Dental Pulp Response to Hydrogen Peroxide and its Potential in the Treatment of Dental Cavities

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Acknowledgements

Abstract

During caries progression, an interaction between the dental pulp, the bacteria and their by-products and the demineralized matrix components can lead to new dentin matrix deposition. The production of tertiary dentin requires a low level of inflammation that enhances the reparative response. There is evidence to suggest that low-grade oxidative stress could have similar results (Lee et al. 2006). It is also known that control of the infection is a prerequisite for vital pulp therapies to be successful. The aim of this project is therefore to explore the potential to use hydrogen peroxide in deep cavities to both eliminate infections and encourage regeneration.

The potential biocidal effect of H_2O_2 to treat dentin infections was assessed by determining the Minimal Inhibitory Concentration (MIC) of H_2O_2 against *E. faecalis, S. anginosus* and *S. mutans.* The viability of dental pulp fibroblasts to these bactericidal concentrations was then studied using an MTT assay. Additionally, a suspension test was carried out to study the inactivation kinetics of the microorganisms when subjected to a clinically relevant exposure time of H_2O_2. Changes in the bacterial cell wall structure were also evaluated using Scanning Electron Microscopy (SEM) imaging. A validated *ex vivo* tooth slice model (Sloan et al. 1998) was also used to study the potential use of H_2O_2 in enhancing a regenerative response. Tooth slices were exposed to H_2O_2 and the dental pulp response was established by viable histological cell counts and immunohistochemistry for inflammatory (TNFα and IL-1β) and regenerative markers (DSPP and PCNA).

Results: MIC of H_2O_2 was 1,250ppm for *E. faecalis, S. anginosus* and *S. mutans*. Dental pulp fibroblast viability was reduced significantly when exposed to bactericidal concentrations of H_2O_2 for 60 seconds or 5 minutes. The bacterial count was not reduced after 5 minutes exposure to 1,000ppm H2O2 and no structural changes were observed using SEM. Tooth slices exposed to 1,000ppm or 300ppm H_2O_2 for 60 seconds or 5 minutes showed no significant reduction in cell counts. Immunohistochemistry showed the presence of inflammation in the vasculature and odontoblast layer, and the expression of dentin extracellular matrix protein DSPP in the odontoblast layer.

In conclusion bactericidal concentrations of H_2O_2 are cytotoxic to dental pulp cells cultured in monolayer. Moreover, at clinically relevant time exposures to H_2O_2 for decontaminating cavity preparations, the bacterial count was not reduced. However, results from this study suggest there may be a potential use for H_2O_2 to induce dental pulp regenerative response.

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Abbreviations

DPP: dentin phosphoprotein

DSP: dentin sialoprotein

DSPP: dentin sialophosphoprotein

DGP: dentin glycoprotein

SIBLINGs: Small Integrin-Binding Ligand N-linked Glycoproteins

ECM: extracellular matrix

TGF-β: transforming growth factor –β superfamily

IGF-1 and -2: insulin growth factor 1 and 2

FGF-2: fibroblast growth factor-2

TGF: transforming growth factors

FGFs: fibroblast growth factors

ILGF: and insulin-like growth factors

DPSCs dental pulp stem cells

PRRs pattern recognition receptors

TLRs: such as the Toll-like receptor family

PAMPS: pathogen-associated molecular patterns

LPSs: lipoteicoic acids, lipopolysaccharides

PMNs: polymorphonuclear leukocytes

IL: interleukins (IL-1β, IL-6, IL-8, IL-10)

TNF: tumor necrosis factors

S. mutans: Streptococcus mutans

E. faecalis: Enterococcus faecalis

SAG: *Streptococcus anginosus group: S. anginosus*, *S. constellatus* and *S. intermedius*

PCR: polymerase chain reaction

ROS: reactive oxygen species

H_2O_2: hydrogen peroxide

O_2^-: superoxide anion

$\cdot OH$: hydroxyl radical

O_2: molecular oxygen

MTA: mineral trioxide aggregate Brain

BHI: Heart Infusion Broth

TSA: Tryptone Soya Agar

CHX: Chlorhexidine digluconate

MICs: Minimum Inhibitory Concentration

MBC: Minimum Bactericidal Concentration

rDPFs: Rat Dental Pulp Fibroblasts

αMEM: Alpha Modified Essential Medium

DMEM: Dulbecco's Modified Eagle Medium

FBS: Foetal bovine serum

MTT: Thiazolyl Blue Tetrazolium Bromide solution

SEM: Scanning Electron Microscopy

H&E: haematoxylin and eosin

TBS: Tris-buffered saline

NHS: Normal Horse Serum

cDNA: Complementary DNA

SD: standard deviation

SE: standard error of the mean

ANOVA: one-way analysis of variance

1. Introduction

1.1 General Introduction

The economic implication of dental emergency visits in the United States is reported to cost almost US$1 billion per year (Allareddy et al. 2014). Dental caries and periodontitis are the most prevalent diseases in the oral cavity (Frencken et al. 2017). If dental caries is left untreated this would ultimately affect the dental pulp causing inflammation first and then necrosis with further damage of the tooth supporting structures.

Caries is a multi-etiological disease that involves the infection of the tooth calcified tissues causing demineralization. During the disease progression, an interaction between the dental pulp, the bacteria and their sub-products, and the demineralized matrix components could lead to new matrix deposition. This depends on the aggressiveness of the lesion and the type of inflammation that provokes (Cooper et al. 2010). Understanding the molecular events during such processes are the basis for regenerative treatment approaches. Additionally, in order for this to be successful, the infection has to be controlled.

This introduction will outline the dental tissues and how they respond to different stimuli, as well as the molecular basis of pulp inflammation. Moreover, it will describe the oral microbiology, some of the organisms responsible for tooth infection, and the current strategies for vital pulp treatment. Finally, the relation between the dentin-pulp complex and its behaviour with hydrogen peroxide will be discussed.

1.2 Dental Tissues

The tooth, as depicted in Figure 1.1, is composed of mineralized tissues, enamel, and dentin, with a core of soft connective tissue called the dental pulp. The supporting tissues, which consist of cement, the periodontal ligament and the alveolar bone, attach the teeth root to the mandible and the maxilla. These allow physiological movement of the teeth that helps to support the forces of mastication.
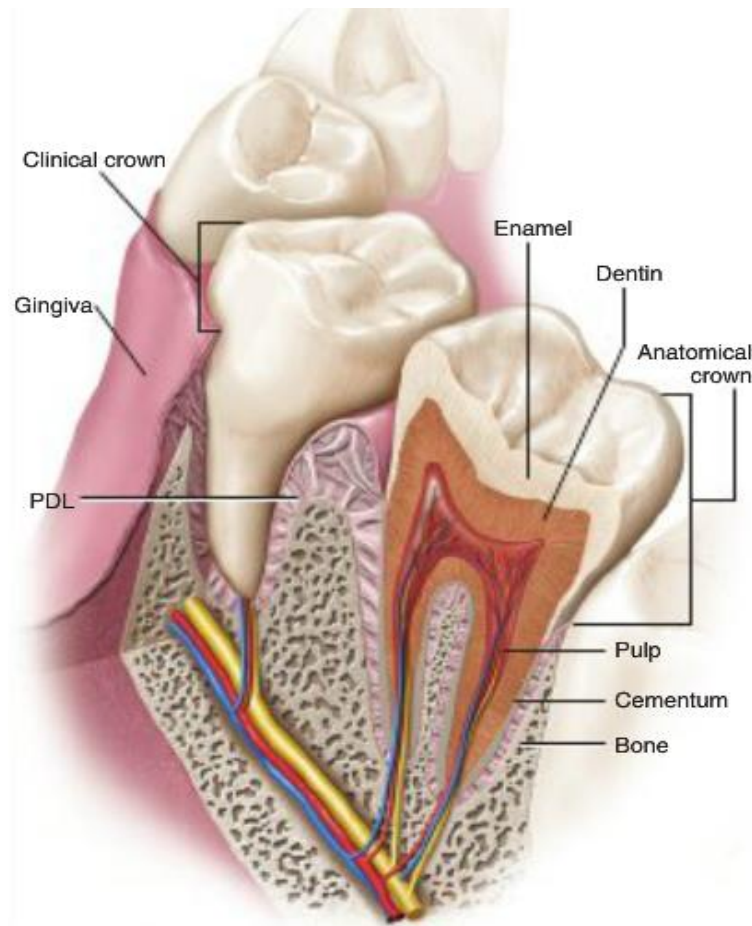
Figure 1.1. The tooth tissues: enamel, dentin and pulp, and the supporting structure: periodontal ligament (PDL), bone and cementum. (Nanci 2018)

Teeth are ectodermal organs derived from a series of interactions between the oral epithelium and mesenchymal cells derived from the neural crest (Chai et al. 2000). Epithelial cells give rise to ameloblasts that are responsible for the enamel production whereas the mesenchymal cells differentiate into all the other terminally differentiated cells that form the tooth, odontoblasts, pulp and periodontal ligament. Tooth location within the dental arch, shape and size are regulated by a precise spatial and temporal expression of signalling molecules, homeobox genes and transcription factors (Thesleff and Sharpe 1997). The enamel covers the coronal part of the tooth, is the hardest matrix of the human body and is composed of 96% mineral, mostly hydroxyapatite and 4% of organic material and water (Nanci 2018). It is inert and acellular since ameloblasts suffer a series of changes once the enamel is completely formed, finally disappearing during tooth eruption. Therefore, the enamel does not have regenerative capability (Nanci 2018).

1.2.1 The Dentin-Pulp Complex

Dentin and pulp share the same embryological origin and once the tooth is completely formed, these two tissues interact closely with each other. Moreover, unlike enamel, dentin continues to be produced after the complete formation of the tooth. This is because, in post-natal dental pulp, a heterogeneous cell population exists that is capable of maintaining the pulp structure and secreting dentin matrix. Therefore, odontoblasts are responsible for dentin existence and the pulp is protected by the dentin and the enamel. Likewise, if the dentin is affected then the pulp will respond accordingly; and any disturbance within the pulp will affect the dentin produced (Hargreaves et al. 2016). These are the main reasons why authors refer to these two tissues as the dentin-pulp complex (Cooper et al. 2010; Smith et al. 2016; Smith and Cooper 2017).

DENTIN

Underneath the enamel is the dentin. This is formed by odontoblasts and is a less mineralized tissue, which is composed of approximately 70% inorganic material, 20% organic material and 10% water (Nanci 2018). Its main characteristic is the presence of s-shaped dentinal tubules that contain the odontoblastic processes. These traverse the whole thickness of the dentin from the pulp chamber to the dentin-enamel junction, making the dentin highly permeable, allowing the diffusion of nutrients through its thickness, as well as enhancing the progression of dental caries and the pulpal response to restorative procedures (Nanci 2018).

Odontoblasts secrete predentin, a layer of unmineralized matrix of 10 to 50 μm thick composed mostly of collagen. As it mineralizes, new matrix is secreted maintaining the thickness of predentine constant. The inorganic component of mature dentin consists of small plates of hydroxyapatite. The organic phase is 90% collagen and 10% of non-collagenous proteins. Most of the structural collagen is type I collagen fibrils, which behaves as a scaffold that holds the mineral, with a small fraction of type III and V collagen.

The non-collagenous matrix proteins of dentin are the phosphorylated proteins: dentin phosphoprotein (DPP), dentine sialoprotein (DSP), dentin glycoprotein (DGP), dentin matrix protein-1 (DMP1) and osteopontin that together form the family of Small Integrin-Binding Ligand N-linked Glycoproteins (SIBLINGs). SIBLINGs act in signalling as they include the RGD motif. Moreover, they play a crucial role in calcification of the predentin by regulating hydroxyapatite crystal formation (Cooper et al. 2010). DPP, DSP and DGP are expressed as a single molecule called dentin sialophosphoprotein (DSPP) that is cleaved immediately after translation (Nanci 2018). DSPP mutations are responsible for dentinal conditions in primary and permanent dentition such as dentinogenesis imperfecta (Schulte and Van Waes 2007). DSPP is synthesized exclusively by odontoblasts and dental papilla cells, and therefore it is widely used as a marker to identify odontoblast differentiation within the dental pulp (Butler et al. 1992; Babb et al. 2017).

Other non-collagenous proteins present in dentin extracellular matrix (ECM) are the non-phosphorylated proteins: osteonectin (SPARC), osteocalcin and small leucine-rich proteoglycans (SLRPs), such as decorin and biglycan, that also play an important role in regulating mineralization. Additionally, bioactive growth factors are present in dentin ECM and are sequestered during the mineralization process. These are members of the transforming growth factor –β superfamily (TGF-β), insulin growth factor 1 and 2 (IGF-1 and -2), fibroblast growth factor-2 (FGF-2) and some angiogenic growth factors (Cooper et al. 2010). Their liberation after demineralization of the dentin triggers the regenerative process.

Classically three types of dentin are described in relation to the moment when it is formed. Primary dentin, which forms most of the tooth, is displayed contouring the pulp chamber. Once the tooth is completely formed, odontoblasts continue to secrete dentin through life along the dentin-pulp border. This type of dentin is called secondary dentin. Tertiary dentin is formed at specific sites in response to diverse stimuli, such as caries, attrition or dental procedures. Tertiary dentin can be formed by upregulation of pre-existing primary odontoblasts stimulated by a mild injury, called reactionary dentin, or reparative dentin formed by odontoblast-like cells that differentiate from stem cells in response to an intense injury (Smith et al. 2012; Nanci 2018).

PULP

The dental pulp is the soft connective tissue that supports the dentin, which consists predominantly of odontoblasts, fibroblasts, undifferentiated ectomesenchymal cells, macrophages and other immune cells. From the periphery to the centre, the dental pulp has four distinctive zones: the odontoblastic zone, the free zone of Weil, a cell-rich zone and the pulp core with the major blood vessels and nerves. The pulp ECM consists mainly of type I and type II collagen and ground substance (glycosaminoglycans, glycoproteins and water) produced by pulp fibroblasts (Nanci 2018).

Odontoblasts are the most unique cells of the dental pulp and are situated forming a layer that lines the periphery of the pulp. Odontoblasts are terminally differentiated from dental papilla cells that are in contact with the basement membrane of the dental epithelium. Odontoblast differentiation implies leaving the cell cycle, they polarize and have transcriptional and translational changes. Finally, as specialized and terminally differentiated cells, they synthesise and secrete dentin matrix. Odontoblast differentiation is caused by a series of coordinated events regulated by transcription factors but ultimately induced by cell-ECM interactions as well as signalling events. The latter are mediated by molecules such as TGFs, FGFs and ILGF that are immobilized in the basement membrane (Ruch et al. 1995; Sloan and Smith 2007).

Mesenchymal stem/progenitor cells have been isolated from the dental pulp, thus termed dental pulp stem cells (DPSCs), and have shown to have high proliferative behaviour, the capacity of self-renewal and multi-lineage differentiation capability (Gronthos et al. 2002). These cells have been shown to generate a dentin-pulp like complex when implanted *in vivo* in an "odonto"-conductive scaffold (Gronthos et al. 2000). It is believed that DPSCs reside in perivascular niches (Shi and Gronthos 2003), and in order to differentiate into odontoblast-like cells, have to be recruited into the site of injury.

1.3 Pulpitis

Pulpitis is the inflammation of the dental pulp and is classified as reversible, symptomatic or asymptomatic irreversible pulpitis (Glickman 2009). Although the diagnosis is established after pain history and clinical and radiographic examination, recent studies have found a correlation between the clinical diagnoses and the real histopathologic state of the pulp (Ricucci et al. 2014). Therefore, this could help to establish better therapeutic strategies.

Pulp inflammation occurs in response to diverse stimuli, such as trauma, restorative procedures and exposure to dental caries. Dentin infection causes the release of bacterial by-products and dentin matrix components, released as a consequence of the acidic environment, that elicit an inflammatory and immune response. Odontoblasts first and then pulp fibroblasts, endothelial cells and stem cells, recognise antigens present in both gram-positive and gram-negative bacteria, this occurs via pattern recognition receptors (PRRs), such as the Toll-like receptor family (TLRs). These recognise pathogen-associated molecular patterns (PAMPS), such as lipoteichoic acids, lipopolysaccharides (LPSs), flagellin and bacterial DNA and RNA. Activated TLRs trigger the immune response via the NF-κB and p38 mitogen-activated protein (MAP) kinase intracellular pathways (Pevsner-Fischer et al. 2007; Hargreaves et al. 2016). Finally, the cells produce cytokines that together with microorganism by-products and the dentin matrix components, act as chemoattractants to immune cells.

First, a focal accumulation of polymorphonuclear leukocytes (PMNs) and monocytes occurs mediated by odontoblasts (the first cells to be in contact with foreign antigens), and dendritic cells. This first response is non-specific, PMNs phagocyte antigens and monocytes differentiate into macrophages. Macrophages act by endocytosis and phagocytosis, ingesting dead cells and foreign bodies and degrading them with lysosomal enzymes. Macrophages also act as antigen presenting cells (Yoshiba et al. 2003) and releasing cytokines and growth factors that perpetuate the immune response (Hargreaves et al. 2016). As caries advances deep into the dentin, the inflammatory response increases as shown in a significant upregulation of the humoral response mediated by B cells and plasma cells (Izumi et al. 1995). Additionally, the release of angiogenic factors stimulates the new capillary vessels formation, together with nerve fibre co-aggregation with antigen-presenting dendritic cells (Yoshiba et al. 2003).

Pulp innervation also plays an important role in the inflammatory response. Bacterial antigens stimulate sensory receptors and induce the afferent neurons to release neuropeptides. These recruit and activate immune cells. This event is termed neuronal inflammation. Interestingly, these neuropeptides could be involved in dentin regeneration by regulating angiogenesis or stimulating osteodentin deposition (El Karim et al. 2009).

