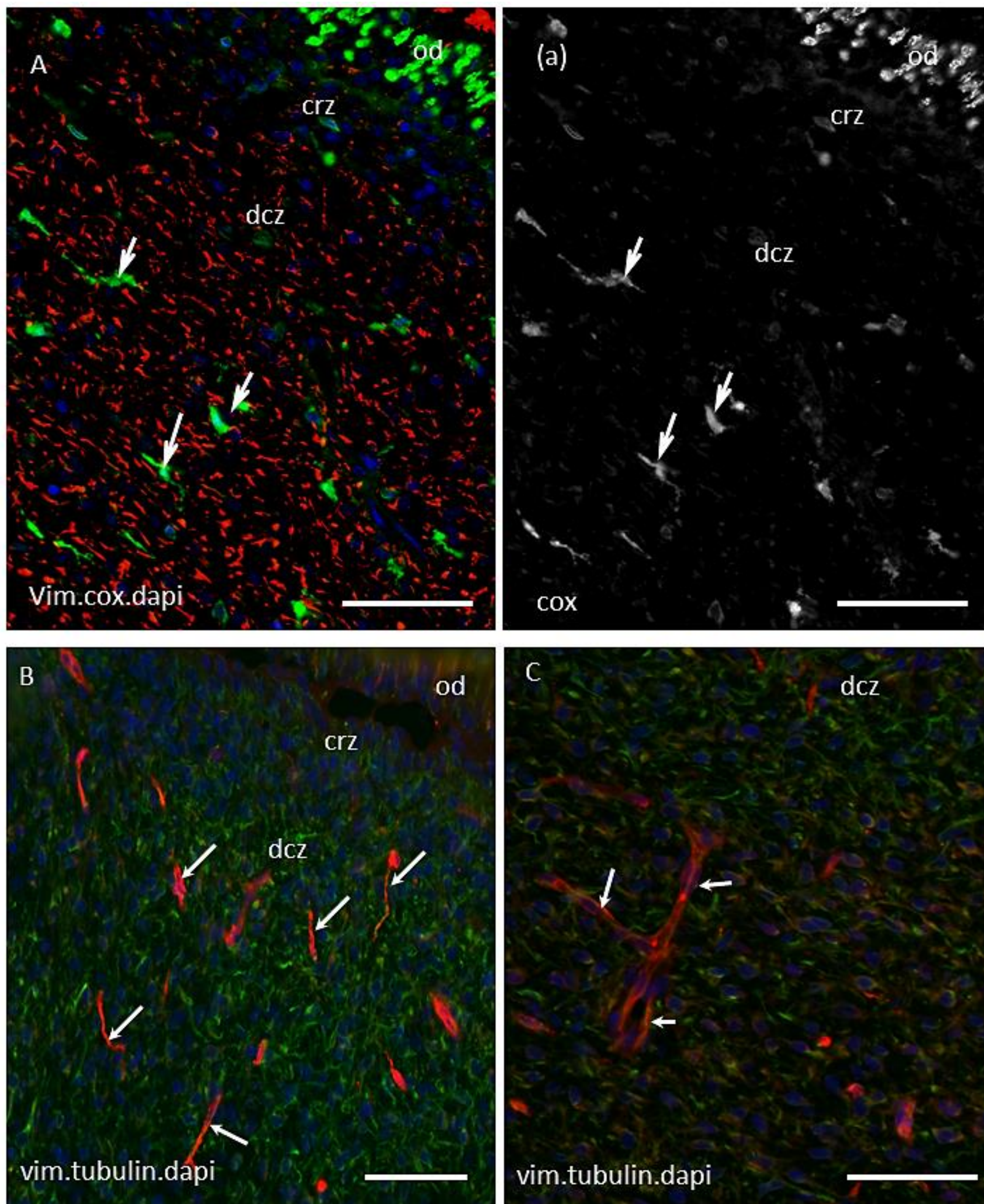


Figure 3-8: Isolated rat incisor pulp (apical half) tissue showing the cellular heterogeneity in the subodontoblast layers. The section stained with antibodies to vimentin (red), cyclooxygenase-1 (green) in A, vimentin (green), tubulin (tub: red) in B and C with dapi as nuclear stain (blue). Note the clear positive IR to the corresponding antibodies observed in some cells and the complete absence in the surrounding cells. All these cell types (with different IR) have very little or no immunoreactivity to vimentin. Calibration bars of 50 µm in all images.

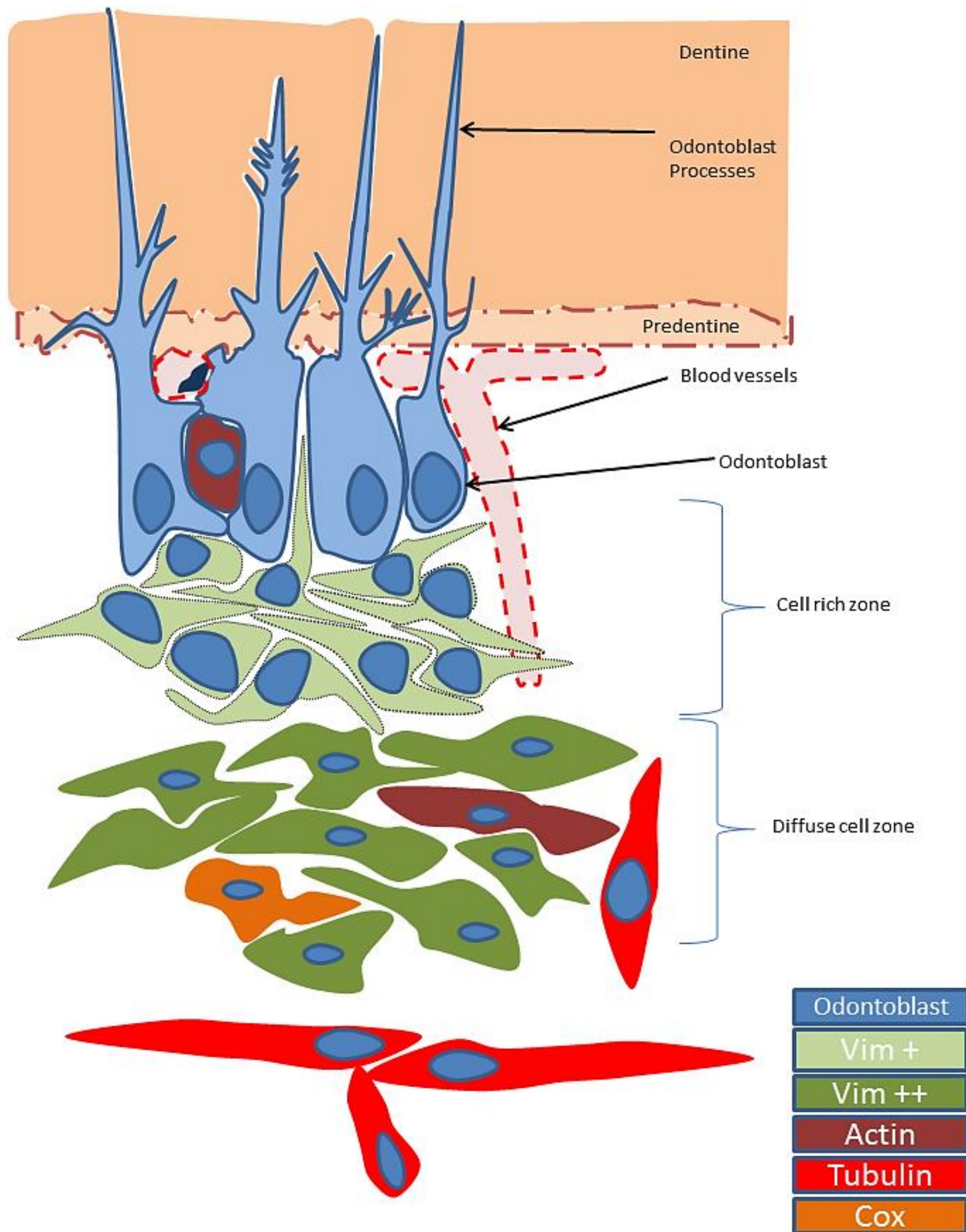


Figure 3-9: A cartoon illustrating structural complexity and cellular heterogeneity within the odontoblast and subodontoblast cell layers of the apical half of rat mandibular incisor.

3.5. Discussion:

Previous studies have reported that in the rat mandibular incisor and during the phase of active dentine deposition, the odontoblasts exhibit a tall columnar shape with varying heights, with nuclei located at the proximal end of their cell bodies, and their Golgi apparatus situated immediately above the nuclei, with the endoplasmic reticulum occupying nearly all of the distal part of the cell body (Takuma and Nagai, 1971, Arana-Chavez and Massa, 2004).

The current study presents a different picture, with the odontoblasts appearing as globular in shape, wide at the proximal part and narrower at the distal part of the cell body. In addition, few sections showed the odontoblast layer to be a single cell layer; rather, it appeared as a stratified or pseudo stratified arrangement of cells which may contain two or more cell rows, with the nuclei arranged in more than one row, giving the appearance of wavy lines. Possible interpretations may be that not all odontoblast cell bodies are in close contact with dentine, raising questions whether some odontoblasts may serve supporting roles alongside active cells that are in contact with the dentine, or that there is some sort of succession, with cells taking turns to deposit dentine matrix while other cells are dormant.

The OCL is generally regarded as odontoblast exclusive, with the occasional antigen-presenting DCs (Tsuruga et al., 1999), which may play a regulatory role in odontoblast function and differentiation (Ohshima et al., 1995, Ohshima et al., 1999). The presence of DCs also highlights the potentially important interface that the odontoblasts occupy. Sections observed in the current study appear to confirm the presence of an additional and hitherto undescribed cell population within the OCL. These cells were morphologically distinct from odontoblasts and were immunoreactive to α-smooth muscle actin and negative to vimentin (the opposite of odontoblasts). It is conceivable that these cells may be DCs, contributing to a complex immunological defence mechanism with the class I MHC odontoblasts acting as antigen recognizing cells (Veerayutthwilai et al., 2007, Keller et al., 2010, Farges et al., 2011, Horst et al., 2011) and the class II MHC-presenting dendritic cells as antigen presenting cells (Yoshiba et al., 2003) and potentially attracted to the area by the odontoblasts (Holland and Botero, 2014, Durand et al., 2006).

Immediately below the odontoblasts is the SOL which may be structurally divided into crz and dcz depending on the cell density. The crz is composed mainly of densely packed cells, which are believed to be capable of differentiation to other cell types, including

odontoblasts when conditions dictate (Fitzgerald et al., 1990, Luukko et al., 2011, Couble et al., 2000). Previous studies have suggested that if odontoblasts are irreversibly damaged or killed, they will be replaced by cells derived from the crz (Fitzgerald et al., 1990) after an increase in its mitotic activity (Murray et al., 2000b, Murray et al., 2002a, Murray et al., 2000a). Cells in the crz exhibit a range of shapes, from polygonal to stellate with cellular processes, that facilitate cellular interactions and actively contribute to complex signalling pathways in the dental pulp (Bongenhielm et al., 1995, Woodnutt et al., 2000, Yamaguchi et al., 2004). Findings from the current study confirm the presence of cellular processes that extend between cells of the crz, with no evidence of any processes directed downwards to the core of the pulp. By contrast, cellular processes were frequently seen to extend from the crz towards the odontoblast layer, extending approximately half the thickness of its pseudo-stratified structure. This finding might indicate previously unrecognized complex cellular interactions between the crz and the odontoblast layer, with the possibility of sensory, defensive, mechanosensitive (Magloire et al., 2009) or other regulatory functions.

Cells of crz in the rat mandibular incisors are found to have poorly developed endoplasmic reticulum and Golgi apparatus, suggesting that they are unlikely to be involved in protein synthesis and secretion. An abundance of well-developed mitochondria (Gotjamanos, 1969b) suggest that these cells have high metabolic rates (Zhai et al., 2003). Cells of the transporting epithelia (Silverthorn et al., 2007) share some histological and ultrastructural features with the crz cells. These include the cellular projections (which increase the surface area) and well-developed numerous mitochondria, all this added to the strong IR to NaKATPase may suggest that these cells are involved in active transport and reabsorption of fluids and ions from the pulp to the OCL. NaKATPase actively transports Na^+ and K^+ ions across the plasma membrane, plays a central role in mediating electrical activity of the nerve cells and has a range of regulatory functions in different cell populations (Banerjee and Chaudhury, 2002). Interestingly, weak staining for NaKATPase was detected in odontoblasts, which are known to be actively involved in transport (Salama, 1992) compared to much stronger IR in the crz cells.

Traditionally, dental pulp interstitial cells have been regarded as a homogenous population of fibroblasts whose major roles were the synthesis and secretion of dental pulp collagen and ground substance (Fries et al., 1994). More recent evidence has

suggested differences in fibroblasts cultured from distinct organs of the body (Fries et al., 1994, Sorrell and Caplan, 2004, Koumas and Phipps, 2002, Phipps et al., 1997), and initiated research into possible heterogeneity of interstitial cell populations within individual tissues, including the dental pulp (Ibuki et al., 2002). In human pulp tissue, different subpopulations of interstitial cells have been reported on the basis of their gene expression profiles (Suguro et al., 2008).

In parallel with the definition of subpopulations, it has become evident that fibroblasts can provide more than just structural support, and roles in balancing proliferation, migration and the synthesis of immune mediators have been suggested (Baglole et al., 2006). Evidence suggests that not only the macrophages, but also pulpal fibroblasts are involved in the modulation of inflammation by producing of cytokines including IL1, IL6, and IL8 (Coil et al., 2004) and as a result, prostaglandins (Nakanishi et al., 2001).

In the current study, at least two subpopulations of heterogeneous dental pulp cells were found sparsely distributed in the diffuse interstitial cell layer.

The observations for COX-1 IR^{+ve} cells suggest its relatively scarce distribution within SOL and the diffuse interstitial cell layers. Previous research indicates that pulp fibroblasts take part in the inflammatory process by cytokine (Coil et al., 2004) and subsequently prostaglandin production (Nakanishi et al., 2001), with the COX enzyme as the rate-limiting enzyme (Zidar et al., 2009).

The SOL is known to contain additional cell types, including macrophages, DCs and immune cells (Luukko et al., 2011). The current observation of cells with different immunoreactivity within the SOL may be of considerable importance. Cells that are immunoreactive to COX-1 might suggest functional and signalling activity. Cells that were immunoreactive to tubulin appeared in two distinct patterns, either as single cells scattered throughout the pulp, or as a specific pattern of aggregated cells, sometimes associated with a narrow empty lumen. This heterogeneity of cells might be associated with a variety of functional and vital roles within the pulp complex which remain unclear.

Data from the current study confirm previous studies on the differences in thickness of the OCL and crz labially and lingually in the apical half of the rat incisor (Ohshima and Yoshida, 1992), with the OCL taller labially than lingually (70-80 µm vs 55-63 µm respectively), and the crz more prominent and thicker labially than lingually (50-60 µm vs 33-40 µm respectively).

One of the added findings from the current work is the expression of α-smooth muscle actin in the cells of the SOL, and extending to the core of the pulp. This expression appears to be less on the labial and increasing in intensity towards the lingual side.

In conclusion, the findings of this study indicate that the OCL and SOL are heterogeneous and more structurally complex than previous reports have suggested. The functional implications of such complexity are currently unclear, but it may highlight important and complex cellular interactions that may involve odontoblast-odontoblast, odontoblast-odontoblast processes and/or odontoblast-subodontoblast cell layers.

Chapter 4. Evidence for nitric oxide and prostaglandin signalling in the regulation of odontoblast function in identified regions of the rodent mandibular incisor

4.1. Introduction

The odontoblasts represent a unique and highly specialised cellular population within the dental pulp, whose primary roles include dentine matrix deposition and mineralization.

Recently, it has been suggested that odontoblasts perform additional sensory and defensive functions, are instrumental in the production of growth factors, and engage in signalling and mechano-transduction throughout the lifetime of the tooth (Allard et al., 2006). The dental pulp as a whole is therefore a complex tissue with formative, defensive and reparative functions, and in common with other tissues, such arrays of function demand cross-talk and regulation at the cellular level and a corresponding network of signalling and control pathways (Silverthorn et al., 2007, Krauss, 2006).

A deep understanding of the fundamental cell-systems physiology and signalling within the dental pulp is foundational to the development and optimisation of clinical therapies to promote healing and repair within damaged teeth and to nurture tissue regeneration in the face of injury. Two fundamental signalling pathways that may provide important insights on functional activity within the dental pulp are the nitric oxide (NO) and prostaglandin (PG) pathways (Goodis et al., 2000, Lin et al., 2002, Di Maio et al., 2004).

Both pathways are believed to play important roles in the regulation of normal physiological processes, inflammatory processes and pathogenesis of some diseases (Waterhouse et al., 1999, Cahlin et al., 2000, Paduch and Kandefer-Szerszeń, 2011, Chuang et al., 2013). There is much evidence of cross-talk between these two pathways in many body systems including the gastrointestinal system (Uno et al., 2004, Calatayud et al., 2002, Sibilia et al., 2008), urinary (Rahnama'i et al., 2010, Wu, 1995, Chuang et al., 2013) and respiratory systems (Strapkova et al., 2006).

With regards to dental pulp, a considerable body of published evidence is available on the PG and NO pathways within the rat incisor (Hirafuji et al., 1980, Borda et al., 2007, Yasuhara et al., 2007), though there is uncertainty whether NO stimulates or inhibits PG production.

4.2. Aim of study

This study sought to develop understanding by exploring the presence of immunoreactivity to COX-1, prostanoid receptors (EP) and nitric oxide synthase 1 (NOS1) in the rat mandibular incisor, and seeking evidence for the presence of complex NO/PG signalling pathways utilizing contemporary immunohistochemical and confocal microscopic technique.

4.3. Materials and methods

Every immunoreactivity image for every antibody presented in this work was considered representative if the observation were repeated in sections from seven different animals. Twenty healthy male Wistar rats (300-600 gm weight) were killed by cervical dislocation and their mandibular incisors (40) were immediately extracted. Samples were collected as described in chapter 3 section 3.3.1, fixed, frozen, sectioned and examined as described in chapter 2 section 2.1.

Specific antibodies against COX1, NOS1, EP1, EP2 and Vimentin were used in this study to detect the presence and exact localisation of the key elements of PG and NO pathways. For a detailed information about the positive and negative controls used in this study see chapter two section 2.1.7.

4.4. Results

In order to provide an overview image and help in the orientation, Figure 4-1 shows a low power image of a section from a demineralised incisor in the central region of the tooth. The components of enamel organ and the components of dentine-pulp complex can be easily identified. The interface between dentine and enamel is marked by arrows. The outer region illustrates the enamel organ with the ameloblasts, stratum intermedium and stellate reticulum. COX-1 IR was strongly represented within the odontoblasts, and to a lesser extent in the ameloblasts. This is seen more clearly in the component image for COX-1 IR (lower left panel). NOS1 IR was observed in the distal region of the odontoblast cell body (OCB) and stronger reactivity in the enamel organ (lower right panel). This is the first time that the exact localisation of COX-1 and NOS1 IR within the dentine-pulp complex of the rat mandibular incisor.

In an attempt to investigate the key elements of PG and NO pathways within the dentine-pulp complex in more details, more specifically COX-1 and NOS1, a demineralised section were utilised (Figure 4-2), in which (A) shows a region of the pulp from the

middle region of the incisor. The interstitial cells of the pulp in the crz and dcz were vimentin IR^{+ve}. This IR was also seen in the distal regions of the OCB and in the odontoblast processes. The nuclei of the odontoblasts appeared to be located in the mesial regions of the cell bodies. The regional distribution of vimentin was seen more clearly in higher magnification images, panels (a) and (b). Vimentin in the distal regions of OCB appeared to be continuous with the that in the odontoblast processes. By contrast, COX-1 IR was localised almost exclusively within the cytoplasm of the odontoblasts, in the region between the nucleus and the vimentin-rich region at the apex of the cell body.

Further investigations utilising a non-demineralised section of pulp tissue from the apical half of the tooth (Figure 4-3). For orientation, the region peripheral to the pulp that would have been occupied by dentine is marked (*). All the morphological zones from the mineralising front to the centre of the pulp can be seen, namely the OCL, crz, dcz and the bundles of big blood vessels running in the centre of the pulp. The section shows the presence of relatively small population of cells with COX-1 IR (red/purple). These cells were sparse in the crz (panel a) but more abundant in the dcz (panel b). A detailed and high magnification examination of these cells (panel c) revealed that the COX-1 IR cells of the dcz (arrows) were not vim IR which clearly suggest a different cell type. Furthermore, COX-1 IR was apparent in the walls of blood vessels as can be seen clearly in panel A.

Another key element of PG pathways which the targets for PG produced by COX-1 were examined using antibodies to prostanoid receptors, type 1 (EP1) and type 2 (EP2) (Figure 4-4). EP2 IR was observed within the OCB and odontoblast processes, with weaker immuno-reactivity in the interstitial cells of the crz (Figure 4-4 A and magnified inset a). IR to EP1 was noted in a small population of cells scattered throughout the pulp and in the small blood vessels as can be seen in (panel b).

In order to explore the presence of other signalling pathways, the distribution of the enzyme NOS1 (enzyme responsible for the production of NO) was examined (Figure 4-5). In (A) the distribution of COX-1 IR was as previously described, whereas NOS1 IR was observed primarily in the distal regions of the odontoblasts and was weakly expressed within the proximal regions of the odontoblast processes within the dentine. Weaker NOS1 IR was observed in the cells of the crz but not the dcz.

More detailed illustrations at higher magnification (Figure 4-6) showing the location of EP2 IR, NOS1 IR and COX-1 IR in the odontoblast and sub-odontoblast regions. Panel A shows that EP2 IR is located primarily in the distal region of the OCB and in the mesial regions of the odontoblast processes. Panel B shows EP2 IR in the crz region. In panel C, NOS1 IR is seen predominantly in the distal region of OCB and weakly in mesial region of odontoblast processes.

In an attempt to explore the IR to the previously mentioned key elements within the blood vessels, Figure 4-7 was prepared. COX-1 IR was observed within blood vessels (panel A) with region of interest (a) shows COX-1 IR associated with the walls of arteries (*) but less so with veins (+) within the dcz. Panels B and C show COX-1 IR associated with the endothelial cells of veins (+), in the arterial wall (*) and in interstitial cells of the dcz. D and the inset (d) show NOS1 IR associated with arteries (*) but not veins.

As a summary, the expression of COX-1 may be taken to suggest a site of production of prostaglandin. The principle locations for COX-1 expression are (i) the central region of the odontoblast, (ii) specific vimentin negative cells in the dcz and (iii) in cells in the wall of both arteries and veins. Receptors for prostaglandin are found on cells in the sub-odontoblastic crz and also, weakly, in the apical region of the odontoblasts and their processes. These observations suggest the possibility of at least three signalling pathways in the pulp involving prostaglandin: odontoblast-subodontoblast, odontoblast- odontoblast process and diffuse cell layer-subodontoblast. An additional prostaglandin signalling system may be associated with the blood vessels. NOS1-IR is located in the apical regions of the odontoblasts and odp and can be regarded a site of production of NO. The precise target for NO is not known, but may be the COX system in the odontoblasts or the sub-odontoblastic cells.

In summary, the odontoblast cell layer is a complex layer presenting in a stratified or pseudo-stratified arrangement with the odontoblast cells appearing in globular shape. Note the presence of a different cell type with α-actin IR within the OCL. There is a vascular network of small arterioles located near the mineralizing front. The SOL is divided into crz and dcz. Some cells in the crz appear to send long processes toward

and into the OCL which suggests the possibility of functional cross talk between these two layers. Other cells in the crz send short horizontal processes with no evidence of processes directed towards the dcz. The dcz shows multiple cell types with different immune-reactivity.

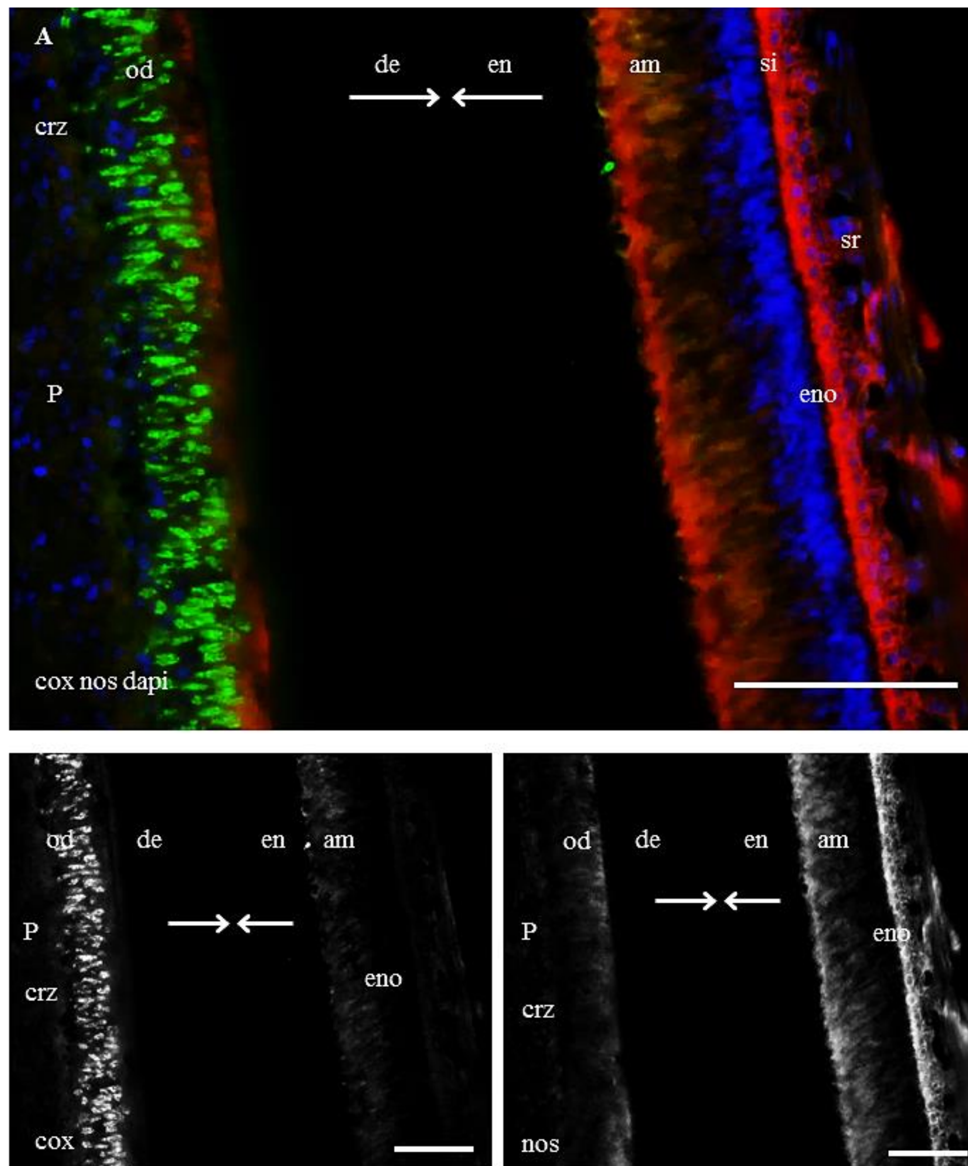


Figure 4-1: **An overview of the cellular and hard tissue components of the apical third of a demineralised rat incisor.** A shows a section stained with antibodies to COX-1 (green), NOS1 (red) and the nuclear stain dapi (blue). Both pulp (P) and enamel organ (eno) can be identified. Within the pulp the odontoblast layer (od) and crz are shown. The interface between the dentine (de) and enamel (en) is indicated by arrows. In the enamel organ the ameloblasts (am), stratum intermedium (si) and stellate reticulum (sr) are shown. The lower panels show the individual images for COX-1 IR and NOS-IR. Calibration bars 200 µm in all images.