CYTOKINES

Cytokines are proteins that mediate paracrine and autocrine signalling. These are pro-inflammatory cytokines, including interleukins (IL-1β, IL-6, IL-8), anti-inflammatory cytokines (IL-10) that limit the immune response to antigens and promotes the development of Tregs, tumor necrosis factors (TNF-α) and interferons (IFN-γ).

Advanced carious lesions stimulate pulp inflammation, which is characterized by a significant increase of cytokines and chemokines released by odontoblasts, fibroblasts, DPSCs and endothelial cells. (Cooper et al. 2010; Michel et al. 2015). The quantity and type of cytokines expressed in a healthy pulp or in reversible and irreversible pulpitis have been subject of research in several studies (Zehnder et al. 2003; Rechenberg et al. 2016; Zanini et al. 2017).

Immune cells activated by bacterial by-products and matrix components secrete TNF-α and Il-1β. The latter is fundamental for pulpitis progression as it stimulates the further production of TNF-α by oral fibroblasts (Piesco et al. 1995). TNF-α induces chemotaxis and activation of leukocytes by influencing the dilatation and an increased permeability of blood vessels that causes the extravasation of these cells into the affected area. As shown in an *in vivo* study, the number of cells expressing TNF-α during pulpitis increases exponentially until the seventh day (Tani‐Ishii et al. 1995). Thus, this

cytokine may be a good marker to assess the progression of the inflammation (Tani‐Ishii et al. 1995; Hirsch et al. 2017). Furthermore, primary dental pulp cells stimulated by *Streptococcus mutans (S. mutans)*, TNF-α, IL-1β and LPSs showed an increased expression, as assessed by polymerase chain reaction (PCR) analysis, of NF-κB pathway components, TLRs and proinflammatory cytokines, after 4 hours of the

stimulation (Patel et al. 2009). Therefore suggesting, that in order to control the inflammation it is necessary to control the infection.

Pulp inflammation causes tissue destruction and if left untreated leads to tissue necrosis and other adverse events. The recruitment of immune cells to the site of infection implies the release of proteolytic enzymes and the release of reactive oxygen species (ROS), such as superoxide anions, hydrogen peroxide (H_2O_2) and hydroxyl radicals that drive further tissue damage (Waddington et al. 2000; Moseley et al. 2002; Cooper et al. 2010).

1.4 Dental Tissue Regeneration

Regeneration within the dentin-pulp complex requires an environment free of microbes and low inflammation (Figure 1.2). Additionally, it is fundamental that there are adequate numbers of cells that secrete the dentin and pulp ECM. These are, either healthy odontoblast to deposit reactionary dentin or dental pulp stem cells with the potential to become odontoblast-like cells and produce reparative osteodentin. Ultimately, the production of tertiary dentin is only possible after a precise molecular signalling cascade that recapitulates tooth development and provides the adequate cell-cell signalling to upregulate the synthesis and secretion of new tissue (Smith et al. 2012). This has to occur in a time and location controlled manner to avoid root canal obliteration (Smith and Lesot 2001).

Several molecules are important for tertiary dentin deposition; these include the mineral phase, which is mainly hydroxyapatite, and the ECM, with collagenous and non-collagenous proteins. The latter have a crucial role in regulating the whole process, by regulating crystal deposition, a function attributed to SIBLINGs and SLRPs, and bioactive growth factors that stimulate odontoblast-like cell differentiation, and dentin production. Moreover, SIBLINGs and Matrix Metalloproteinases (MMPs) act synergistically in the regenerative process. SIBLINGs are related to the mineralization process once the inflammation has cleared, MMPs are related to the catalytic process helping in dentin regeneration by the cleavage of pro-collagen molecules to allow fibrillation. MMPs also play a role in the cleavage of DSPP into DSP and DPP and in the activation of growth factors (Michel et al. 2015).
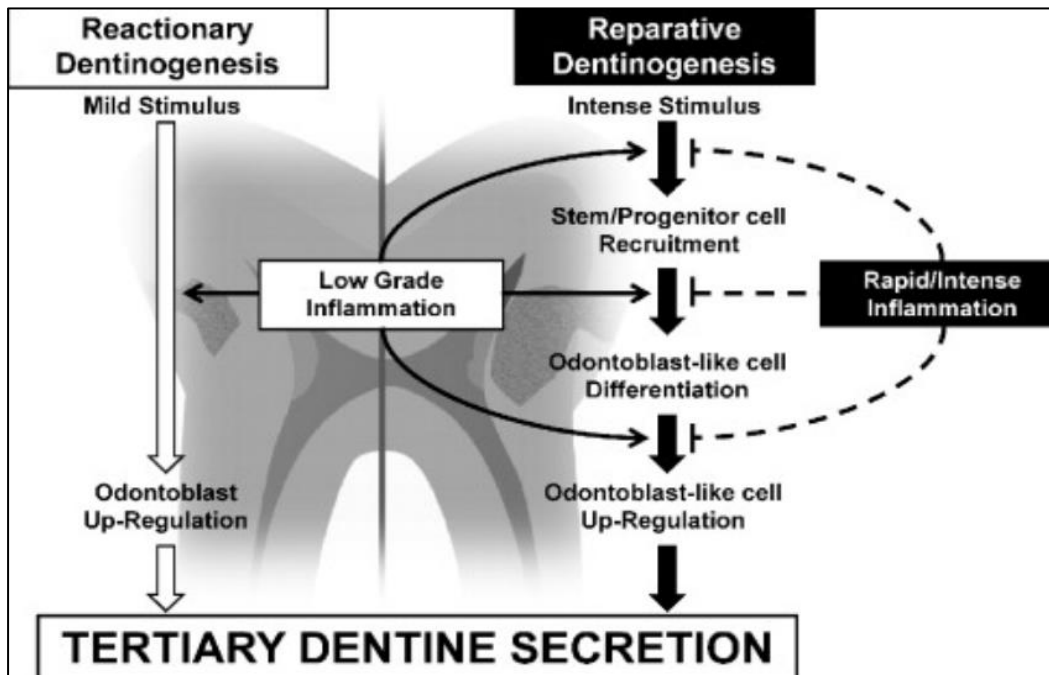
Figure 1.2. The interplay between tertiary dentinogenesis and inflammation intensity. A mild stimulus that elicits a low-grade inflammation stimulates odontoblasts to synthesise and secrete reactionary dentine, and it also stimulates the progenitor cells recruitment, the odontoblast-like differentiation and the production of reparative dentine. Whereas a rapid or intense inflammatory response, generated as a result of intense stimuli, halts the regenerative response (Cooper et al. 2010).

It has now become clear, that the molecular events during inflammation are crucial to the regenerative response (Goldberg et al. 2008). For instance, it has been shown that low levels of TNF-α can induce dental pulp stem cells to differentiate into odontoblast-like cells via the p38 MAPK pathway (Paula-Silva et al. 2009). Furthermore, for DPSCs stimulated with low doses of TNF-α, the levels of alkaline phosphatase, Osteopontin, Osteocalcin, Osterix and Runx2 were up-regulated, suggesting a promotion of mineralization. On the contrary, when stimulated with high doses of TNF-α, DPSCs osteogenic differentiation was arrested (Qin et al. 2015). Moreover, *in vitro* studies showed the ability of IL-1β to stimulate osteoblasts to synthesise bone matrix. Interestingly, however, mesenchymal stem cells were unable to proliferate or differentiate when they were exposed to this pro-inflammatory cytokine (Lange et al. 2010). This suggests that at early stages of inflammation, these cytokines kick start the regenerative process whilst with chronic inflammation or severe inflammation they have an inhibitory effect on regeneration (i.e. cell proliferation and differentiation) (Cooper et al. 2010).

Therefore, in its early stages, inflammation can be a desirable response. A low level of inflammation could be beneficial to fight the infection by the release of antimicrobial peptides and ROS. There is evidence to support the concept that low levels of oxidative stress can stimulate a regenerative response. A study by Lee et al. has shown that H_2O_2 applied at low concentrations enhanced the differentiation of a pre-odontoblast cell line as assessed by the early differentiation marker alkaline phosphatase (ALP). Additionally, in order to test a late marker of differentiation, the extent of matrix mineralization was assessed by Alizarin red S staining, where the pre odontoblasts treated with H2O2 showed significantly higher mineralization when compared to the untreated controls (Lee et al. 2006).

1.5 Oral Microbiology

The oral cavity hosts an abundant and heterogeneous population of microorganisms that can coexist in the form of biofilms colonizing the surfaces of teeth, prostheses, gums and tongue. Biofilm formation is vital for many of these organisms as they depend on metabolic symbiosis. The oral flora is mostly composed of *Streptococci* and *Actinomyces* species (Jenkinson and Lamont 1997). *Streptococci* express surface protein adhesins that bind to multiple substrates, such as other bacteria, molecules present in saliva, host cells or ECM molecules. Most importantly, adhesins bind to the acquired pellicle of the tooth surface initiating the dental plaque formation. As the dental plaque matures, the flora becomes more diverse, comprising rods, cocci and filaments (Jenkinson and Lamont 2005). When fermentable carbohydrates enter the oral cavity, the bacteria from the dental plaque metabolize them, producing organic acids that when reaching a critical pH value, can demineralize the tooth surface (Featherstone 2000). If the dental plaque is removed, the hydroxyapatite crystals can capture ions from the dental pellicle remineralizing the enamel surface. If this does not occur, the demineralization progresses into dental caries, cavity formation and eventual exposure of the dentin.

1.5.1 Infection of the Dentin-Pulp Complex

Once the dentin is exposed, bacteria from the oral cavity can colonize the dentinal tubules putting the dental pulp at risk of infection. Dentinal tubules are conical in shape, with the larger diameter next to the pulp chamber (mean diameter of 2.5µm) and the narrower diameter at the surface next to the enamel or the cementum (mean diameter of 0.9µm) (Garberoglio and Brännström 1976). Oral *Streptococci* have an average size of 1 to 2.0µm. It is easy to speculate that once the dentin is exposed to the oral cavity, the dentinal tubules provide a direct access to the dental pulp. This, however, is not the case as in vital pulp situations dentin permeability is significantly reduced as the dentinal tubules become obstructed by fluids, the odontoblastic processes, nerve fibres and collagen fibrils reducing its physiologic diameter to 5 to 10% of its anatomical value (Michelich et al. 1978). Moreover, fluid components such as antibodies (IgG) and components of the complementary system may halt bacterial invasion (Hahn and Overton 1997). Additionally, dentin permeability can decrease due to dentinal sclerosis, tertiary dentin deposition or smear layer that obstructs the tubule lumen (Michelich et al. 1978).

The microflora of carious dentin varies depending on the depth of the cavity and the location within the tooth, whether coronal or root caries. In shallow cavities of the crown, the predominant species are facultative anaerobes, such as *S. mutans.* In contrast, microflora isolated from deep coronal cavities are dominated by obligate anaerobic organisms, such as *Propionibacteium, Eubacterium and Bifidobacterium,* and facultative species, such as *Actinomyce*s and *Lactobacillus* (Love and Jenkinson 2002). Root surface caries is characterized by the presence of *Actinomyces* species and *S. mutans* in shallow, middle and deep dentin, whereas *Lactobacilli* and Gram-negative organisms are rarely isolated in these cavities.

1.5.2 Periapical Infections

As previously described, if left untreated, dentinal caries will continue the process of invasion deep into the tissue finally provoking pulp inflammation and necrosis and further periapical lesions, such as abscess or granuloma depending on the virulence of the bacteria invading the root canal system (Trowbridge and Stevens). Abscesses present a diverse range of organisms, in particular, the presence of members of the

Streptococcus anginosus group (SAG) and other pyrogenic organisms have been found in clinical isolates (Fisher and Russell 1993). SAG bacteria comprise of *S. anginosus*, *S. constellatus* and *S. intermedius*, Gram-positive cocci, which are part of the normal human commensal flora. They are microaerophilic, but their growth increases in the presence of carbon dioxide (Facklam 2002). This group exhibit hyaluronidase and chondroitin sulphatase activity, which may be the reason behind their ability to invade tissues and behave as opportunistic pathogens (Whiley et al. 1992).

Enterococcus faecalis (*E. faecalis*) species, a Gram-positive facultative anaerobic coccus, which is also a member of the normal commensal flora, is a well-known opportunistic pathogen frequently related to nosocomial infection. *E. faecalis* is the most commonly isolated species in refractory apical periodontitis, i.e. endodontic failure (Love 2001; Barbosa-Ribeiro et al. 2016), as well as dental caries (Kouidhi et al. 2011) as it can invade the dentin tubules in variable depths and survive under different environmental conditions. These organisms adhere to collagen and hydroxyapatite by protein adhesins and lipoteichoic acids (Hubble et al. 2003; Kayaoglu and Ørstavik 2004). Their ability to form biofilms protects them from the immune response and antimicrobials. Additionally, they have shown to obtain energy from hyaluronan and dentinal tubule fluids giving them the ability to survive under extreme conditions, making *E. faecalis* an interesting candidate to test biocides (Rosen et al. 2018).

1.6 Current Treatments.

The main goal of the current strategies to treat the infected dentin is to maintain pulp vitality. The pulp provides the tooth with innervation, nutrition and immunological defence. Moreover, the tooth with its pulp intact has better mechanical resistance than a non-vital tooth (Iglesias et al. 2003) and thus, devitalizing the tooth could reduce its likelihood of long-term survival. The use of vital pulp therapies would, therefore, be more desirable than endodontic treatments where the inflamed pulp is completely eliminated and the root canal filled with thermoplastic materials to prevent further infection in the periapical tissues.

Vital pulp therapies include indirect pulp capping, stepwise caries excavation, direct pulp capping and pulp chamber pulpotomy. In these treatments the aim is to maintain pulp vitality by controlling inflammation, eliminating the infection and promoting a tertiary dentin deposition (Cohenca et al. 2013). For this purpose different materials are used, for example, calcium hydroxide is used as capping material since the early twentieth century (Zander 1939). This material is highly alkaline which creates a bactericidal effect whilst generating a low-grade inflammation that enhances the regenerative response from the dental pulp. It has several drawbacks, however, as its inability to adhere to dentin, which prevents the creation of a long-term seal against micro-leakage (Cohenca et al. 2013). Other materials have been used more recently, that promote better outcomes, such as mineral trioxide aggregate (MTA) and bioactive capping materials. Unfortunately, these materials have not drastically improved vital pulp therapy success rates and therefore clinicians often prefer endodontic procedures to treat pulpitis (Zanini et al. 2017).

Several studies have explored the possibility of liberating bioactive growth factors from the calcified dentin using different acidic solutions to enhance the natural repair response (Smith et al. 2001; Sloan and Lynch 2012; Sadaghiani et al. 2016). There is, however, still a need in these situations to find a strategy to disinfect the dentin from organisms whilst stimulating the pulp to produce tertiary dentin.

1.7 Hydrogen Peroxide

Reactive oxygen species (ROS) are metabolic intermediates produced by eukaryotic cells, primarily in the mitochondria, as a defence mechanism against invading microorganisms. ROS include superoxide anion (O_2^-), H_2O_2, hydroxyl radical ($\cdot OH$) and molecular oxygen (O_2). These molecules can cause damage to lipids, proteins and DNA. Both mammalian cells and microorganisms naturally produce enzymatic and non-enzymatic antioxidants as a defence mechanism against ROS (Radi 2018). When ROS production overcomes the antioxidant capacity, damage to the cells occurs in a situation termed oxidative stress, which is considered to be the cause of several diseases such as cancer and atherosclerosis (Thannickal and Fanburg 2000). Interestingly, ROS are implicated in cell signalling (Finkel 1998). The difference between having a toxic effect or a physiologic has been hypothesised to be concentration related.

H_2O_2 bactericidal effects have been harnessed in endodontic procedures as an irrigant in concentrations ranging from 3 to 5 %. However, it is in disuse due to its low tissue-dissolving capacity and because its bactericidal effect is less effective than other irrigants. Moreover, by using H_2O_2 as an intracanal irrigant there is a risk of provoking a periapical embolism, thereby causing pain and probably tissue damage (Hargreaves and Cohen 2011).

However, research of the effects of H_2O_2 in the dental pulp cells has been increasing in the last decade because it is the main component of tooth bleaching agents (Fukuyama et al. 2008; Soares et al. 2015; Benetti et al. 2017). There is evidence that suggests that H_2O_2 molecules can penetrate through the enamel and reach the dentin-pulp complex (Camargo et al. 2007). As previously stated, low concentrations of H_2O_2 stimulated the differentiation of a pre odontoblast cell line showing how physiological levels of oxidative stress may enhance a reparative response from the dental pulp (Lee et al. 2006). A recent in vivo study, showed how the pulp response was concentration-dependent when bleaching agents were applied to the enamel surface, where high concentrations caused pulp necrosis whilst lower concentration induced a regenerative response (Benetti et al. 2017).

It can, therefore, be speculated that the use of H_2O_2 at low concentrations could potentially stimulate tertiary dentin deposition when applied in deep cavities, whilst preventing the perseverance of dentin infections.

1.8 The *ex vivo* Tooth Slice Model

The use of ex vivo models to study biological behaviour in pre-clinical studies offers several advantages over *in vitro* or *in vivo* models. Firstly, *ex vivo* models allow to study the cells whilst maintaining the tissue architecture and therefore provide the mean to assess the cell-matrix interactions. This is of special importance in the study of dentinogenesis in the dentin-pulp complex, where odontoblasts have to be cultured in contact with the dentin matrix in order to maintain their phenotype and function (Munksgaard et al. 1978). Moreover, the dental pulp ECM provides support and nutrition to allow the survival of the cells for longer periods (Sloan et al. 1998). Secondly, although *in vivo* models provide the means to evaluate tertiary dentinogenesis in a precise timescale of the biological events (Smith et al. 1995; Babb et al. 2017), other systemic events may influence on the results.

A validated *ex vivo* rodent tooth slice model, capable of maintaining tissue architecture and cell viability for up to 14 days in semi-solid agar medium, has been developed 20 years ago (Sloan et al. 1998). This model provided the platform to assess tertiary dentine production after the influence of growth factors (Sloan and Smith 1999; Sloan et al. 2000), or the influence of fluoride on dentin mineralization (Moseley et al. 2003). Recently, the culture conditions of the *ex vivo* tooth slice model were modified in order to develop a co-culture model with *S. anginosus* group (Roberts et al. 2013). Additionally, the model was used to create a mixed species pulpal infection *ex vivo* model, which can be used to study microorganisms colonization mechanisms and to assess the antimicrobial and anti-inflammatory efficacy to treat pulp infection (Nishio Ayre et al. 2018).

1.9 Aims and Objectives

The production of tertiary dentin requires a low level of inflammation that enhances a reparative response. There is evidence to suggest that low-grade oxidative stress could have similar results. It is also known that control of the infection is a prerequisite for vital pulp therapies to be successful.

The aim of this project is therefore to explore the potential to use hydrogen peroxide in deep cavities situations to both eliminate infections and encourage regeneration.

In order to achieve this aim, first, the role of hydrogen peroxide as a biocide will be assessed, by testing the susceptibility of microorganisms that are related to the dentin-pulp complex infections. Next, the cytotoxicity of primary pulp cells, when treated at bacteriostatic and bactericidal concentrations, will be evaluated. Additionally, in order to accurately assess the pulp response to different concentrations of H_2O_2, a validated *ex vivo* tooth slice model (Sloan et al. 1998) will be used.

Ultimately, the aim of this project is to better understand the dentin-pulp complex response to hydrogen peroxide at the cellular and molecular level, as well as its effect on microorganisms associated with tooth infections.

2. Methods

2.1 Preparation of Bacterial Stocks

S. anginosus, E. faecalis and *S. mutans* (DSM20523) were obtained from frozen stock from the culture collection of the Oral Microbiology Unit, at Cardiff University Dental Hospital. *S. anginosus* and *E. faecalis* were clinical isolates and their identity was previously validated (Nishio Ayre et al. 2018). The three strains were streaked and cultured on Tryptone Soya Agar (TSA) (Oxoid Ltd., Basingstoke, UK) to obtain single colonies. Bacterial suspensions were obtained using Brain Heart Infusion Broth (BHI) (Oxoid Ltd., Basingstoke, UK) and cultured overnight in an incubator at 37°C and 5% CO_2.

2.2 Gram Stain

Using a sterile loop, an inoculum of bacteria was taken from an overnight bacterial suspension in BHI and spread over a slide in an area of approximately 1 cm^2. After allowing the smear to air dry, the slide was passed through the flame of a Bunsen burner two or three times to fix the bacteria. Then the sample was stained by first flooding with crystal violet for 30 seconds, then rinsing with water and subsequently flooding with Iodine for 30 seconds and rinsing with water again. To decolorize, acetone was poured drop-by-drop for 5 to 10 seconds and the sample rinsed with water immediately. Finally, in order to counter-stain the sample, the sample was flooded with fuchsine for 30 seconds and rinsed with tap water. The sample was left to air dry and viewed with an X100 oil immersion lens in an upright microscope (Nikon Eclipse 50i).

2.3 Minimum Inhibitory Concentrations (MICs)

Chlorhexidine digluconate (CHX) is the most widely used cavity disinfectant (van Rijkom et al. 1996). Therefore, CHX was used as a control for the experimental procedures in this study.

Overnight cultures of S. *anginosus, E. faecalis* and *S. mutans* were prepared in BHI broth and diluted to 10^8 CFU/mL (absorbance at 600nm 0.08 to 0.1). The inoculum was further diluted in double strength broth in order to obtain a final concentration of 10^6 CFU/mL.

Serial dilutions in distilled water of the biocides were prepared in 96 well plates (Sarstedt) as follows:

a) Serial dilutions of CHX

CHX 20% w/v (Sigma) was diluted in sterile distilled water to a final concentration of 32µg/mL. Serial dilutions of CHX in sterile distilled water were prepared in a 96 well plate (Sarstedt) with concentrations ranging from 16 µg/mL to 0.5 µg/ml of CHX, with a volume of 50µl in each well.

b) Serial dilutions of H_2O_2

H_2O_2 30% w/w (Sigma Aldrich) was diluted in sterile distilled water to a final concentration of 20,000 ppm. Serial dilutions were prepared in a 96 well plate (Starstedt) with concentrations ranging from 10,000ppm to 312ppm of H_2O_2, with a volume of 50µL in each well.

Then, 50µl of the bacterial inoculum in double strength broth was added to the biocide solutions, with each well having a total volume of 100µL and a total of six different concentrations of biocide (starting at 8 µg/mL of CHX or 5,000ppm of H_2O_2); and a final concentration of 5 x 10^5 CFU/mL of bacteria per well. Single strength sterile broth was used as a negative control, and bacterial suspensions of each strain at a concentration of 5 x 10^5 CFU/mL were used as positive controls.

Absorbance was measured at 600nm with a FLUOstar Optima Microplate reader (BMG Labtech LTD, Aylesbury, UK) after incubation for 24 hours at 37°C and 5% CO_2. The MIC was determined for each microorganism, as the lowest concentration of antimicrobial agent that inhibits its growth (Hasselmann 2003). The well giving the absorption nearly equal to blank with more than 90% difference in OD value as compared to the growth control was considered the MIC90.

2.4 Minimum Bactericidal Concentration (MBC) of H_2O_2

For each strain studied, TSA plates were divided into quarters representing different concentrations of serial dilutions of H_2O_2. To determine the MBC one loop of inoculum was taken from the well of the MIC plate that showed growth with the higher concentration of H_2O_2, and then three subsequent higher concentrations. These were spread onto the TSA plate and incubated for a further 24 hours at 37°C and 5% CO_2 under aerobic conditions.

The MBC was determined as the lowest concentration of H_2O_2 for which no growth was detected after visual inspection of the MBC assay plate. This procedure was performed for each replicate of the MIC experiment.

2.5 Cell Culture

Rat Dental Pulp Fibroblasts (rDPFs) were obtained from a frozen stock at the Cardiff University Dental Hospital. Cryopreserved cells were thawed and cultured, with a seeding density of 5,000 cells/mm^2 in Alpha Modified Essential Medium (αMEM), containing ribonucleosides and deoxyribonucleosides and phenol (Gibco, Thermo Fisher Scientific, USA). The medium was supplemented with 10% foetal bovine serum (FBS) (Invitrogen, UK), 100 units/mL of penicillin, 0.1μg/mL of streptomycin and 0.25μg/mL of amphotericin B (Antibiotic/Antimycotic, Sigma-Aldrich). Cells were maintained at 37 °C and 5% CO_2 in a humidified incubator and media was changed every 3 to 5 days.

When confluence was reached, rDPFs were subcultured at a seeding density of 5,000 cells/mm^2 in vented T75 plastic culture flasks. In order to do so, first media was removed and cells washed with 10ml of PBS to reduce the protein content. Then, to allow detachment of the cells, 2 to 4 mL of accutase was added and incubated at 37 °C for 4 minutes. Cell detachment from the plastic was confirmed by observation under a light microscope (Nikon Eclipse TS100). To stop the accutase enzymatic activity and prevent cell damage, 5ml of media was added to the flask. Next, the flask content was transferred to a universal tube and centrifuged at 1500 g for 5 minutes to obtain a pellet of cells. This was then re-suspended in 5ml of media. To count the cells, 10μL of the cell suspension was mixed with 10μL of 0.4% Trypan blue solution (Sigma, UK) and 10μL was placed into a cytometer to count the viable cells under a microscope. The average cell count of the four corners of the cytometer was multiplied by 10,000 (to convert the volume to mL) and by 2 (to account for the dilution factor of the trypan blue) and the number of cells per ml was obtained. Then the volume to take from that suspension was calculated in order to seed 5,000 cells/cm^2 whether to maintain the stock to use in experimental procedures.

Before starting the experiments, rDPFs were tested negative for mycoplasma infection.

2.6 Cell Viability after Treatment with H$_2$O$_2$ or CHX

In order to assess the dental pulp cell response to H$_2$O$_2$ or CHX, an MTT assay was carried out as follows: one 96 well plate (Sarstedt) was set up for each time point (5 min or 60 seconds treatments), rDPFs passage numbers 5 to 7 were seeded at a seeding density of 5000 cells/cm^2, avoiding the outer wells. The 96 well plates were then incubated for 24 hours at 37°C and 5% CO$_2$ to allow the cells adhere to the plastic surface.

Treatment duration of 5 minutes or 60 seconds was tested as it was considered as a clinically relevant exposure time to the biocide.

Biocide solutions were prepared at the test concentrations in non-supplemented αMEM to avoid protein precipitation by the CHX. All solutions that were to be applied to the

rDPFs in this experiment including MTT, PBS, treatment solutions and Triton X were filtered through 0.2µm pore syringe filters.

After 24 hours the medium was removed, and 100µL of medium containing the test concentrations for each treatment (Table 2.1) were added to each well for 5 minutes or 60 seconds. Cells without treatment applied were used as a negative control and treatment with 1% Triton X was used as a positive control. After each time point, the treatment was removed, followed by a wash with phosphate buffer solution (PBS), which consisted of adding 100µL of PBS to each well, gently pipetting back and forth and removing it. Finally, each well was filled with 100µL of phenol free αMEM (Gibco, Thermo Fisher Scientific, USA) supplemented with 10% FBS and 20µL of Thiazolyl Blue Tetrazolium Bromide solution (MTT) (5mg/mL; Sigma) and incubated for four hours at 37°C and 5% CO_2. Next, the medium containing the MTT solution was removed carefully to prevent clearing away the formazan crystals, which were dissolved by means of adding 100µL of Dimethyl Sulfoxide (DMSO) to each well and incubating it for 30 minutes at 37°C and 5% CO_2.

Finally, the absorbance was measured at 570nm in FLUOstar Optima Microplate reader (BMG Labtech LTD. Aylesbury, UK). Averages of the replicates were calculated before the statistical analysis.

Table 2.1. Treatment concentrations applied to rDPFs in MTT assay.

Chlorhexidine (µg/ml)	Hydrogen peroxide (ppm)
32	19,000
16	9,500
8	4,750
4	2,350
2	1,188

2.7 Suspension Test

A suspension test was carried out to investigate if non-bactericidal and potentially biocompatible H_2O_2 concentrations reduced the load of planktonic *E. faecalis, S. anginosus* and *S. mutans*. One colony of each strain was inoculated into approximately 10mL of BHI broth and cultured overnight at 37°C in aerobic conditions. These were then centrifuged 3 times at 4000g for 10 minutes and re-suspended in buffer solution (PBS) to reach a concentration of 10^8 CFU/mL (absorbance at 600nm 0.08 to 0.1).

100μL of the inoculum was added to an Eppendorf tube containing 900μL of H_2O_2 solution of either 1,000ppm or 500ppm (Sigma Aldrich diluted in sterile distilled water) and cultured in the incubator at 37°C. After a contact time of 5 minutes, an aliquot of 100μL was neutralized in 900μL of a solution containing 20g/L of sodium thiosulphate and 500U/mL of catalase from bovine liver (Sigma, Dorset UK) at room temperature (~ 20°C) for 5 minutes. Four further serial dilutions were made (1:10) in PBS in order to count the residual viability. The drop counting method was used by plating three drops of 10μL of each of the last four dilutions into TSA and incubating them for 24 to 48 hours at 37°C in aerobic conditions (Fraud et al. 2001). An untreated suspension was serially diluted and counted in a similar way as a control. The microbiocidal effect was expressed as log_{10} reduction (mean of three experiments), determined by log_{10} of the control minus log_{10} of the treated solution expressed in CFU/mL (Fraud et al. 2001).

The neutralizer used was controlled to verify it did not have a biocidal effect. In this case, 100μL of test suspensions were added to 900μL of neutralizer without using biocide. Then serial dilutions were made and the drop counting method was used to verify there was not a log reduction in this case.

2.8 Scanning Electron Microscopy (SEM) Investigation

SEM was used to investigate any structural damage to the microorganisms tested (*E. faecalis, S. anginosus* and *S. mutans*) after treatment with 1,000ppm H_2O_2 for 5 minutes.

Suspensions of the three strains investigated were prepared in a buffer solution as described in the previous section. A 1mL aliquot of each was added to 900mL of 1,000 ppm H_2O_2 for 5 minutes. Then the samples were fixed by diluting the treated suspension 1:10 in 2% glutaraldehyde solution in 0.1 M of sodium cacodylate buffer (pH7.4). After 2 hours of incubation, the suspension was transferred to a 0.2µm polycarbonate filter membrane using a vacuum/pump filtration system. Then the excess glutaraldehyde was washed by transferring the membranes into 0.1 M sodium cacodylate buffer (pH7.4) for 3 minutes. The samples were then dehydrated by passing them through a series of ascending concentrations of ethanol (50, 70, 80, 95, 100 and 100% v/v) during 3 minutes each. After the final ethanol step, the membranes were immersed in the drying agent hexamethyldisilazane (HMDS) for 5 minutes. Next, the membranes were transferred to individual petri dishes and left overnight in a bell jar to allow the HMDS to evaporate. Finally, the membranes were cut into small squares and mounted on stainless steel stubs using adhesive discs. Samples were coated with a 10nm Au:Pd film using a Quorum Q150TS sputter coater prior to analysis. Microscopy was performed on a Tescan Maia3 field emission gun scanning electron microscope (FEG-SEM) fitted with an Oxford Instruments XMAXN 80 energy dispersive X-ray detector (EDX). Images were acquired using the secondary electron and backscattered electron detectors. Samples were dispersed as a powder onto 300 mesh copper grids coated with holey carbon film.

2.9 *Ex vivo* Tooth Slice Treatment with H_2O_2

28-day-old male Wistar rats were collected freshly sacrificed (under schedule 1 of the UK Animals Scientific Procedures Act, 1986) from the Joint Biological Services Unit, Cardiff University. In a period no longer than half an hour incisors were carefully extracted. Teeth were then cut into thick transverse sections of approximately 2mm using a bone saw (TAAB, Berkshire, UK) with a segmented diamond edged blade using PBS as a coolant. Next, the slices were put into a 24 well plate with DMEM supplemented with 10% v/v FBS and 1% v/v Antibiotic/Antimycotic solution, and incubated at 37°C and 5% CO2 for 24 hours in order to allow the recovery of the tissue after dissection and cutting.

After 24 hours, media was removed from each well, and 500µL of treatment with concentrations of 1,000ppm or 300 ppm of H_2O_2 solution in DMEM was added for each time point: 5 minutes or 60 seconds (Table 2.2). Tooth slices without exposure to H_2O_2 were used as a negative control. Finally, the samples were incubated for a further 24 hours in a humidified incubator at 37°C and 5% CO_2 before histological processing.

2.10 Histological Tissue Processing

Media was removed and tooth slices were fixed in 10% w/v neutral buffered formalin at room temperature for 24 hours. Next, formalin was removed and 2 mL of 10% w/v formic acid was added to each well for 48-72 hours, changing it at 24 hours to avoid saturation and to allow total demineralization of the samples. The demineralization process was performed under constant agitation in a rocker.

Once demineralized, the samples were transferred into individual biopsy cassettes. Then the tooth slices were dehydrated through a series of ascending concentrations of ethanol, then xylene and two steps of molten wax, in an automatic tissue processor (Leica ASP300s, Nussloch, Germany). Finally, the slices were embedded in paraffin wax oriented in such way that the coronal-apical length was placed at an angle of 90° to the cutting surface, therefore the histologic sections could hold a transversal cut of the root showing dentin and the root canal with the dental pulp inside. These samples were then used to perform cell counting and immunohistochemistry.

2.11 Histological Cell Counting

Wax blocks with the treated and control samples were cut into sections of 4μm thickness using a microtome (Shandon Finesse, Thermo Electron Co., Cheshire, UK). Sections were mounted on polystyrene slides (Fischer Scientific, UK) and placed in an incubator at 60°C to improve adhesion of the tissue sections to the slide. The slides were then placed on an automated tissue stainer (Model Linistainer GLX, Thermo Shandon). There, they were washed with xylene, alcohol and water before being stained with haematoxylin and eosin (H&E). They were then immersed through alcohol and xylene. Finally, a coverslip was placed using DPX mounting medium (Cellpath, Powys, UK).

Slices images were captured at x20 magnification using a light microscope (Olympus AX70) and a 5.0MP digital camera (Paxcam 5+, IL, USA), connected to Pax-it imaging software (Pax-it, IL, USA). Images were merged using ImageJ software (National Institutes of Health, Maryland, UK). Four samples of each treatment (Table 2.2) were then analysed to count the number of cells using a macro: blue and green fields were extracted from the images and the moments threshold method was applied to separate the pulp cells. Adjacent cell nuclei were isolated running the watershed function and particles ranging from 3 to 100μm^2 in size were counted. The data was normalised to the pulp area to yield the mean number of cells/mm^2 ± standard error.

Table 2.2 Treatments applied to tooth slices.

H_2O_2 concentration (ppm)	Time of exposure
1,000	5 minutes
1,000	60 seconds
300	5 minutes
300	60 seconds
Negative control: no treatment.	