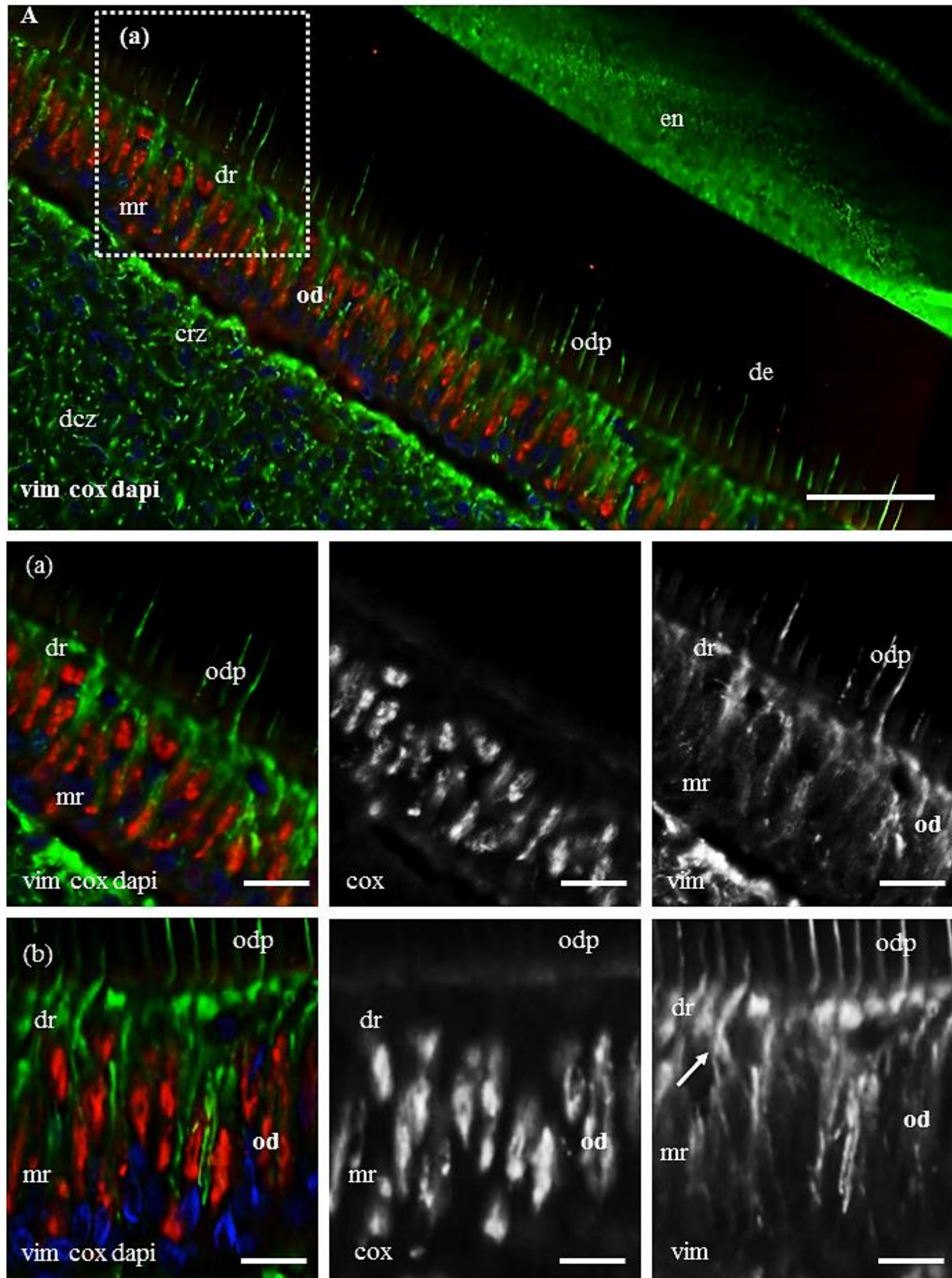


Figure 4-2: A demineralised section form the apical half of the tooth showing the location of COX-1 IR and vimentin IR within the odontoblasts. Section was stained for COX-1 (red), vimentin (vim: green) and dapi (blue). Images at higher magnification are also shown from a region of interest (a). (b) Shows a different region of a different tooth illustrating the same staining pattern. Abbreviation are: dentine (de), enamel (en), odontoblast processes (odp), odontoblast (od), distal region (dr), mesial region (mr), cell-rich zone (crz), diffuse cell zone (dcz). Calibration bar in A 50 µm and in 15 µm in (a) and (b).

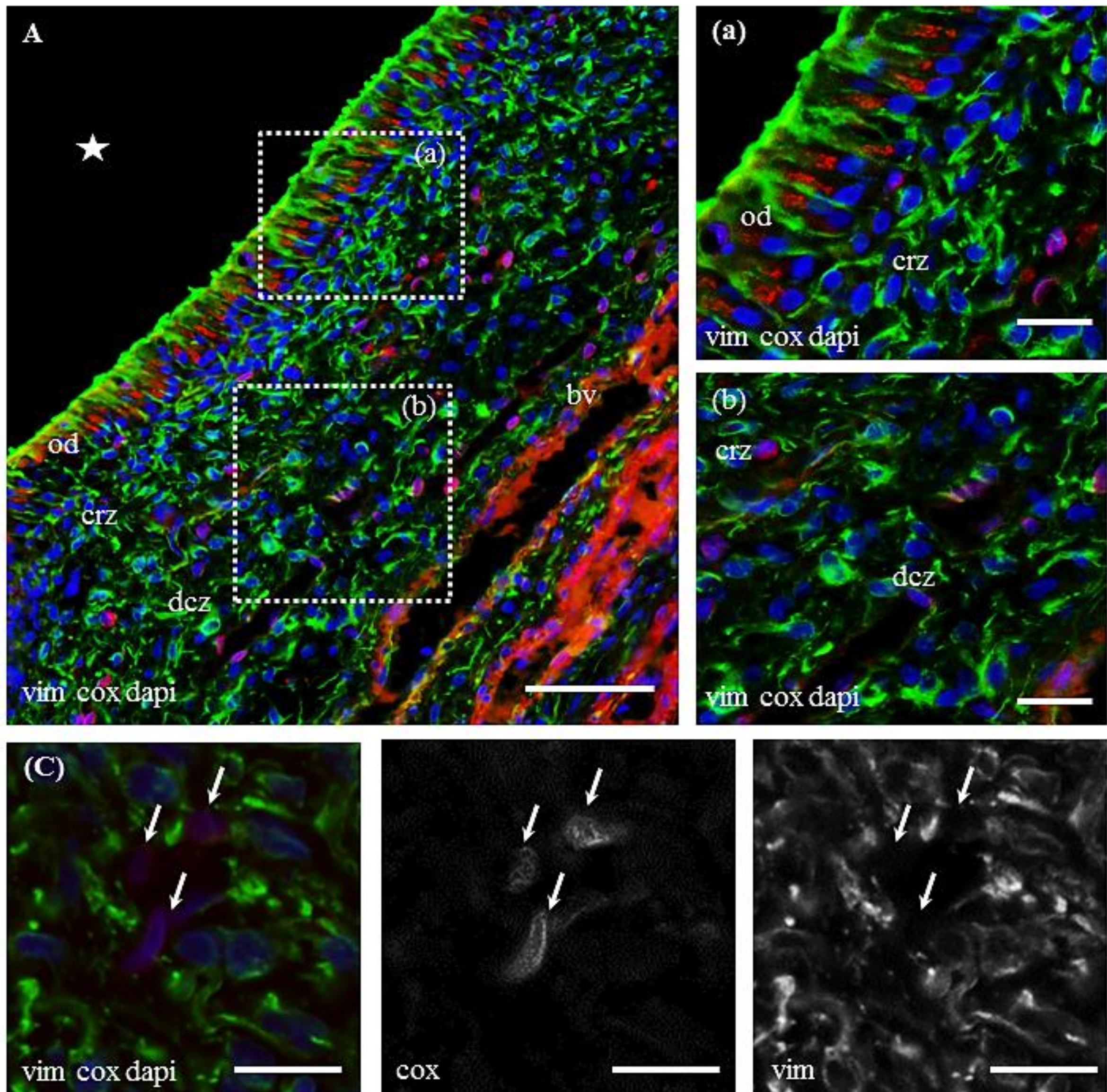


Figure 4-3: Overview of the different cell layers within the apical half of a non-demineralised rat incisor pulp. A shows a section stained with antibodies to vimentin (green), COX-1 (red) and dapi (blue). The odontoblast layer (od) is readily identified. Immediately below the odontoblasts is a sub-odontoblast layer that can be separated into a crz immediately below the odontoblast layer and a dcz. Higher magnification of regions marked (a) and (b) are shown in the adjacent panels showing the presence of COX-1 IR cells (red) in the OCL and dcz but fewer in the crz. (C) Show a similar region from a different preparation at high magnification, where three COX-1 IR cells are indicated by the arrows. The same arrows are shown in the vimentin image but these cells do not stain for vimentin. Abbreviation are: odontoblast (od), cell-rich zone (crz), diffuse cell zone (dcz). Calibration bars: 100 µm in A, 25 µm in (a) and (b) and 15 µm in (c).

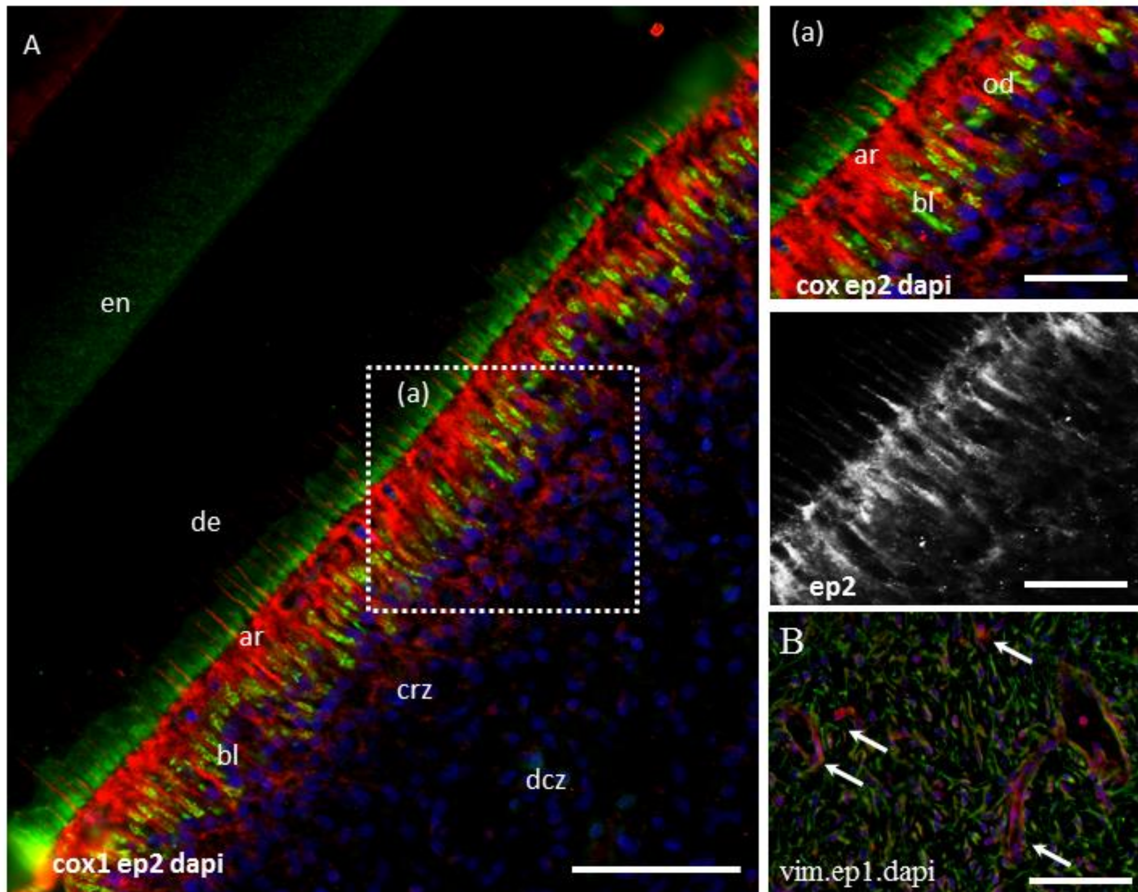


Figure 4-4: Distribution of COX-1 and the prostanoid receptors EP1 and EP2 in a demineralised section form the apical half of the rat mandibular incisor. Section stained with antibodies to COX-1 (green), EP2 (red) in A and a, EP1 (red) in B and dapi (blue). The region of interest (a) is shown in the adjacent panels with the individual images for COX 1-IR and EP2-IR illustrated. These images show that COX-1 IR was seen in the central region of the OCB just above the nuclei, and EP2 IR seen in the distal region (dr) of the OCB and weaker IR observed in the mesial region (mr) of OCB, cell rich zone (crz) and the odontoblast processes (odp). Section B shows EP1 IR in a small population of cells and the small blood vessels (arrows). Calibration bar is 150 µm in A and 80 µm in (a and B).

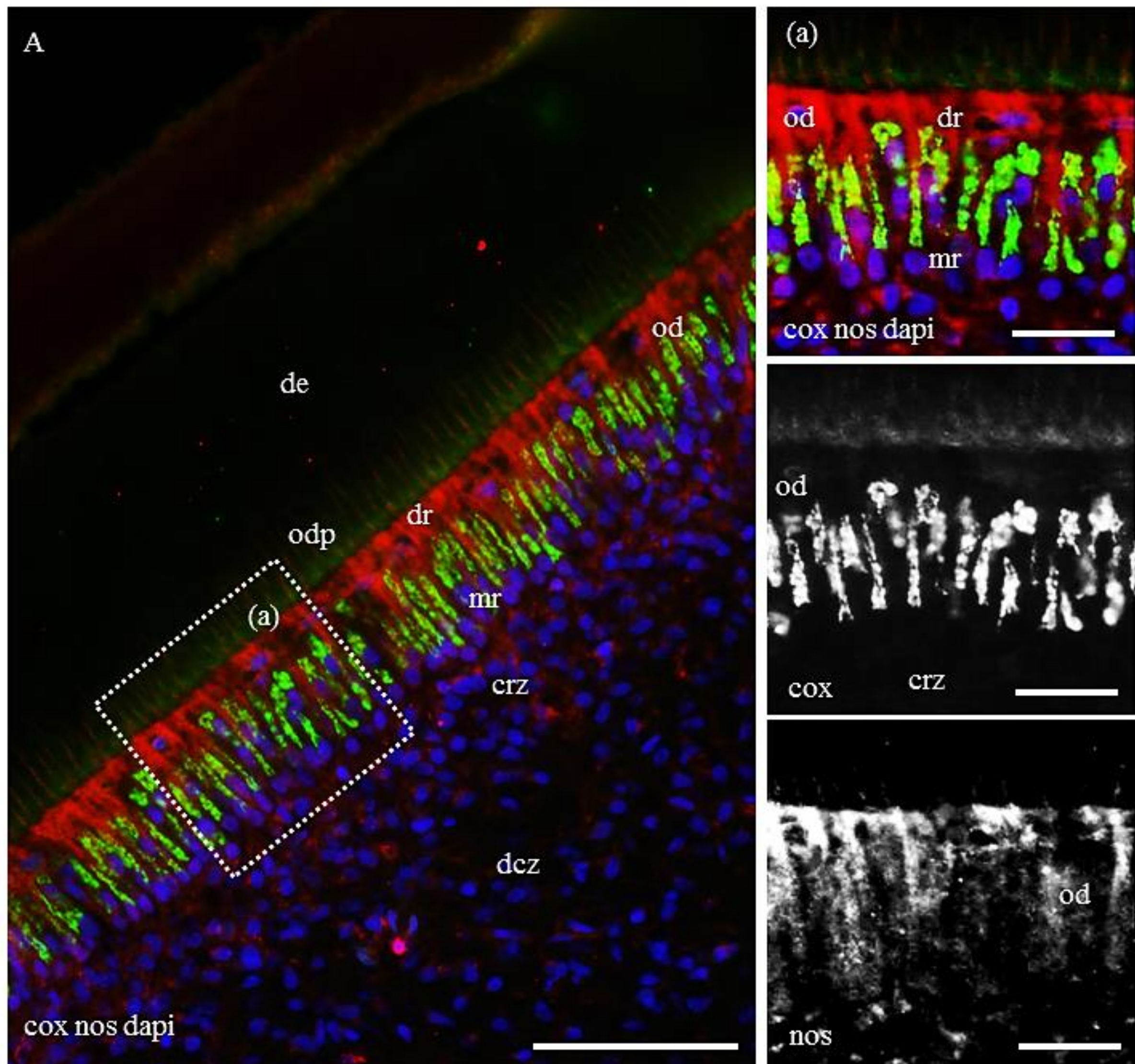


Figure 4-5: Distribution of COX-1 IR and NOS1 IR a demineralised section from the apical half of rat mandibular incisor. Section stained with antibodies to COX-1 (green), NOS1 (red), and dapi (blue). A shows a low power image. Images from a region of interest (a) are shown at higher magnification and separated into individual COX-1 and NOS1 components. Abbreviation are: dentine (de), odontoblast processes (odp), odontoblast (od), distal region (dr), mesial region (mr) cell-rich zone (crz), diffuse cell zone (dcz). The high regional expression of COX-1 IR and NOS1 IR can be easily seen. Calibration bar in A 150 µm and in (a) 80 µm.

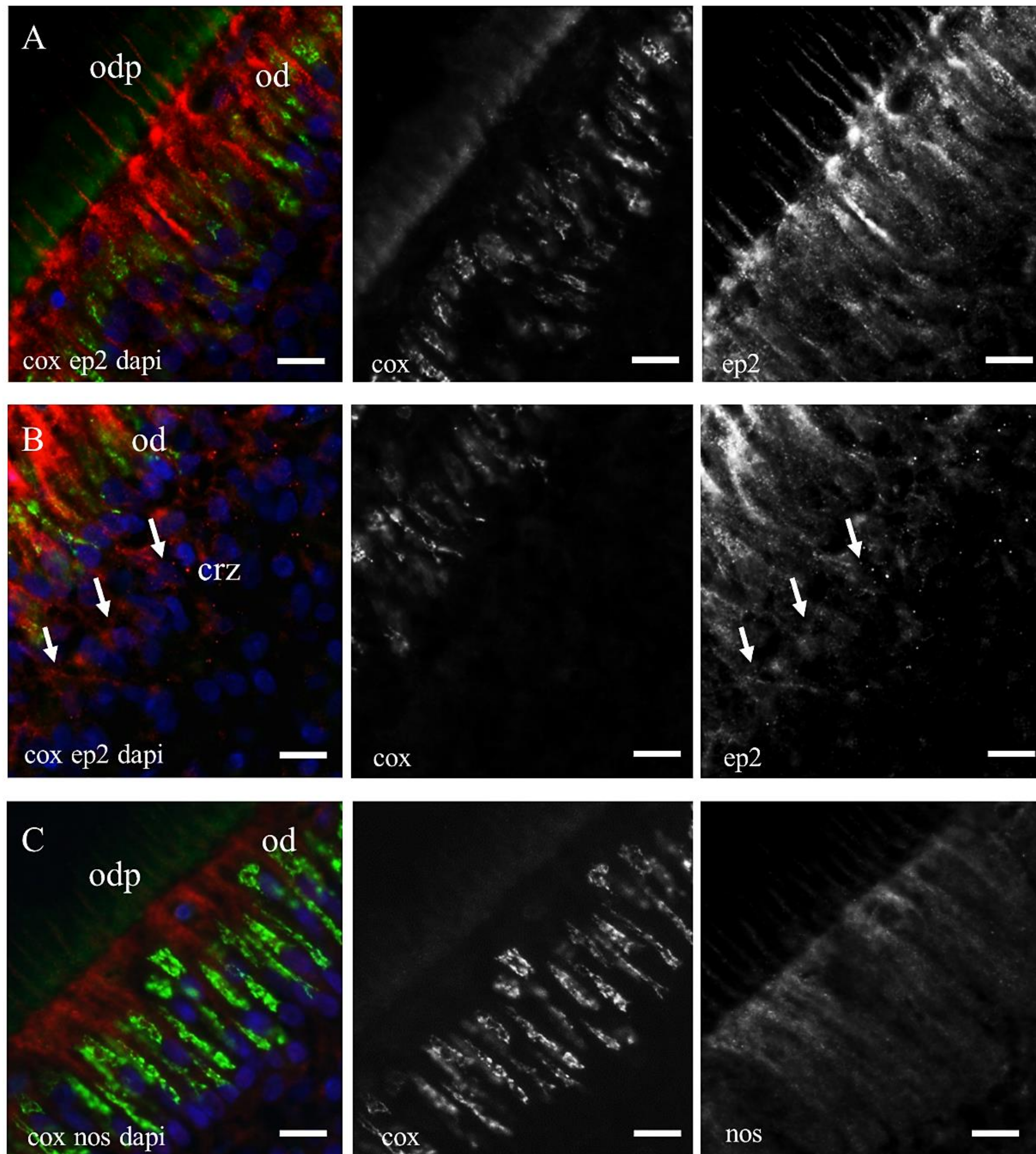


Figure 4-6: High power images illustrating the intracellular locations of COX-1 IR, NOS1 IR and EP2 IR within odontoblasts. A shows a section stained for COX-1 IR (green) and EP2 IR (red). COX-1 IR was as described previously. EP2 IR was located primarily in the distal region of the odontoblast and this was found to extend into the odontoblast processes (odp). B shows a region of cell-rich zone (crz) with relatively weaker EP2 IR (red). C illustrates the distribution of NOS1 IR (red), which appears to be located primarily to the distal region (dr) of the odontoblast cell bodies (OCB) with only a faint IR appearing in the odp. Calibration bars 15 µm in A, B and C.

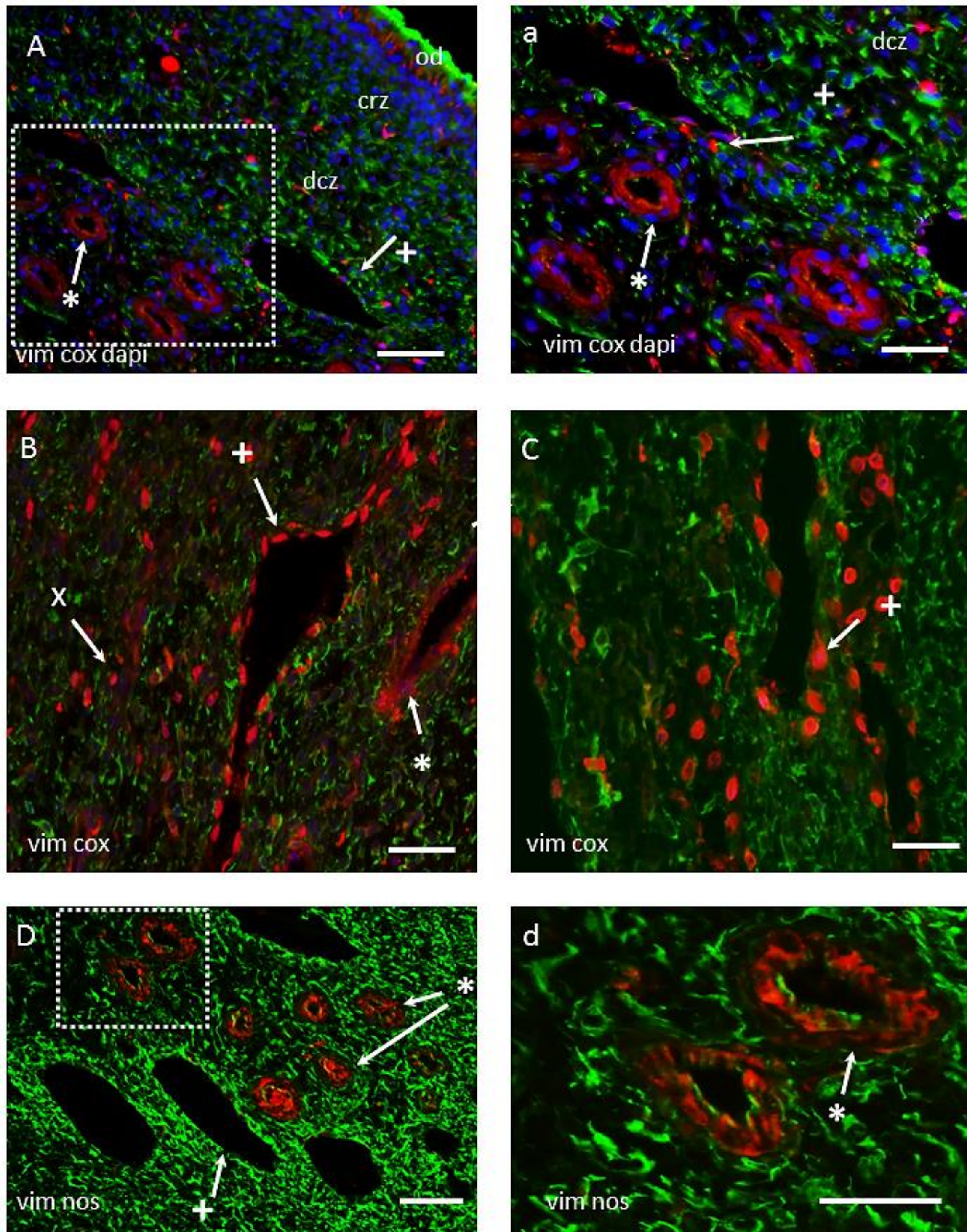


Figure 4-7: Distribution of COX-1 IR and NOS IR within the major blood vessels of the rat mandibular incisor pulp. A, a, B and C are sections stained with antibodies to COX-1 (red) and vimentin (vim: green) (A and a are also stained with dapi (blue)). The boxes in A and D identify regions of interest that are shown at higher magnification in the adjacent panel. In A and B typical arteries are shown by (*) and veins (+). The (x) points to interstitial cells that are COX-1 IR. D and d were stained with antibodies to NOS1 (red) and vimentin (vim: green). Arteries are shown by (*) and veins (+). Abbreviation are: cell-rich zone (crz) and diffuse cell zone (dcz). Calibration bars in A 250 µm and adjacent panel 100 µm; B, 80 µm; C 30 µm and D, 100 µm with adjacent panel 80 µm.

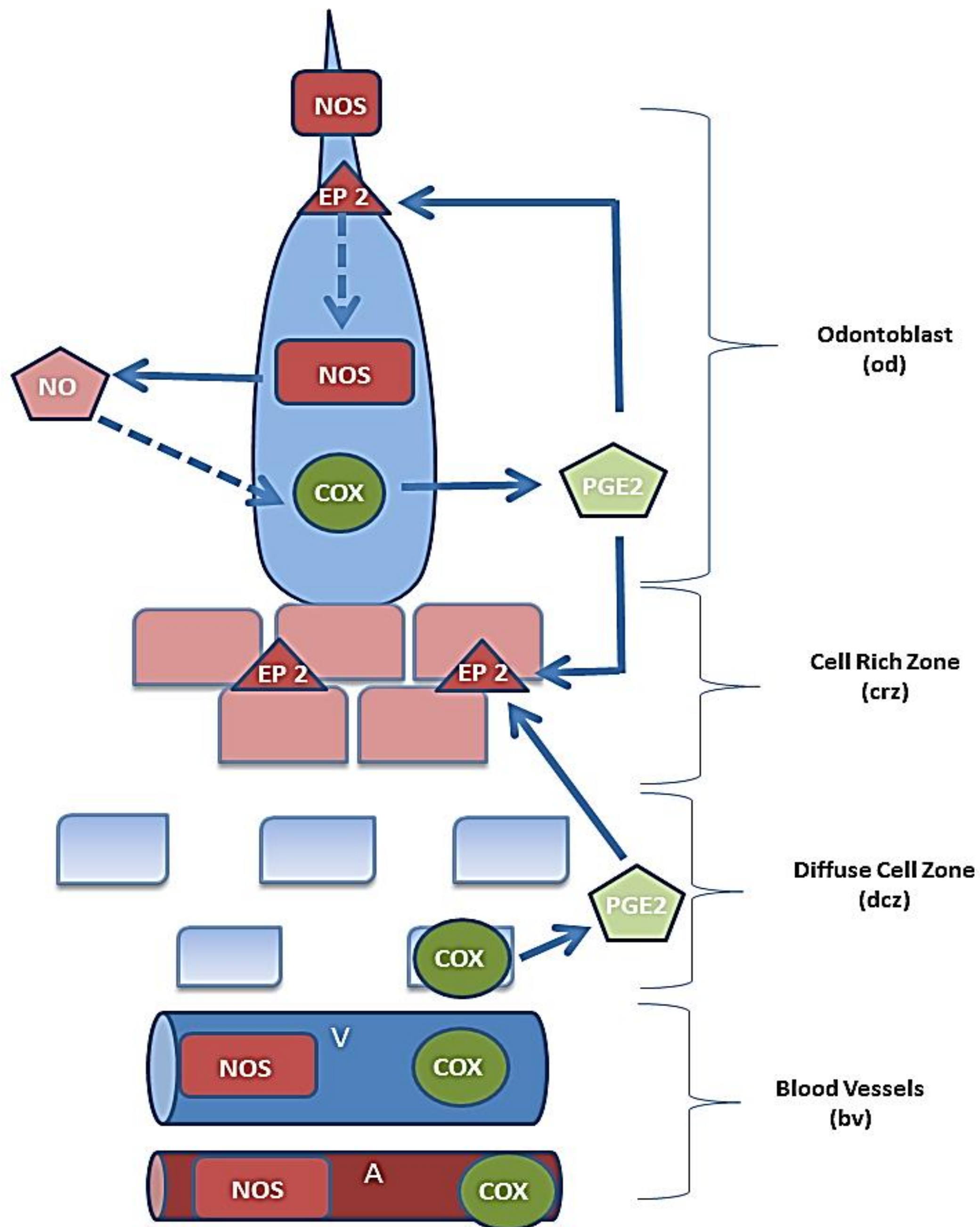


Figure 4-8: Cartoon illustrating the distribution of COX-1 and NOS in the odontoblasts and sub-odontoblast layers of the rodent pulp in the central region of the incisor.