2.12 DAB-Immunohistochemistry TNF-α, IL-1β, DSP and PCNA

Treated and control tooth slices (Table 2.2) were fixed, decalcified, dehydrated and embedded in paraffin wax as previously described in section 3.10. Embedded samples were cut into 5µm sections, mounted onto poly-L-Lysine coated slides and incubated at 65°C for 45 minutes to allow the samples to adhere to the slide. The samples were then washed in xylene 3 times for 5 minutes to remove the paraffin wax and then rehydrated through a series of 5-minute washes in ethanol: 3 times 100 % v/v, 2 times 70% v/v and 1 time 50% v/v. Endogenous peroxidase activity within the tissues was quenched by pouring a drop hydrogen peroxide 3% v/v in Tris-buffered saline (TBS) over the sample during 15 minutes; and then washed three times for 3 minutes each with TBS (pH 7.4, 9g/L sodium chloride, 1.2g/L Tris buffer in distilled water). To prevent non-specific binding, a drop of blocking solution of 3% v/v normal horse serum (NHS)(Vector Laboratories, Burlingame, CA) in TBS was poured for thirty minutes. No antigen retrieval was performed.

Primary antibodies for TNF-α, IL-1β, DSPP and PCNA (Table 2.3) (Santa Cruz Biotechnology, Heidelberg, Germany) were diluted 1:50 in TBS containing 5% v/v NHS. Sections were incubated with the primary antibody solution during one hour and then washed 3 times for 5 minutes in TBS. Immunoreactivity was then performed using Vectastain® Universal Quick Kit (Catalog No. PK-8800, Vector Laboratories, Burlingame, CA). The negative control was primary antibody exclusion. Positive controls were rat lung for TNF-α, IL-1β and rat skin for PCNA. Samples stained for TNF-α, DSPP and IL-1β were counterstained for with Haematoxylin for 30 seconds and washed in tap water for 5 minutes. Samples stained to localise PCNA were counterstained with fast green FCF (Sigma Aldrich) for 5 minutes. Finally, the samples were dehydrated with 100% ethanol and xylene and mounted with DPX. Images were analysed with an upright microscope (Nikon 50i) and captured with a digital camera and StCamSWare v3.10 imaging software.

Table 2.3 List of primary antibodies used for Immunohistochemistry

Antibody	Type	Code
TNF-α	Monoclonal IgG$_1$	SC-52746
IL- 1β	Polyclonal IgG	SC_7884
DSPP	Monoclonal IgG$_{2b}$	SC_73632
PCNA	Polyclonal IgG	SC_7907

2.13 Primer Design and Validation for RNA gene expression analysis of PCNA and DSPP genes

Primers to quantify the gene expression of PCNA and DSPP for Rattus norvegicus were designed using the National Center for Biotechnology (NCBI). Three pairs of primers (listed in Table 2.4) were selected for each gene. The selection criteria were that the amplified product had to span an exon exon junction, the percentage of CG nucleotides had to be between 50% and 60% and there had to be low self-complementary (<5).

Table 2.4 Primer sequences designed for qPCR analysis

Gene	Primer sequence (5'->3')	Product length (Bp)	Melting temperature (°C)	GC-content (%)	NCBI reference sequence
PCNA (1)	Forward –ACC TCA CCA GCA TGT CCA AAA Reverse – CTA CGC AGC TGT ACT CCT GTT	198	57.9 59.8	47.6 52.4	NM_022381.3
PCNA (2)	Forward – TTG GAA TCC CAG AAC AGG AGT Reverse – GTC CCG GCA TAT ACG TGC AA	74	57.9 59.4	47.6 55	NM_022381.3
PCNA (3)	Forward – GCA TGG ATT CGT CTC ACG TC Reverse – TTG GAC ATG CTG GTG AGG TTC	115	59.4 59.8	55 52.4	NM_022381.3
DSPP (1)	Forward - GCA GAG CCA AAA TCA GGG ATT A Reverse – ATG GTG TCC GTT GCT GTC TT	112	58.4 57.3	45.5 50	NM_012790.2
DSPP (2)	Forward - GCC ATT CCG GTC CCT CAG TT Reverse - CTC ATT CTG TGC TGC GGT TC	102	61.4 59.4	60 55	NM_012790.2
DSPP (3)	Forward - AAA ATC TGC CGA CGT ACC CT Reverse - CAA CTC ATT CTG TGC TGC GGT	58	57.3 59.8	50 52.4	NM_012790.2

Primers (Eurofins Genomics, Ebersberg, Germany) were diluted in nuclease-free water (Promega, Southampton, UK) to a stock concentration of 100pmol/μL (1μM).

In order to determine the efficiency of the designed primers, total rat RNA was converted to complementary DNA (cDNA) using a G-storm™ GS1 Thermal Cycler (Genetic Research Instrumentation, Braintree, UK). First, RNA concentration was determined using a NanoVue Spectrophotometer (GE Healthcare Life Sciences, Buckinghamshire, UK). RNA sample purity was determined by the ratio of absorbance at 260/280nm and considered pure if the value was above 1.7.

cDNA was then synthesised by reverse transcription polymerase chain reaction (RT-PCR) as follows: 1μg of total rat RNA was added to 1μl of random primers and the volume adjusted to 15μl with RNAse-free water. This solution was then incubated at 70°C for 5 minutes to allow annealing of the random primers to the RNA. The product of this reaction was added to a master mix that contained: 5μL of MMLV reaction buffer, 1.25μL of dNTPSs (deoxynucleotide triphosphates, from a 10mM stock solution of comprising dATP, dCTP, dGTP and dTTP), 0.6 μL of RNase inhibitor, 1μl of M-MLV reverse transcriptase and 2.15μL of nuclease-free distilled water (all reagents used were sourced from Promega, Southampton, UK). This mix had a final volume of 25μL. Next, this solution was incubated for 1 hour at 37°C allowing the reverse transcriptase to extend the random primers at the expense of the dNTPSs, and therefore synthesise the double-stranded cDNA.

Following RT-PCR, the newly synthesised cDNA was serially diluted 1:5 in nuclease-free water (starting with 30ng of cDNA) in qPCR plates (Primerdesign, Chandler's Ford, UK). Each well contained 5μL of cDNA solution and was combined with 10μL of FAST 2x qPCR SYBR® Green MasterMix (Primerdesign, Chandler's Ford, UK), 2μL of forward and 2μL of reverse primers and 1μL of nuclease-free water. The negative control involved the substitution of cDNA with nuclease-free water. The plates were first heated to 95°C for 20 seconds; then the PCR stage consisted of 40 cycles of 95°C for 1 second and 55°C for 20 seconds. Finally the melt curve analysis stage consisted of increasing the temperature to 95°C for 15 seconds, then decreasing it to 60°C for 60 seconds and heating it up to 95°C for 15 seconds. The reaction was carried out in a QuantStudio™ 6Flex Real-Time PCR system with QuantStudio™ Real-Time PCR Software v1.2 (ThermoFischer Scientific, Loughborough, UK). Primer efficiency was

calculated using the formula below, using the slope of the dilution curve plotted against cycle number and had to be en the range of 90% to 110% and a melting curve showing a single peak.

$$Efficiency\ (\%) = \left(10^{\frac{-1}{slope}} - 1\right) \times 100$$

2.14 Statistical Analysis.

The data generated was expressed as mean values ± standard deviation (SD) or standard error of the mean (SE) and analysed using SPSS (IBM SPSS Statistics 2015 for Windows, Version 23.0. Armonk, IBM Corp.). The Kolmogorov-Smirnov test was used to determine normal distribution and Levene's test was used to test for homogeneity of variances. A one-way analysis of variance (ANOVA) was performed to determine the relative significance of the differences between the means when data were normally distributed and variances were not different. If these requirements were unmet, a Kruskal-Wallis test was used and the Mann Whitney U test to study differences within groups. Confidence intervals were set at 95% and a p-value less than or equal to 0.05 was considered significant.

3. Results

3.1 Gram stain

As shown in Figure 3.1 after staining, the three strains under study, *E. faecalis, S. anginosus* and *S. mutans* appear as chains of gram-positive cocci with a diameter between 1 and 2µm.
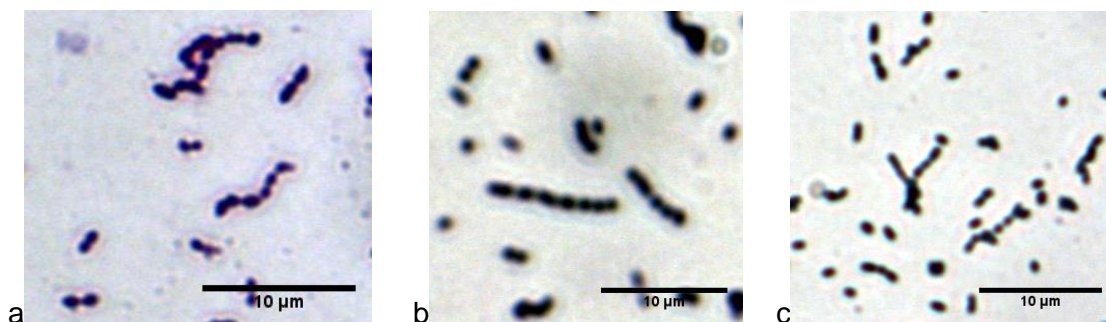

Figure 3.1. Images showing gram stain results for a) *E. faecalis*, b) *S. anginosus* and c) *S. mutans.* (Scale bar = 10 µm)

3.2 MICs

The MIC was determined for each microorganism, as the lowest concentration of antimicrobial agent that inhibits its growth (average of n=3 with 3 internal replicates). This was represented by an OD value similar to the blank control (broth alone). Following incubation during 24 hours at 37 °C and 5% CO_2 in the presence of various H_2O_2 dilutions in distilled water, *E. faecalis, S. anginosus* and *S. mutans* growth was inhibited at 1,250ppm H_2O_2. Moreover, this value can be described as the MIC90 as the OD is reduced by approximately 90% when compared to the growth control.

After incubation in serial dilutions of CHX, the MICs showed different results within the three strains. *E. faecalis* growth was inhibited at 8µg/mL, *S. anginosus* at 2µg/mL and *S. mutans* at 1µg/mL.

Control wells containing bacterial suspensions in BHI broth showed no inhibition of bacterial growth and blank control wells containing broth alone did not show any growth.

3.3 MBCs

After 24 hours incubation at 37°C and 5% CO_2, the minimum concentration in which *E. faecalis* growth was undetectable was 2,500ppm (double the MIC value) of H_2O_2, whereas for *S. anginosus* and *S. mutans* this was 1,250ppm (equal to the MIC value).

3.4 Evaluation of Cell Viability after Treatment with H_2O_2 and CHX

To investigate the effects of H_2O_2 and CHX on rDPFs, an MTT assay was performed as described in section 2.2.6. Data was normalized to the untreated control before the statistical analysis was performed. Gaussian distribution of data was not confirmed ($p<0.05$), and so a Kruskal-Wallis test was used to analyse differences between the ranks of the groups. The Mann Whitney U test was used to explore the differences between the groups.

H_2O_2 treatment for 5 minutes

As shown in Figure 3.2, all the treated groups showed a significant difference when compared to the untreated group ($p<0.001$), the cells exposed to the least concentrated H_2O_2 (1,188ppm) had a 2-fold decrease in percentage viability compared to the untreated sample ($p<0.001$). Moreover, the three higher concentrations (19,000ppm, 9,500ppm and 4,750ppm) showed no significant difference ($p=1$) to the cells treated with Triton X 1% v/v (positive control). Thereby, rDPFs were significantly affected by the treatment for 5 minutes with H_2O_2 at all the concentrations tested.
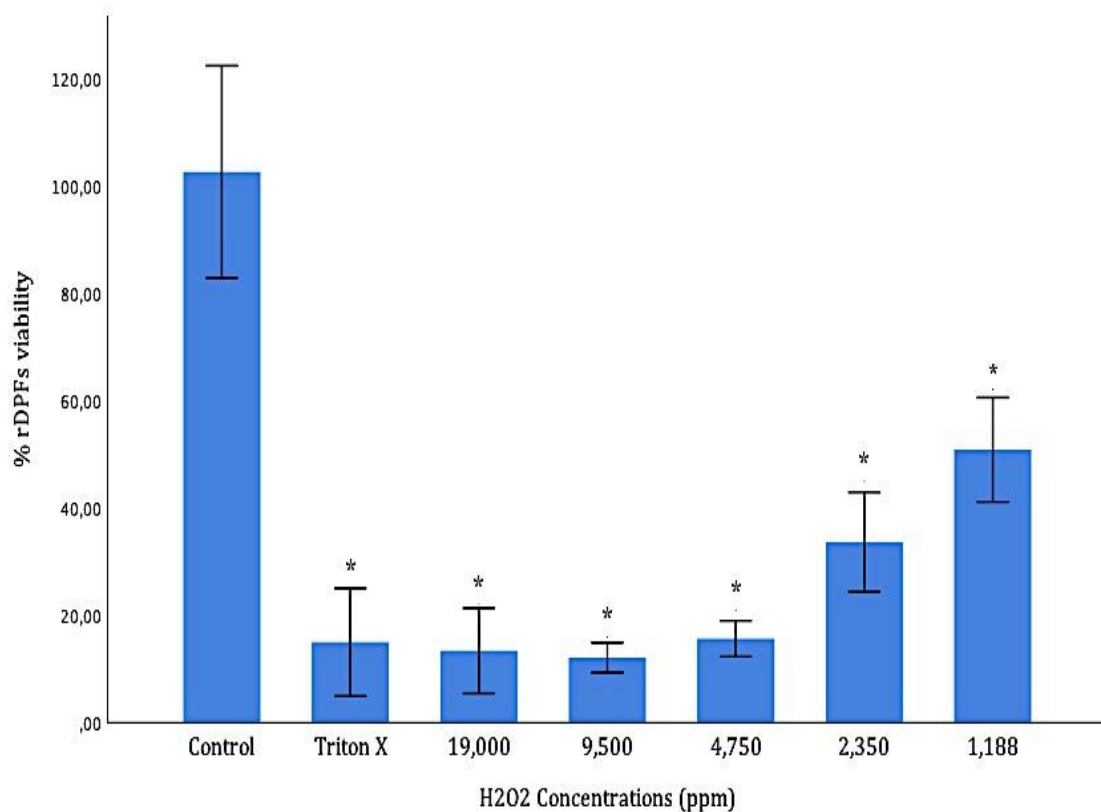
Figure 3.2. rDPFs were treated with different concentrations of H_2O_2 for 5 minutes. Then the cell viability was assessed by means of an MTT assay. Data was normalized to the untreated control before statistical analysis. Data show the mean values ±SD (n=12 per treatment, *p < 0.001)

CHX treatments for 5 minutes

The data after normalization to the negative control (untreated) deviated from normality (p<0.05), thus a Kruskal-Wallis test was used showing enough evidence that there was a statistical difference between the groups (p<0.001). As shown in Figure 3.3, the three lower concentrations used (8µg/mL, 4µg/mL and 2µg/mL) showed no significant difference when compared to the untreated cells (p= 1, 0.88 and 1 respectively). At the 2 higher concentrations (32µg/mL and 16µg/mL) there was a 2-fold decrease in the percentage cell viability (p<0.001). Thus, rDPFs were unaffected when exposed to concentrations lower than 8µg/mL.
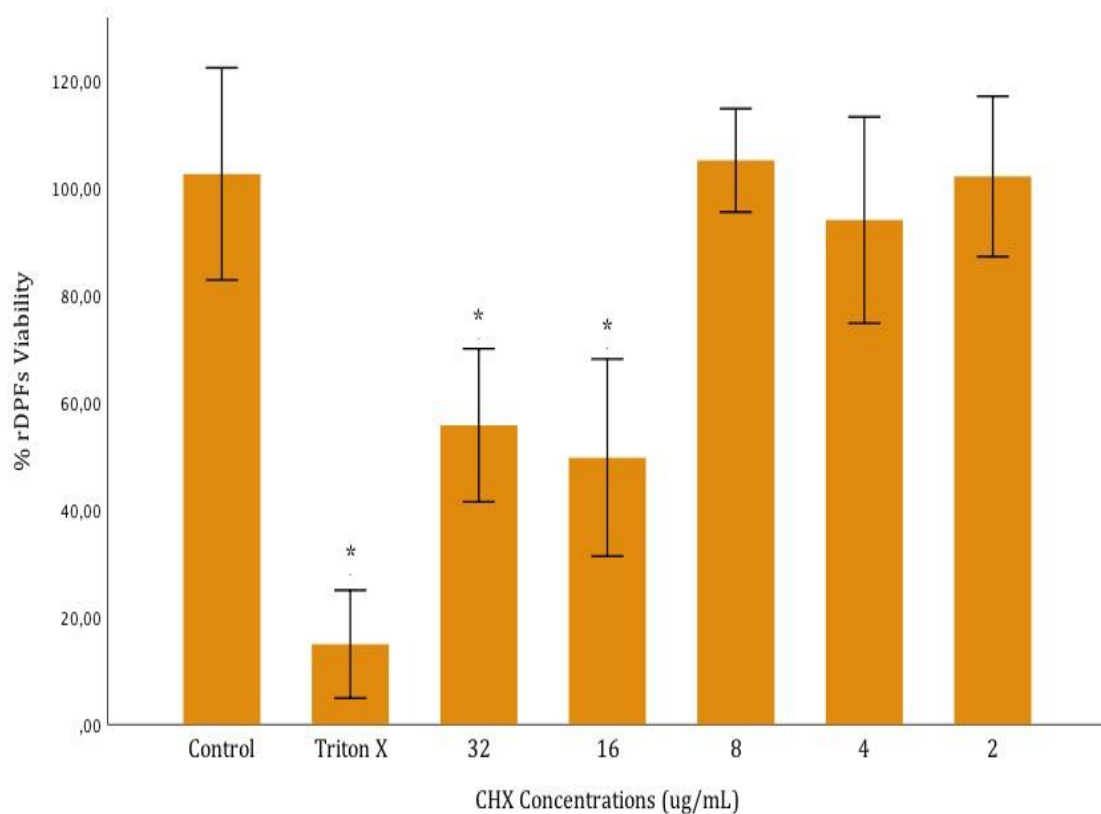
Figure 3.3. rDPFs treated with different concentrations of CHX for 5 minutes. Bars represent the mean value for the percentage cell viability ±SD. (n=10 per treatment, *p<0.001).

H_2O_2 treatment for 60 seconds

The percentage viability of rDPFs treated with H_2O_2 during 60 seconds was significantly different from the percentage viability of the untreated cells (p<0.001). The graph in Figure 3.4 shows that the three higher concentrations (19,000ppm, 9,500ppm and 4,750ppm) affected the cell viability in an equal way as the positive control (p=1). Despite the percentage of viable rDPFs treated with the lower concentration of H_2O_2 used (1,188ppm) was 3-fold higher than the positive control (p<0.001); they were significantly affected when compared to the untreated fibroblasts (2,2-fold, p<0.001). Therefore, although the trend suggests a significantly higher percentage of cell viability at low concentrations, all the concentrations tested affected the rDPFs viability.
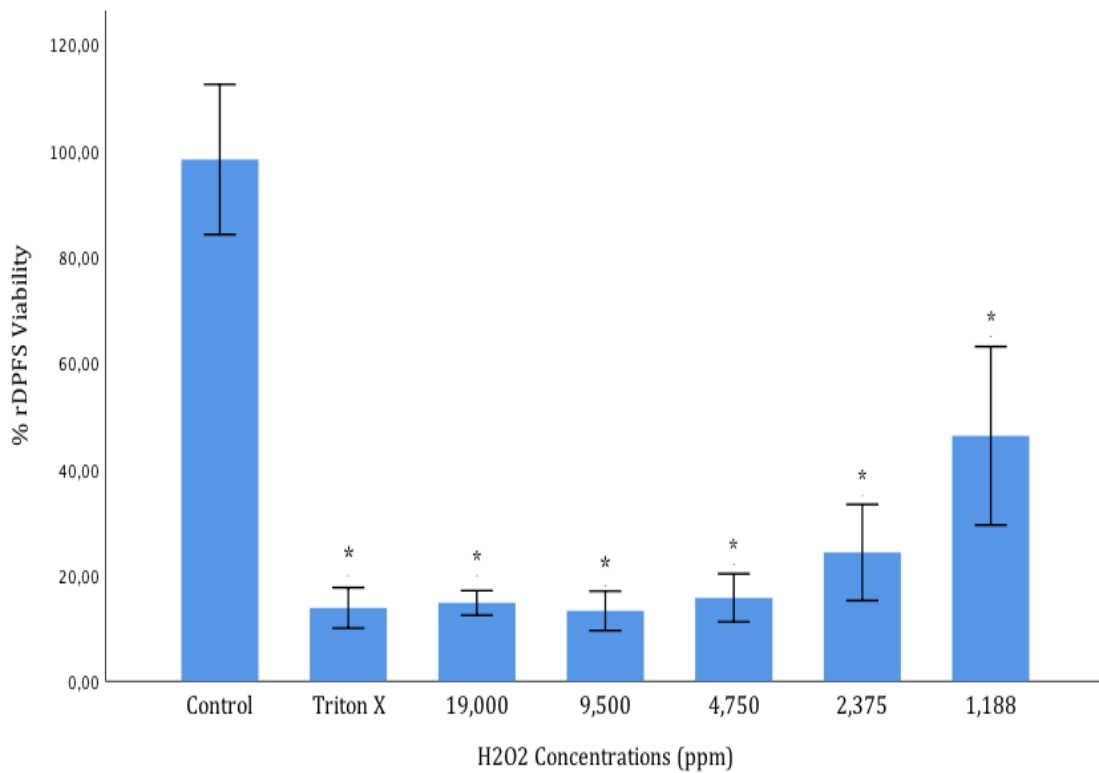
Figure 3.4. rDPFs were treated with different concentrations of H_2O_2 for 60 seconds. Data shows the mean values ±SD (n=10 per treatment). Cells were significantly affected by all the treatment concentrations tested (*p<0.001).

CHX treatment for 60 seconds

Figure 3.5 shows that cells treated with CHX at the four lower concentrations (16μg/mL, 8μg/mL, 4μg/mL and 2μg/mL) for 60 seconds. The results show no significant difference in percentage viability when compared to the untreated samples (p>0.05).
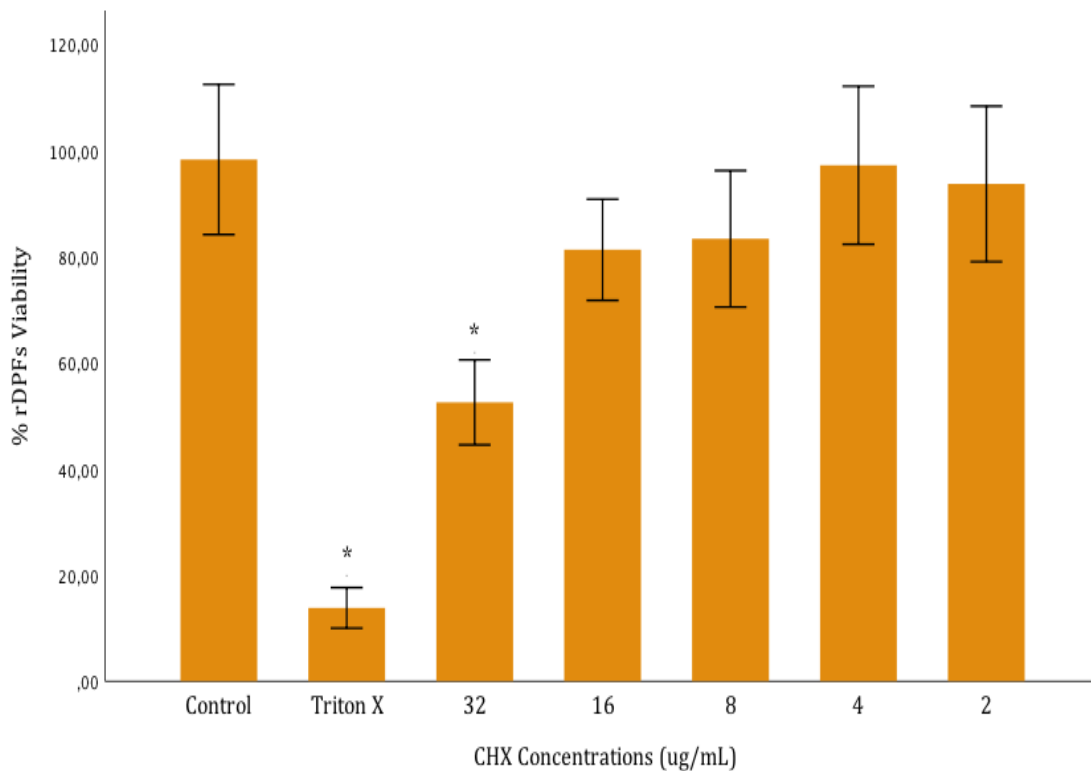
Figure 3.5. rDPFs treated with different concentrations of CHX for 60 seconds. Bars represent the mean value for the percentage cell viability ±SD (n=10 per treatment, *p < 0.001).

3.5 Effect of H_2O_2 on Microorganism Viability

As shown in Table 2.1, after 5 minutes of treatment, H_2O_2 had no bactericidal effect at either of the concentrations assessed. The neutralizer used (20g/L of sodium thiosulphate and 500U/mL of catalase from bovine liver) also had no bactericidal effect.

Table 2.1. Effect of H_2O_2 on planktonic microorganisms using a quantitative suspension test.

| Concentration (ppm) | Mean log reduction (SD) in CFU/mL for each strain 5 minutes contact time | | |
	E. faecalis	*S .anginosus*	*S. mutans*
1000 (pH4.6)	1.63 (0.1)	1.07 (0.27)	1.40 (0.16)
500 (pH5.8)	1.80 (0.09)	1.34 (0.19)	1.06 (0.2)

3.6 SEM Imaging after Treatment with H_2O_2

E. faecalis, S. anginosus and *S. mutans* treated for 5 minutes with H_2O_2 at 1,000ppm and the untreated control were fixed as described in section 2.2.8. Representative images taken with SEM are shown in Figures 3.6, 3.7 and 3.8. The number of cells was equal in the treated and untreated samples in the three strains investigated.
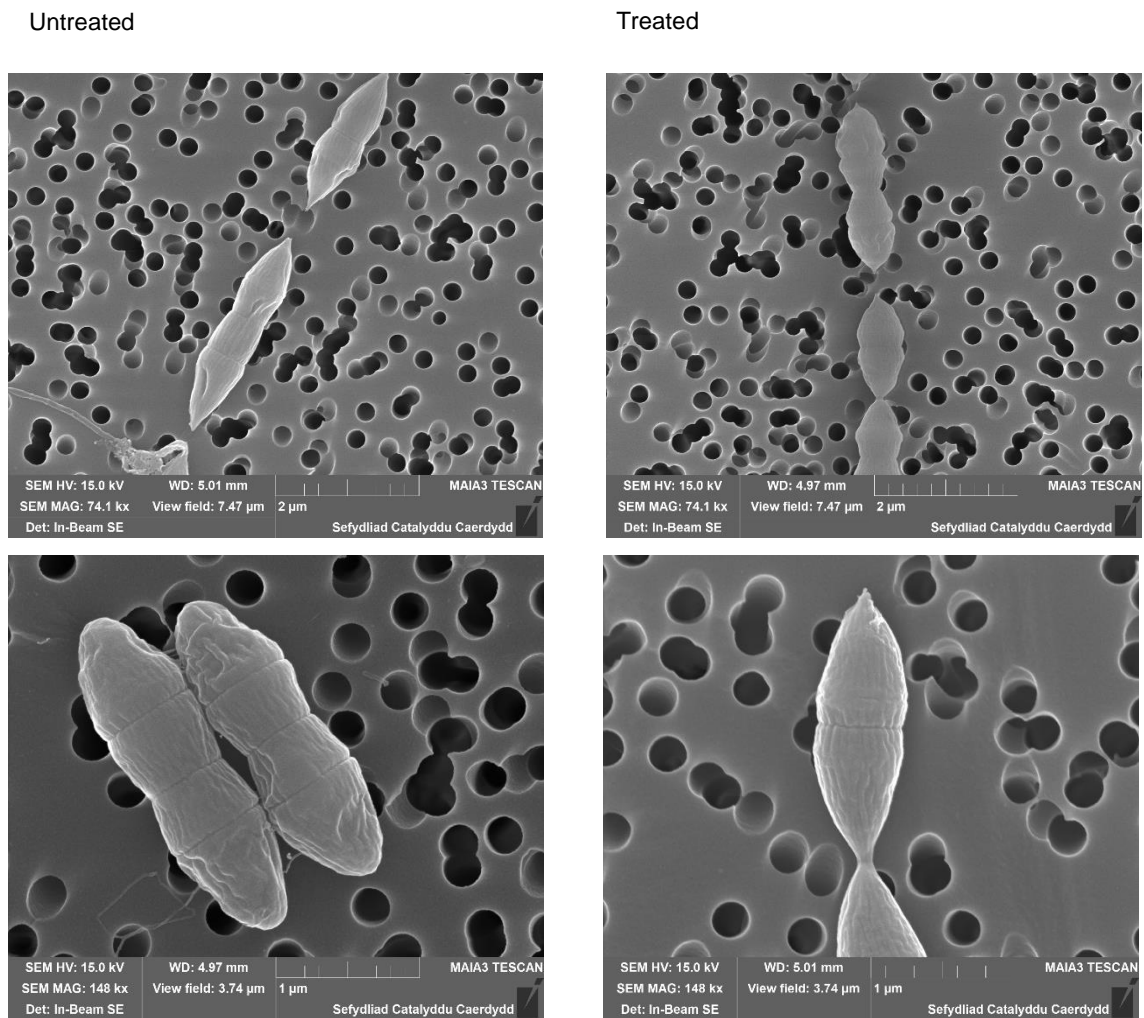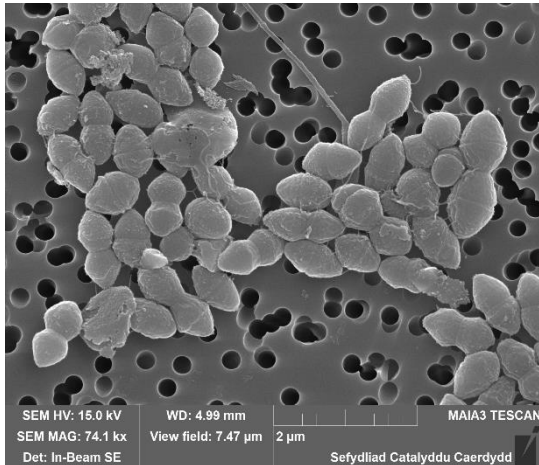
Untreated   Treated

Figure 3.6. SEM images of untreated and treated (with H_2O_2 for 5 minutes) *E. faecalis*. The upper images were taken with a magnification of 74.1kx and the lower with a higher magnification of 148kx. No changes in the morphology and structure of the cell were observed between the treated and untreated samples.
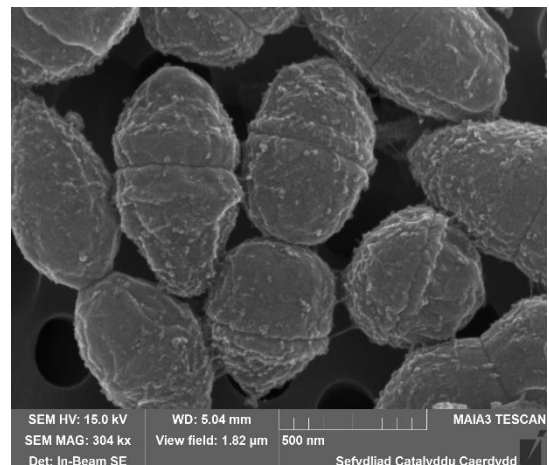
Untreated   Treated

Figure 3.7. SEM images of untreated and treated (with H_2O_2 for 5 minutes) *S. anginosus*. The upper images were taken with a magnification of 74.1kx and the lower with a higher magnification of 304kx. A discrete increased roughness was observed in the treated samples, however no differences were seen in terms of size and morphology.

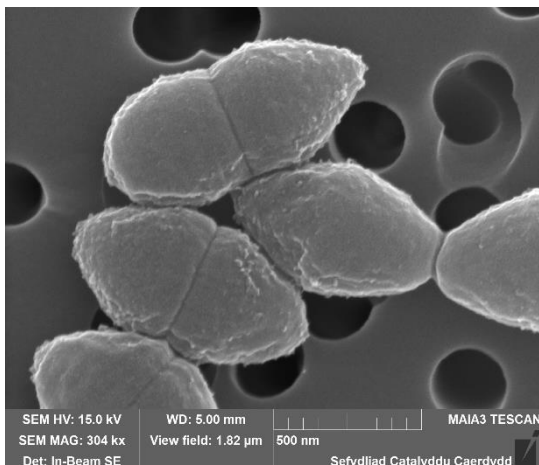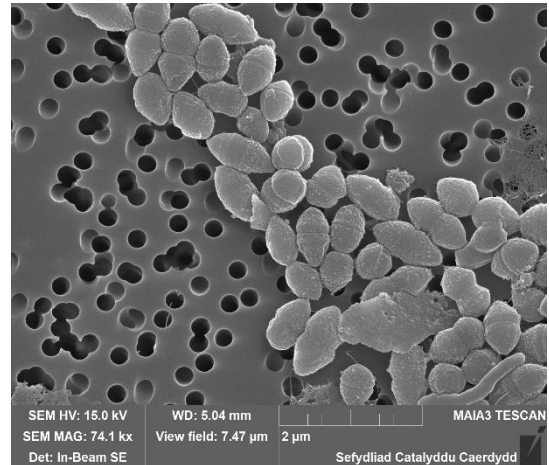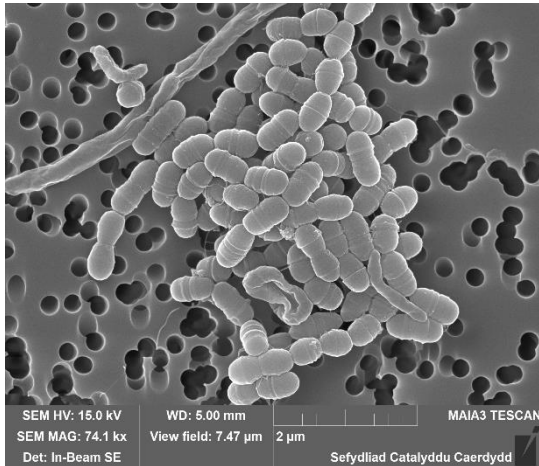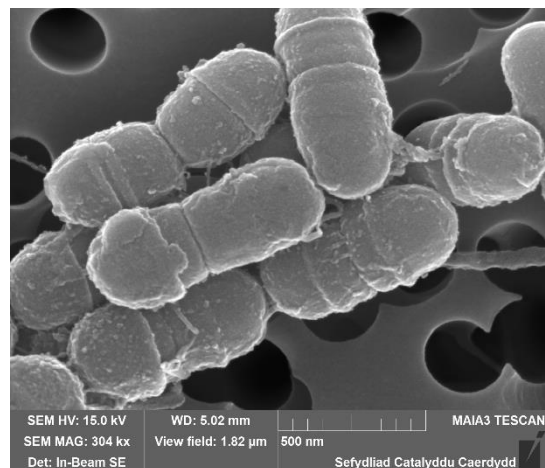Untreated   Treated
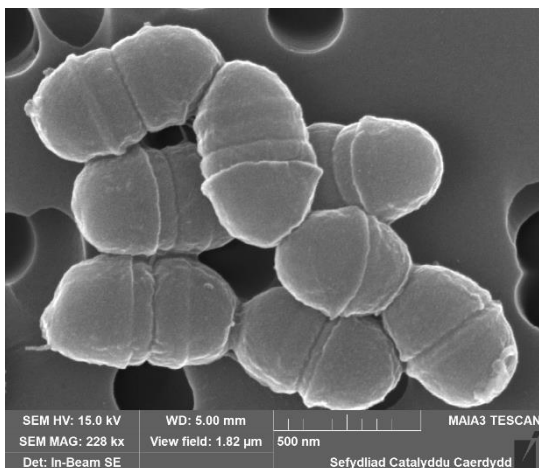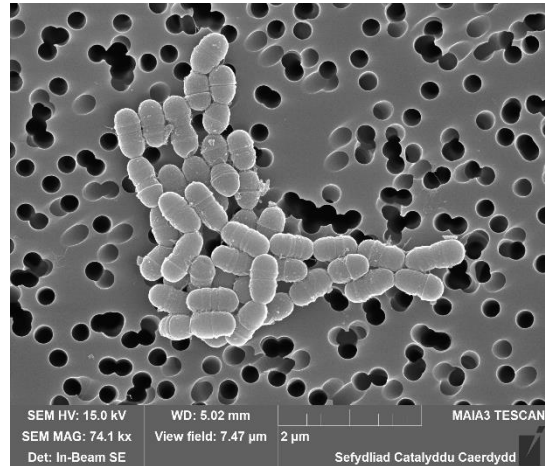
Figure 3.8. SEM images of untreated and treated (with H_2O_2 for 5 minutes) *S. mutans*. The upper images were taken with a magnification of 74.1kx and the lower with a higher magnification of 148kx. A discrete increased roughness is appreciated in the treated samples, however no difference is seen in terms of size and morphology.