4.5. Discussion:

The current study provides immunohistochemical evidence for the expression of COX-1, NOS1 and EP2 immunoreactivity within the apical and middle regions of the rat mandibular incisor.

Both COX and NOS exist in constitutive and inducible forms (Chandrasekharan et al., 2002, Nathan, 1992), with the inducible form usually being detectable only in inflamed tissues (Holt et al., 2005, Huang et al., 2005). By contrast, the constitutive forms of both enzymes are usually present at detectable levels in almost all healthy tissues (Nakanishi et al., 2001, Di Maio et al., 2004); both enzymes catalysing its own pathway with final products of PG and NO respectively.

It is well-recognised that molecular-level cross-talk occurs between the COX and NOS pathways (Cuzzocrea and Salvemini, 2007, Clancy et al., 2000), with NO and PG acting as intracellular messengers and/or inflammatory mediators. This cross-talk may help to regulate tissue homeostasis, vasodilation and vascular permeability, and participate in pathological processes including chronic inflammation (Dolan et al., 2000). However, the literature is still divided on whether NO stimulates or inhibits PG (Clancy et al., 2000).

It has been variously reported that NO may activate or inhibit PG production in a range of healthy and damaged cellular systems (Clancy et al., 2000, Mollace et al., 2005, Salvemini et al., 1993). The exact mechanism of these effects may be through changing COX genes expression (Salvemini et al., 1993), activation or blockage of the COX pathway (Clancy et al., 2000, Zou et al., 1997) or by enhancing the enzymes responsible for PG downregulation (Mollace et al., 2005).

Similarly, PG activates NOS activity through an increase in the hydrolysis of phosphoinositides by Pilocarpine (Borda et al., 2002, Borda et al., 2007).

Evidence from the current study shows the localization of COX-1 IR in three distinct regions within the dental pulp, first within the OCB as a uniform and well localized region located immediately above the nucleus, second: in a relatively small cell population within the dcz noting that these cells are vimentin negative, unlike the majority of cells surrounding them; and third in the walls of pulp blood vessels.

To obtain a fuller picture of the PG system within the dental pulp, EP2 IR which represents the target receptors for PG was investigated. Images showed that EP2 IR was

widespread and diffuse, with strongest representation in the distal part of odontoblast cell bodies, with relatively weaker IR in the crz immediately subjacent to OCL.

Collectively, it is possible that PG synthesised in the OCL may be liberated to act on the EP2 receptors either within the OCL or on EP2 receptors located on cells subjacent to the odontoblasts. Furthermore, PG produced by the COX-1 IR cells within the dcz may work on the EP2 receptors in the crz immediately beneath the odontoblasts, whereas the PG produced in the walls of blood vessels may act locally to control pulpal blood flow or pressure (Vane and McGiff, 1975) as seen in other tissues (Hintze and Kaley, 1977, Förstermann et al., 1986).

NOS1 distribution was diffuse and seen at the distal part of OCB close to the dentine, in odontoblast processes and in the walls of blood vessels particularly the arteries. The primary target for NO is not clear, however, it is logical to believe that the NO produced from the NOS1 IR regions in the distal part of OCB may further activate or inhibit COX activity within the odontoblast or subodontoblast region, whereas the NO produced from the walls of blood vessels may work to regulate pulpal blood flow (Lohinai et al., 1995) possibly in conjunction with PG.

Findings from this study suggest complex interactions between PG and NO pathways at the cellular levels including odontoblast-odontoblast, odontoblast-subodontoblast and odontoblasts-odontoblasts process through the viable dentine. These interactions might help to regulate or control odontoblast and subodontoblast cell functions and might be involved in other sensory and regulatory functions within that area as there are no nerves in the odontoblast and subodontoblast cell layers.

Finally, it is important to mention that the PG and NO signalling has not been investigated directly in this work, but the expression of the key enzymes and receptors for these pathways were investigated.

Chapter 5. Effects of Lipopolysaccharide, ATP and nitric oxide on PGE_2 release from rat dental pulp tissue explants

5.1. Introduction

Prostaglandin belongs to the prostanoid class of fatty acid derivatives. Arachidonic acid is liberated from the membrane phospholipids, a reaction catalysed by phospholipases. Arachidonic acid (AA) is then metabolised into PGG_2 and PGH_2 catalysed by enzymes called cyclooxygenases (COX-1 and COX-2). These undergo a final conversion to PGE_2 by prostaglandin E synthase enzyme (Helliwell et al., 2004, Murakami and Kudo, 2004).

Once formed, PGE_2 is released from the cell and exerts its biological action on a specific group of receptors on the cell surface. PGE_2 is produced in many tissues, including bronchi, vascular, brain, gastrointestinal, uterine and bladder smooth muscles, placenta, kidney, testis, mesenchymal stem cells, macrophages and monocytes (Kargman et al., 1996, Attar and Bulun, 2006, Chen and Bazan, 2005, Coceani et al., 2005). An increased PGE_2 production has been reported in response to inflammation, arthritis, cancers, tissue injury and fever (Ivanov and Romanovsky, 2004, Attar and Bulun, 2006, Molloy and McCarthy, 2005, Fahmi, 2004, Ghilardi et al., 2004). Inhibition of PGE_2 production is sometimes useful and achieved by administration of non-steroidal anti-inflammatory drugs (NSAIDs) (which inhibit cyclooxygenases) and by corticosteroids (which inhibit phospholipases) (Vane, 1971, Chang et al., 1987).

PGE_2 has many biological actions including vasodilation (Vane and McGiff, 1975), anti and proinflammatory actions, modulation of sleep/wake cycles, stimulation of bone resorption, regulation of sodium excretion and renal hemodynamics, facilitation and inhibition of salt and water absorption from the kidney (Vila, 2004, Breyer and Breyer, 2001, Peti-Peterdi, 2005), tissue specific relaxation or contraction of smooth muscles (Tilley et al., 2003, Carbillon et al., 2001), and sensitization of peripheral nerves (Zeilhofer, 2005). It also acts as an inflammatory mediator (Molloy and McCarthy, 2005, Fahmi, 2004, Hata and Breyer, 2004) and activates mitosis and differentiation of cancer cells (Hull et al., 2004, Brueggemeier et al., 2003).

5.1.1. Effect of LPS on PG signalling

Bacterial LPS is regarded as the main constituent of the external membrane of Gram negative bacterial populations with the exception of *Sphingomonas* (Kawahara et al., 2000). It represents the most widely used stimulant to induce inflammation in any tissue

both *in vivo* and *in vitro* due to its ability to stimulate an innate or humoral immune response in almost all eukaryotes, with subsequent production of pro-inflammatory cytokines (De Schepper et al., 2008, Bannerman, 2009). Primarily, LPS targets resident macrophages, peripheral monocytes, neutrophils, DCs and TLR-4 (Muzio et al., 2000, Zhang et al., 1999, Kitchens, 1999).

A highly evident modulatory effect of LPS on PGE_2 biosynthesis has been reported. Infusion of LPS has been found to increase PGE_2 secretion in various tissues including bovine mammary gland (Piotrowska-Tomala et al., 2012, Piotrowska-Tomala et al., 2015) and in astrocytes by inducing COX-2 expression and COX-1 inhibition (Font-Nieves et al., 2012). Moreover, it has been found that the proteins responsible for PGE_2 synthesis were up or down regulated in response to LPS administration, which may affect the PGE_2 efflux or influx during the inflammatory process (Ohkura et al., 2012).

5.1.2. Effect of NO on PG signalling

NO is a crucial intracellular messenger and local mediator that, under normal physiological conditions, contributes in many cellular, vascular, neuronal and immune functions (Mollace et al., 2005). Following inflammatory stimuli, NO is released in high concentrations and found to have detrimental effects, including: decreasing the ability of DC to present antigens (Holt et al., 1993), elimination of T lymphocyte mitosis (Merryman et al., 1993) and the propagation of some diseases (Moilanen et al., 1999). The key enzyme in the synthesis of NO is NOS with three isoforms, two constitutive including: neuronal NOS (NOS1 or nNOS) and endothelial NOS (NOS3 or eNOS), and one inducible isoform (NOS2 or iNOS). The constitutive forms are responsible for the production of NO in small amounts and for short durations under normal conditions, while the inducible form is responsible for NO production in high concentrations and for prolonged periods in response to inflammatory stimuli.

NO and PGE_2 pathways are present together in almost all instances, sharing similar steps and fundamentally participate in similar pathophysiological events (Wu, 1995).

There is considerable accumulated evidence to suggest a constant cross-talk between PG and NO pathways. The first crucial link was reported by Salvemini in 1993 (Salvemini et al.), who showed that increased biosynthesis of PG in response to inflammation was almost completely induced by NO. This raised the hypothesis that the COX enzymes served as an endogenous receptor for NO. The outcome of this cross-talk happens at

multiple levels and is not straightforward, as the endogenous NO and synthetic NO donors have been implicated in switching on/off PG pathways (Colasanti and Suzuki, 2000) in diverse physiological and pathological systems depending on the severity of the stimulus, levels of NO produced and the cell type responsible for PG formation.

NO has been found to stimulate COX activity or increase PGE_2 synthesis in dental pulp (Borda et al., 2007), sheep seminal vesicles (Salvemini et al., 1993), macrophages (Von Knethen and Brüne, 1997, Perkins and Kniss, 1999) and in bovine mammary gland (Piotrowska-Tomala et al., 2015).

By contrast, NO has been implicated in the inhibition or decreasing of PGE_2 synthesis in chondrocytes (Amin et al., 1997), LPS-stimulated macrophages (Habib et al., 1997), guinea-pig urothelium (Nile and Gillespie, 2012) and guinea-pig bladder (Nile et al., 2010).

More detailed reports suggest a stimulatory effect of NO on COX-1 and inhibitory effect on COX-2 (Minghetti et al., 1996, Guastadisegni et al., 1997, Clancy et al., 2000).

Conversely, PGE_2 may play a role in activating NOS (Borda et al., 2002, Chen et al., 1997, Borda et al., 2007) or in reducing NOS expression and activity (Minghetti et al., 1997).

5.1.3. Effect of ATP on PG signalling

ATP exists in millimolar amounts inside nearly all cells and can be released to the extracellular spaces once the cell is damaged or lysed (Hamilton and McMahon, 2000, Ding et al., 2000). ATP is frequently described as the “ubiquitous intracellular source of energy”. In addition to this vital role, ATP acts as a signalling molecule and participates in a wide variety of cellular activities through its interaction with specific receptors called purinergic receptors (Burnstock and Verkhratsky, 2012). Furthermore, ATP has been shown to participate in acute and chronic inflammatory conditions (Peng et al., 2009, Gourine et al., 2007, Khakh and North, 2006).

Positive correlations have been found between ATP and PGE_2, where an increase in PG biosynthesis was observed following treatment with ATP or ATP analogues in different biological systems including, guinea pig bladder (Nile et al., 2010), golden hamster cremaster muscle (Hammer et al., 2001) and human leg (Mortensen et al., 2009). On the other hand, decreasing PGE_2 biosynthesis was found to inhibit ATP release from tissues (Tanaka et al., 2011).

5.1.4. Aim of study

The main aim of this study is to investigate the amount of PGE_2 released from explanted dental pulp tissue of the rat mandibular incisor, and to closely evaluate the changes in PGE_2 release in response to incubation with LPS, NO, ATP and NO+ATP for different periods of time.

5.2. Methods

5.2.1. Tissue collection:

32 Wistar rats were killed in a CO_2 chamber (SMART BOX, Auto CO_2, System, Euthanex Corp.) before surgical extraction of their mandibular incisor teeth. Under the operating microscope and using a high speed dental handpiece and diamond burs under constant water cooling, a groove was made along the lateral wall of each mandibular incisor tooth until the pulp tissue became visible through the dentine. Care was taken to avoid exposure to the pulp and to minimise any trauma. Using a scalpel, the lateral walls were broken carefully removing the hard walls, trying to expose the pulp tissue before careful removal with tweezers. Immediately after removal, pulp tissues were transferred to a container filled with Dulbecco's Modified Eagles Medium (DMEM) (D 6429, Sigma-Aldrich) containing 1% Foetal bovine serum (FBS) (Sigma Aldrich, UK) and 50 (µg/ml, IU/ml) penicillin-streptomycin (Sigma Aldrich, UK). The total number of collected pulp tissues were (n= 32). 20 pulps were randomly divided into four groups (n=5) and assigned to test the effect of short term incubation with different stimuli on PGE_2 release. The remaining 12 pulps were divided into four groups (n=3) and assigned to test the effect of long term incubation with different stimuli on the PGE_2 release. Every single pulp tissue was then carefully placed in wells of standard tissue culture plates (24 wells plate) with DMEM. The weight of pulp tissues ranged from 11-15 mg. Any DMEM solution mentioned throughout this thesis contained 1% FBS and 50 (µg/ml, IU/ml) penicillin-streptomycin.

5.2.2. Sample preparation:

I. Short term effect

- All the work of changing and collecting the media was done in a class 2 hood (BH-EN 2003, Faster, UK).
- At the beginning of each experiment (T=0 minutes), each single pulp tissue was placed in a well with 400 µL DMEM and incubated (MCO-17AIC, Sanyo, Japan)

at 37˚C with 5% CO_2 gas (Huang et al., 2006, Agha-Hosseini et al., 2010) for 15 minutes.

- At T=15 minutes: the bathing media were aspirated, disposed of and replaced with new media, keeping the temperature and gasification with CO_2 continuously controlled.
- At T=30 minutes: the bathing media were again aspirated, disposed of and replaced with new media under the same conditions previously.
- At T=45 minutes: the bathing media were collected, divided into two 200 µL aliquots, and immediately stored in a -20˚C freezer. These were defined as the "unstimulated sample" 1 (U1). The media were then replaced with new fresh media.
- At T=60 minutes: bathing media were collected again, divided into two 200 µL aliquots, and immediately stored in a -20˚C freezer. These were defined as the unstimulated sample 2 (U2).
- U1 and U2 were collected to determine the endogenous PGE_2 release from the tissue.
- Pulp tissues were randomly allocated in four groups, each group contained 5 pulps.
- At T=60 minutes: The media were again replaced with fresh media before adding a further reagent: For the first group of pulps, ATP analogue (2'(3')-O-(4-benzoylbenzoyl) adenosine-5-triphosphate tri (triethylammonium) salt (100 µM) (Sigma Aldrich, UK) was added. For the second group of pulp tissues, the nitric oxide donor (diethylamine NONOate diethylammonium salt (NONOate) (10 µM) (Sigma Aldrich, UK) was added. For the third group, Lipopolysaccharides (LPS) from *Escherichia Coli* serotype 026:B6 (LPS) 10 µg/ml (Sigma Aldrich, UK) was added. For the fourth group, both NONOate and ATP were added together.
- At T=75 minutes: bathing media were again collected, divided into two 200 µL aliquots, and immediately stored in a -20˚C freezer. These were defined as the "stimulated sample" (S). The media were then replaced with new fresh media without additions.
- At T=90 minutes: bathing media were once again collected, divided into two 200 µL aliquots and immediately stored in -20˚C freezer. These were defined as the

"recovery sample" 1 (R1). The media were then replaced with new fresh media without additions.

- At T=105 minutes: bathing media were collected, divided into two 200 µL aliquots and immediately stored in a -20˚C freezer. These were defined as the "recovery sample" 2 (R2).
- All the collected bathing media (U1, U2, S, R1, R2) were stored in a -20˚C freezers for later use.
- The pulp tissues were finally removed from media, carefully dried by gentle touching with tissue paper and weighed in milligrams with a digital balance (OHAUS, Northern balance, UK) to an accuracy of four decimal places.

II. Long term effect.

The experiment was designed to determine the PGE_2 in the supernatants across longer incubation periods with each stimulant as follows:

a) T=60 min, U sample collected.
b) T=120 min, S1 sample collected.
c) T=180 min, S2 sample collected.
d) T=240 min, S3 sample collected.
e) T=300 min, R sample collected.

5.3. Results

This study employed an *in vitro* pulp stimulation model on rat dental pulp explants. This included the incubation of pulp tissue in a nutrient medium (0.5 ml DMEM) at 37˚C with 5% CO_2 in a CO_2 incubator. The detailed description of the methodology and equipment utilized in this experiment were provided in chapter 2, sections 2.2. In the absence of any external stimuli, the dental pulp released a relatively small amount of PGE_2 (64-90 pg/mg of tissue, over 15-60 minutes). This basal PGE_2 concentration was almost the same in samples collected after 15 minutes and one hour.

Two time scales have been chosen to detect the effect of the incubation materials on PGE_2 release from the dental pulp, to detect the effect-time relation and to determine whether there are differences in the levels of PGE_2 detected from comparable tissues.

5.3.1. Effect of LPS

For this group, the mean PGE_2 released from the unstimulated pulp explants was ≈ 62 pg/mg. Incubation of pulp tissue (n=5) with 10 µg/ml of LPS for 15 minutes resulted in a highly significant increase in PGE_2 release (Figure 5-1) to the mean of ≈ 83 pg/mg compared to unstimulated values ($P<0.01$). Fifteen minutes after washing the tissue and changing the media to terminate stimulation, the PGE_2 concentration output (R samples) did not return to basal levels and remain as high as ≈ 83 pg/mg of tissue.

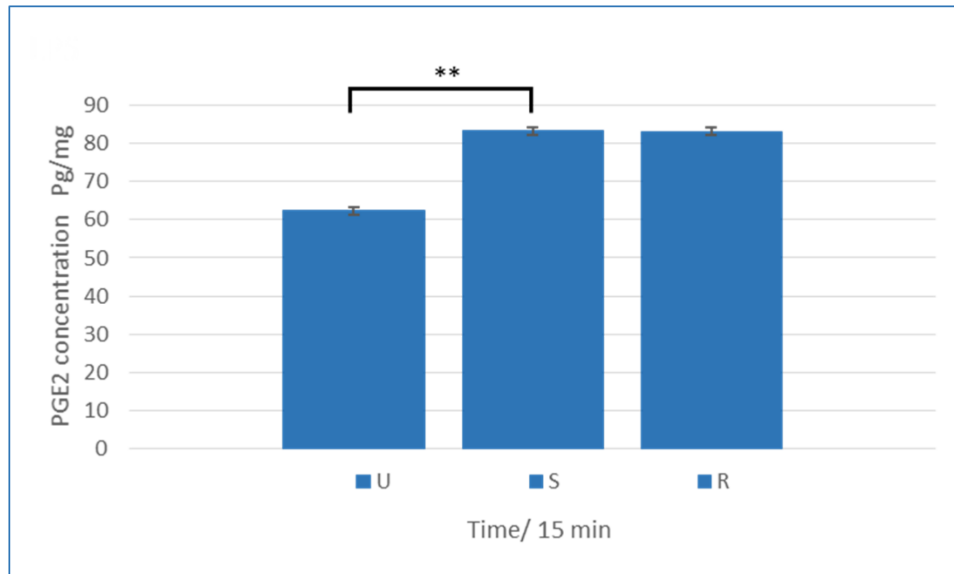


Figure 5-1: Short term effect of LPS on PGE_2 concentration (15 minutes intervals). Values represented by the bars represent the means ± standard error of means (SEM). Where marked by horizontal lines, a student *t* test was carried out, (*) refers to significant differences, in which **$P<0.01$.

For the one-hour incubation group, unstimulated mean values were ≈ 64 pg/mg. PGE_2 concentrations in S1 samples (after 60 minutes incubation with LPS, T=120 minutes) showed a slightly higher concentration than that after 15 minutes (mean ≈ 88 pg/mg) (Figure 5-2). This increase was statistically significant compared to basal levels.

S2 samples (120 minutes exposure, T= 180 minutes) showed a further statistically significant increase in the amount of PGE_2 released into the incubating media (mean ≈ 96 pg/mg) (Figure 5-2).

S3 samples (180 minutes exposure, T= 240 minutes), showed a greater and highly significant increase in PGE_2 concentration to the mean of ≈ 121 pg/mg concentration. (Figure 5-2).

Sixty minutes after the cessation of the stimulus (R samples, T= 300 minutes), PGE_2 values were not lower than S3 values. Levels were as high as that of the last stimulated sample (S3) to the mean of ≈ 125 pg/mg.

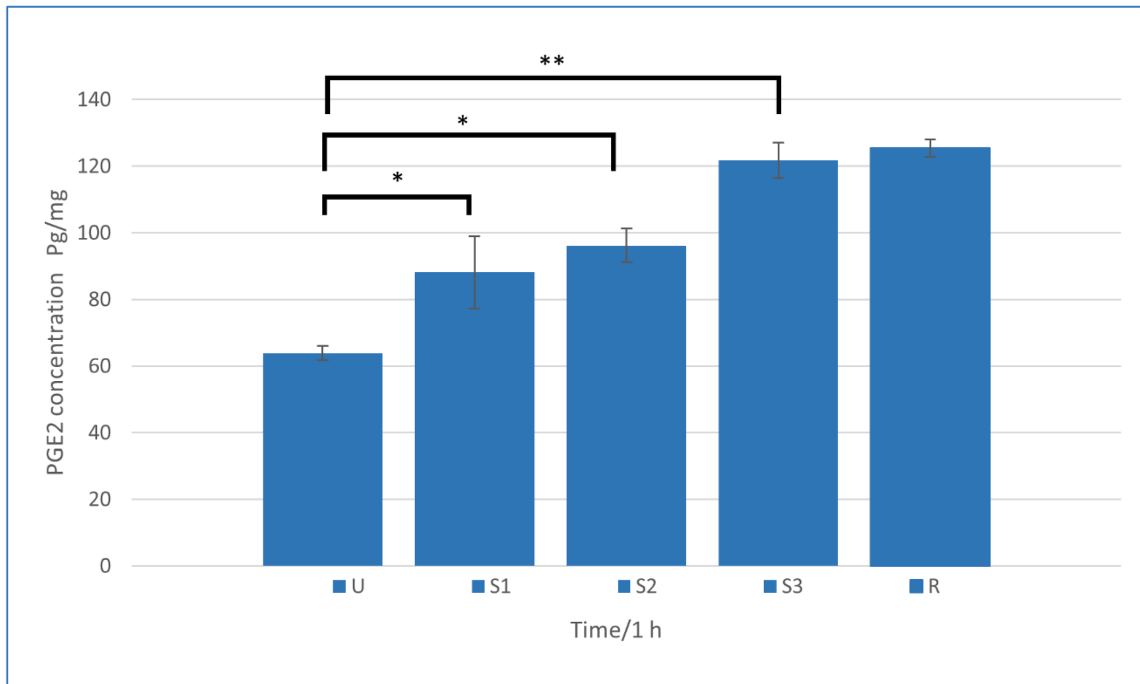


Figure 5-2: Long term effect of LPS on PGE_2 concentration (60 minutes intervals). Values represented by the bars represent the means ±SEM. Where marked by horizontal lines, a student *t* test was carried out, (*) refers to significant differences, in which * $P<0.05$ and ** $P<0.01$.

The cumulative effects of LPS on PGE_2 release are illustrated in Figure 5-3. In this figure the total amounts of PGE_2 released from the dental pulp are represented on a time scale, so PGE_2 release after two hour exposure to LPS represent the amount measured at S1 and S2 added together and the total amount released after three hours represent the PGE_2 measured at S1, S2 and S3 all together to determine the magnitude of the pulp response to LPS.

This cumulative view is only suitable for this section (treatment with LPS), because it is accompanied with increased PGE_2 release and is non-suitable for the next sections as they are associated with decreased PGE_2 levels.

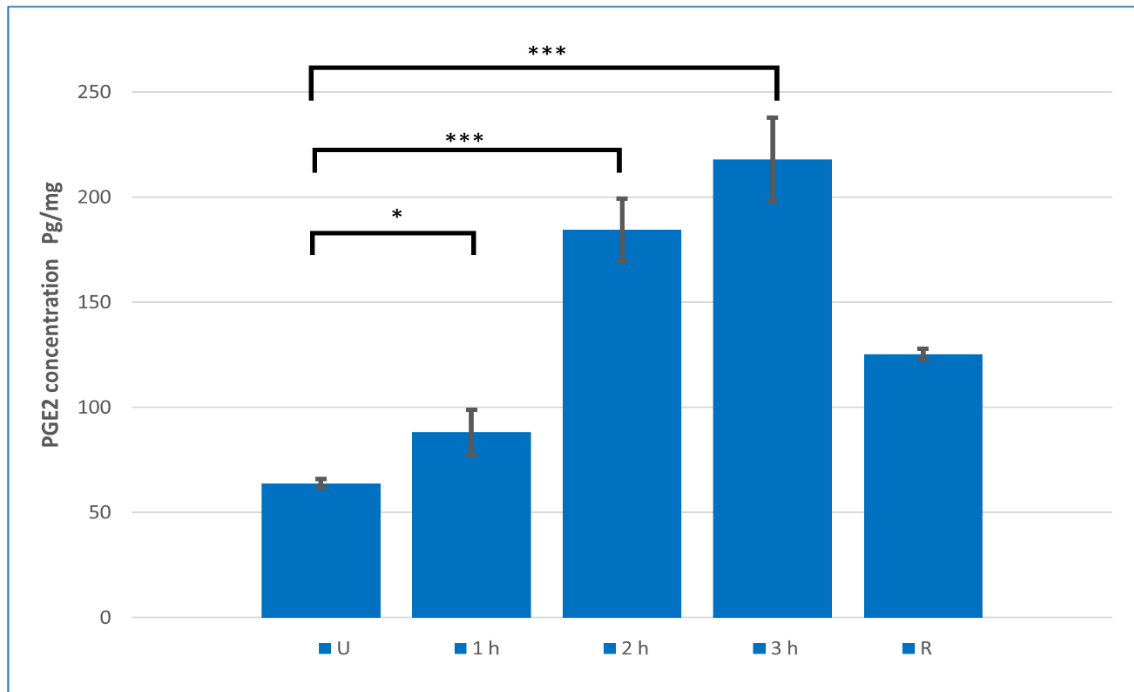


Figure 5-3: Cumulative effect of LPS on PGE_2 concentration. Values represented by the bars represent the means ±SEM. Where marked by horizontal lines, a student *t* test was carried out, (*) refers to significant differences, in which *P<0.05 and *** P<0.0001.

Based on this view, the PGE_2 released after two hours of exposure to LPS was about three times that of the basal levels and more than double that after one hour. The difference between basal and two hours PGE_2 concentrations was highly statistically significant (P<0.0001)**.** A similar observation was made for 3 hour samples. However, Figure 5-3 shows a different view regarding the recovery of the pulp tissue, with samples collected one hour after withdrawal of stimulation reduced to almost half of the 3 hour values.

Based on the finding of this experiment, treatment with LPS can result in a time-dependant increase in PGE_2 release from explanted rodent pulp tissue. This increase continues as long as the stimulus persists. It was also noted that the PGE_2 release did not drop to baseline levels within 1 hour of stimulus withdrawal. On the other hand, a cumulative view on the data show a higher magnitude of pulp response in the form of increased PGE_2 levels and a clear attempt of pulp tissue to re-establish the basal levels after stimulus termination in the form of decreased PGE_2 levels.

5.3.2. Effect of NO

For this group, the reported mean PGE_2 released from the unstimulated pulp explants was ≈ 88 pg/mg. To assess whether NO has an effect on the PGE_2 release from the dental pulp, an experiment was designed, where 10 μM of NO donor was added to the

incubation media containing the dental pulp explants (n= 5). The amounts of PGE_2 released into the bathing media were assessed and are shown in Figure 5-4. Detected concentrations from the stimulated samples after 15 minutes showed a marked and highly significant decrease compared to unstimulated basal levels (mean of ≈ 56 pg/mg). After washing the tissue and changing the media to terminate the stimulus, the output of PGE_2 assessed (R samples) showed slight recovery (to the mean of ≈ 61 pg/mg).