3.6 Dental Pulp Cells Quantification

As shown in Figure 3.9, after treatment of the tooth slices with 1,000ppm or 300ppm of H_2O_2 for 5 minutes or 60 seconds, no significant differences were demonstrated in viable cells between the treatment and control groups.
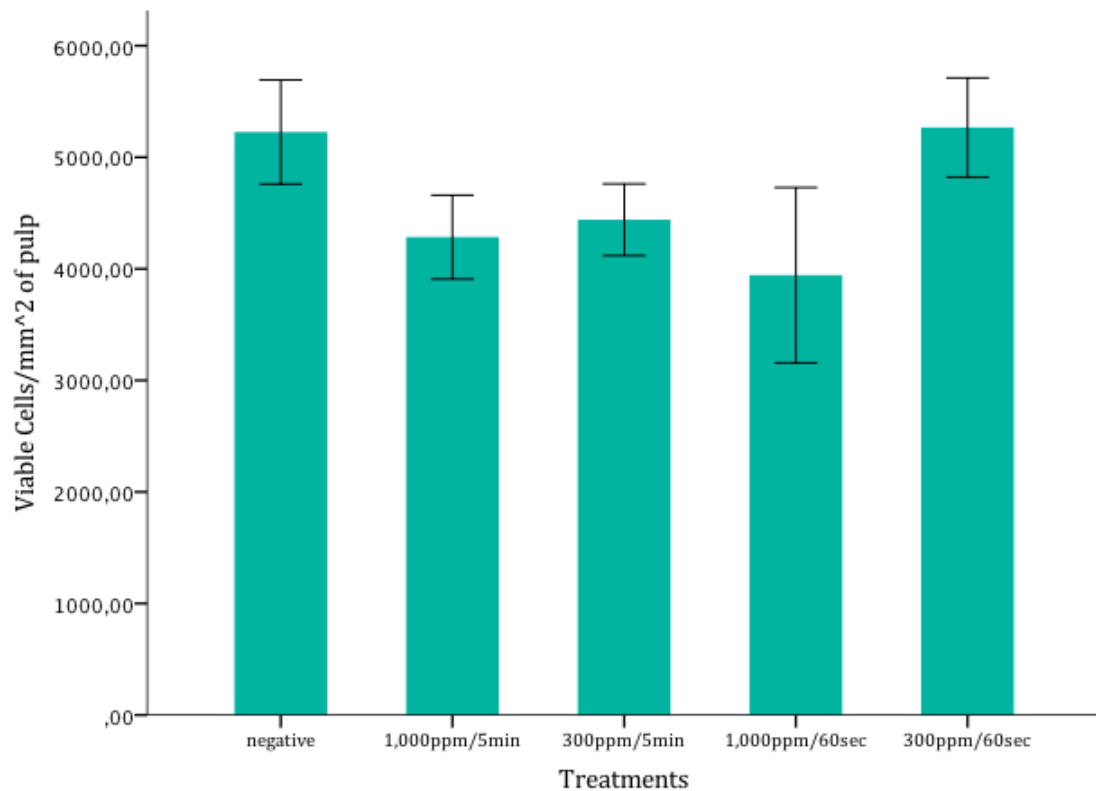
Figure 3.9. Viable cells counted per mm^2. Tooth slices were treated with 1,000ppm or 300ppm H_2O_2 for 5 minutes or 60 seconds. No significant reduction was accounted for the treated samples when compared to the negative control ($p > 0.05$). Bars show mean values ± SE

3.7 Localization of TNF-α, IL-1β, PCNA and DSPP by Immunohistochemistry

In order to assess the inflammatory response to the treatments with H_2O_2 (Table 2.2), the samples were subjected to immunohistochemical detection of TNF-α and IL-1β, as these are representative cytokines of dental pulp inflammation. Additionally, detection of DSPP was performed to identify odontoblasts, whether primary or newly differentiated ones; and PCNA to identify proliferative activity in the dental pulp cells.

As shown in Figure 3.10, the inflammatory cytokines assessed were differentially expressed. TNF-α was highly expressed in the odontoblast layer and in the perivascular area of the treated samples, showing a similar staining to the rat lung positive control (Figure 3.12). The untreated samples did not show immunopositivity for TNF-α. IL-1β had negative staining in all the samples. Primary antibody exclusion was also negative for staining.

Immunohistochemical detection of PCNA showed no proliferation in the dental pulp in the untreated samples or in the treated ones (Figure 3.11). DSPP immunohistochemical localization showed staining in the odontoblast layer in untreated and treated samples.

Staining was negative when the primary antibody was omitted showing the absence of nonspecific binding of the secondary antibody (Figure 3.10). Positive controls were Rat lung for TNF-α and IL-1β, and skin was used as a control of PCNA. All positive controls demonstrate positive staining (Figure 3.12).
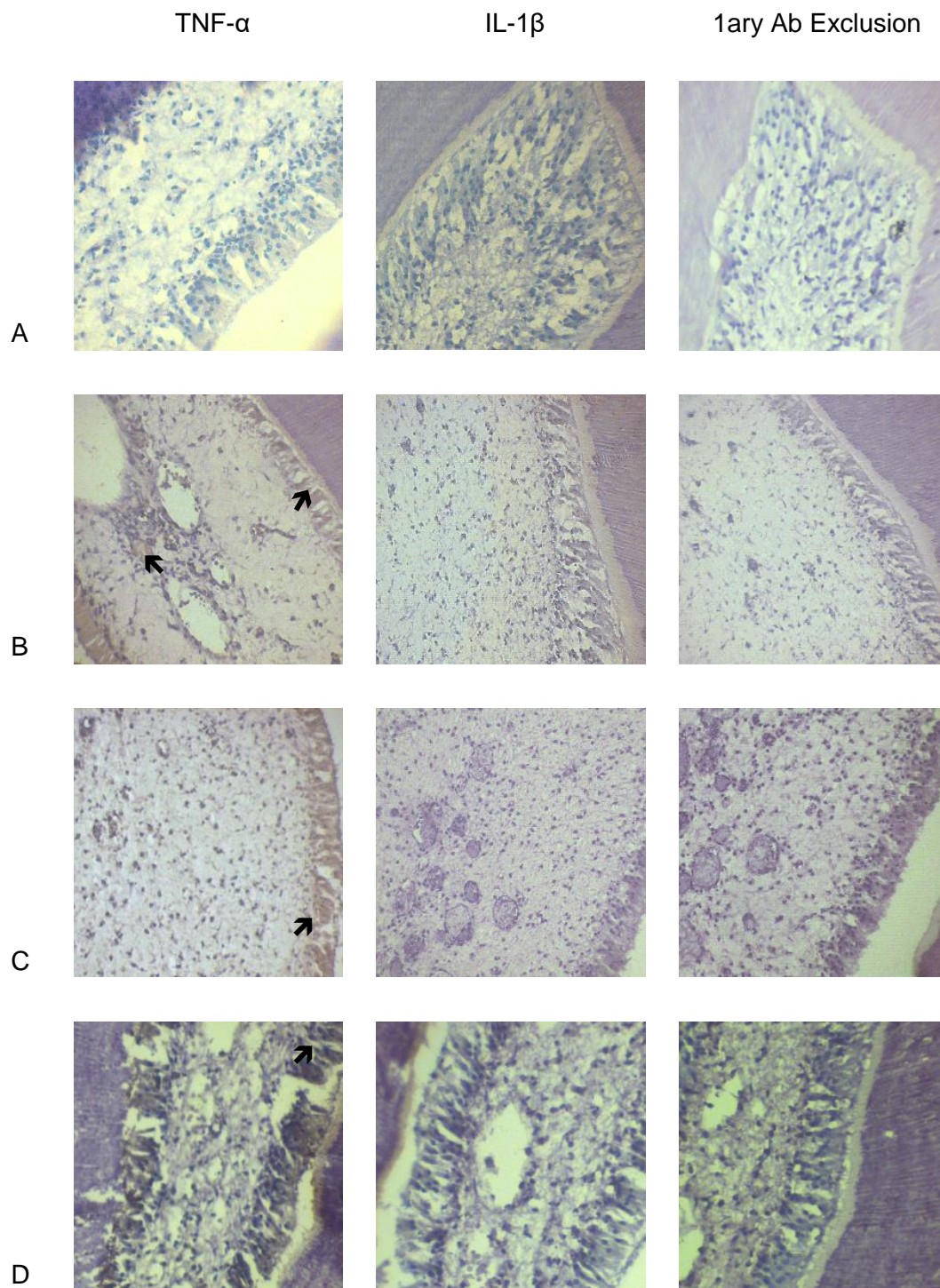
Figure 3.10. Cytokine expression. A) Negative control, B) 5 minutes/1,000ppm, C) 60 seconds/1,000ppm, and D) 60 seconds/300ppm. Control samples showed no expression of TNF-α, while staining was positive for all the samples treated with H_2O_2. None of the samples showed immunopositivity for IL-1β. Primary antibody exclusion controls were negative for staining. Black arrows highlight positive staining for TNF-α.
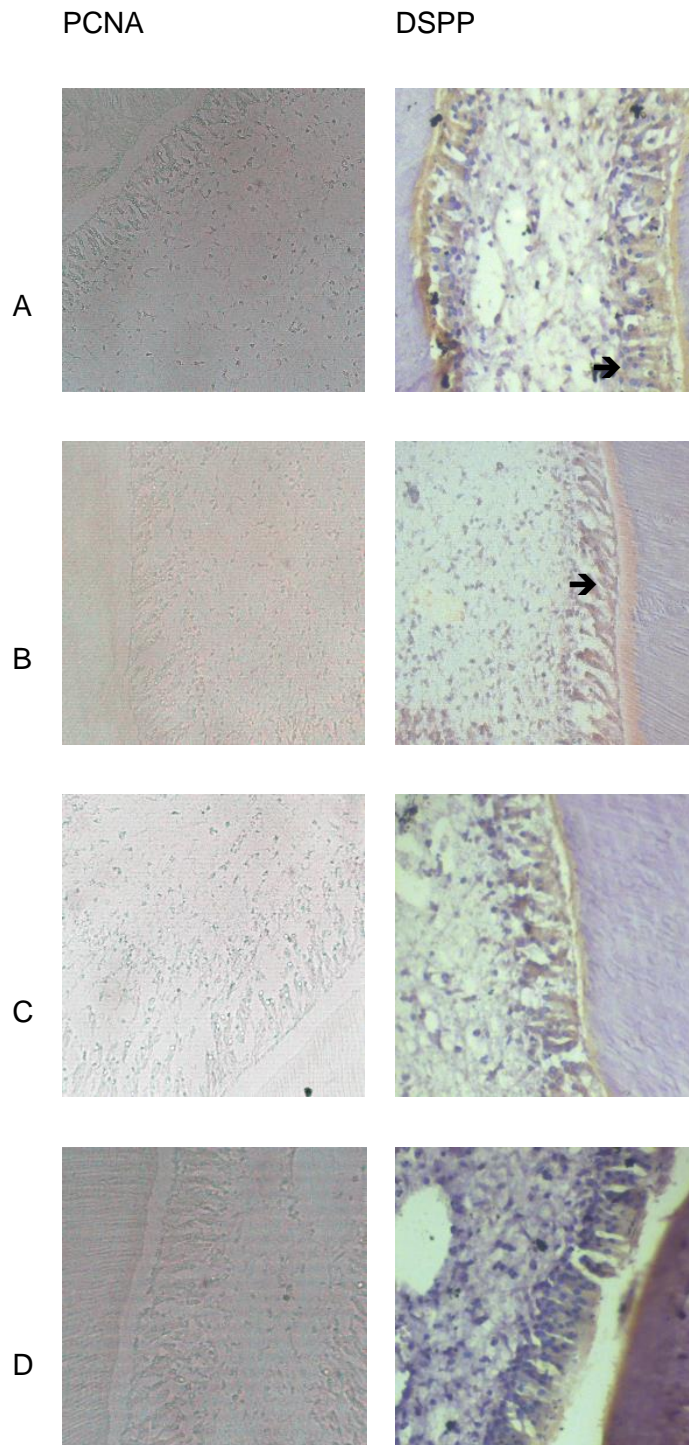
Figure 3.11. Immunohistochemistry for PCNA and DSPP: A) control samples, B) 5minutes/1,000ppm, C) 60 seconds/1,000ppm, and D) 60 seconds/300ppm. Black arrows highlight positive staining of DSPP.

| Rat Lung – TNF-α | Rat Lung IL-1β | Rat Skin PCNA |
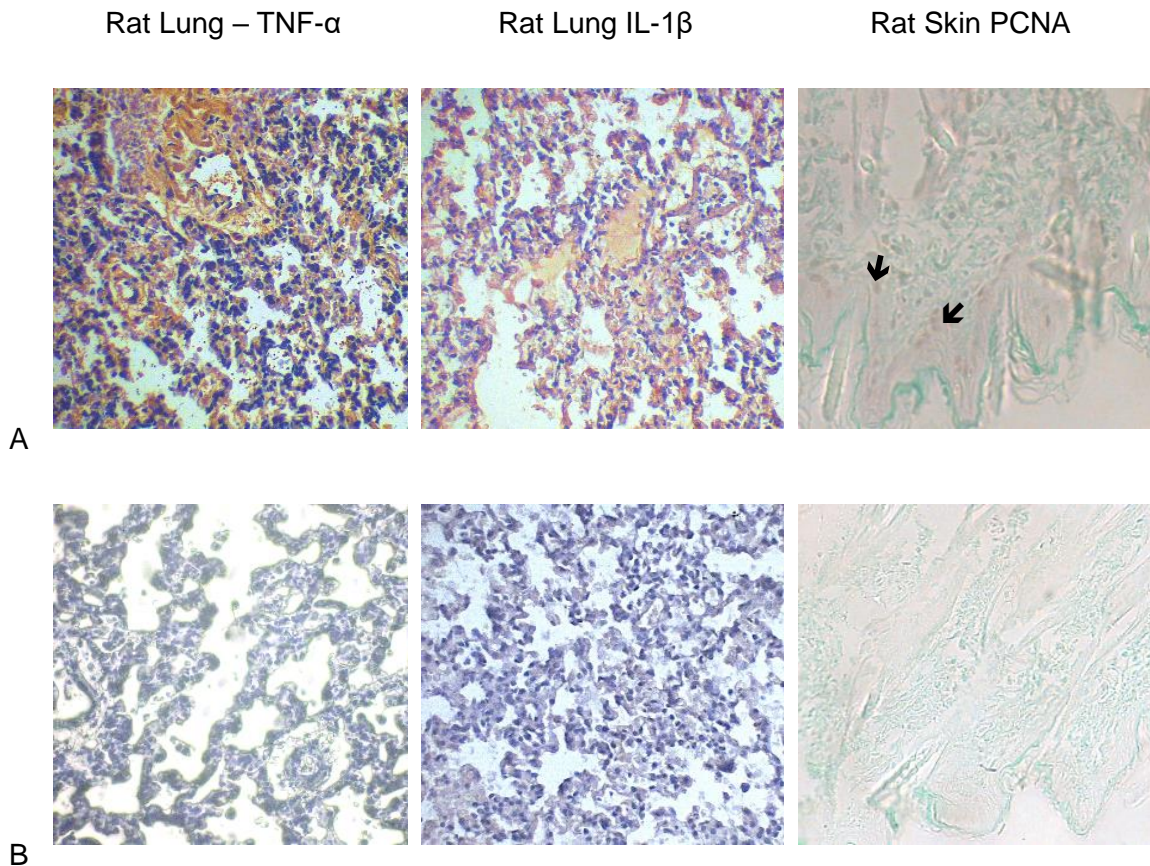
Figure 3.12. IHC controls. A) Positive controls: rat lung for TNF-α and IL-1β, and rat skin for PCNA. All showing positive staining (black arrows highlight cell nuclei in rat skin). B) Same tissues with primary antibody exclusion.

3.8 Primer validation for RT-qPCR analysis

Melt curves resulting from the designed primer validation are illustrated in Tables 3.2. And standard curves are shown in Table 3.3. DSPP (2) was the only pair of primers that showed acceptable efficiency (90%), and a single peak in the melting curve. Moreover, it has a linear standard curve (r^2= 0.991) providing a high correlation between Cq and the target copy number.

Table 3.2. Melt curves

PCNA (1)

DSPP (1)

PCNA (2)

DSPP (2)

PCNA (3)

DSPP (3)
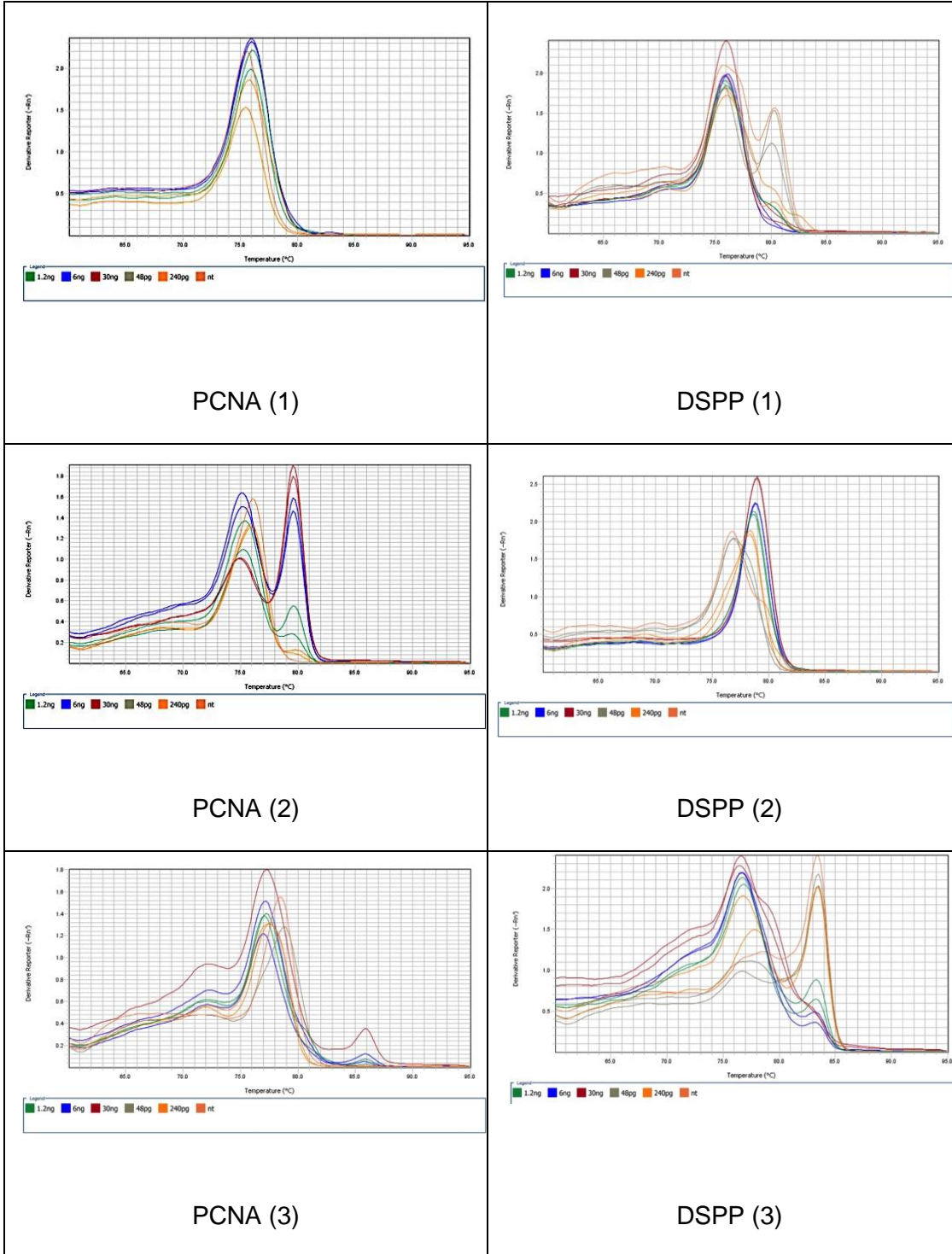
Table 3.3. Standard curves. Eff: percentage efficiency, r^2: linear correlation and slope for each pair of primers tested.

PCNA (1) eff=125%, r^2=0.907 Y = -2.839x + 29,636	DSPP(1) eff=363%, r^2=0.919 Y= -1.501x + 30,538
PCNA (2) eff=175%, r^2=0.989 Y= -2.277x + 29,029	DSPP(2) eff=90.150%, r^2=0.991 Y= -3.583x + 29,982
PCNA (3) eff=205%, r^2=0.968 Y= -2.063x + 30,778	DSPP(1) eff=280%, r^2=0.963 Y= -1.722x + 28,789
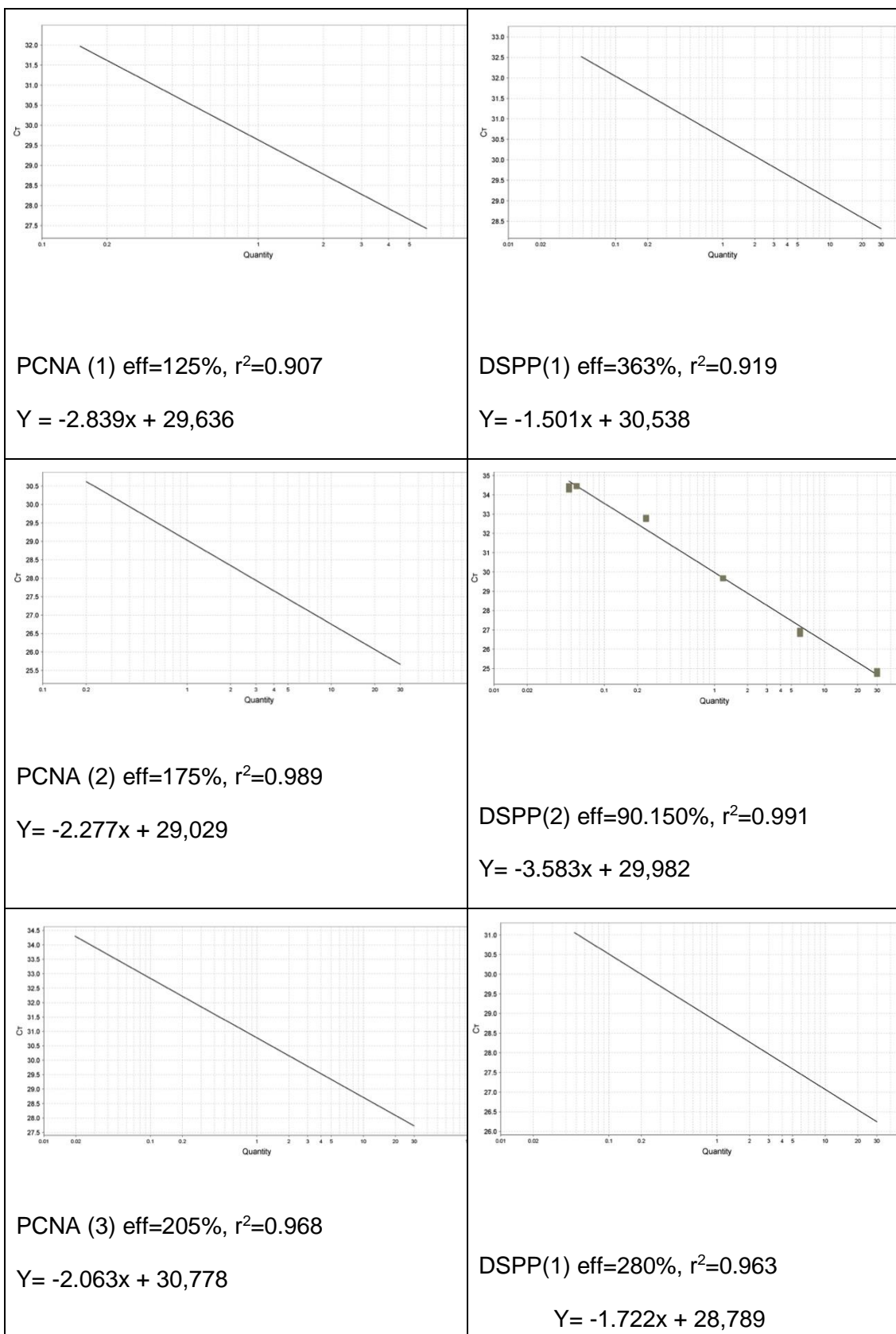
4. Discussion

4.1 General discussion.

The present study was designed to explore the potential to use H_2O_2 in deep carious cavities to both eliminate dentin-pulp complex infections and encourage regeneration. The development of strategies to treat dental caries is of interest because caries is a major public health problem, affecting the vast majority of adults (Petersen 2008) and generating a considerable economic burden (Allareddy et al. 2014).

Current treatments of dental cavities involve removing the affected tissues and decontaminating the residual dentin prior to restoring the anatomy and function of the tooth. Despite the continuous optimization of dental materials, there is still a high rate of failure in current treatments (Lucarotti et al. 2005). One of the main causes affecting the success in dental restoration is the persistence of bacterial remnants in the cavity walls that may lead to recurrent caries and pulpal inflammation (Nedeljkovic et al. 2015).

In dental caries progression, the dental pulp is able to secrete tertiary dentin stimulated by a mild inflammatory response. This can be reactionary dentin when it is secreted by primary odontoblasts or reparative dentin when it is produced by newly differentiated odontoblast-like cells (Cooper et al. 2010). The capacity of dentin to regenerate has been the subject of research for several decades and is clinically exploited by the use of materials that promote the natural response. However, these strategies have not yet succeeded in decreasing the failure rates in caries treatments (Zanini et al. 2017).

Hydrogen peroxide effect on dental pulp cells is gaining interest because it is the major component of tooth bleaching agents. It has been shown in *in vitro* studies, that dental pulp cells may be stimulated by low doses of ROS to express dentinogenic markers; thus reacting in a comparable way to low-grade inflammation (Lee et al. 2006; Soares et al. 2015). However, much uncertainty still exists between the relationship between H_2O_2, oxidative stress and dental pulp regeneration, and what is less clear is its potential use in deep cavities to induce the production of tertiary dentin.

4.2 The potential use of H_2O_2 as a biocide

The first question in this study sought to determine the justification of using H_2O_2 as a cavity disinfectant. Therefore, it was crucial to find the concentration that inhibited the growth of the microorganisms found in dentin-pulp complex infections. Once this range was established, dental pulp cell viability was determined when treated to these concentrations at clinically relevant exposure times. The results of this study have shown that concentrations greater than 1,250 ppm of H2O2 are required to inhibit the growth of the three strains evaluated and that at those concentrations there is a significant loss of viability of the rDPFs.

The MICs of CHX found for *E. faecalis* and *S. mutans* in this study was similar to the one found in previously published investigations (Jarvinen et al. 1993; Kitagawa et al. 2016). Additionally, rDPFs viability was not affected at bactericidal concentration, confirming that it is biocompatible and safe to use as a cavity disinfectant.

The results from the suspension test showed that H_2O_2 had no antibacterial activity against planktonic cells of *S. mutans, S. anginosus* and *E. faecalis*. Where concentrations as high as 1,000ppm showed no $\log_{10}$ reduction within 5 minutes. SEM images obtained after applying the same conditions showed in a general scan of the treated samples that the microorganisms were not significantly reduced in quantity when compared to the untreated sample.

The microorganisms tested here are unable to produce catalase enzyme, therefore their capacity to cope with oxidative stress in such high concentration of molecular H_2O_2 might be due to other mechanisms (Flahaut et al. 1998). This is of particular concern because surviving microorganisms at sub-MIC concentrations could generate tolerance to the biocide (Maillard 2007). In particular, *E. faecalis* mechanisms to adapt to oxidative stress was previously studied and it was shown that this microbe resistance could be related to new protein synthesis (Flahaut et al. 1998). Furthermore, by this acquired mechanism, *E. faecalis* could increase its virulence, allowing it to become an opportunistic pathogen (Riboulet et al. 2007). This is important because leaving microorganisms attached to the dentin walls could lead to recurrent caries lesion and further dental pulp damage. But also, using sub-MIC concentrations could

lead to the development of an intrinsic resistant microorganism with decreased susceptibility to other biocide applications (Maillard 2007).

These findings, while preliminary, suggest that at biocidal concentrations, H_2O_2 is highly cytotoxic to dental pulp cells and that at a clinically relevant time exposure at those concentrations does not inactivate the bacteria. Therefore, its clinical use in deep cavities at these concentrations is discouraged.

4.3 Hydrogen peroxide and its potential regenerative effect

The second objective of this project was to identify a relation between H_2O_2 and tertiary dentin production. As previously discussed in the introduction (section 1.7), *in vitro* studies showed that pre-odontoblastic cells (MDPC-23) exposed low concentrations of H_2O_2 showed an enhanced regenerative response, and maintain an odontoblast phenotype (Lee et al. 2006; Soares et al. 2016). Other studies that used primary human dental pulp cells exposed to low concentrations of H_2O_2 showed the ability to form mineralized tissues (Matsui et al. 2009) and to overexpress DSPP (Min et al. 2008).

These studies used monolayer cell-cultures to assess the response to oxidative stress, but the presence of the ECM may affect the diffusion of the H_2O_2 and reduce its cytotoxic effect (Wataha 2012). Moreover, cell-ECM interactions are crucial to study a regenerative response because the matrix contains bioactive molecules responsible for mediating the cellular behaviour (Smith et al. 2016). Therefore, the rat *ex vivo* tooth slice model can help in assessing the dentin-pulp complex response to H_2O_2 in a way that more closely mimics the *in vivo* situation.

Firstly, the dental pulp cells viability was assessed after treating the tooth slices with two different concentrations of $H_2O_{2.}$ An interesting finding in this study was that the number of cells/mm^2 was not reduced after all the treatments tested. On the contrary, the same conditions resulted toxic to rDPFs cultured in monolayer. This finding further supports the importance of the use of *ex vivo* models, showing how the presence of the

ECM and the cells being held in their natural structural environment can affect their response to drug testing. But most important, it implies that H_2O_2 might have potential use as a cavity disinfectant because in a clinical situation the cells are protected by the ECM and the dentin remnant and higher concentrations of the biocide could be used.

Immunohistochemistry results in this project are in line with those of previous studies, showing that the samples treated with H_2O_2 expressed high levels of TNF-α. Thus, suggesting an inflammatory response to oxidative stress. In this case, TNF-α was expressed mostly in the odontoblast layer. This is interesting because, as previously demonstrated, TNF-α can potentiate the effect of TGF-β to induce an upregulation of the p38-MAPKinase (Simon et al. 2010); and therefore could be implicated in tertiary dentin secretion on behalf of primary odontoblasts. Moreover, TNF-α can induce dental pulp cells to express mineralization associated proteins and to differentiate into odontoblast-like cells (Paula-Silva et al. 2009).

Interestingly, Il-1β showed negative staining in all the samples suggesting the absence of expression of this inflammatory marker. However, it could be argued that this negative result might be due to the lack of optimization of the immunohistochemistry technique. In this study, tissues were fixed in formalin and no antigen retrieval step was used, therefore the absence of staining could also be caused by the inability of the primary antibody to bind to the cytokine. A previous study that showed by immunohistochemistry that IL-1β is highly expressed in inflamed human dental pulps, showed as a histological finding that this cytokine was highly expressed in the odontoblast layer of healthy pulps as well (Alessandra Cecília Oliveira et al. 2009). To overcome these inconsistencies, cytokine expression at a gene level could be quantified by RT-qPCR. To implement such an experiment, tooth slices would have to be cut into thicker sections in order to have more tissue mass for the RNA extraction.

Immunohistochemical detection of DSPP, showed staining in the odontoblast layer in the negative control and treated samples, thus suggesting that 24 hours after treatment, primary odontoblasts were unaffected by any concentration of H_2O_2 or duration of the exposure applied (Table 3.1). However, DSPP was absent in other areas of the pulp, suggesting that treatment with H_2O_2 did not propitiate an odontoblast-like differentiation. Moreover, PCNA expression was not detected in either of the treatments; it is possible therefore that the treatment did not enhance a

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proliferative response from the DPSCs, or that incubation of the samples for 24 hours after the treatment was not enough to allow such behaviour.

These results agree with a recently published study, that used a controlled molar-damage *in vivo* model to study the timescale of reparative dentinogenesis, where PCNA was overexpressed 3 days after damage and that DSPP expression by newly formed odontoblasts was shown 5 days after damage as assessed by immunohistochemistry and *in situ* hybridisation respectively (Babb et al. 2017). Sloan et al. (1999) used the *ex vivo* tooth slice model to assess the dentin-pulp complex response to TGF-β, showing a significant increase in the predentin thickness assessed by histological examination after 7 days of culture. Thus, although there was no evident proliferative activity nor differentiation from the pulp cells in this study, this negative result could be caused because the post-treatment incubation time was insufficient to evaluate a reparative response and not due to a negative response to the H_2O_2 treatment.