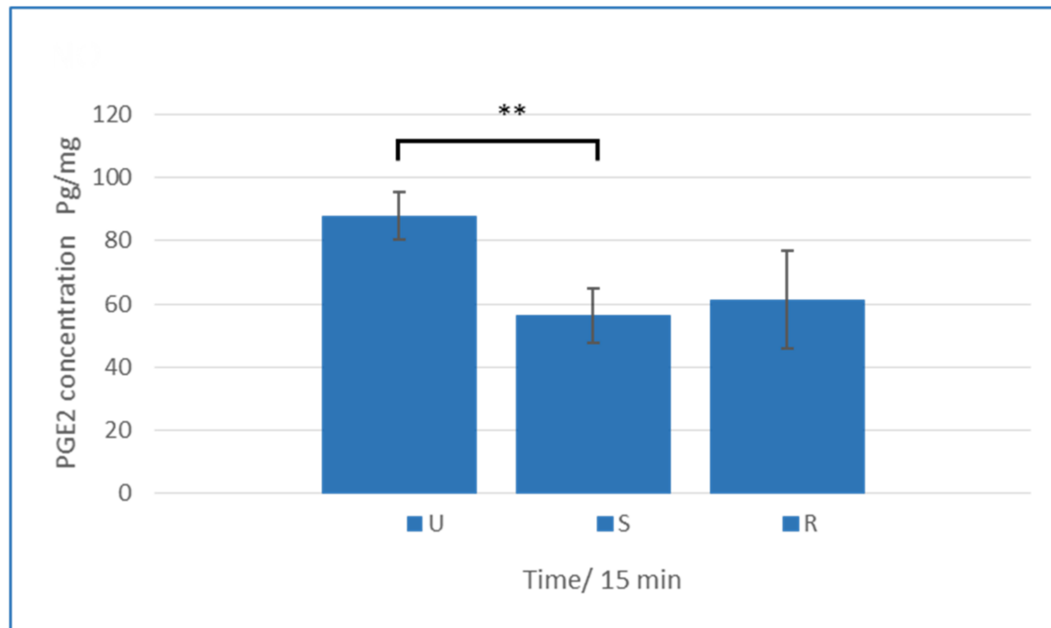


Figure 5-4: Short term effect of NO donor on PGE_2 concentration (15 minutes intervals). Values represented by the bars represent the means ±SEM. Where marked by horizontal lines, a student *t* test was carried out, (*) refers to significant differences, in which $^{**}P<0.01$.

For the one-hour incubation group, unstimulated mean values were ≈ 74 pg/mg. Pulp explants (n=3) were incubated with NO for one hour duration. PGE_2 levels measured from S1 samples (60 minutes incubation with NO, T= 120 minutes) showed a slight but highly significant decrease (mean ≈ 65 pg/mg) (Figure 5-5). A dramatic and highly significant decrease in PGE_2 concentration was found when assessing the S2 samples (120 minutes exposure, T= 180 minutes) to the mean of ≈ 14 pg/mg.

S3 samples (180 minutes exposure, T= 240 minutes) showed a further highly significant decrease (sharp compared to basal and slight compared to S2) to the mean of ≈ 11 pg/mg (Figure 5-5).

R samples (One hour after the cessation of stimulation, T= 300 minutes), showed a detectable increase of PGE_2 levels to the mean of ≈ 35 pg/mg from basal (nearly half the starting basal levels).

The difference between T=15 (S) in (Figure 5-4) and T=60 in (Figure 5-5) may be due to the high standard deviation in the T=15 value or due to short term inhibitory effect of NO.

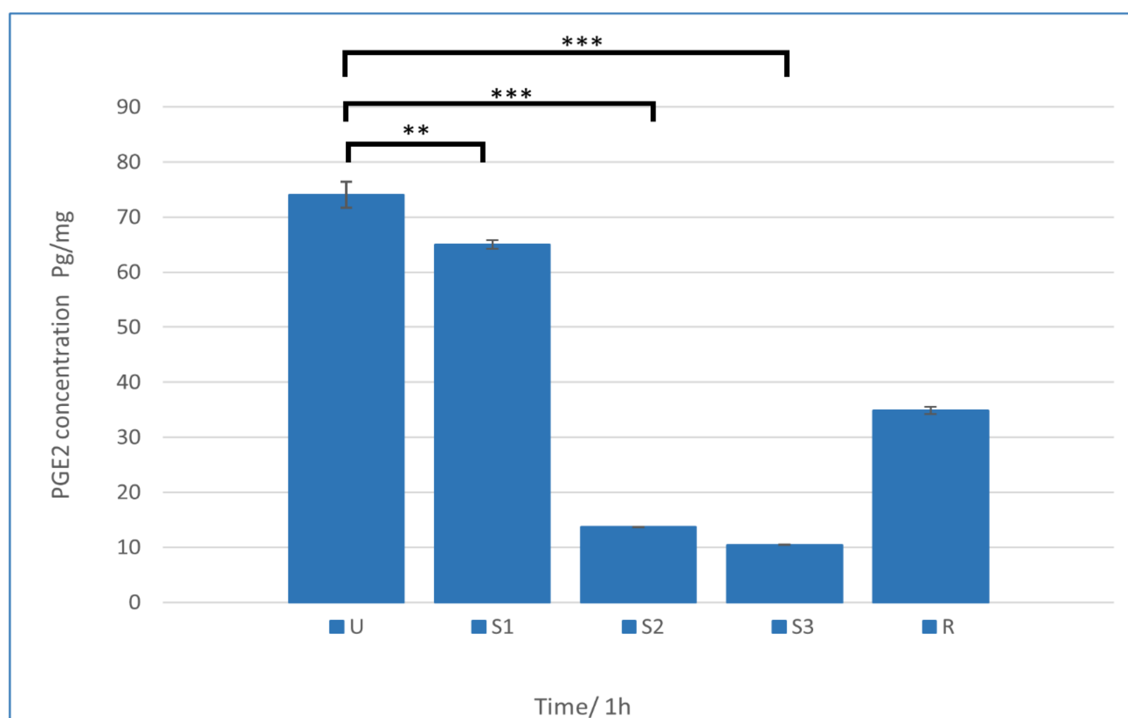


Figure 5-5: Long term effect of NO donor on PGE_2 concentration (60 minutes intervals). Values represented by the bars represents the means ±SEM. Where marked by horizontal lines, a student *t* test was carried out, (*) refers to significant differences, in which ***P<0.0001, **P<0.01.

These observations have demonstrated that NO (in this case NO donor) has a highly significant inhibitory effect on PGE_2 release from the dental pulp explants both on short and long term treatments, followed by a relatively slow increase in PGE_2 levels after stimulus termination.

5.3.3. Effect of ATP

Unstimulated samples released a mean of ≈ 71 pg/mg PGE_2, whereas concentrations determined from samples collected after 15 minutes of pulp incubation with ATP (n=5) showed a marked and highly significant decrease in PGE_2 concentration to the mean of ≈ 40 pg/mg tissue (Figure 5-6). Samples collected 15 minutes after the stimulus discontinuation showed nearly the same PGE_2 concentration as the ATP affected samples to the mean of ≈ 38 pg/mg.

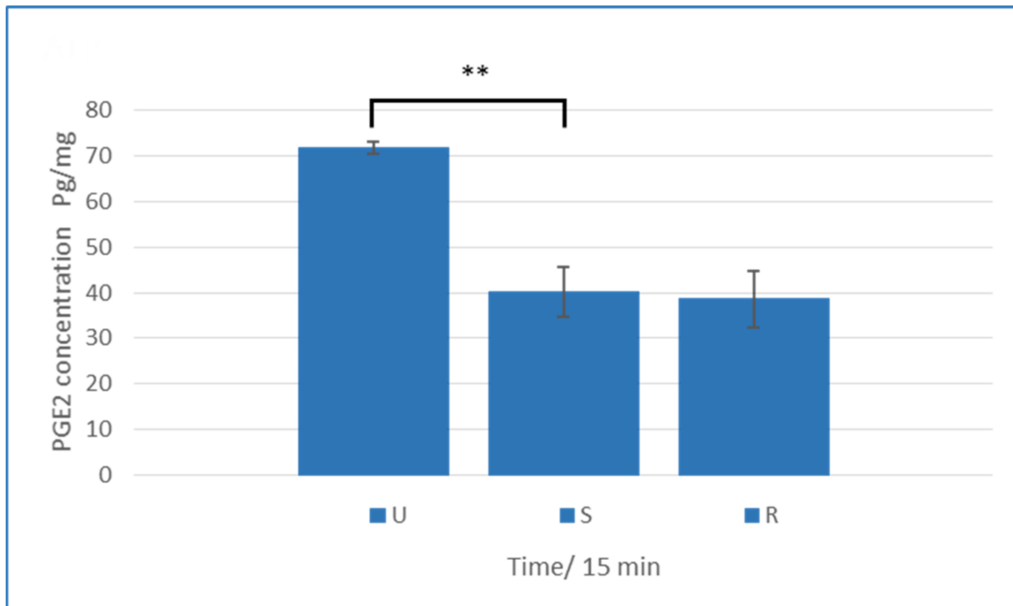


Figure 5-6: Short term effect of ATP on PGE_2 concentration (15 minutes intervals).

Values represented by the bars represents the means ±SEM. Where marked by horizontal lines, a student t test was carried out, (*) refers to significant differences, in which ***P*<0.01.

Figure 5-7 shows the PGE_2 concentration of samples collected from pulp tissues incubated with 100 µM of ATP analogue for one hour time intervals (n=3). Reported PGE_2 concentration released from unstimulated pulp explants were ≈ 90 pg/mg of tissue. Determination of PGE_2 concentration in S1 samples (60 minutes incubation with ATP, T=120) showed a large and highly significant decrease to the mean of ≈ 57 pg/mg. S2 samples (120 minutes exposure, T=180) showed a similar highly significant levels of PGE_2 as with S1 sample (mean ≈ 63 pg/mg). Similar observations were made for S3 samples (180 minutes exposure, T=240 minutes, mean ≈ 59 pg/mg).

However, 60 minutes from termination of the stimulus (R samples, T= 300 minutes) showed a detectable increase (almost back to basal) in PGE_2 concentration to the mean of ≈ 86 pg/mg.

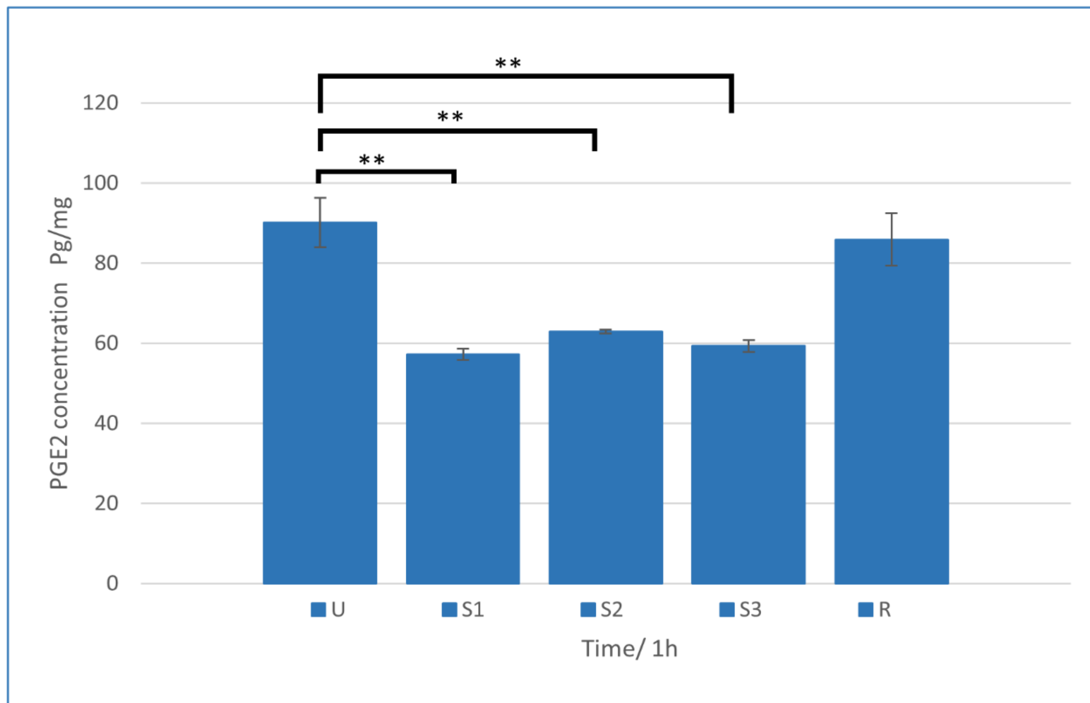


Figure 5-7: Long term effect of ATP on PGE_2 concentration (60 minutes intervals).
Values represented by the bars represents the means ±SEM. Where marked by horizontal lines, a student *t* test was carried out, (*) refers to significant differences, in which $^{**}P<0.01$.

Taken together, it can be assumed that ATP (in this case the ATP analogue) has a marked highly significant inhibitory effect on PGE_2 release from the dental pulp explants. This inhibition starts as early as 15 minutes and continues steadily for as long as three hours (limit of this study). Furthermore, the pulp explants fail to recover from the inhibited status 15 minutes after stimulus withdrawal, but remarkably recovers to almost untreated levels within one hour.

5.3.4. Effect of NO+ATP

Unstimulated samples released ≈ 71 pg/mg PGE_2. In this series of experiments, the combined effect of NO donor and ATP analogue on the PGE_2 output from dental pulp explants was tested. Figure 5-8 presents the short term effect of both previously mentioned stimulants on the PGE_2 release from dental pulp explants (n=5).

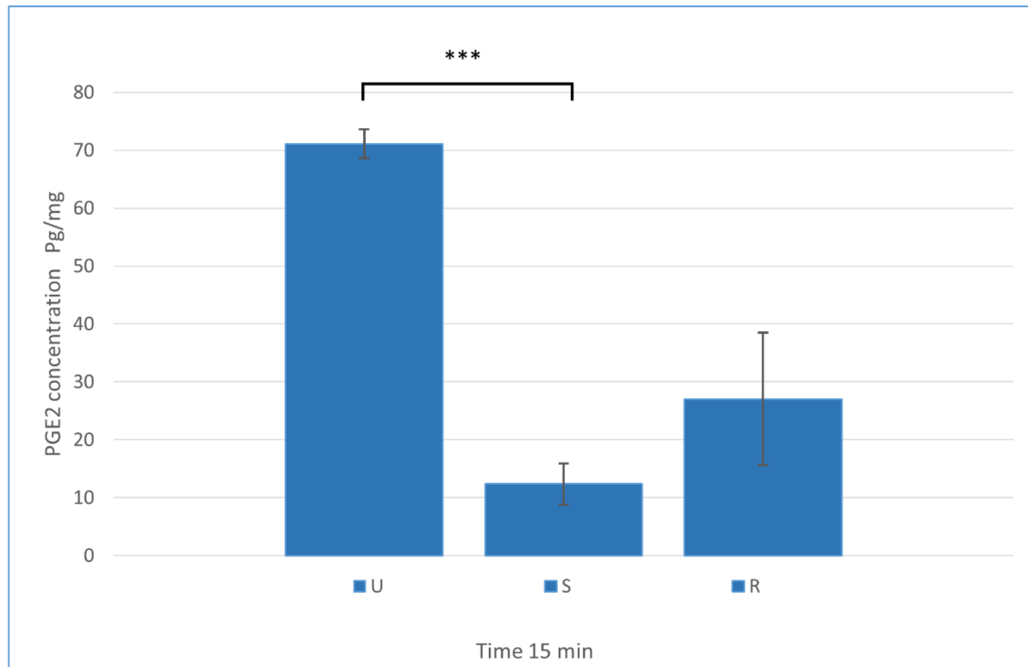


Figure 5-8: Short term combined effect of NO+ATP on PGE_2 concentration (15 minutes intervals). Values represented by the bars represents the means ±SEM. Where marked by horizontal lines, a student *t* test was carried out, (*) refers to significant differences, in which ****P*<0.0001.

A marked and highly significant ($P<0.0001$) decrease in PGE_2 concentration was observed in the samples collected after 15 minutes incubation with the NO+ATP to the mean of ≈ 12 pg/mg. A slight pulp recovery was observed in samples collected 15 minutes after stimulus termination to the mean of ≈ 27 pg/mg. Although the bar chart shows a slightly faster recovery of pulp tissue in this case compared to recovery after NO or ATP alone, the PGE_2 concentration was still far below that in each separately.

For the longer incubation group, the unstimulated pulp explants released ≈ 66 pg/mg PGE_2. After longer exposure of pulp explants (n=3) to NO+ATP, PGE_2 concentrations in S1 samples (60 minutes exposure to NO+ATP, T=120 minutes) showed a highly significant ($P<0.01$) decrease in the amount of PGE_2 measured to the mean of ≈ 45 pg/mg (Figure 5-9).

S2 samples (120 minutes exposure, T=180 minutes) showed a remarkable and highly significant ($P<0.0001$) reduction in PGE_2 concentration to the mean of ≈ 12 pg/mg. The PGE_2 concentrations measured from S3 samples (180 minutes exposure, T=240 minutes) showed a fairly similar concentrations as that of S2 samples (≈ 10 pg/mg) but remarkably lower and highly significant ($P<0.0001$) concentration compared to baseline levels (Figure 5-9).

Measurements of PGE_2 concentration in R samples (60 minutes after stimulus withdrawal, T=300 minutes) showed fairly good pulp recovery in the form of increased PGE_2 concentrations to almost two thirds that of baseline levels ≈ 43 pg/mg (Figure 5-9).

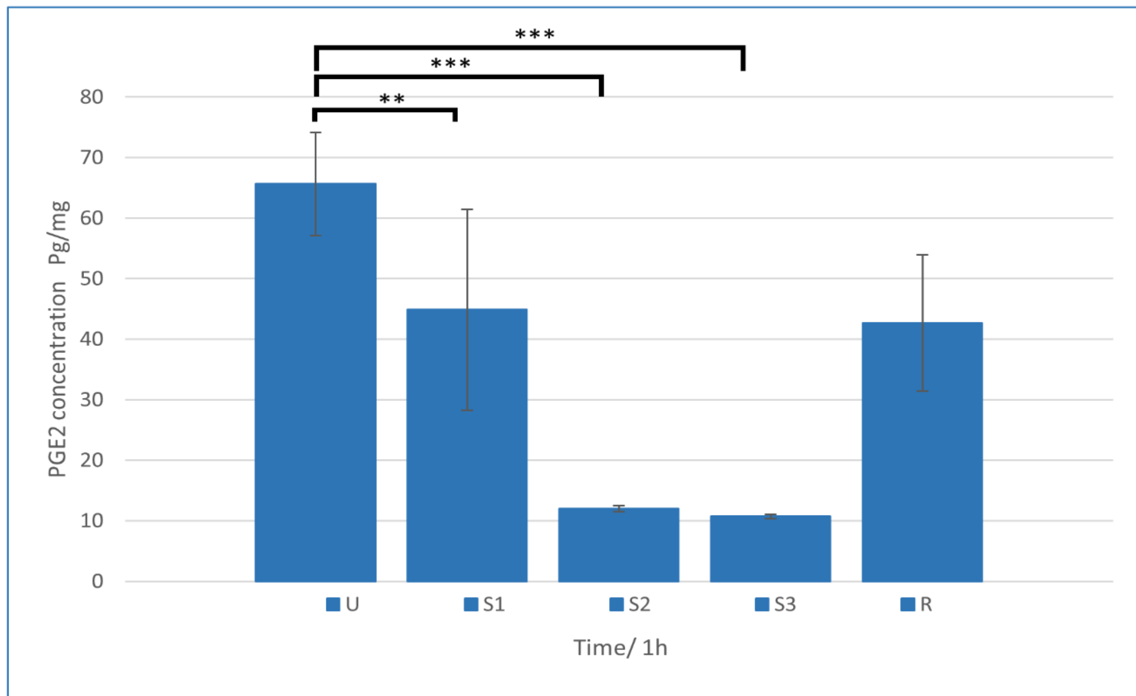


Figure 5-9: Long term combined effect of NO+ATP on PGE_2 concentration (60 minutes intervals). Values represented by the bars represents the means ±SEM. Where marked by horizontal lines, a student *t* test was carried out, (*) refers to significant differences, in which ** $P<0.01$, *** $P<0.0001$.

Overall, these results suggest that combining NO and ATP will provide a combined, highly significant inhibitory effect on the PGE_2 release from the dental pulp to a very low levels, with the lowest levels detected after two hours of incubation. The dental pulp appears to recover moderately and steadily, without reaching the basal level even after one hour of stimuli termination.

5.4. Discussion

Historically, it has been suggested that the dental pulp can release detectable amounts of PGE_2 (Hirafuji et al., 1980). Despite considerable research targeting PGE_2 in the dental pulp, its physiological and pathological roles are not fully understood. This may be attributed to the complexity of PG pathways within the dental pulp, involving two or three pathways that synthesise different PGs from different cell types. Moreover, the presence of multiple prostanoid receptors (at least four with many variants), each of which is linked to unique pathways, adds to the diversity of the roles of PGs in

physiological and/or pathophysiological conditions. As mentioned in the introduction, cross-talk between different pathways may alter or regulate the action of PGs in the dental pulp, which further adds to the complexity of PG pathways within the dental pulp.

The results of this study suggest that three or four different substances interact and modulate the amount of PGE_2 released from dental pulp explants. This may help to clear some of the ambiguity of PGE_2 pathways. To the best of our knowledge, this is the first time that the direct effects of LPS, NO donor, ATP analogue and both NO and ATP incubation on PGE_2 release from dental pulp explant has been studied, and especially after short and relatively long term exposures.

However, it is important to highlight that the current data represent an *in vitro* model which may not accurately reflects the normal biological tissue which is far more complex with more complicated interactions.

5.4.1. Effect of LPS on PGE_2

Histological signs of dental pulp inflammation can be observed long before microbes invade the pulp, suggesting the role of microbial products and components in the induction of tissue changes. Virulence factors from Gram negative bacteria usually come from cell-membrane components, and LPS is probably the most prominent pro-inflammatory agent.

The fact that dentine is highly tubular and that odontoblast cellular processes occupy these tubules to a variable extent, gives a clear inference of which dental pulp cells will encounter bacteria and their toxins first. Thus the odontoblast can be regarded as the first cellular line of defence within the dental pulp, with many fundamental roles in the tooth's defence systems (see chapter one, section 1.6.4). Data shown in chapter four demonstrates the distribution of COX-1 enzyme IR within the dental pulp and the clear detection of COX-1 in odontoblasts, sparsely within the fibroblast population and within the walls of blood vessels, may provide an idea of where this PGE_2 release comes from.

Data from the current study suggests a detectable increase in the amount of PGE_2 released from dental pulp tissue explants incubated with LPS over a relatively short time period (as short as 15 minutes). The dental pulp continues to release elevated levels of PGE_2 measured in the incubation media after longer incubation periods (1, 2 and 3 hours) with almost double the amount as compared to baseline levels. This may reflect a relatively

quick and steadily increased response of the dental pulp to the inflammatory stimuli until they are terminated.

These findings appear to be in accordance with the literature (Petrini et al., 2012, Chang et al., 2003a, Chang et al., 2003b) where the stimulatory effect of LPS on PGE_2 biosynthesis has been well documented.

On the other hand, it was somewhat surprising that the termination of the stimuli did not result in a decrease in PGE_2 levels to baseline levels after 15 and 60 minutes. This non-recovery state of the dental pulp may reflect the persistence of the inflammatory status of the pulp or that PGE_2 continues to be produced at a high level for a longer time period to counteract the damage resulting from the inflammatory process. The knowledge of dual pro and anti-inflammatory roles of PGE_2 may further explain this apparent non-recovery state. It is feasible to assume that the relatively fast elevation of PGE_2 released from the dental pulp after short-term contact with LPS may be to evoke pro-inflammatory actions of PGE_2 like macrophage and neutrophil attraction, thus facilitating defence mechanisms against the effects of LPS. The continuity of elevated levels of PGE_2 released from the dental pulp during exposure to LPS may exert pro-inflammatory effects. The elevated levels of PGE_2 after the stimulus termination may be caused by the effort of the dental pulp to counter the harmful effects of inflammation in an attempt to limit unnecessary tissue breakdown through the anti-inflammatory effects of PGE_2 (See Chapter 5, section 1.9.3), or may simply reflect the need of dental pulp tissue for more time to re-establish unstimulated basal levels.

Interestingly, looking at the data obtained from this experiment in a different way provided a slightly different finding. The cumulative effect of LPS on PGE_2 release (Figure 5-3), provide more evidently elevated levels as a response to longer incubation times. In addition, some recovery from the LPS-mediated effects was very clear this time in the form of decreased levels of PGE_2 collected after the stimulus was discontinued.

It has been suggested that LPS-mediated events are initiated by targeting resident macrophages, peripheral monocytes, neutrophils and DCs (Muzio et al., 2000, Zhang et al., 1999, Kitchens, 1999). Primarily, LPS interacts with cluster of differentiation 14 (CD14) receptors (Wright et al., 1990) and TLRs, specifically TLR-4 (Qureshi et al., 1999, Takeda and Akira, 2005) and TLR-2 (Hattar et al., 2013). These interactions are followed by the activation and overexpression of COXs. Although COX-2 is claimed to

be the main source of PGE_2 in inflammation (Dubois et al., 1998), there are some suggestions of COX-1 involvement based on the observation of COX-1 in the resident inflammatory cells and induction of COX-1 in the LPS-induced inflammatory conditions (McAdam et al., 2000). It is believed that COX-1-derived PGE_2 production is dominant in the early phase of acute inflammatory conditions in human, while COX-2 activation and associated PGE_2 production occurs after hours (Smyth et al., 2009).

5.4.2. Effect of NO on PGE_2

Here, the experiment was designed to test the cross-talk and interactions between the NO and PGE_2 pathways, since they have many similarities (discussed previously) and cross interactions reported in many tissues. Elements of these pathways are found in almost every tissue and findings from Chapter four of this thesis showed the coexistence of the constitutive enzymes of both pathways (COX-1 and NOS1) in a close proximity to each other, mainly in the odontoblast cells and the blood vessels, suggesting that NO produced by the action of NOS1 may affect COX-1 activity nearby either directly or indirectly.

Results from the current study showed a clear inhibitory effect of NO on the amounts of PGE_2 released from the dental pulp to almost two thirds that of the resting levels after brief incubation of pulp explants with NO donor. A small drop in PGE_2 levels was reported after one hour incubation, followed by a sharp decrease after two and three hours of incubation. These findings are in agreement with the literature in different tissues (Amin et al., 1997, Habib et al., 1997, Nile and Gillespie, 2012, Nile et al., 2010, Stadler et al., 1993) where the inhibitory effects of NO on PGE_2 biosynthesis have been reported.

It has been assumed that the inhibitory effect of NO on PG pathway is partly due to the inactivation of COXs by the nitration effect of NO (Goodwin et al., 1998, Gunther et al., 1997), decreasing the expression of COX-2 (Clancy et al., 2000) and/or inhibition of COX-2 (Minghetti et al., 1996), interference with Acetyl Choline-mediated PGE_2 synthesis (Nile and Gillespie, 2012), in addition to the modulatory effect of NO on the AA availability which further affect the PGE_2 substrate availability (Mollace et al., 2005). Nevertheless, NO was found to cause increased COX activity and PG synthesis in other experiments (Borda et al., 2007).

The modulatory effects of NO on PGE_2 pathway appears to be due to the possibility that COXs represent an endogenous receptor for NO as suggested by (Salvemini et al., 1993).

A relatively low level of recovery was observed 15 minutes after stimulus withdrawal, but this improved to less than half baseline after one hour.

5.4.3. Effect of ATP on PGE_2

Although the main function of ATP is the release of energy to satisfy tissue needs, it also has a signalling role, especially in neurons after a tissue injury, adding to its suggested role in inflammation and some pathophysiological conditions. It is believed that ATP exerts its actions by acting on specific receptors called purinergic receptors (P_2) (Kobayashi et al., 2006) which exist in eighteen subtypes (seven P_2X, nine P_2Y and two P_2U families) (Abbracchio et al., 2006). Evidence for the presence of these receptors within the dental pulp has been reported and found to be in close proximity to the distribution of COX enzymes in addition to the pulp nerves both myelinated and unmyelinated (Alavi et al., 2001, Cook et al., 1997).

It was hypothesised that ATP can influence the availability of AA within the tissue (which is the substrate molecule for PGE_2 synthesis) with a dual effect, either enhancing availability by acting on P_2Y receptors (Strokin et al., 2003), or inhibiting the AA release by acting on P_2U subtypes (Xing et al., 1992), thus modulating the PGE_2 pathway indirectly.

It is an interesting finding that incubation of pulp tissue with ATP analogue resulted in a significant decrease in the PGE_2 released into the medium to nearly half of the basal levels, following a short incubation period (15 minutes). Longer incubation (1 hour, T=120 minutes) resulted in similar diminished levels of PGE_2, but prolonged incubation (T=180 and T=240 minutes) failed to decrease these levels further.

The inhibitory effect of ATP on PGE_2 release from the dental pulp explants reported in this study appears to be in agreement with that reported in older research (Xing et al., 1992), but contradicts with ATP stimulatory effect on PGE_2 in other tissues (Nile and Gillespie, 2012, Nile et al., 2010).

The dental pulp failed to re-establish the basal PGE_2 levels 15 minutes after stimulus withdrawal. However, complete re-establishment of the basal levels of PGE_2 have been reported after one hour of stimulus termination, which is the fastest and the only recovery back to basal levels reported in this study.

The inhibitory effect of ATP on PGE_2 and the complete pulp recovery to baseline level may be attributed to the effect of ATP on AA availability only, where as soon as ATP is

discontinued the AA becomes available again and the process of releasing PGE_2 continues. It has been reported that ATP can lead to increased synthesis and release of NO (Ray et al., 2002) which inturn may lead to decreased PGE_2 release as reported by the current study section 5.3.2.

5.4.4. Dual effect of NO+ATP on PGE_2

The sharpest drop in PGE_2 concentration occurred after 15 mins of incubation with NO+ATP. Different response was evident after one hour incubation with ATP+NO with higher levels of PGE_2, this may be partially due to rapid breakdown of NO.

It is logical to observe this strong inhibitory effect of both stimuli on PGE_2 concentrations in the bathing media, because individually, each one of the stimuli caused a fairly strong inhibition.

The clinical significance of the results obtained from this study is that it may help to control the inflammatory responses within the dental pulp, reducing the signs and symptoms of inflammation, limiting the tissue destruction in the course of inflammation and modulating the immune response in a favoured direction.

5.5. Conclusion

The data collected from this study suggest that great complexity exists in the control and regulation of PGE_2 biosynthesis and release which involve the contribution of other pathways and inflammatory mediators within the dental pulp than has been hitherto described.

Within the limitations of this study, it has been found that LPS has a strong stimulatory effect on PGE_2 synthesis and release from the rat mandibular incisor dental pulp explants over a short or long exposure of time, with no obvious evidence of the pulp's ability to re-establish basal levels within one hour of stimulus withdrawal.

It has also been observed that NO and ATP, both alone or in combination, have a variable inhibitory effect on the synthesis and release of PGE_2 from rat mandibular incisor dental pulp explants over short or long exposure time, with variable pulp recovery speed.

Finally, the continuous production of PGE_2 from the dental pulp over a 5 hours period of incubation with different agents may suggest that freshly harvested rodent pulp tissues may remain viable and capable of examination for periods up to 5 hours in the laboratory setting. However, no further analysis of the tissue was undertaken to detect degenerative

or other effects that may have occurred within the tissues to explain the continued release of PGE_2.

It is important to mention some of the limitations of the current study, including the use of rat model, organ culture procedure, concentrations of the materials used, materials and kits used, and the individual variabilities.

Chapter 6. Differential regulation of pro-inflammatory and anti-inflammatory genes following exposure to LPS, NO, ATP and PGE_2

6.1. Introduction

The dentine-pulp complex shows a great similarity to other connective tissues within the body, however it has considerable complexity and a number of unparalleled features, due to its enclosure within the hard and non-compliant shell of the tooth in all directions except the small apical foramen. This encirclement may limit the pulp's ability to neutralise and recover from microbial invasion. This certainly represents the most obvious challenge facing the dental pulp, as it faces the onslaught of injuries including dental caries, operative dental surgery and trauma.

The host's defences against such challenges are in the form of a series of molecular and cellular events in the course of the inflammatory process. The main aim of this process is firstly to attract immunocompetent cells from the circulatory system, to eliminate the pathogens and remove any degradation products from the tissue and secondly to try to limit tissue destruction, while switching on repair and regeneration processes within the tissue, through the stimulation of specific resident cellular responses.

Immune responses (both innate and adaptive) prevail in the dental pulp and represent the main weapon in the face of inflammation. Triggering of the local production of cytokines is the most common outcome of the innate immune response, which will aid in attracting phagocytic leukocytes as a pro-inflammatory measure. The innate immune response was believed to be non-specific, however more specific responses have been reported and are thought to be enacted via Toll-like receptors (TLRs) (Hans and Hans, 2011, Mahanonda and Pichyangkul, 2007).

Continuity of the injuries and the attachment of pathogen-associated molecular patterns (PAMPs) to TLRs on innate immune cells can influence and trigger the adaptive immune response (Iwasaki and Medzhitov, 2004).

Adaptive immune responses are not evoked to take the place of innate immune responses, but usually develop around them. The evolution of the adaptive response is dependent on the stimulation of innate immune cells, particularly dendritic cells, with their antigen-presenting abilities. TLRs on the inactive dendritic cell surface detect PAMPs and convey information through signalling pathways to activate the dendritic cells, which leads to the

secretion of chemokines and cytokines crucial for the differentiation of T-cells (Hornung et al., 2002).

Although these two immune responses were evoked to defend the tissues, their effects, combined with the non-compliant environment of the pulp may result in exacerbation of tissue damage and endanger pulp vitality.

When dental caries breaches through protective enamel, it encounters dentine. Dentine demineralisation resulting from caries progression leads to the release of bioactive molecules (Smith et al., 2012a, Graham et al., 2006, Tomson et al., 2007), which together with bacterial LPS can trigger the odontoblasts, the first cellular defence of the dental pulp, and the nearby innate immune cells.

The triggering of odontoblasts and pulp fibroblasts can lead to the activation of the nuclear factor kappa B ($NF\text{-}_{k}B$) intracellular signalling pathway (Lee and Burckart, 1998) which inturn leads to upregulation of many adhesion molecules and pro-inflammatory cytokines responsible for localised or systemic responses to LPS exposure (Bierhaus et al., 1999, Ohtsuka et al., 2001). These cytokines are synthesised by various tissues and immune cells and have powerful signalling abilities. They can interact with specific cellular receptors to regulate the genetic expression of the target and molecular responses (possibly by involvement of a second signalling pathway) in a synergistic way, with the stimulation of a series of events that lead to the release of other related molecules. The combined effects of these cytokines and the related molecules will affect and regulate the innate and adaptive immune response.

The release of pro-inflammatory cytokines within a diseased tissue (including the dental pulp) will provide diverse, wide-ranging effects that usually focus on the control of pathology, reinforce the resolution of inflammation and aid in the activation of tissue repair and regeneration. One of the major effects of these cytokines is the development of a chemotactic gradient, which will enhance the chemotaxis and activation of immune cells (Brennan et al., 2008, Reing et al., 2008) underpinning the adaptive and innate immune responses.

Effector cells of innate immunity (neutrophils and macrophages) aid in phagocytosis at the early phase of inflammation following the first encounter with the pathogens. Natural killer (NK) cells are then extravasated to the affected site in response to cytokines (Maghazachi, 2005). NK cells interact with resident immature DCs which will lead to

mutual stimulation and increase in cytokine production by these cells (Kikuchi et al., 2004). The further production of interferons (Hahn et al., 2000) can stimulate macrophages and promote a T-cell response (Trinchieri, 1995). Mature DCs present the antigen to T-cells, which can be found in healthy pulp in contrast to B-cells which have been found to be absent in healthy pulp (Jontell et al., 1998). However, with the persistent inflammatory process and development of prolonged infection, the early inflammatory infiltrate of macrophages, monocytes and neutrophils intensifies with the accumulation of T and B cells and with the development of an adaptive immune response.

Control of the inflammatory processes is of crucial importance and represents an essential tool to limit unnecessary tissue damage. Research focused on this topic suggests that repair and regeneration of the dental pulp occurs only after resolution or full control of the infection and inflammation (Baumgardner and Sulfaro, 2001, Rutherford and Gu, 2000). On the other hand, it has been suggested that the inflammatory process is an essential prerequisite to facilitate repair and regeneration (Goldberg et al., 2008).

Despite the fact that there is a good clinical valuation for the effect of inflammation on disease progression and management results, the relationship between the biological events of pulp inflammation and the disease progression are currently inadequately understood. This produces a big challenge to clinical diagnosis and management of pulpal disease (Petersson et al., 2012, Mejare et al., 2012), especially as regenerative approaches to enhance tooth vitality become established (Hargreaves et al., 2013, Law, 2013, Trope, 2008). The significance of recognising the cellular and molecular events of pulpal inflammatory processes is currently explained further with the acknowledgement that there is an impressive interaction between inflammatory, pathological and regenerative events.

6.2. Aim of study

To analyse the effect of carefully selected inflammatory stimuli and mediators on selected anti and pro-inflammatory molecules utilizing quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) and to explore a possible cross-linkage between different pathways by the inclusion of key-role enzymes and receptors from these pathways in an effort to further elucidate the complexity associated with pulp inflammation and pathology.

Genes of COX 1, COX 2, EP1, EP2, NOS1 were chosen because they represent the key elements of the targeted pathways (PG and NO). IL1, IL6 genes and their receptors were chosen because of the close relation between these cytokines and PG pathway, while TLR-4 gene was chosen because it is the primary receptor for LPS.

6.3. Materials and methods

6.3.1. Sample collection

6.3.1.1. Untreated:

13 Wistar rats (age= 9 weeks, weight 260-400 G) were killed in a CO_2 chamber before surgically extracting the mandibular incisor tooth from each rat (n= 13). Under the operating microscope and using a high speed dental handpiece and diamond burs under constant water cooling, a groove was made along the lateral wall of the mandibular incisor tooth until the pulp tissue became visible. Care was taken to avoid exposure to the pulp and to minimise any trauma. Using a scalpel, the lateral walls were broken carefully removing the hard walls, trying to expose the pulp tissue before careful removal with tweezers. Immediately after removal, pulp tissues (mean weight = 14 mg) were suspended with 500 µL RNA stabilization agent (RNAlater, Cat. No. 76106, Qiagen, Germany) (Wang et al., 2009) for 24 hours at 4˚C.

6.3.1.2. Treated (test and controls):

28 Wistar rats were managed as in section 6.3.1.1 and 56 fresh pulps (mean weight=14 mg) collected and stored submerged in DMEM. For each rat, the right mandibular incisor was assigned for the control group and the left for the test group for direct comparison. The pulps (test and control) were divided into four groups (n=7), each group being incubated in a well plate (one pulp per well filled with 500 µL DMEM + FCS + antibiotics). For the first test group, ATP analogue (2'(3')-O-(4-benzoylbenzoyl) adenosine-5-triphosphate tri(triethylammonium) salt (100 µM) (Sigma Aldrich, UK) was added. For the second test group, a nitric oxide donor (diethylamine NONOate diethylammonium salt (NONOate) (10 µM) (Sigma Aldrich, UK) was added replacing the incubation media with fresh addition of NONOate every 20 minutes. For the third test group, LPS from *Escherichia Coli* serotype 026:B6 (10 µg/ml) (Sigma Aldrich, UK) was added. For the fourth test group, PGE_2 100 ng/ml (Sigma Aldrich, UK) was added. All four test groups were incubated with the specified chemicals for three hours while the

corresponding control groups were incubated with DMEM only. Upon finishing the incubation, the pulp tissues were collected and stored in 500 μL RNAlater at 4˚C.

The detailed description of the methods and materials used in this work is presented in chapter two, section 2.3. For each rat, the right mandibular incisor was assigned for the control group and the left for the test group for direct comparison. The pulps (test and control) were divided into four groups (n=7). One test group was incubated with LPS, one with NO, one with ATP and the last one with PGE_2, while their corresponding control groups were incubated with nutrient media (+ FCS + antibiotics) only. At the end of the incubation period (three hours), pulps were removed, total RNA extracted, reverse transcribed to cDNA and PCR procedure accomplished.

The accuracy of PCR quantification depends on two important parameters: linearity and efficiency. These parameters were measured using double standard curve samples generated by progressive dilution of cDNA samples. For quality control within each run, all samples were tested in duplicate with the inclusion of internal negatives, internal and external positives as described in chapter 2, section 2.3.9.

For a quantitative approach, a fold change views were prepared. This was accomplished by a comparison between the Ct value of the treated and non-treated samples after normalisation with the reference gene Ct. The fold change is equivalent to the value of $2^{-\text{delta delta Ct}}$, where delta delta Ct (ΔΔCt) = ΔCt (treated sample) - ΔCt (non-treated sample).

6.4. Results

In order to calibrate the qRT-PCR results, a relative standard curve was included for each run. This was prepared from a serial dilution of cDNA template from the control tissue and used as positive internal controls. The correlation coefficient (R^2) for the standard curves ranged from 0.97-1.00 and the efficiency ranged from 90-110 % as seen in Figure 6-1.

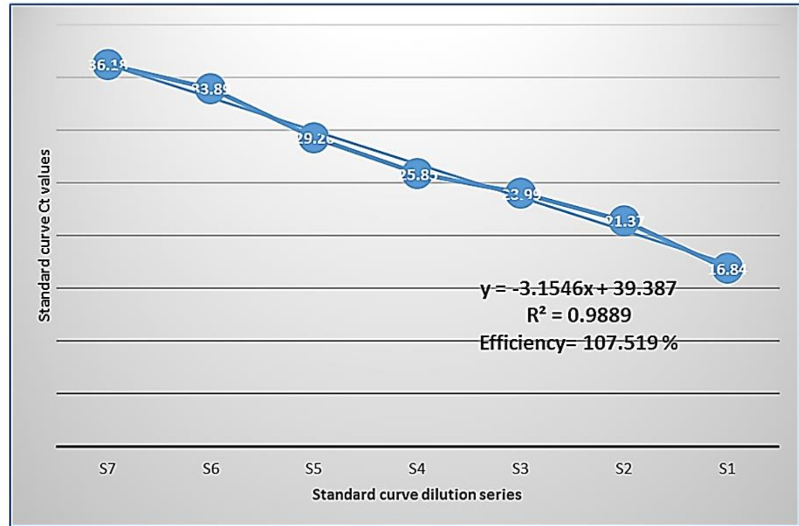


Figure 6-1: A representative of plot of standard curve threshold values (*Ct*) versus the log of the amount of total cDNA added to the reaction (0.0005-50 ng) showing the R^2 and efficiency values.X axis represent the serial dilution of target cDNA where S1 represent the neat cDNA, S2 represent S1 diluted ten times and so on.

A standard curve amplification plot was obtained by plotting the *Ct* versus the fluorescence for each dilution. No amplification or fluorescence was observed in non-template control (NTC) and RT^{-ve} mRNA as shown in Figure 6-2 (arrow). This indicated the complete absence of any genomic DNA contamination. This plot was obtained by the use of a specific primer for β-actin with a total cDNA input ranging from 0.0005-50 ng/µl as serial dilution of β-actin. The relative quantity of β-actin transcript from each concentration is reflected at the exponential phase of the amplification curve (Figure 6-2) and determined by the *Ct* value.

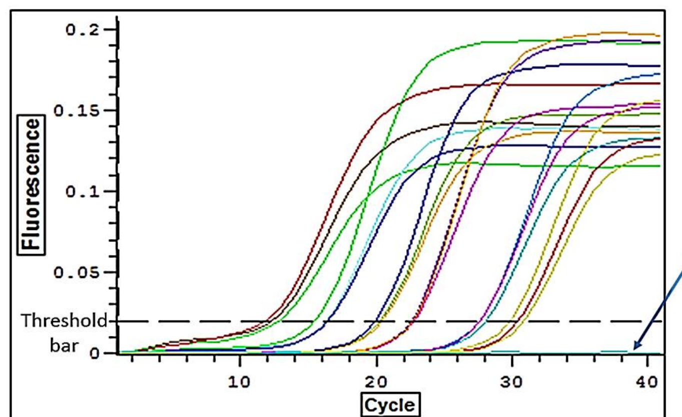


Figure 6-2: Fluorescence amplification versus cycle number for β-actin serial dilutions. The curves represent the highest to lowest input mRNA from left to right. Triplicates were used for each input amount and are reflected by overlapping curves on the graph. The threshold bar is the point at which the mRNA levels were estimated. The arrow points to the amplification of RT-ve mRNA (no amplification).

As a part of the standardisation requirements, a melting curve was generated for each gene, where the data was acquired between 55 and 90˚C and analysed by the Opticon monitor software. Melting curves showed distinct and fully overlapping melting peaks for all genes which indicates the specificity of the primers and the amplification of a unique products. Figure 6-3 shows the melting curve for β-actin with the peaks at 86˚C which confirms the specificity of the primer set. The absence of any peak for the NTC reaction suggests the complete absence of any other PCR product as well as the zero-possibility of any primer dimers formation during the PCR amplification.

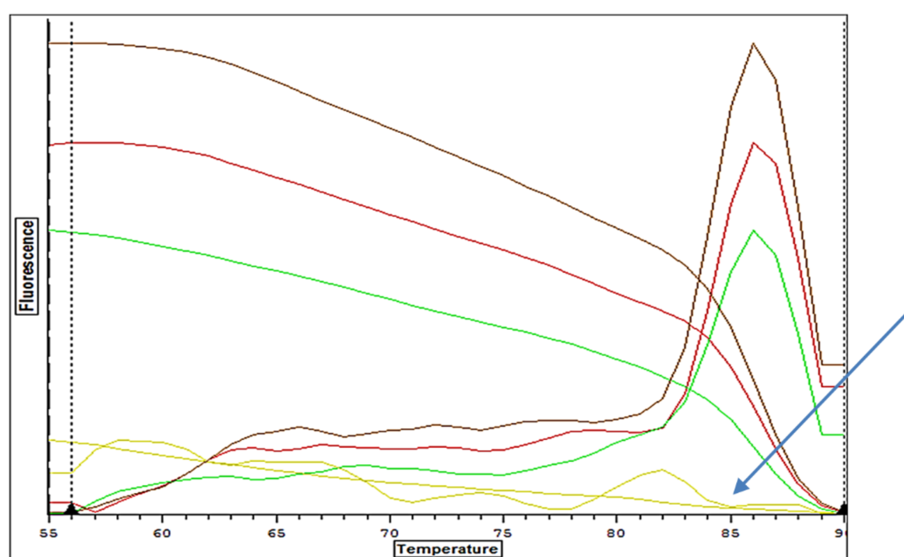


Figure 6-3: Fluorescence versus temperature (melting curve) for β-actin. After amplification, the samples were slowly heated in order to detect the loss of fluorescence that occurs at the melting temperature which is recognised by a specific melting peak for each PCR product. All the peaks are located at the same temperature point. This pattern refers to the specificity of the product, as any product has one melting temperature and confirms the specificity of the primers. Note that the peaks are not at the same height because each peak represents a different concentration. The arrow refers to the NTC and RT^{-ve} reaction.

To determine whether or not the targeted genes in this chapter were expressed in normal animals, freshly harvested pulp tissue (n=3) was used and total RNA extracted. Utilizing conventional PCR technique and gel electrophoresis, this RNA revealed that all the targeted genes are present in single bands, within the correct base pair (bp) length as shown in Figure 6-4.

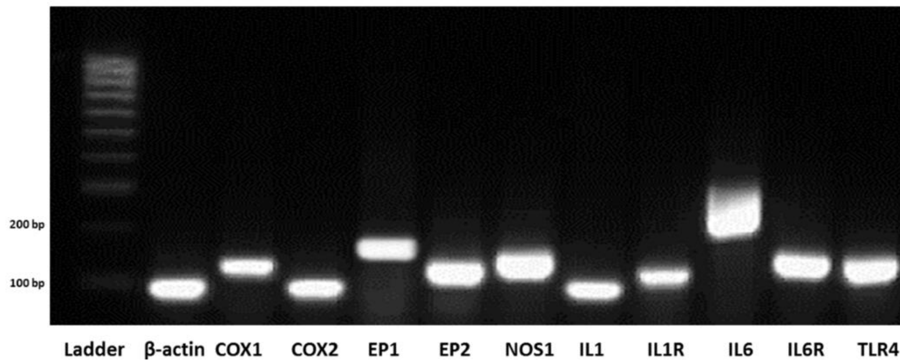


Figure 6-4: Conventional PCR (end point) gel electrophoresis of rat mandibular incisor dental pulp. It shows bands of appropriate base pair (bp) (as correlated to a ladder to the left) for all the target genes. β-actin was used as positive internal control.

To provide understanding of the presence of target genes under normal physiological conditions, normal rat dental pulp (n=10) was explored, where 50 ng cDNA, specific set of primers for each gene and SYBR green chemistry were utilized to perform qRT-PCR reaction. All genes were detectable at the mRNA level, and in measurable quantities (Figure 6-5). The gene for β-actin was expressed at very high basal levels.

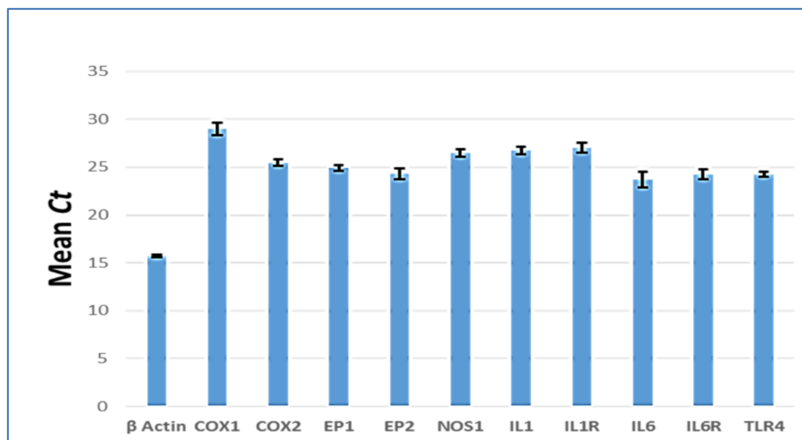


Figure 6-5: qRT-PCR quantitation for the target genes within normal rat dental pulp. Samples operated in duplicates in the presence of β-actin as a normaliser. The level of gene expression was calculated after normalising against β-actin in each sample and is presented as relative mRNA expression units. Note that *Ct* values are inversely proportional to the actual mRNA expression. Values are mean ± SEM (n=10 per gene).

6.4.1. Effect of LPS

To determine whether incubation of rat dental pulp tissue (n=14, 7 control + 7 test) with LPS has an effect on the expression of target genes or not, dental pulp tissues from seven left mandibular incisors were incubated with a nutrient media and LPS for three hours while the corresponding right mandibular incisor pulps were incubated with nutrient media alone. At the end of the experiment, pulp tissues were retrieved, RNA extracted

and converted to cDNA. 50 ng of cDNA from both treated and corresponding non-treated pulps were used in the qRT-PCR reaction.

A direct comparison between the mRNA levels of the treated and non-treated samples is displayed in Figure 6-6. The mRNA coding for the reference gene (β-actin) was unaffected, while those for all target genes were upregulated as compared to values of their respective untreated controls. The upregulation of all genes (except IL6 and TLR-4) was found to be extremely statistically significant (*P*<0.0001), while the upregulation was very statistically significant for IL6 (*P*<0.01) and significant for TLR-4 (*P*<0.05).

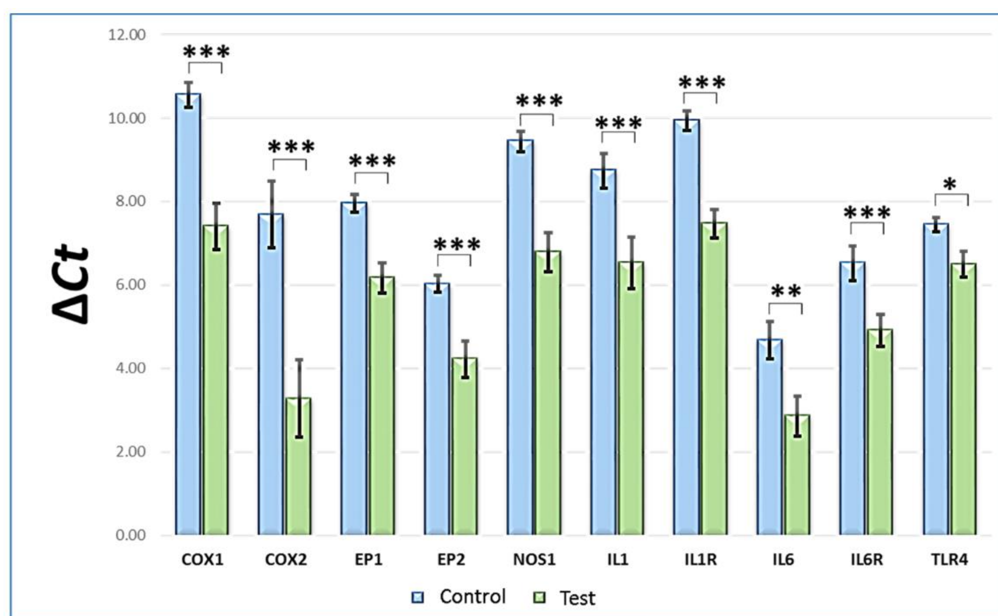


Figure 6-6: qRT-PCR quantitation for the target genes within LPS-treated and corresponding non-treated controls. Samples were operated in duplicates in the presence of β-actin as a normaliser. The level of gene expression was calculated after normalising against β-actin in each sample and is presented as relative mRNA expression units. Note that *Ct* values are inversely proportional to the actual mRNA expression. Values are mean ± SEM (n=7 per gene). Student *t* test was used to detect the level of significance, (*) refers to significant differences, in which * *P*<0.05, ***P*<0.01 and *** *P*<0.0001.

COX-1 gene shows almost nine-fold upregulation, which represents the second highest value after COX-2 in which the upregulation was more than twenty one fold. NOS1 was the third expressing more than six-fold upregulation, followed by IL1R at more than 5 fold. TLR-4 gene upregulation was the lowest with less than two fold, while all other genes displayed between 3-5 fold upregulation as shown in Figure 6-7.

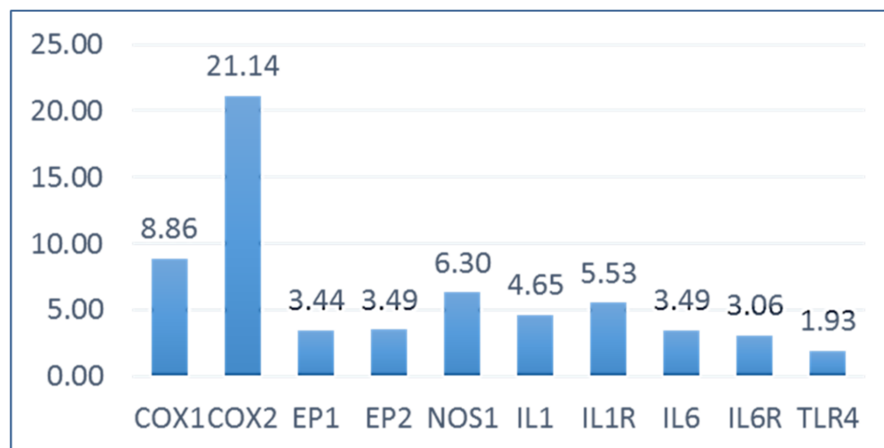


Figure 6-7: Illustration of the upregulation of target genes in response to LPS incubation by fold changes. Fold change value is the fold differences of the target genes in the test samples relative to the control samples and can be calculated according to the formula fold change = $2^{-\Delta\Delta Ct}$.

For the purpose of direct visualisation of the changes in the target genes histologically, immunohistochemistry experiments were accomplished to explore normal (n=3) and LPS incubated (n=3) pulp tissues using specific antibodies (described in Chapter 2 Table 2-1). COX-1 immunoreactivity (IR) in the normal pulp was found in a localised area within the odontoblast cell bodies (OCB), in few cells within the pulp and the walls of the blood vessels as shown in Figure 6-8A, B and C respectively. No COX-1 IR was found in the subodontoblast layer (SOL). In LPS-incubated pulp tissues, COX-1 IR appeared to be less or non in OCBs, however a larger population (compared to normal) of cells in SOL and the cells of the bulk of the pulp, in addition to blood vessels show IR to COX-1 as illustrated in Figure 6-8D, E and F respectively. COX-2 IR was seen in very few cells scattered within the bulk of the pulp in normal tissues as seen in Figure 6-8G, while no IR was observed in the blood vessels or anywhere else. In LPS-incubated pulps, COX-2 IR was observed in a relatively larger population of cells within the bulk of the pulp and walls of blood vessels (Figure 6-8H and I).

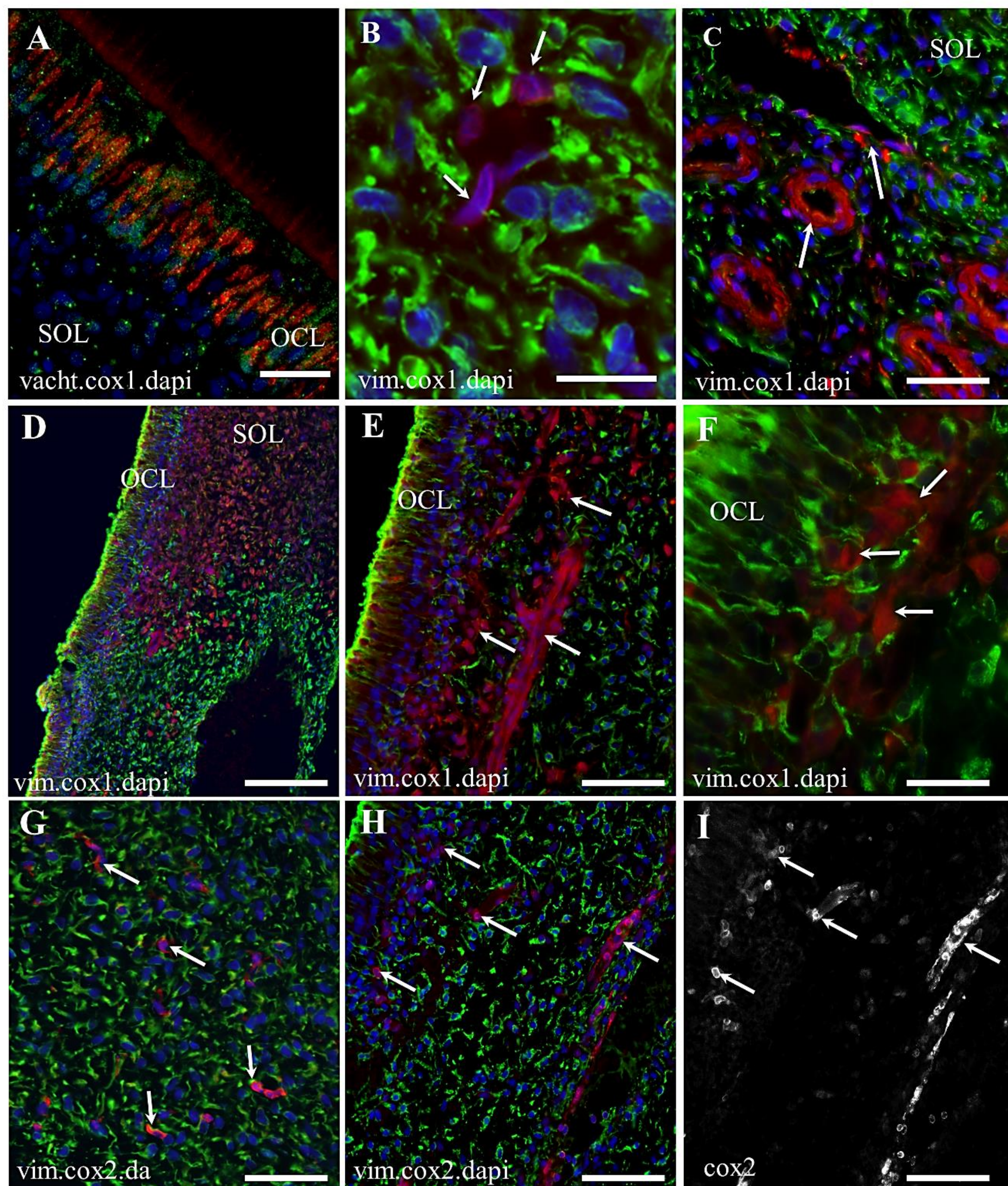


Figure 6-8: An overview of the immunoreactivity for COX-1 and COX-2 in normal and LPS-incubated pulps. A shows a demineralised section stained with antibodies to COX-1 (red), vacht (green) and the nuclear stain dapi (blue), where COX-1 IR has been found in specified regions within the OCBs. B shows COX-1 IR in few cells within the bulk of the pulp (arrowed), while COX-1 IR in the blood vessels is shown in C. In LPS-incubated pulps, COX-1 IR was found mainly in the SOL and the blood vessels as seen in D, E and F. Freshly extracted pulp tissue (normal) is shown in G, where few pulp cells were found to be COX-2 IR+ve with no reported IR in blood vessels. In LPS-incubated pulps (H) more cells within the bulk of the pulp and the blood vessels have been shown to be COX-2 IR+ve as can be seen clearly in (I) which represent the COX-2 IR component image. Arrows refer to points of interest. Calibration bars are 75 µm in A and C, 25 µm in B and F, 200 µm in D and E, 100 µm in G, H and I.

IR to EP1 receptors was seen in the walls of blood vessels and few cells in normal pulps (Figure 6-9A). However, exploring pulp tissues incubated with LPS showed that EP1 IR was spread among more cells within the bulk of the pulp (Figure 6-9B). EP2 IR was seen in the distal part of the OCBs, weakly in the odontoblast processes (ODP) and SO area (Figure 6-9C) with limited success in detecting this IR in the LPS-incubated pulp tissues. Figure 6-9D and E show the IR to NOS1 in normal pulp which can be observed in the distal part of OCBs, walls of blood vessels and weakly in the ODP and SO area. NOS1 IR in LPS incubated pulp was found to be mainly in the subodontoblast cell layers (SOL) and in scattered cells within the bulk of the dental pulp in addition to the blood vessels as shown in Figure 6-9F, f, G and g.

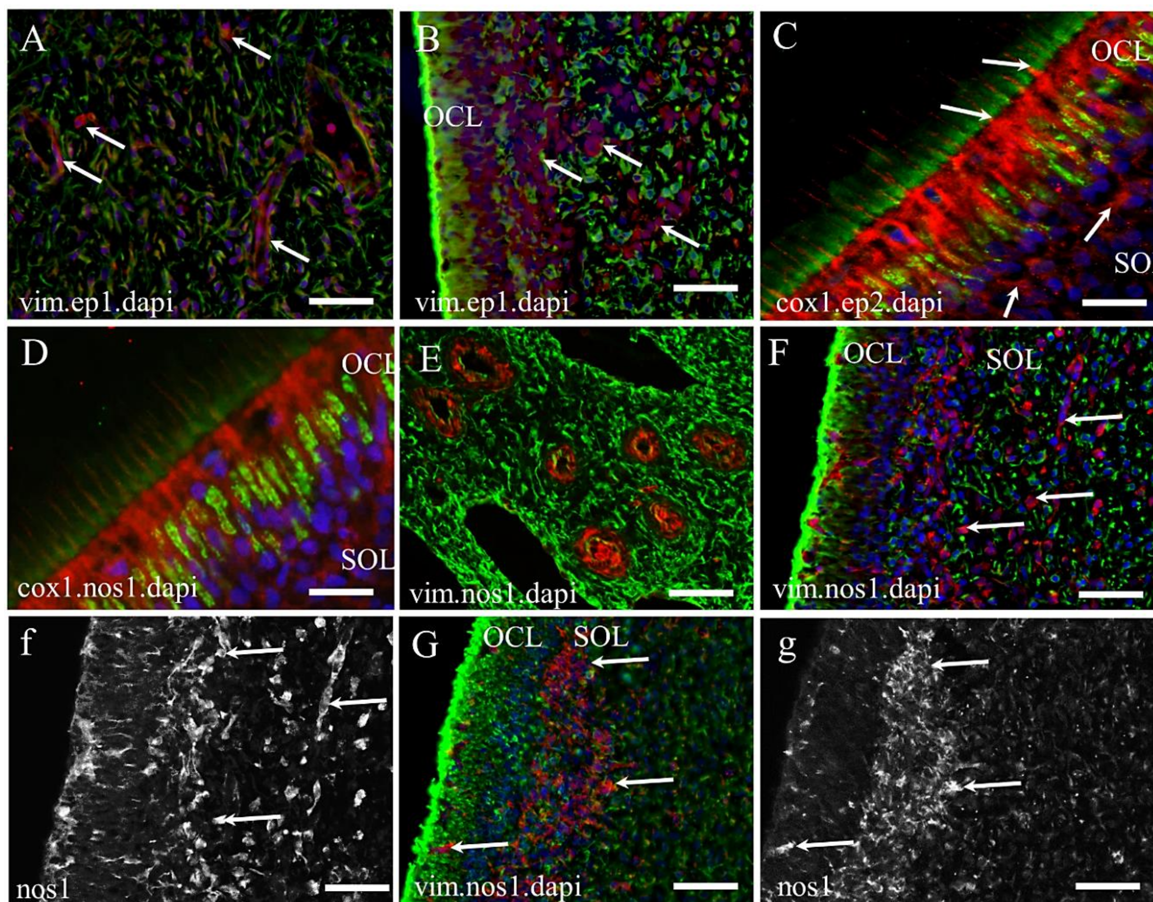


Figure 6-9: Immunoreactivity for EP1, EP2 and NOS1 in normal and LPS-incubated pulps. A and B show extracted pulp tissues stained with antibodies to EP1 (red), vim (green) and dapi (blue), where A shows EP1 IR in normal untreated pulps and B shows EP1 IR in LPS-incubated pulps. C is a demineralised section stained with EP2 (red), vim (green) and dapi (blue) shows EP2 IR in the OCB and SOL. D and E are images from demineralised sections stained with antibodies to NOS (red), vim (green) and dapi (blue) in normal pulps. F and G are from LPS-incubated pulps stained with NOS1 (red), vim (green) and dapi (blue) In LPS-incubated pulps, with their NOS1 component images to the right of each one (f and g respectively). Calibration bars are 75 µm in all images.

Weak IR was observed for IL1β antibody in the odontoblast cell layer (OCL) (Figure 6-10A and a) and the walls of relatively big blood vessels in the central part of the normal pulp (Figure 6-10B and b). Disappearance of IL1β IR from the OCL was observed in LPS-incubated pulp samples, while this IR start to appear sometimes in small clumps of cells in the SO area (Figure 6-10C and c) or as large numbers of cells with +ve IR within the same cell layer (Figure 6-10D and d). IL1 receptor IR was observed weakly in the OCL and the walls of small blood vessels within the normal pulps (Figure 6-10E and e), with limited success in detecting IL1 receptor IR in LPS-incubated pulp.

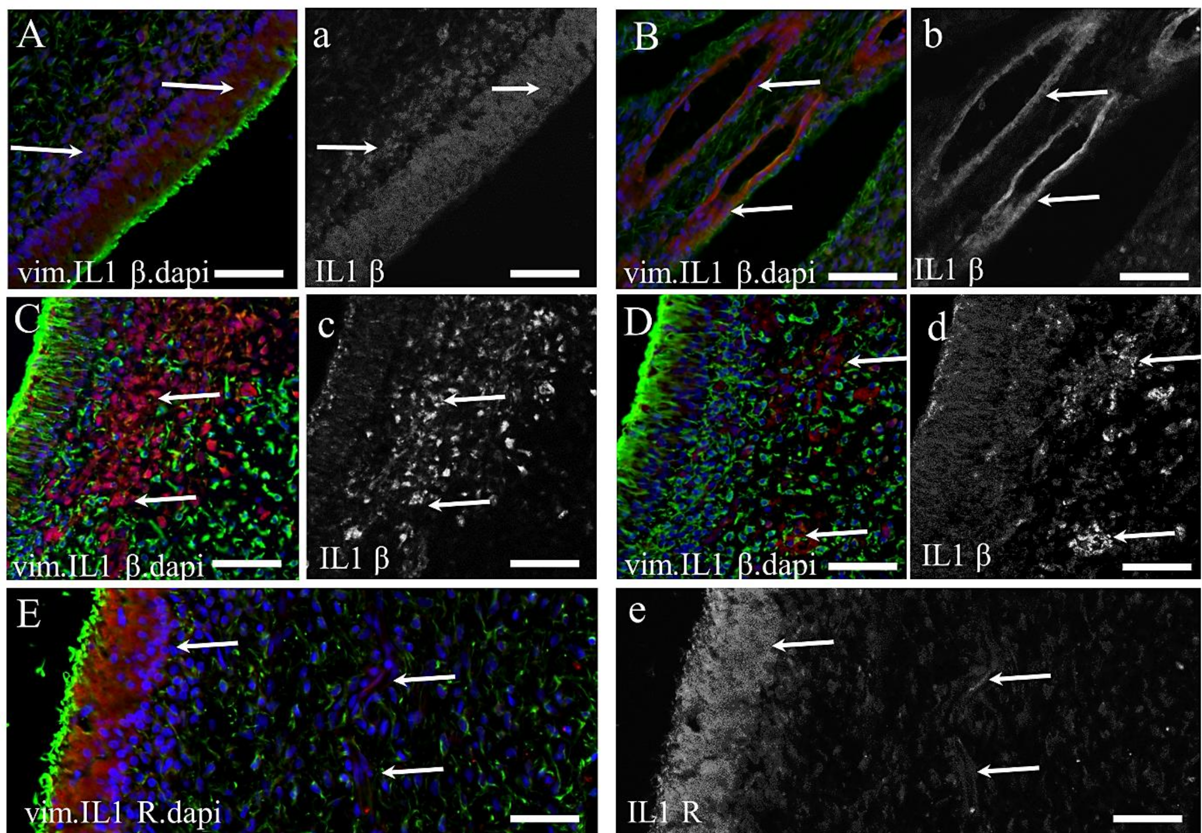


Figure 6-10: Immunoreactivity for IL1 β and IL1 R in normal and LPS-incubated pulps. A, a, B and b shows extracted pulp tissue stained with antibodies to IL1 β (red), vim (green) and dapi (blue), shows IL1 β IR in the OCL and blood vessel walls within the normal untreated pulps. C, c, D and d are stained with IL1 β (red), vim (green) and dapi (blue) shows IL1 β IR in the SOL. E and e are stained with antibodies to IL1 R (red), vim (green) and dapi (blue) in LPS-incubated pulps. Calibration bars are 65 µm in all images.

Weak IR was observed against IL6 antibody and was found to be located in the OCL and the walls of blood vessels (Figure 6-11A), with limited observation of IR within LPS-incubated tissues. Figure 6-11B, b, C and c shows IR against IL6 receptor antibody and is shown to be mainly within the OCL, the thick walls of blood vessels and weakly in few, scattered cells within the bulk of the pulp. IL6 receptor IR in LPS-incubated pulp tissues

was the same as that in normal pulp, but the number of the IR^{+ve} cells within the bulk of the pulp was slightly greater as shown in Figure 6-11D, d, E, e, F and f.

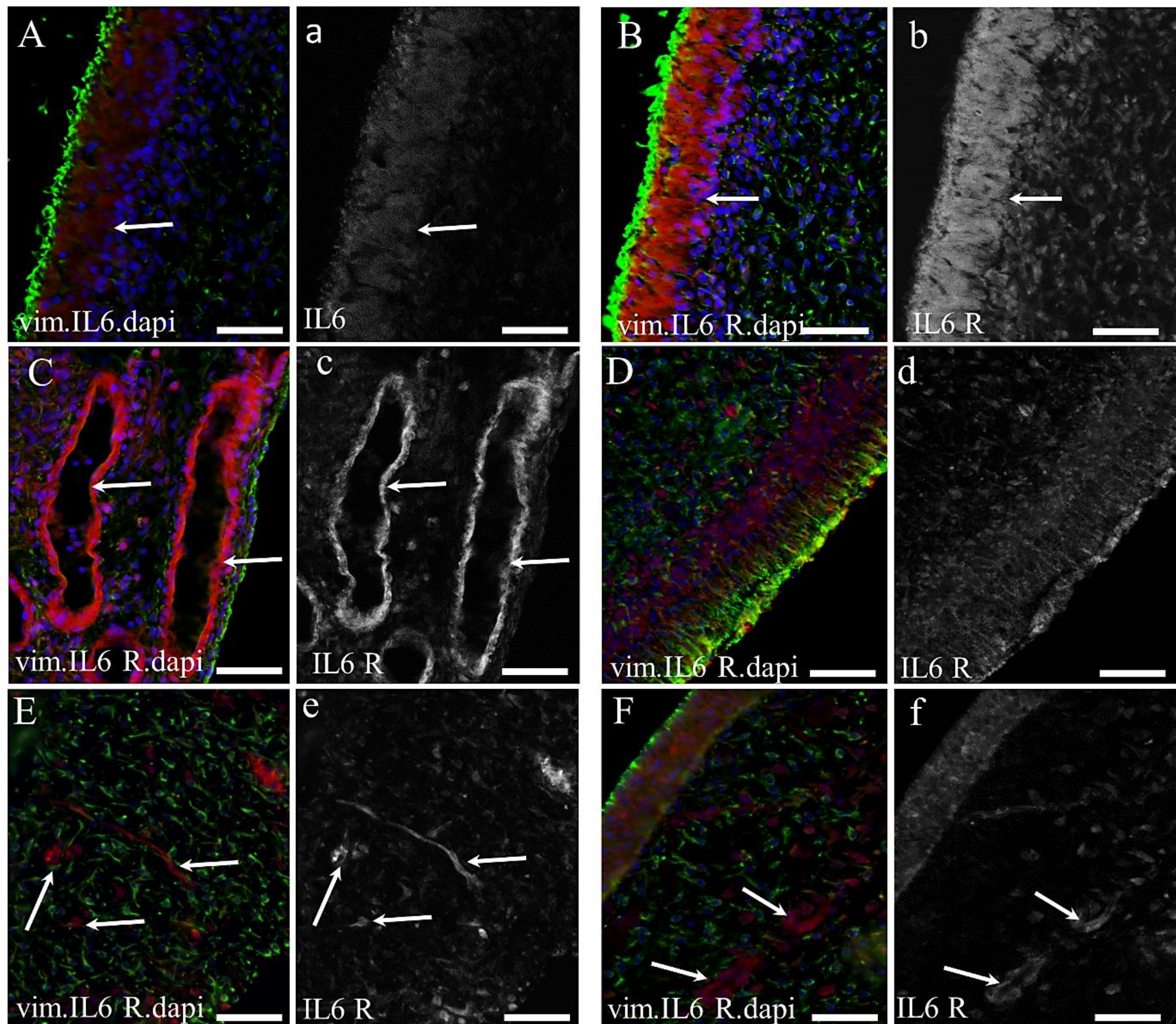


Figure 6-11: Immunoreactivity for IL6 and IL6 R in normal and LPS-incubated pulps. A and a shows extracted normal pulp tissue stained with antibodies to IL6 (red), vim (green) and dapi (blue) with weak IR in OCL. B, b, C and c shows IL6 R IR in the OCL and blood vessel walls within the normal untreated pulps. D, d, E, e, F and f are stained with IL6 R (red), vim (green) and dapi (blue) shows IL6 R IR in the SOL, walls of blood vessels and few cells within the bulk of LPS-incubated pulps. Calibration bars are 65 µm in all images.

6.4.2. Effect of NO

To explore the effect of incubation of rat dental pulp tissue (n=14, 7 control + 7 test) with NO on the gene expression of target genes, mRNA levels of the target genes were calculated from test and control samples.

After normalisation with the reference gene, a direct comparison between the mRNA levels of the treated and non-treated samples is displayed in Figure 6-12. The mRNA

coding for the reference gene (β-actin) was stable. Excluding TLR-4, all target genes were upregulated as compared to values of their respective untreated pulp samples. Despite the upregulation of nine genes, only that of COX-1, NOS-1 and IL6R were found to be statistically significant ($P<0.05$).

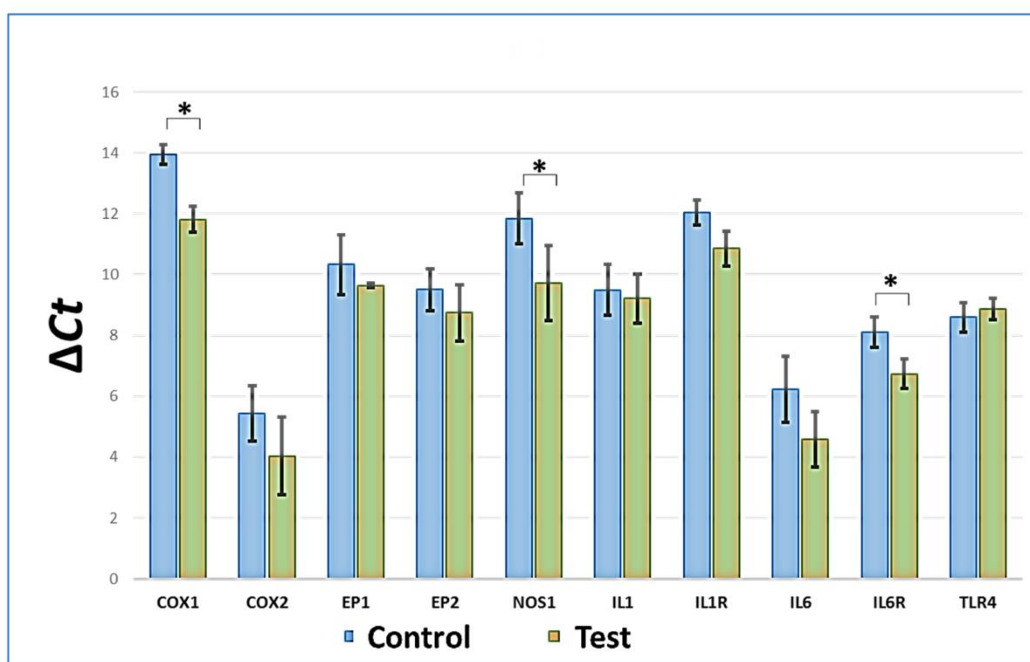


Figure 6-12: qRT-PCR quantitation for the target genes within treated (NO-incubated) and corresponding non-treated rat dental pulps. Samples operated in the presence of β-actin as a reference gene. The level of gene expression was calculated after normalising against β-actin in each sample and is presented as ΔCt. Note that Ct values are inversely proportional to the actual mRNA expression. Values are mean ± SEM (n=7 per gene). Student *t* test was used to detect the level of significance, (*) refers to significant differences, in which * $P<0.05$.

COX-1 and NOS-1 showed more than four-fold increase in gene expression which represent the highest change among all target genes, followed by IL6 with more than three folds increased expression. COX-2, IL1R and IL6R comes next with more than two fold changes, then EP1, EP2 and IL1 showing more than one fold change, while TLR-4 occupies the bottom of the list with the least fold change of less than one fold. The overall fold changes of all target genes are represented in Figure 6-13.

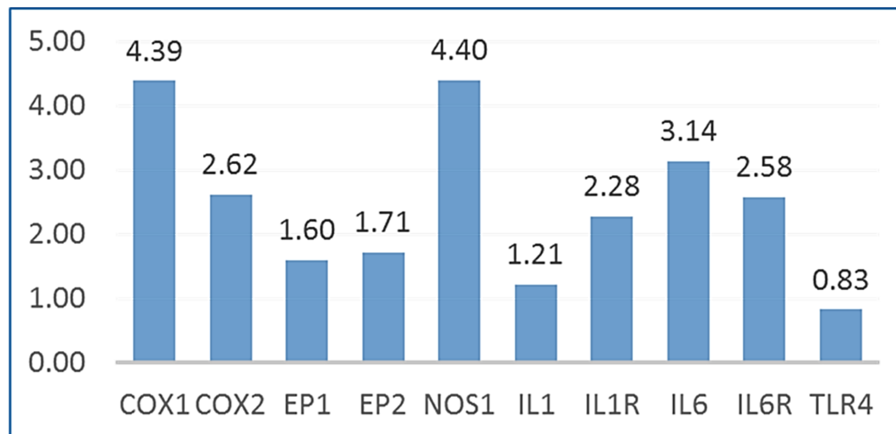


Figure 6-13: Interpretation of the modulating effect of NO incubation on the expression of target genes in terms of fold changes.

For more clarity, an immunohistochemistry staining of normal and NO-incubated pulps was accomplished using antibodies for the most affected target genes.

Antibodies for COX-1, COX-2 and vimentin were used to stain sections from NO-incubated pulps (n=3) shown in Figure 6-14, while the expression of these antibodies in normal tissues have been shown previously in Figure 6-8 (A, B and C for COX-1, and G for COX-2). COX-1 IR was found in a well localised area within the OCB (Figure 6-14A), cells within the bulk of the pulp (Figure 6-14B) and walls of blood vessels (Figure 6-14C). Although COX-1 IR in the OCB appeared to be the same as in normal tissue, this IR appeared to be more intense in the walls of blood vessels and the IR^{+ve} cells within the pulp appeared to be present in larger numbers forming a bigger population compared to normal pulps Figure 6-14B and its component black and white image. The COX-1 IR^{+ve} cells within the bulk of the pulp appeared to be heterogeneous depending on the intensity of IR, in which some cells were intense and pure COX-1^{+ve} (Figure 6-14 D + sign), and others showed pure but weaker COX-1 IR (Figure 6-14 D # sign) with some cells having mixed IR to COX-1 and vimentin (Figure 6-14D * sign). COX-2 expression appeared to be more intense than in normal pulps and concentrated in the SOL cells and the walls of blood vessels while no COX-2 IR was found in the OCL. Figure 6-14 (E, F and G) shows numerous COX-2 IR cells in NO-incubated pulps compared with few scattered COX-2 IR cells in normal pulp (images illustrated in Figure 6-8 G).

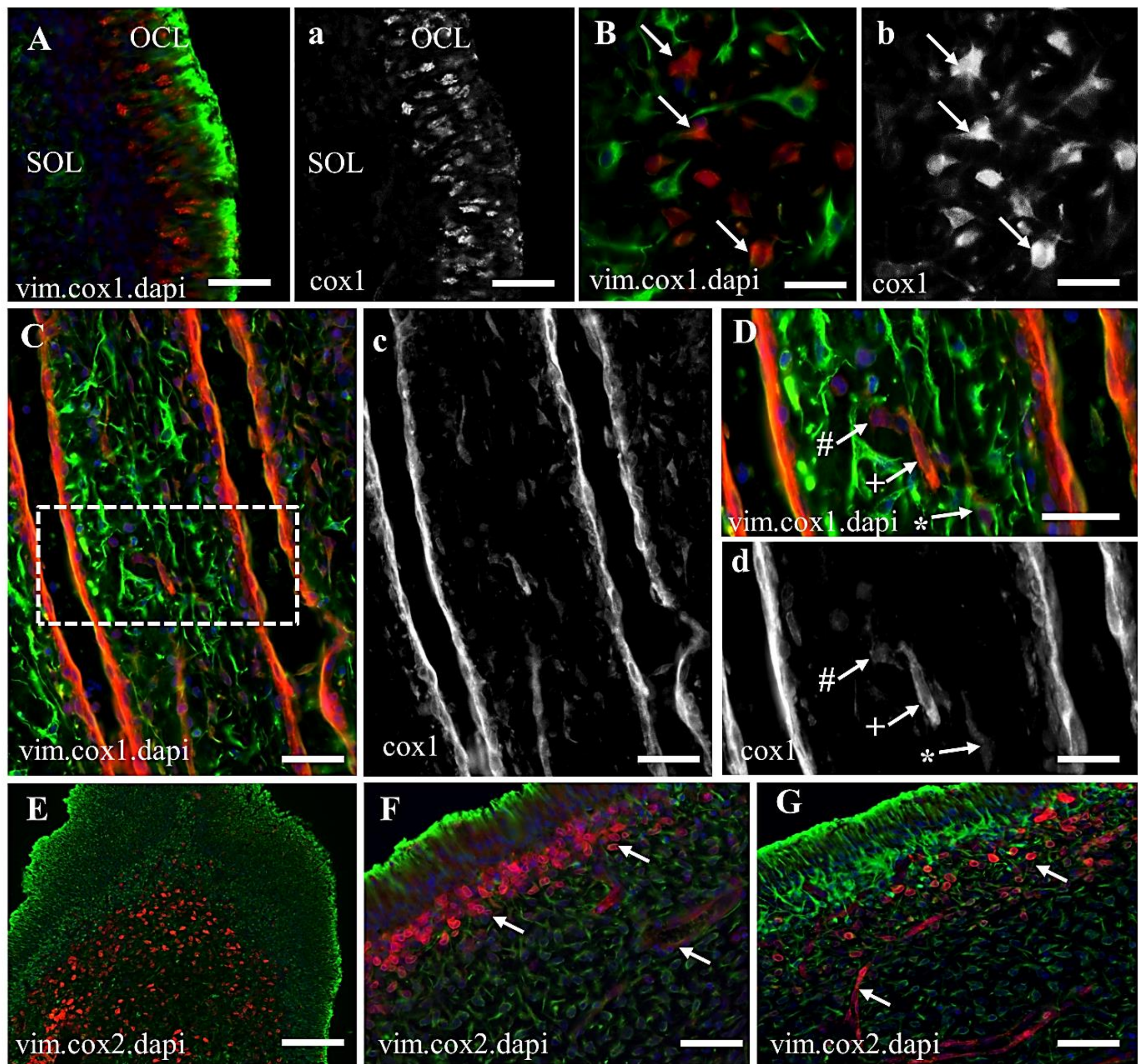


Figure 6-14: Immunoreactivity to Cox-1 and COX-2 within NO-incubated pulps. A, B and C are sections from extracted rat pulp stained with antibodies for COX-1 (red), vimentin (green) and dapi (blue). A shows COX-1 IR in OCL, B show COX-1 IR in pulp cells and C showing intense IR in the walls of blood vessels. More details can be seen in the component images (a, b and c) to the right of each individual image. Area of interest in image C (marked by rectangle) is magnified and shown in D in which few COX-1 IR cells are shown, one cell shows pure COX-1 IR stronger IR than the others (panel (D and d) marked with + sign), another cell with pure and weaker COX-1 IR (panel (D and d) marked with # sign) and the last one with mixed IR to vimentin and COX-1 (panel (D and d) marked with * sign). COX-2 IR is shown in panels E, F and G where this IR appears to be concentrated in the SOL and the walls of blood vessels. Calibration bars are 65 µm in A, a, F and G, 35 µm in B, b, D and d, 60 µm in C and c, and 100 µm in E.

NO-incubated pulp section stained with antibodies against NOS-1 showed a positive IR in the SOL close to the OCL with very weak IR in OCB (Figure 6-15A) and the walls of blood vessels (Figure 6-15B and C). Few cells are scattered in the bulk of the pulp (Figure 6-15D) with variable NOS-1 IR can be seen. It appears that NOS-1 IR changed

from OCB in normal (shown in Figure 6-9D) to SOL in NO-incubated pulps with more interstitial pulp cells NOS-1 IR^{+ve} in the NO-incubated pulps.

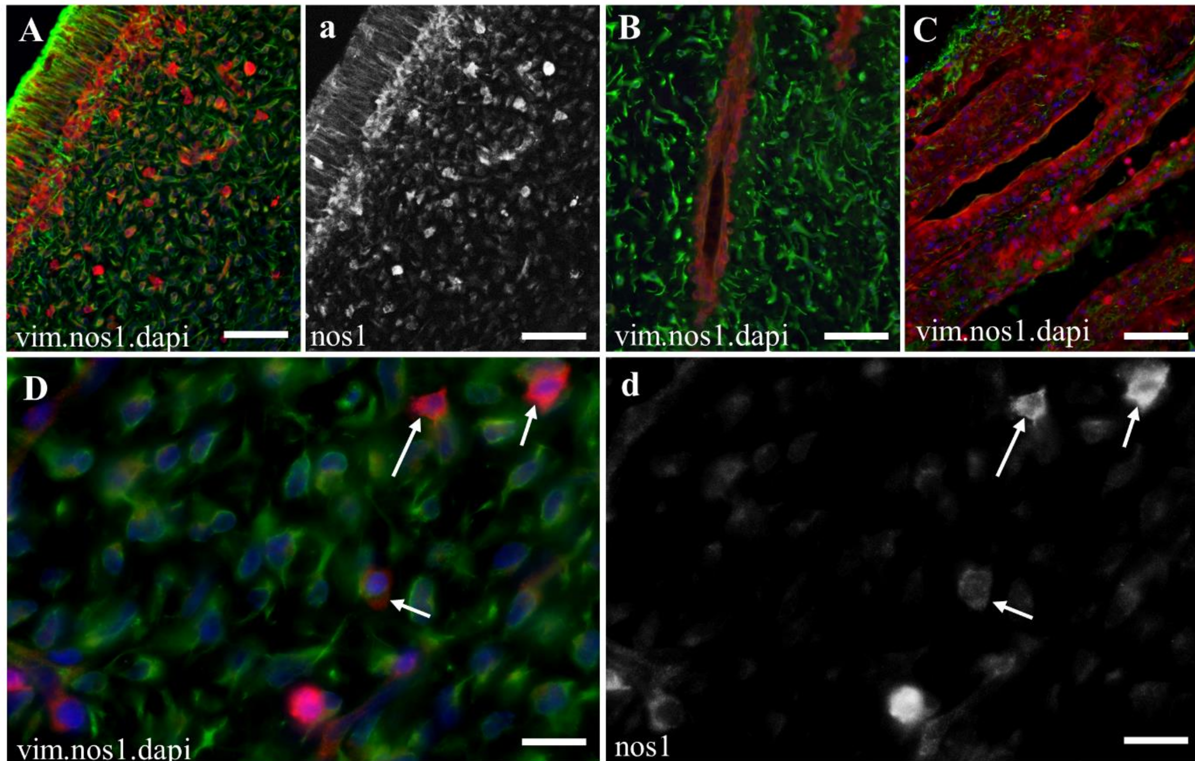


Figure 6-15: **Immunoreactivity against NOS-1 antibody in NO-incubated pulp tissues.** Sections are stained with antibodies for nos1 (red), vim (green) and dapi (blue). A shows the very weak NOS-1 IR in OCB, stronger IR in SOL with IR^{+ve} cells scattered within the pulp with further illustration in the component panel (a). B and C showing IR within the walls of blood vessels. In D, few IR^{+ve} cells can be seen scattered within the pulp with variable intensity (arrows).

6.4.3. Effect of ATP:

Measuring the mRNA expression level of target genes in normal and ATP-incubated pulps (n=14, 7 control + 7 test) revealed slight and non-significant over expression of COX-1, NOS-1 and IL6R genes. Slight and non-significant downregulation of COX-2 and IL1R was observed while significant downregulation was reported with EP1. Very significant down regulation of IL1 and TLR-4 and extremely significant down regulation of EP2 was observed, while IL6 remained unaffected. The detailed comparison of the effect of ATP incubation on mRNA level can be seen in Figure 6-16.

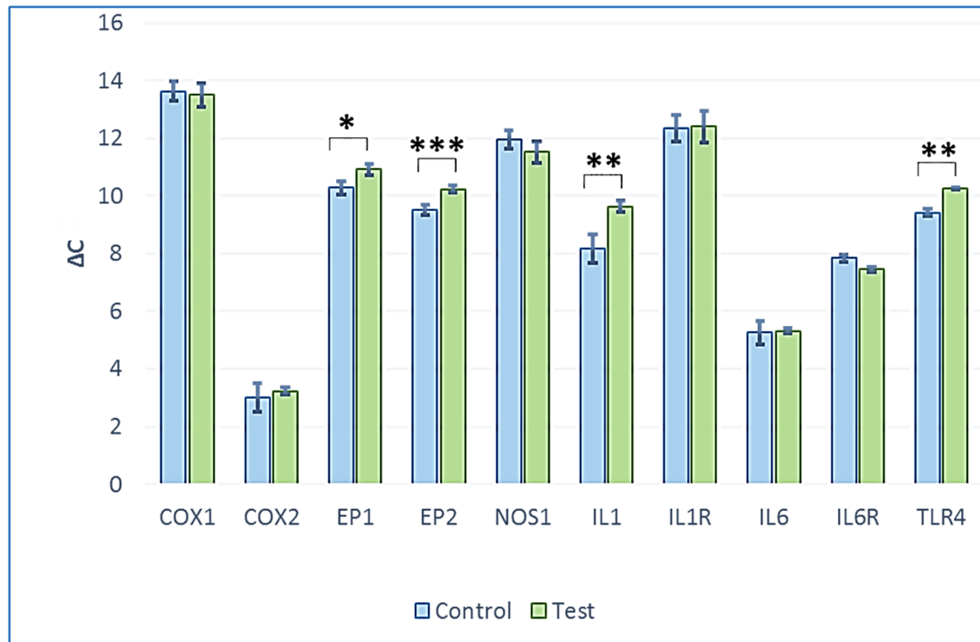


Figure 6-16: qRT-PCR quantitation for the target genes within treated (ATP-incubated) and corresponding non-treated rat dental pulps. Samples operated in the presence of β-actin as a reference gene. The level of gene expression was calculated after normalising against β-actin in each sample and is presented as ΔCt. Note that Ct values are inversely proportional to the actual mRNA expression. Values are mean ± SEM (n=7 per gene). Student *t* test was used to detect the level of significance, (*) refers to significant differences, in which * $P<0.05$, ** $P<0.01$ and *** $P<0.0001$.

In terms of fold changes, COX-1, NOS1 and IL6R represent the highest fold changes of more than one-fold upregulation, though statistically non-significant. Changes of all the other genes comes with less than one fold as shown in Figure 6-17.

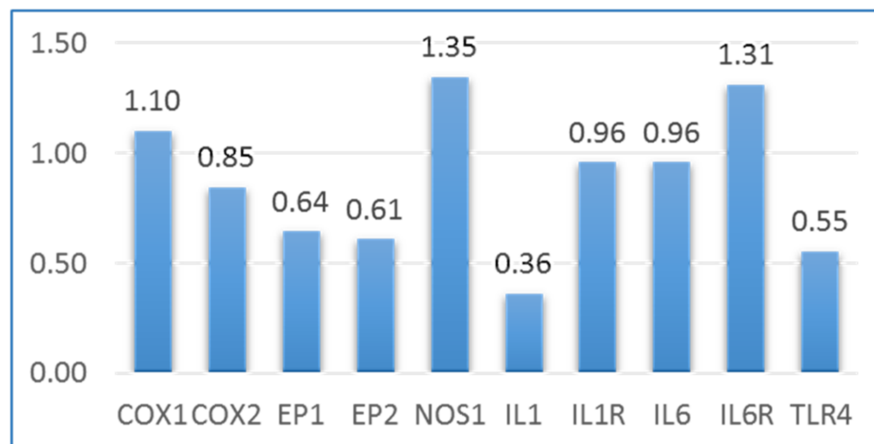


Figure 6-17: Illustration of the changes in the expression of target genes in response to ATP incubation in terms of fold changes.

Immunohistochemistry sections showed no difference in the IR of the target genes between the normal and ATP-incubated pulp tissues.

6.4.4. Effect of PGE_2

Since it has been observed that the pulp tissue released an increased amount of PGE_2 under experimental inflammatory condition as seen in chapter five section 5.3.1, then an experiment was designed to assess the effect of increased PGE_2 on the target genes at a molecular level. The mRNA level of the target genes was evaluated in PGE_2-incubated pulps (n=7) and compared to that of their corresponding normal pulps (n=7). It was found that gene expression of COX-1, COX-2, NOS1, IL1R and IL6R was upregulated to varying degrees, none of which were statistically significant, with the exception of COX-2 and NOS1 which were significant and very significant respectively as shown in Figure 6-18. On the other hand, the gene expression of EP1, EP2, IL1 and TLR-4 was found to be downregulated in varying degrees, though this was non-significant for all genes except TLR-4 (Figure 6-18).

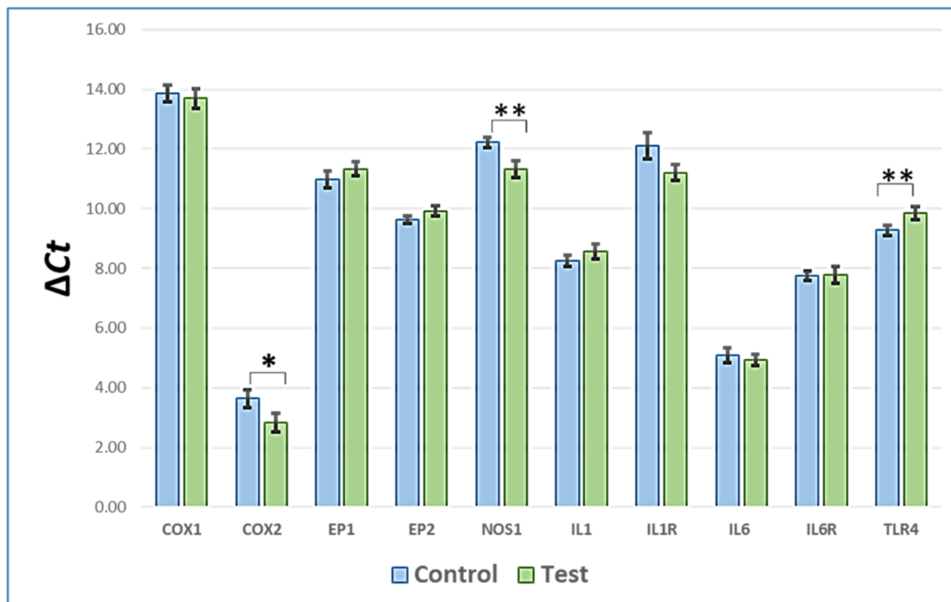


Figure 6-18: qRT-PCR quantitation for the target genes within treated (PGE_2-incubated) and corresponding non-treated rat dental pulps. Samples operated in the presence of β-actin as a reference gene. The level of gene expression was calculated after normalising against β-actin in each sample and is presented as Δ*Ct*. Note that Ct values are inversely proportional to the actual mRNA expression. Values are mean ± SEM (n=7 per gene). Student *t* test was used to detect the level of significance, (*) refers to significant differences, in which * $P<0.05$ and ** $P<0.01$.

To further illustrate the changes of the target genes in response to incubation with PGE_2, Figure 6-19 shows these changes in terms of fold. The highest fold changes were observed with COX-2, NOS1 and IL1R close to two folds. Slightly over one fold changes

were observed with COX-1 and IL6 while nearly no changes were reported with all the remaining genes.

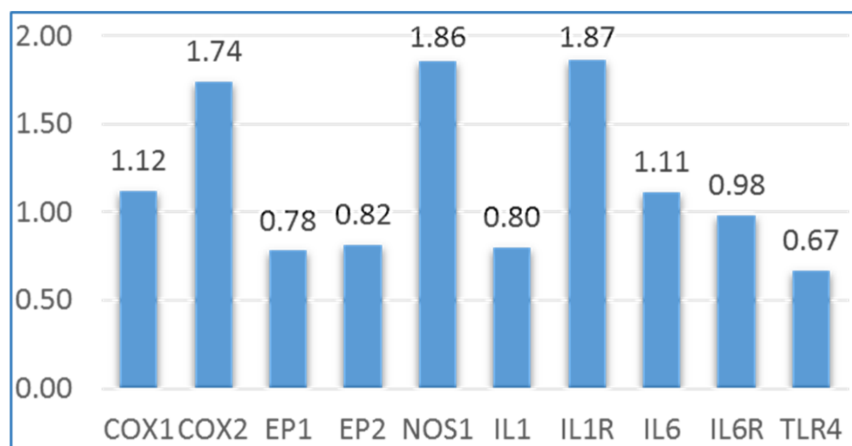


Figure 6-19: Fold changes illustration of the changes in the mRNA level of target genes in response to PGE_2 incubation in terms.

Immunohistochemistry sections show no difference in the IR of the target genes between the normal and PGE_2-incubated pulp tissues.

6.5. Discussion

The results from this chapter demonstrate that all target genes are constitutively expressed in the normal rat dental pulp. The use of rat dental pulp and experimentally inducing inflammation by exposure to inflammatory materials may not reflect the exact *in vivo* environment of pulp inflammation in humans or rats, but it represents a valuable model for studying the inflammatory reactions within a tissue in a lab-based environment.

The dental pulp is a complex system, which is subject to harmful stimuli from its surroundings including bacterial infections, mechanical and thermal stimuli. In all events the pulp becomes damaged, whether reversibly or irreversibly depending on the severity and persistence of the insult. Reversible damage usually includes the injury or death of damaged cells followed by healing induction (Bronckers et al., 1996), often including reactionary and reparative dentinogenesis (Smith, 2002). Irreversible damage usually includes a series of pathological events which may end with partial or complete pulp necrosis.

6.5.1. Effect of LPS

In the event of a progressing carious lesion, the primary resident bacterial species are Gram-positive which represent about 70% of the bacterial population in deep caries (Bjørndal and Larsen, 2000). This percentage changes as the lesion advances, leading to

an increased population of Gram-negative bacteria (Hamilton, 2000). Since the evolution and maturation of microbial populations is usually accompanied by the liberation of toxins and metabolites as soluble molecules, it is logical to think that bacteria can be recognised by host defence systems through the interaction with these molecules (Rainard and Riollet, 2006) and hence the use of LPS which is the major product of Gram-negative bacteria.

LPS binds to a special cell membrane receptor called CD14 (Moore et al., 2000), which is a glycoprotein usually expressed by neutrophils (Detmers et al., 1995), macrophages (Kim et al., 2013), myeloid cells (Wright et al., 1990), monocytes (Sakuta et al., 2001) and odontoblasts (Botero et al., 2006), an interaction that is enhanced by the presence of LPS-binding protein LBP (Triantafilou and Triantafilou, 2002). However, CD14 has no transmembrane domain which prevents its participation in intracellular signalling (Haziot et al., 1996). Another contributor is the molecule MD-2, which is a soluble molecule capable of binding to the extracellular portion of TLR-4 (Shimazu et al., 1999) with high affinity to bind both TLR-4 and LPS (Visintin et al., 2001). TLR-4 is regarded as a receptor for bacterial LPS (Poltorak et al., 1998) and is expressed in macrophages (Hoshino et al., 1999), dendritic cells (Medzhitov, 2001), endothelial cells (Faure et al., 2000) and the odontoblast (Botero et al., 2006, Mutoh et al., 2007). The CD14-LPS-LBP complex is presented to the TLR-4-MD-2 complex and bond together (Triantafilou and Triantafilou, 2002) and this complex recognition process leads to the recruitment of adapter myeloid differentiation factor 88 (MyD88). This binds to the intracellular portion of TLR-4 and initiates a series of events and reactions leading to the activation of nuclear factor kB (NF-kB) (Lee and Burckart, 1998) and the formation of interferon β (INF-β) (Seya et al., 2005) which eventually enhances pro-inflammatory mediators and cytokine expression including NO, PG, IL1, IL6 and increased polymorphonuclear leukocytes (PMNL) cells recruitment and migration (Alves-Filho et al., 2006).

The effect of LPS on the dental pulp was explored in this chapter by studying normal gene expression and following LPS incubation. Results from this chapter show a relatively quick pulpal activation at a transcriptional level, evident by the elevated target gene expression compared to non-incubated pulps. All genes showed an extremely significant upregulation (except IL6 and TLR-4 which it is believed are due to high standard error values) in response to three-hour incubation with LPS. Although the upregulation of COX-2 in response to LPS has been reported before (Font-Nieves et al.,

2012), the magnitude was not as high as reported here. However, the upregulation of COX-1 in the presence of LPS within the dental pulp reported in this chapter is novel and has never been reported before, although reported in other tissues (Onodera et al., 2004, Bezugla et al., 2006), and this appears to be in contrast to what has been reported in previous literature (Font-Nieves et al., 2012). LPS appears not to upregulate COXs only, but also changes their localisation within the dental pulp as demonstrated immunohistochemically. This upregulation and re-localisation can be an explanation for the increase in PG release from the dental pulp in response to LPS incubation reported in Chapter 5, section 5.3.1.

The upregulation of prostanoid receptors observed in this chapter can further enhance the effect of PG by providing more receptors for interaction, thus enhancing the PG pathway which has both anti and pro-inflammatory roles. This upregulation has not been reported previously.

Overexpression of NOS1 enzyme gene observed in this chapter appears to be in agreement with the literature (Kawashima et al., 2005) and may be translated to an increase in NO production, which seems logical, as NO is regarded as a key factor and mediator in local inflammatory conditions (Okada et al., 1999) and it has been reported to be involved in the progression of experimental pulpitis (Kawashima et al., 2005). NO may interact with PG causing inhibition of PG release (as reported in Chapter 5 section 5.3.2) or enhancing PG synthesis directly through upregulation of COX enzymes (Goodwin et al., 1999).

The coexistence of both IL1 and IL6 with their receptors together in the rat dental pulp and the fact that all of them are upregulated following exposure to LPS may reflect their involvement in the advancement of the inflammatory process. The upregulation of IL1 and IL6 appears to be in agreement with the literature (Nakanishi et al., 2001, Lin et al., 2002), however the upregulation of their respective receptors has not been reported before within the dental pulp. This induction of both the cytokines and their receptors may refer to an amplified response to LPS incubation as both cytokines contribute to the inflammatory processes.

It is well addressed at the beginning of the discussion section of this chapter that TLR-4 is the key receptor in modulating the tissue response to LPS. According to results from this chapter, TLR-4 expression within the dental pulp is significantly upregulated after

incubation with LPS and appears to be in accordance with the literature (Mutoh et al., 2007, Font-Nieves et al., 2012, Botero et al., 2006).

6.5.2. Effect of NO

Upregulation of all target genes except TLR-4 was observed, but not all changes were statistically significant. NO is well known as a biological mediator molecule of the immune response to harmful stimuli (Moilanen et al., 1999) and has crucial roles in the course of inflammation with close relation to the progression of the inflammatory process (Speranza et al., 2004).

The upregulation of PG pathway elements (COX-1, COX-2, EP1 and EP2) seems to highlight an important functional link between NO and PG pathways. The upregulation of COXs upon pulp interaction with NO may further strengthen the hypothesis that COXs represent endogenous receptors for NO. This may further explain the multifaceted nature of NO (Salvemini et al., 1993). This induction of the COXs and their receptors may refer to an overall stimulation of the PG pathway, which may in turn affect NOS activity in a reciprocal way (Borda et al., 2007). The effect of NO on COXs reported in this chapter is in accordance with the literature (Cuzzocrea and Salvemini, 2007), however very little is known about the effect of NO on prostanoid receptors.

Surprisingly, the addition of NO to the pulp explants elicited an induction effect on NOS1 expression, which may be due to indirect effects on the PG pathway (Borda et al., 2007), or due to positive feedback of NO on NOS1 to produce further amounts of NO.

Another important finding from this study was the varying upregulation of IL1, IL6 and their corresponding receptors upon exposure to NO. The effect on IL1 was weaker than any other gene and has been poorly reported in the literature. However, the induction of IL6 was reported in the literature in other tissues and hypothesised to be mediated by cGMP-dependant pathway via the modulation of NF-kB activity (Siednienko et al., 2011, Makris et al., 2010). Very little is known about the effect of NO on the IL1R and IL6R in any tissue and particularly the dental pulp.

The response of TLR-4 gene to NO in this study appears to be the first reported within the dental pulp research field, in which TLR-4 gene is slightly downregulated (although non-significant) upon exposure of the pulp explants to NO donor.

6.5.3. Effect of ATP

The main functions of ATP are energy supplier, participation in metabolic activities, acting as a key mediator in the advancement of cardiovascular diseases (Burnstock, 2002) and acting as a trigger for various activities in the course of systemic inflammation (Cauwels et al., 2014). In addition, extracellular ATP may serve as a vital signalling molecule, especially in injured tissue. Its sources include platelets, neuronal, inflammatory and endothelial cells (Gordon, 1986). ATP acts on purinergic receptors and indirectly regulates the process of inflammation (Virgilio and Solini, 2002). By acting on a specific receptor, ATP can lead to short-term upregulation of COX-2 gene (Lin et al., 2009). Other than the transient effect of ATP on COX-2, it is not yet clear whether ATP has an effect on the remaining target genes.

Results from this chapter demonstrate the greatest effect of ATP was a downregulating effect on prostanoid receptors, IL1 and TLR-4 with a slight non-significant effect on the remaining genes. In contrast with what has been reported by Lin *et al* (2009), the effect of ATP on COX-2 was slight downregulation. The mild effect of ATP recorded in this chapter may be due to the incubation time or due to the concentration used. Unfortunately, very little is known about the effect ATP on the dental pulp. However, results from Chapter 5 section 5.3.3 shows that ATP has an inhibitory effect on PGE_2 release which may further support the downregulation of ATP on COX-2 reported in the current chapter.

6.5.4. Effect of PGE_2

The role of PGs as inflammatory mediators is well documented in the literature (Waterhouse et al., 1999, Moriyama et al., 2005), and their responsibility for a wide range of housekeeping actions such as homeostasis and cytoprotection (Dubois et al., 1998) is also well-recognised. The detailed actions of PGE_2 were discussed in Chapter 5 section 1.9.3.

The upregulation effect of PGE_2 on NOS1 observed in the current study is reported for the first time and is in agreement with the increased NOS activity reported in the literature (Borda et al., 2002, Borda et al., 2007). This upregulated NOS1 could be the primary cause for the upregulation effect of PGE_2 on COXs reported in the current study (Cuzzocrea and Salvemini, 2007). The slight downregulation of IL1 appears to be in contrast with reports in other tissues, though the upregulation of IL1R is in agreement with the report of Sheibanie *et al* (2004). The very minor upregulation of IL6 reported in

the current study may contradict observations in other tissues (Gruber et al., 2000), but can be explained as a result of the downregulation of prostanoid receptors (Li et al., 2002).

The effect on TLR-4 is of great importance as TLR-4 is regarded as one of the key mechanisms by which cells recognise a pathogen. Exposure to PGE_2 causes a significant downregulation of mRNA expression of TLR-4, which although reported in other tissues (Degraaf et al., 2014) is only on a protein level not on mRNA level. The inhibition of TLR-4 can lead to diminished ability of the host to respond to the pathogens which may have a protective role in protecting the tissues from further damage and limits the inflammatory process.

Finally, it is important to highlight the fact that dental pulp from rat mandibular incisor can be regarded as a good source of mRNA even after 3 hour period of incubation, with evident tissue viability demonstrated by the response of the target genes with up or down regulation within that time scale.

Chapter 7. General discussion

From many aspects, dental pulp may be regarded as a unique tissue. That it is a loose connective tissue of mesenchymal origin is not unique, but the presence of highly specialised odontoblasts is. Odontoblasts align peripherally in a homogenised palisaded pattern in intimate contact with and encased by dentine, into which their cytoplasmic processes extend-another unique characteristic. This encasement means that the pulp has little opportunity to expand in response to inflammation (Heyeraas and Berggreen, 1999). The control of pulpal blood pressure and flow is of critical importance to its survival, and this is regulated by the action of chemical mediators and nerve fibres in the walls of pulpal blood vessels (Iijima and Zhang, 2002). Another unique property of the dental pulp is the absence of a collateral blood supply, making it reliant on a single microcirculatory system that progressively diminishes with age (Ikawa et al., 2003) and may hinder the pulp's ability to withstand insults and recover.

Although the dental pulp is encased within dentine, enamel and an investing periodontium, it is quite responsive to external stimuli. The details and purpose of its responses to external stimuli are incompletely understood.

The tooth is subject to a range of stimuli and insults, and after exposure of dentine, pulp tissue may be especially vulnerable to thermal, mechanical and microbial challenges. The effect of these stimuli is cumulative and may eventually harm the pulp both in reversible and irreversible ways.

The single greatest threat to the preservation of vital pulp functions is dental caries, and this is known to elicit a wide range of cellular and molecular reactions in response both to bacteria and their toxins, and the proteins liberated from dentine by the acidic carious process. One element of this is inflammation.

The advancing carious lesion will induce pulpal inflammation and consequently either necrosis or repair and regeneration, all of which requires sophisticated control and regulation (Cooper et al., 2010). Responses may be different to relatively mild and intense stimuli, indicating that control systems are at work (Cooper et al., 2010).

Elements of this include innate and adaptive immune responses (Jontell et al., 1998). Features of such responses include identified populations of MHC-I and MHC-II cells with their inherent ability to present antigens to antigen-recognising cells and to trigger

immune responses. One of these antigen-recognising cells is the odontoblast (Horst et al., 2011, Farges et al., 2011), and with its TLRs (Jiang et al., 2006, Mutoh et al., 2007), the odontoblast, or populations of odontoblasts can orchestrate differential responses to both Gram-negative and Gram-positive bacteria (Veerayutthwilai et al., 2007). More specifically, TLR-2 is crucial for the recognition of Gram-positive bacterial components (Underhill et al., 1999), while TLR-4 is responsible for the detection of Gram-negative bacterial components (Takeda and Akira, 2005). These two receptors are present in odontoblasts as well as the fibroblasts in the sub-odontoblast area (Farges et al., 2009).

In shallow to moderate carious lesions, Gram-positive bacteria dominate (Love and Jenkinson, 2002). However, once the caries has progressed more deeply, a shift in the microflora occurs, manifested as an increase in the anaerobic Gram-negative bacteria at the expense of aerobic Gram-positives (Hamilton, 2000). LPS is regarded as the most common bacterial cell wall component of Gram-negatives and is regarded as the most immune-stimulatory component of these microorganisms (Akira et al., 2006).

Once the presence of pathogenic elements is detected, an innate immune response will be initiated within the pulp, marked by the secretion of inflammatory mediators, chemokines (Bachmann et al., 2006), antimicrobial peptides (Pazgier et al., 2006) and pro-inflammatory cytokines (Veerayutthwilai et al., 2007). These will recruit immuneocompetent cells and inhibit dentine formation and calcification (Botero et al., 2006). The main immune-competent cells invading the affected area are immature DCs (Durand et al., 2006), resident and/or recruited macrophages and neutrophils. These cells, in addition to the odontoblasts and fibroblasts, will secrete a wide range of pro-inflammatory mediators, including: NO, IL1, IL6, TNF-α and PG (Kawashima and Suda, 2008, Kawashima et al., 2005). These mediators and molecules will interact with each other and determine the fate of pulpal inflammation, with the interaction between these molecules and mediators leading to further increase in their production in a vicious cycle. Therefore, within the low-compliance environment of the dental pulp, it is extremely important to regulate these mediators to avoid pulp necrosis and enable the conditions for pulp survival and regeneration (Nakashima et al., 2009). The elimination or control of inflammation is prerequisite for uncovering of the regenerative abilities of the pulp (Rutherford and Gu, 2000).

In the current collection of studies, the well-established and readily available rat mandibular incisor dental pulp was employed as an experimental model. It is recognised

that rat incisor pulp has many similarities, as well as differences, when compared to human tissues. Their continuous growth makes them an excellent model to study the different stages of hard tissue formation as well as the life cycle of cells in the dental pulp, with all stages from early development to maturation being observable in the same tooth (Ohshima and Yoshida, 1992). In addition, it has been reported that a single rodent tooth can provide adequate amounts of RNA for multiple gene expression analysis (McLachlan et al., 2003).

At present, the detailed structural and physiological relationships of cells within the pulp are incompletely understood, and efforts to promote reparative or regenerative therapies may depend on such knowledge.

Through the sections of this thesis, I have attempted to address some of these issues in the following way. In Chapter 3, the structural and histological architecture of the dental pulp was explored. Chapter 4 focused on the rather interesting finding of immunoreactivity to elements of the PG and NO pathways within the pulp. Functional correlation between the former two pathways as well as the effect of some stimulants on PGE_2 release was investigated in Chapter 5. Finally, Chapter 6 focused on the effect of the same stimulants on PG pathway elements as well as testing the relationship with other key molecules in the inflammation at the mRNA level.

In Chapter 3, work was focussed on developing a better understanding of cell populations, alignments and interrelations in different parts of the pulp. Underpinning this work was a desire to understand how structure may relate to function. Data from this work has illustrated the possibility of a heterogeneous cellular architecture in defined regions of the pulp, most strikingly in the odontoblast cell layer, which may previously have been considered relatively homogeneous although shows regional differences. In addition, cellular heterogeneity (in terms of different IR to different antibodies) was observed in cells that structurally appeared to be homogenous, notably the fibroblast population within the bulk of the pulp, e.g. cells IR^{+ve} to COX-1 or COX-2 and different from the surrounding cells with only vimentin IR. Furthermore, the IR to a single antibody appeared in varying degrees within the IR^{+ve} cells, including the cells with variable intensity and purity for COX-1 IR observed and illustrated in Figure 6-14 (C, c, D and d). Added to the heterogeneity, is the cellular communication between different cell layers within the pulp, in which cells from the SOL were seen to send cellular extensions between the OCB. Although this communication can be regarded as direct

structural communication between two distinct cell layers, it sheds little light on likely functionality.

Based on the observed complexity within the dental pulp, IR to key elements of PG and NO pathways and the previously reported interactions in other tissues, the interactions of these two pathways and their corresponding elements within the dental pulp were chosen to be the focus of this thesis, with observations under normal physiological conditions and in response to different stimuli. Nevertheless, other pathways and elements with a possible relation to the former two pathways were also explored for the possibility of interactions and in an attempt to reveal some of the hidden mechanisms.

In chapter 4, immunohistochemical methods were employed to investigate aspects of these relationships, where areas of COX-1 and COX-2 IR may be regarded as PG producing sites, and similarly, areas with NOS-1 IR were considered as NO producing centres.

Although this work revealed for the first time the precise localisation of IR to some elements of the PG and NO pathways, such as COX-1, NOS1 and prostanoid receptors, the observation of COX-2 IR cells in the normal pulp was the most mysterious as this has previously been associated only with inflammation. It has been reported before in other tissues (Vane et al., 1998) and is believed to have constitutive functions in regulating the blood flow in the states of crisis. This identification of COX-2 in normal pulp was further confirmed with q-RT-PCR. A possible interpretation is that the rat dental pulp may always have some degree of inflammation, possibly due to the daily attritional activity as its wearing incisal edge. It is also possible that it has a general housekeeping function, with roles including the regulation of blood flow and vascular tone in states of daily stress. In addition, COX-2 IR in experimentally inflamed pulp was less than expected, although gene expression showed a dramatic increase in COX-2 expression which may indicate that this increase was only on a transcriptional level within the given time scale.

Following successful immune-localisation of the key enzymes and receptors, a functional cross-talk between PG and NO was investigated in chapter 5 utilising ELISA technique. It has been suggested in this study that experimentally-induced inflammation led to an increase in PG release, whereas NO caused inhibition of PGE_2 release although it caused upregulation of COXs. A possible explanation for this is that NO may stimulate COXs but only on a transcriptional level within the given time scale, or it could lead to the

release of other types of PG not PGE_2. Similarly, PGE_2 caused upregulation of NOS1. This functional link between the two pathways was further highlighted by the close proximity of their producing elements within the dental pulp. Although this reciprocal relation has been reported before in the dental pulp (Borda et al., 2007) and other tissues (Borda et al., 2002), this is the first time that such links have been investigated with the array of techniques employed in these studies.

To test whether the chosen stimulants have an effect on PG and NO elements, in addition to a selection of other molecules with key roles in inflammation, q-RT-PCR was utilised to test this at an mRNA level. The wide selection of genes and stimulants explored in this study allowed investigation of some of the complex network of interactions between different molecules both under physiological and experimentally-inflamed conditions. It also highlights the fact that some of the basic interactions that were reported previously were not only due to direct effects but also indirect effects. For example, the LPS upregulatory effect on COX-2 may be both direct and indirect through the production of NF-kβ and MyD88 which also upregulates COX-2 (Gorina et al., 2011), or through the activation of IL1 which also involves COX-2 upregulation (Holt et al., 2005, Nakanishi et al., 2001), followed by a further interplay between PGE_2 and IL1.

Additional complexity could be uncovered when examining the stimulatory effect of LPS on IL1 which may lead to upregulation of IL6 (Deb et al., 1999, Tsakiri et al., 2008) followed by a reciprocal stimulatory effect of IL6 on IL1 (Minogue et al., 2012). At the same time IL6 caused an increase in PGE_2 release (LIU et al., 2006) and PGE_2 causes an increase in IL6 (Li et al., 2002) as well as other cytokines (Sheibanie et al., 2004). These experiments also led to some less-expected results, including the mild effect of LPS on TLR-4 gene expression, but this was in accordance with published literature (Farges et al., 2009).

Observations from the current study may help to improve understanding of the complexity of the inflammatory process within the dental pulp, in an effort to regulate and control this process and to limit unnecessary tissue damage in the face of insult. This control will aid in the maintenance of pulpal integrity and health in cases of inflammatory conditions and/or regenerate and repair the damaged pulp tissue in the course of inflammation. There is growing interest by researchers and clinicians to understand and implement therapies for the regeneration and repair of the dental pulp (Murray et al., 2007, Sloan and Smith, 2007).

It is recognised that in all of these investigations, the challenging and confounding effects of direct microbial infection were not included.

Chapter 8. Suggestions for future work

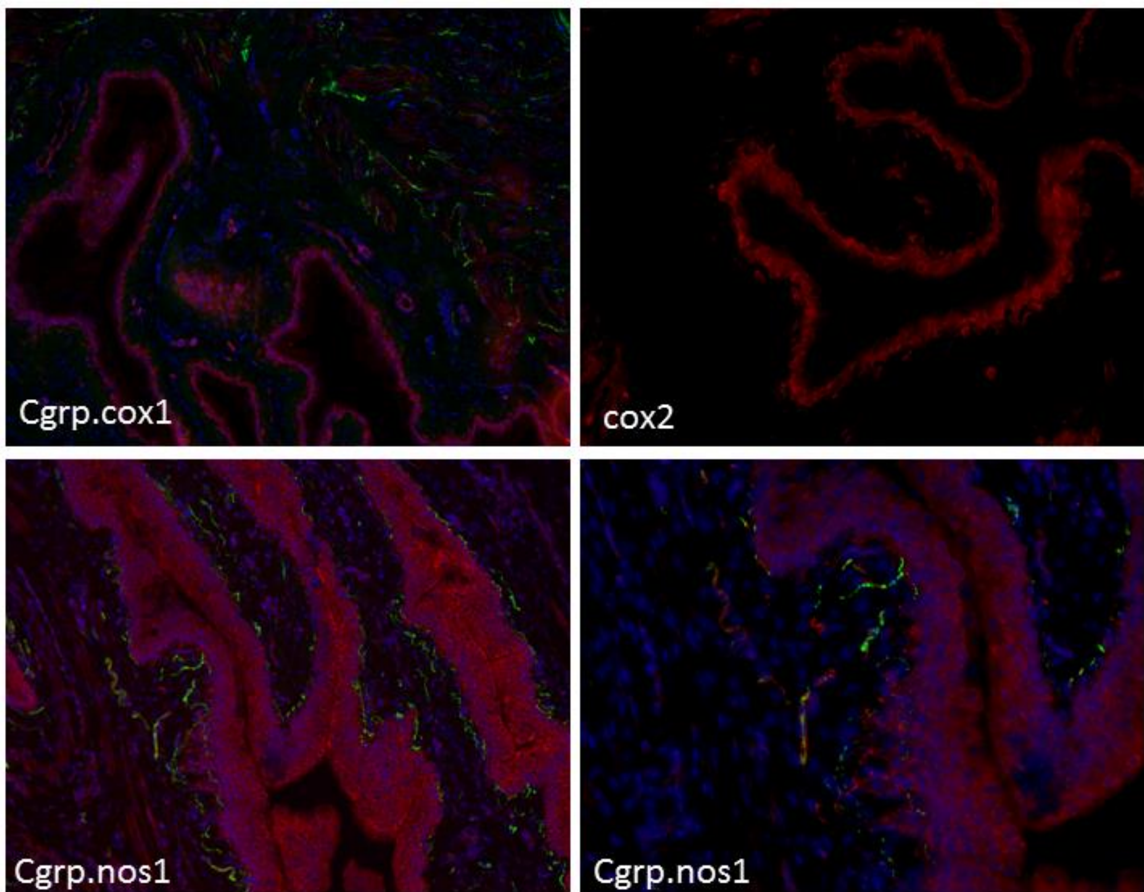
Key findings in the current collection of investigations highlight opportunities for further research that may be considered within the following areas:

1. Investigation of additional elements of the PG pathway, such as PG transporters, as well as other types of PG in experimentally-inflamed and NO-incubated pulp tissues to detect whether these transporters will change their expression during the course of inflammation and to test the effect of stimulants on other types of PG.
2. Analysis of the effects of different COX inhibitors on PG release under the influence of different stimulants to recognise the enzymes responsible for PG production under specific conditions. This line of investigation builds on studies reported in Chapter five and may help to develop novel therapeutic approaches to modulate inflammatory processes within the dental pulp.
3. Evaluation of the effects of different stimulants on PG release using COX and EP knockout rats to analyse changes in response. This line of investigation builds on studies reported in Chapter five and may help to provide insight on key elements of the pulp's inflammatory response in the face of injury and opportunities for its therapeutic modulation.
4. Investigation of the amount of PGE2 release from the dental pulp utilizing a longer incubation time and longer recovery time. This will help (added to findings from chapter five) to provide a better understanding to the way the PG pathway is stimulated and recovered in response to external stimulations.
5. Comparing results from pulp tissues and different cell lines to detect whether laboratory cell lines are beneficial as tissue replacements for investigation and therapy and to test their ability to reflect the behaviour of original tissue responses. This work would build on investigations reported in Chapter five and six and may provide evidence to support or undermine the use of such models in the study of pulp tissue structure and function.

Repeating elements of the work included in this thesis on human dental pulp specimens to examine cellular relationships, diversity and communication (Chapters three and four), as well as functional and molecular interactions between PG and NO pathways under different incubation conditions and with tissue specimens of different ages. This work may lead to the development of novel drugs and materials that may be of value in

promoting the preservation of vital pulp functions, or the repair and regeneration of tissues that have been damaged.

Appendix:



External positive immunohistochemistry images for some key antibodies used in this thesis.

The work I am illustrating is where I am the first author.

A. Published work: Two papers, first author in one and second in the other one.

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Complexity of Odontoblast and Subodontoblast Cell Layers in Rat Incisor

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Keywords: Odontoblast, Pulp biology, Fibroblast, Cell biology, Complexity, Cell signalling

ABSTRACT

Structural and functional interrelationships between odontoblast (OCL) and sub-odontoblast cell layers (SOCL) are relevant to tissue formation and regulation in health and disease, but are incompletelyunderstood. This study employed contemporary immunohistochemical techniques to gain important new insights on tissue complexity within the apical half of the rodent mandibular incisor.

Whole incisors were removed from freshly killedWistar rats, fixed in 4% paraformaldehyde,demineralized, and prepared for standard immunohistochemistry. Tissues were labelled with primary antibodies to vimentin, α actin, NaKATPase, alpha tubulin and COX 1, and secondary antibodies alexafluor 488 and 594, before examination by fluorescence microscopy.

Odontoblasts presented in stratified or pseudo-stratified arrangements, rather than simple columnar form. Additional stellate cells,α-actin immunoreactive, were observed predominantly in the distal half of the OCL.

Cells in the cell rich zone (crz) also showed complexity, with high cell density, and cells presenting with large nuclei and sparse cytoplasm. These cells showed intense NaKATPase immunoreactivity.

Some of these cells sent processes towards and into the OCL, whilst other processes remained within the crz. No processes directed downwards.

The OCL of the rat mandibular incisor presents a hitherto undescribed complexity, in terms of cellular arrangement and composition, the functional significance of which is not immediately apparent.

The observation that cellular processes from crz tothe OCL and lateral processes within crz suggest functional connectivityand cell to cell communication within the layer. The intense immunoreactivity to NaKATPase suggests functional specification and a high degree of tissue activity. Structural observations will require further investigations.

INTRODUCTION

The odontoblasts represent a specialized cell population with primary roles in the secretion and mineralization of dentine matrix. Further roles include regulation dentinal of dentine permeability Bishop [1], involvement of immuneresponses Horst et al.

B. Conference participation:

I had participated in the 2015 IADR/AADR/CADR General Session (11-14 March 2015) in Boston- USA with two posters (first author in one and second author in one) which was published in online version of Journal of Dental Research.

1. Complexity of Odontoblast and Sub-Odontoblast Cell Layers in Rat Incisor.

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Affiliation: [1] Centre for Oral Health Research, [2] Institute of Cellular Medicine, [3] School of Dental Science.

Objectives: Odontoblast and sub-odontoblast cell layers (OCL and SOL) have been studied extensively. However complex structural interrelationship and functional interactions between these layers are not understood. The aim of this study was to use rat mandibular incisor to highlight this complexity in both structure and function.

Methods: Pulp tissues were surgically extracted, fixed in 4% paraformaldehyde, demineralized, and prepared for standard immunohistochemistry. Antibodies to vimentin, α actin, NaKATPase, and PGP 9.5, were used followed by alexafluor 488 and 594, and then examined using fluorescent-confocal microscopy.

Results: The observations suggest that the odontoblasts are not simple columnar cell, but stratified or pseudo-stratified. In addition, there appear to be other cell types within OCL, these are stellate and positive to αactin located predominantly in the distal half of OCL. There is avascular network of small arterioles, with capillaries network located near the mineralizing front. Cells in SOL also show more complex structure, the cell density is high there, composed of cells with large nuclei and sparse cytoplasm. These cells show intense NaKATPase immunoreactivity. Some of these cells send processes towards and into the OCL, other processes remain within SOL. No processes where seen to enter to the diffuse cell layer of the bulk of the pulp. In keeping with other studies, no nerves were found in the OCL and SOL.

Conclusion: OCL demonstrate structural complexities and heterogeneity for which the functions are not immediately apparent. SOL has similar complexities. The observations that these cells send processes into OCL suggest some functional connectivity. Also lateral processes may suggest cell to cell communication within the layer. In additions, the intense immunoreactivity to NaKATPase suggests functional specification and high degree of activity.

Complexity of Odontoblast and Sub-Odontoblast Cell Layers in Rat Incisor

3182

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Introduction and Aim of the study

Structural and functional interrelationships between the odontoblast and sub-odontoblast cell layers (od & sub-od) are relevant to the understanding of tissue formation, function and regulation in health and disease, but are incompletely understood.

This study employed contemporary immuno-histochemical techniques to gain new insights on the structural and functional complexity within the od and sub-od in the apical half of the rodent mandibular incisor.

Methods

Pulp tissues were removed from the lower incisors of freshly culled Wistar rats, fixed in 4% paraformaldehyde, demineralised (4-6 weeks)[1], and prepared for standard immuno-histochemistry. Tissues were labelled with primary antibodies to vimentin, α-actin, COX-1, tubulin, NaKATPase, and secondary antibodies alexafluor 488 and 594 before examination by fluorescence-confocal microscopy. Images were analysed with Cell F and Image J software.

Results

Odontoblasts presented in stratified or pseudo-stratified arrangement, (Figure 1-A), rather than simple columnar formation as described previously[2]. Additional cell types were observed within the od, which were stellate in form, positive to α-actin, and located predominantly in the distal half of the od as shown in Figures 1-B and 1-C (arrows).

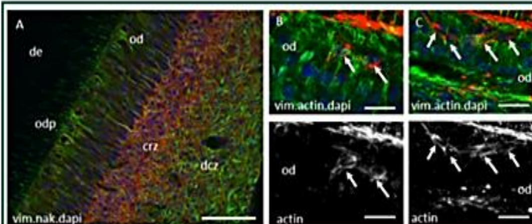

Figure 1: A overview of the cellular components and dentine of the apical half of demineralised rat incisor tooth. B & C show the α-actin IR cells within the od.

The sub-od can be divided into cell rich and diffuse cell zones (crz & dcz). Cells in the crz also showed complexity, with high cell density, and cells presenting with large nuclei and sparse cytoplasm. These cells showed intense NaKATPase immunoreactivity (Figure 2-A). Some of these cells send processes towards and into the od (Figure 2-A & B, arrowed), whilst other processes remained within the crz. No processes were seen to enter the dcz within the body of the pulp.

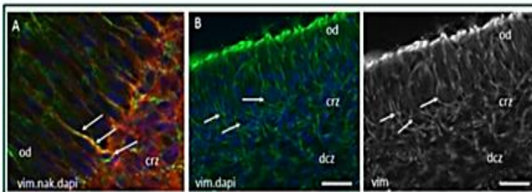

Figure 2: A demineralised and B non-demineralised sections of rat mandibular incisor showing cellular processes between the od and crz.

Cells within the dcz close to the core of the pulp showed cellular heterogeneity with the observation of COX-1 IR cells (Figure 3-A), tubulin IR cells (Figure 3-B) and tubulin IR structures or aggregates of cells (Figure 3-C).

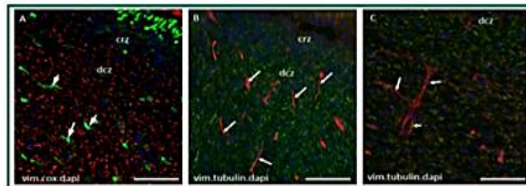

Figure 3: Isolated pulp tissue from the apical half of a non-demineralised rat incisor, showing cellular heterogeneity in the sub-od area.

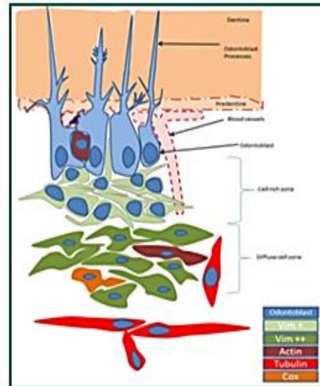

Figure 4: A cartoon summarizing the structural complexity and cellular heterogeneity within the od and sub-od cell layers of the apical half of the rat mandibular incisor.

Discussion

The pseudo-stratified appearance of the od may suggest that not all odontoblasts are in contact with the dentine or that the odontoblasts may work in a successive manner, with some odontoblasts secreting dentine while others rest.

The presence of different cell types within the od, the cellular communication between crz and the od and among the cells of crz themselves, in addition to cellular heterogeneity within the dcz may indicate the complex functional, sensory, defensive and/or regulatory roles of these cells.

Conclusion

- The od of the rat mandibular incisor presents a hitherto undescribed complexity, in terms of cellular arrangement and composition.
- The observation that cells within the sub-od send processes into the od suggests functional connectivity, and the identification of lateral processes may also suggest cell-to-cell communication within the layer.
- The intense immunoreactivity of crz to NaKATPase suggests functional specification and a high degree of tissue activity.
- The occurrence of such features within the human pulp demands further investigation.

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وزارة التعليم العالي والبحث العلمي

2. I participated in the 17th Biennial ESE Congress- Barcelona, Spain; held in September 2015 by submitting a full paper to Wladimir Adlivankine ESE Prize, my participation was an oral presentation.

Evidence for nitric oxide and prostaglandin signalling in the regulation of odontoblast function in identified regions of the rodent mandibular incisor

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3. I had participated in the International Federation of Endodontic Associations (IFEA) conference (3-6 June 2016) with two posters, first author in one of them and second author in the other one.

Title: Immunolocalisation and gene Expression of nitric oxide synthase, cyclooxygenase and prostaglandin E2 receptors in the rat mandibular incisor

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Affiliations: 1 Centre for Oral Health Research, 2 Institute of Cellular Medicine, 3 School of Dental Sciences, Newcastle University, Newcastle upon Tyne, NE2 4BW, UK.

Objectives: Therapies to promote pulp repair and regeneration after injury should be underpinned by a deep understanding of normal tissue behaviour, cellular cross-talk and regulation. This study employed immunohistochemical and quantitative reverse transcription polymerase chain reaction (q-RT-PCR) methods to investigate the expression of nitric oxide synthase 1 (NOS1), cyclooxygenase 1 (COX1) and prostaglandin E2 receptors (EP1 and EP2) within the dental pulp to explore the possibility of complex nitric oxide/prostaglandin signalling pathways in the rat mandibular incisor.

Methods: Fresh pulp tissue was obtained from freshly culled Wistar rats (9 weeks age), fixed in 4% paraformaldehyde, demineralized, and prepared for standard immunohistochemistry using fluorescence-confocal microscopy. q-RT-PCR was conducted on pulp tissues extracted from animals of same age.

Results: COX1 immunoreactivity (IR) observed in specific regions within the odontoblasts just above the nuclei and in distinct population of cells within the pulp core. NOS1-IR and EP2-IR was observed in the distal part of odontoblasts and weakly in the cell rich zone. Gel electrophoresis images showed clear products bands within the specified base pair lengths. q-RT-PCR showed a clear products curve with a very distinguishable threshold cycles (*Ct*) values.

Conclusion

This investigation provided evidence of complex cellular signalling and cross-talk between different cellular layers within the rodent incisor dental pulp, which may be important in regulation and control of odontoblasts and subodontoblasts functions.

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Immunolocalisation and gene Expression of nitric oxide synthase, cyclooxygenases and prostanoid receptors in the rat mandibular incisor

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Introduction

Therapies to promote pulp repair and regeneration after injury should be underpinned by a deep understanding of normal tissue behaviour, cellular cross-talk and regulation. This study employed immunohistochemical and quantitative reverse transcription polymerase chain reaction (q-RT-PCR) methods to investigate the expression of nitric oxide synthase 1 (NOS1), cyclooxygenase enzymes (COX-1 and COX-2) (collectively called COXs) and prostanoid receptors (EP1 and EP2) within the dental pulp to explore the possibility of complex nitric oxide/prostaglandin signalling pathways in the rat mandibular incisor.

Methods

- Fresh pulp tissue was obtained from freshly culled 9 week old Wistar rats, fixed in 4% paraformaldehyde, demineralized or the pulp extracted, and prepared for standard immunohistochemistry using fluorescence-confocal microscopy.
- Conventional and q-RT-PCR was conducted on pulp tissues extracted from animals of the same age utilising gel electrophoresis and SYBR green technology respectively.

Results

COX-1 immunoreactivity (IR) was found in only discrete regions of the odontoblast cytoplasm (Figure 1 A), in some cells of the subodontoblast cell layer and in the walls of centrally-located blood vessels (Figure 1 B). COX-2 IR were found only in a small population of cells within the pulp (Figure 1 C). EP1 IR was found in the walls of blood vessels and few cells (Figure 1 E). EP2 and NOS1 IR was observed in the distal part of the odontoblast cell body, weakly in the proximal region of odontoblast processes and in cells of the subodontoblast cell layer (Figure 1 D and F). Well defined bands of appropriate base pair for the target genes were detected as by PCR gel electrophoresis Figure 2. q-RT-PCR provided further confirmation, detecting abundant evidence of target genes after amplification (Figure 3).

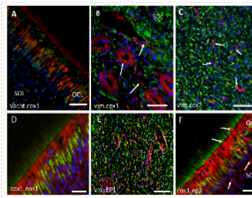

Figure 1: Distribution of COX-1, COX-2, EP1, EP2 and NOS-1 in sections from the rat mandibular incisor dental pulp. Calibration bars are 60 µm in A and 40 µm in all other images.

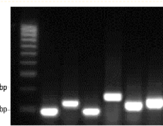

Figure 2: Conventional PCR gel electrophoresis of rat mandibular incisor dental pulp (n=5), show bands of appropriate base pair (bp) (as correlated to a ladder to the left) for all the target genes. β-actin was used as positive internal control.

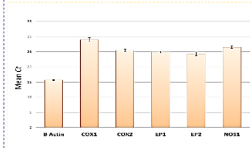

Figure 3: q-RT-PCR quantitation for the target genes within normal rat dental pulp. Samples operated in duplicates in the presence of β-actin as a normaliser. The level of gene expression was calculated after normalising against β-actin in each sample and is presented as relative mRNA expression units. Note that *Ct* values are inversely proportional to the actual mRNA expression. Values are mean ± SE (n=10 per gene).

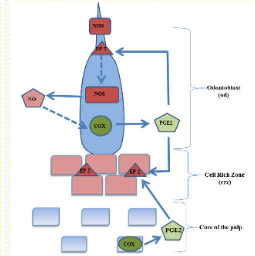

Figure 4: Cartoon illustrating the distribution of COXs, prostanoid receptors and NOS in the odontoblasts and sub-odontoblast layers of the dental pulp of the rodent mandibular incisor.

Discussion

Although the presence of COXs, prostanoid receptors and NOS in the dental pulp has been described before (Borda et al. 2007), their precise location is uncertain. The current study provides new immunohistochemical evidence for the exact location of COX-1, COX-2, NOS-1, EP1 and EP2 IR within the apical half of the rat mandibular incisor dental pulp, in addition to the genetic expression of these genes.

It is possible that prostaglandin (PG) synthesised within the odontoblast cell layer may be liberated to act on EP2 receptors, either within the odontoblast cell layer or on cells subjacent to the odontoblasts. Furthermore, PG produced by the COX-1 IR cells within the pulp core may work on EP2 receptors in the cells immediately beneath the odontoblasts, whereas the PG produced in the walls of blood vessels may act locally to control pulpal blood flow or pressure as seen in other tissues (Beaudin et al. 2014).

It is logical to believe that the nitric oxide (NO) produced from the NOS-1 IR regions may further activate or inhibit COXs activity within the odontoblast or subodontoblast region, whereas the NO produced from the walls of blood vessels may work to regulate pulpal blood flow, as reported in other tissues (Yoshioka et al. 2015).

Conclusion

- Immunoreactivity to COX-1, COX-2, NOS1, EP1 and EP2 are present in specific regions of the rat mandibular incisor dental pulp.
- Components of PG signalling pathway are located in close proximity to each others and to NOS component of NO pathway.
- There is evidence of possible complex cellular and molecular interaction between odontoblasts, odontoblast and subodontoblast cells and between odontoblasts and their processes through the dentine.
- It is suggested that these interactions may regulate or control odontoblast and subodontoblast cell functions and might be involved in other sensory and regulatory functions within the odontoblast and subodontoblast cell layers that are devoid of neural components.
- To the best of our knowledge, this is the first time to exactly localise the immunoreactivity to COX-1, NOS-1, EP1 and EP2 within the normal rodent dental pulp.

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