Therefore, the present study suggests that H_2O_2 might elicit a regenerative response from the primary odontoblasts to secrete reactionary dentine. These maintain their phenotype and express TNF-α, which could potentiate the stimulation to produce matrix by growth factors present in the ECM. Moreover, although a reparative response was not evident in other areas of the pulp, the cell viability was not reduced and therefore it will be worth further investigating that potential.

4.4 Study limitations and future work

One of the drawbacks of using an *ex vivo* model to study the inflammatory response, is the lack of circulatory system making the model a static system. However, the model provides the means to study specific dentinogenic events without systemic variations. The major limitation of this project was the time constraint, which led to the inability to assess the reparative response in longer culture periods and to quantify the cytokine and regenerative markers at a gene level. For this purpose, a first attempt to validate primers for PCNA and DSPP was performed as shown in sections 2.13 and 3.9 resulting in the validation of a pair of primers for DSPP.

Future work will address these questions, focusing on following a reasonable dentinogenesis timescale that could be assessed with the *ex vivo* rat tooth model. Moreover, in future investigations, it might be useful to work with a range of lower H_2O_2 concentrations, but with longer periods of exposure, in an attempt to mimic the natural occurring oxidative stress, and thus, to be able to study the molecular mechanisms underlying a potential dentinogenic response. The use of an *ex vivo* model in this situation can be justified by the fact that dental pulp stem cells are a heterogeneous population, which have high variability in the proliferative and differentiation capabilities (Alraies et al. 2017). *Ex vivo* models for oxidative stress have already been designed in other tissues. For example, a porcine *ex vivo* retina culture model for oxidative stress was developed, but in this case with the purpose of understanding eye disorders caused by ROS (Schnichels et al. 2016).

4.5 Conclusions

The aim of the present research was to examine the dentin-pulp complex response to hydrogen peroxide at the cellular and molecular level, as well as its effect on microorganisms associated with tooth infections.

The most obvious finding to emerge from this study is that, whilst biocidal concentrations of H_2O_2 decreased significantly the cell viability of dental pulp cells cultured in monolayer, did not affect the viability of dental pulp cells in the *ex vivo* tooth model. This finding highlights the importance of the ECM in influencing drug diffusion through the tissue architecture. But most important, it suggests a potential use of H_2O_2 in cavity disinfectant formulations. However, this would require further investigation on the inactivation kinetics of the microorganisms involved in dentin infection.

The short incubation time after the treatment of the tooth slices might have limited the possibility of the proteins involved in tertiary dentin production to be expressed. Future work will address these limitations and intend to add data of expression at the gene level.

In spite of these limitations, this study suggests that after treatment with H_2O_2, primary odontoblasts maintain their viability and phenotype, while expressing TNF-α, a cytokine that could stimulate the secretory production of such cells. And thus, suggesting an autocrine regulation of the odontoblast layer in response to oxidative stress. Therefore, these results suggest a potential of hydrogen peroxide as a mediator in the regenerative processes and a possible clinical use for that purpose. In conclusion, the results from this study support to pursue the research of the effect of oxidative stress on the dentin-pulp complex.

5. References

Alessandra Cecília Oliveira, S. et al. 2009. Interleukin-1 beta and interleukin-8 in healthy and inflamed dental pulps. *Journal of Applied Oral Science* 17(5), pp. 527-532. doi: 10.1590/S1678-77572009000500031

Allareddy, V. et al. 2014. Hospital-based emergency department visits involving dental conditions: Profile and predictors of poor outcomes and resource utilization: Profile and predictors of poor outcomes and resource utilization. *The Journal of the American Dental Association* 145(4), pp. 331-337. doi: 10.14219/jada.2014.7

Alraies, A. et al. 2017. Variation in human dental pulp stem cell ageing profiles reflect contrasting proliferative and regenerative capabilities. *BMC Cell Biol.* 18(1), doi: 10.1186/s12860-017-0128-x

Babb, R. et al. 2017. Axin2-expressing cells differentiate into reparative odontoblasts via autocrine Wnt/β-catenin signaling in response to tooth damage. *Sci Rep* 7(1), pp. 3102-3102. doi: 10.1038/s41598-017-03145-6

Barbosa-Ribeiro, M. et al. 2016. Antimicrobial Susceptibility and Characterization of Virulence Genes of Enterococcus faecalis Isolates from Teeth with Failure of the Endodontic Treatment. *Journal of Endodontics* 42(7), pp. 1022-1028. doi: 10.1016/j.joen.2016.03.015

Benetti, F. et al. 2017. Hydrogen peroxide induces cell proliferation and apoptosis in pulp of rats after dental bleaching in vivo: Effects of the dental bleaching in pulp. *Archives of Oral Biology* 81, pp. 103-109. doi: https://doi.org/10.1016/j.archoralbio.2017.04.013

Butler, W. et al. 1992. ISOLATION, CHARACTERIZATION AND IMMUNOLOCALIZATION OF A 53-KDAL DENTIN SIALOPROTEIN (DSP). *Matrix* 12(5), pp. 343-351. doi: 10.1016/S0934-8832(11)80030-2

Camargo, S. E. A. et al. 2007. Penetration of 38% Hydrogen Peroxide into the Pulp Chamber in Bovine and Human Teeth Submitted to Office Bleach Technique. *Journal of Endodontics* 33(9), pp. 1074-1077. doi: 10.1016/j.joen.2007.04.014

Chai, Y. et al. 2000. Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis. *Development* 127(8), pp. 1671-1679.

Cohenca, N. et al. 2013. Vital Pulp Therapy. *Dental Clinics of North America* 57(1), pp. 59-73. doi: 10.1016/j.cden.2012.09.004

Cooper, P. R. et al. 2010. Inflammation–regeneration interplay in the dentine–pulp complex. *Journal of Dentistry* 38(9), pp. 687-697. doi: https://doi.org/10.1016/j.jdent.2010.05.016

El Karim, I. A. et al. 2009. Neuropeptides Regulate Expression of Angiogenic Growth Factors in Human Dental Pulp Fibroblasts. *Journal of Endodontics* 35(6), pp. 829-833. doi: 10.1016/j.joen.2009.03.005

Facklam, R. 2002. What Happened to the Streptococci: Overview of Taxonomic and Nomenclature Changes. *Clinical Microbiology Reviews* 15(4), p. 613. doi: 10.1128/CMR.15.4.613-630.2002

Featherstone, J. D. B. 2000. THE SCIENCE AND PRACTICE OF CARIES PREVENTION. *The Journal of the American Dental Association* 131(7), pp. 887-899. doi: 10.14219/jada.archive.2000.0307

Finkel, T. 1998. Oxygen radicals and signaling. *Current Opinion in Cell Biology* 10(2), pp. 248-253. doi: 10.1016/S0955-0674(98)80147-6

Fisher, L. E. and Russell, R. R. B. 1993. The Isolation and Characterization of Milleri Group Streptococci from Dental Periapical Abscesses. *Journal of Dental Research* 72(8), pp. 1191-1193. doi: 10.1177/00220345930720080501

Flahaut et al. 1998. The oxidative stress response in Enterococcus faecalis : relationship between H 2 O 2 tolerance and H 2 O 2 stress proteins. *Letters in Applied Microbiology* 26(4), pp. 259-264. doi: 10.1046/j.1472-765X.1998.00325.x

Fraud, S. et al. 2001. Comparison of the mycobactericidal activity of ortho- phthalaldehyde, glutaraldehyde and other dialdehydes by a quantitative suspension test. *Journal of Hospital Infection* 48(3), pp. 214-221. doi: 10.1053/jhin.2001.1009

Frencken, J. E. et al. 2017. Global epidemiology of dental caries and severe periodontitis – a comprehensive review. *Journal of Clinical Periodontology* 44(S18), pp. S94-S105. doi: 10.1111/jcpe.12677

Fukuyama, Y. et al. 2008. Hydrogen peroxide induces expression and activation of AMP‑activated protein kinase in a dental pulp cell line. *International Endodontic Journal* 41(3), pp. 197-203. doi: 10.1111/j.1365-2591.2007.01337.x

Garberoglio, R. and Brännström, M. 1976. Scanning electron microscopic investigation of human dentinal tubules. *Archives of Oral Biology* 21(6), pp. 355-362. doi: 10.1016/S0003-9969(76)80003-9

Glickman, G. N. 2009. AAE Consensus Conference on Diagnostic Terminology: Background and Perspectives.

Goldberg, M. et al. 2008. Inflammatory and immunological aspects of dental pulp repair. *Pharmacological Research* 58(2), pp. 137-147. doi: 10.1016/j.phrs.2008.05.013

Gronthos, S. et al. 2002. Stem Cell Properties of Human Dental Pulp Stem Cells. *Journal of Dental Research* 81(8), pp. 531-535. doi: 10.1177/154405910208100806

Gronthos, S. et al. 2000. Postnatal human dental pulp stem cells (DPSCs) in vitro and invivo. *Proceedings of the National Academy of Sciences of the United States of America* 97(25), p. 13625. doi: 10.1073/pnas.240309797

Hahn, C.-L. and Overton, B. 1997. The effects of immunoglobulins on the convective permeability of human dentine in vitro. *Archives of Oral Biology* 42(12), pp. 835-843. doi: 10.1016/S0003-9969(97)00080-0

Hargreaves, K. M. et al. 2016. *Cohen's pathways of the pulp*. 11th edition. ed. Saint Louis, Missouri : Elsevier.

Hargreaves, K. M. and Cohen, S. 2011. *Cohen's pathways of the pulp*. 10th ed. ed. St. Louis: St. Louis : Mosby Elsevier.

Hasselmann, C. 2003. Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. *Clin. Microbiol. Infect.* 9(8),

Hirsch, V. et al. 2017. Inflammatory cytokines in normal and irreversibly inflamed pulps: A systematic review. *Archives of Oral Biology* 82, pp. 38-46. doi: 10.1016/j.archoralbio.2017.05.008

Hubble, T. S. et al. 2003. Influence of Enterococcus faecalis proteases and the collagen‐binding protein, Ace, on adhesion to dentin. *Oral Microbiology and Immunology* 18(2), pp. 121-126. doi: 10.1034/j.1399-302X.2003.00059.x

Iglesias, K. et al. 2003. Reduction in tooth stiffness as a result of endodontic access and loss of different dental crown surfaces. *J. Dent. Res.* 82, pp. 134-134.

Izumi, T. et al. 1995. Immunohistochemical study on the immunocompetent cells of the pulp in human non-carious and carious teeth. *Archives of Oral Biology* 40(7), pp. 609-614. doi: 10.1016/0003-9969(95)00024-J

Jarvinen, H. et al. 1993. In vitro susceptibility of Streptococcus mutans to chlorhexidine and six other antimicrobial agents. *Antimicrobial Agents and Chemotherapy* 37(5), p. 1158. doi: 10.1128/AAC.37.5.1158

Jenkinson, H. and Lamont, R. 1997. Streptococcal adhesion and colonization. *Crit. Rev. Oral Biol. Med.*

Jenkinson, H. F. and Lamont, R. J. 2005. Oral microbial communities in sickness and in health. *Trends in Microbiology* 13(12), pp. 589-595. doi: 10.1016/j.tim.2005.09.006

Kayaoglu, G. and Ørstavik, D. 2004. Virulence Factors ofEnterococcus faecalis: Relationship to Endodontic Disease. *Critical Reviews in Oral Biology & Medicine* 15(5), pp. 308-320. doi: 10.1177/154411130401500506

Kitagawa, H. et al. 2016. Evolution of resistance to cationic biocides in Streptococcus mutans and Enterococcus faecalis. *Journal of Dentistry* 47, pp. 18-22. doi: 10.1016/j.jdent.2016.02.008

Kouidhi, B. et al. 2011. Antibiotic resistance and adhesion properties of oral Enterococci associated to dental caries. *BMC Microbiol.* 11, doi: 10.1186/1471-2180-11-155

Lange, J. et al. 2010. Action of IL-1 beta during Fracture Healing. *J. Orthop. Res.* 28(6), pp. 778-784. doi: 10.1002/jor.21061

Lee, D. et al. 2006. Effects of hydrogen peroxide (H 2 O 2) on alkaline phosphatase activity and matrix mineralization of odontoblast and osteoblast cell lines. *Cell Biology and Toxicology* 22(1), pp. 39-46. doi: 10.1007/s10565-006-0018-z

Love, R. M. 2001. Enterococcus faecalis – a mechanism for its role in endodontic failure. *International Endodontic Journal* 34(5), pp. 399-405. doi: 10.1046/j.1365-2591.2001.00437.x

Love, R. M. and Jenkinson, H. 2002. Invasion of dentinal tubules by oral bacteria. *Crit. Rev. Oral Biol. Med.*

Lucarotti, P. S. K. et al. 2005. Outcome of direct restorations placed within the general dental services in England and Wales (Part 1): Variation by type of restoration and re-intervention. *Journal of Dentistry* 33(10), pp. 805-815. doi: 10.1016/j.jdent.2005.03.008

Maillard, J. Y. 2007. Bacterial resistance to biocides in the healthcare environment: should it be of genuine concern? ,

Matsui, S. et al. 2009. Stimulatory Effects of Low-concentration Reactive Oxygen Species on Calcification Ability of Human Dental Pulp Cells. *Journal of Endodontics* 35(1), pp. 67-72. doi: 10.1016/j.joen.2008.08.034

Michel, G. et al. 2015. Is Pulp Inflammation a Prerequisite for Pulp Healing and Regeneration? *Mediators of Inflammation* 2015(2015), doi: 10.1155/2015/347649

Michelich, V. et al. 1978. Dentin Permeability: A Comparison of Functional Versus Anatomical Tubular Radii. *Journal of Dental Research* 57(11-12), pp. 1019-1024. doi: 10.1177/00220345780570110301

Min, K.-S. et al. 2008. Hydrogen Peroxide Induces Heme Oxygenase–1 and Dentin Sialophosphoprotein mRNA in Human Pulp Cells. *Journal of Endodontics* 34(8), pp. 983-989. doi: https://doi.org/10.1016/j.joen.2008.05.012

Moseley, R. et al. 2002. Comparison of the antioxidant properties of HYAFF ®-11p75, AQUACEL ® and hyaluronan towards reactive oxygen species in vitro. *Biomaterials* 23(10), pp. 2255-2264. doi: 10.1016/S0142-9612(01)00360-X

Moseley, R. et al. 2003. The Influence of Fluoride Exposure on Dentin Mineralization Using an in Vitro Organ Culture Model. *Calcified Tissue International* 73(5), pp. 470-475. doi: 10.1007/s00223-003-0022-8

Munksgaard, E. C. et al. 1978. Biosynthesis of phosphoprotein by rat incisor odontoblasts in in-vitro culture. *Archives of Oral Biology* 23(7), pp. 583-585. doi: 10.1016/0003-9969(78)90275-3

Nanci, A. 2018. *Ten Cate's oral histology : development, structure, and function*. 9th edition. ed. St. Louis, Missouri : Elsevier.

Nedeljkovic, I. et al. 2015. Is secondary caries with composites a material-based problem? *Dental Materials* 31(11), pp. e247-e277. doi: 10.1016/j.dental.2015.09.001

Nishio Ayre, W. et al. 2018. Enterococcus faecalis demonstrates pathogenicity through increased attachment in an ex vivo polymicrobial pulpal infection.

Patel, M. et al. 2009. Phenotype and behaviour of dental pulp cells during expansion culture.

Paula-Silva, F. et al. 2009. TNF-alpha Promotes an Odontoblastic Phenotype in Dental Pulp Cells. *J. Dent. Res.* 88(4), pp. 339-344. doi: 10.1177/0022034509334070

Petersen, P. E. 2008. World Health Organization global policy for improvement of oral health - World Health Assembly 2007. *International Dental Journal* 58(3), pp. 115-121. doi: 10.1111/j.1875-595X.2008.tb00185.x

Pevsner-Fischer, M. et al. 2007. Toll-like receptors and their ligands control mesenchymal stem cell functions. *Blood* 109(4), pp. 1422-1432. doi: 10.1182/blood-2006-06-028704

Piesco, N. et al. 1995. REGULATION OF CYTOKINE EXPRESSION IN PULP FIBROBLASTS. *J. Dent. Res.* 74, pp. 545-545.

Qin, Z. et al. 2015. High dose of TNF-α suppressed osteogenic differentiation of human dental pulp stem cells by activating the Wnt/β-catenin signaling. *Journal of Molecular Histology* 46(4), pp. 409-420. doi: 10.1007/s10735-015-9630-7

Radi, R. 2018. Oxygen radicals, nitric oxide, and peroxynitrite: Redox pathways in molecular medicine. *Proceedings of the National Academy of Sciences of the United States of America* 115(23), pp. 5839-5848. doi: 10.1073/pnas.1804932115

Rechenberg, D. et al. 2016. Biological Markers for Pulpal Inflammation: A Systematic Review. *PLoS One*.

Riboulet, E. et al. 2007. Relationships between Oxidative Stress Response and Virulence in Enterococcus faecalis. *Journal of Molecular Microbiology and Biotechnology* 13(1-3), pp. 140-146. doi: 10.1159/000103605

Ricucci, D. et al. 2014. Correlation between Clinical and Histologic Pulp Diagnoses. *Journal of Endodontics* 40(12), pp. 1932-1939. doi: 10.1016/j.joen.2014.08.010

Roberts, J. L. et al. 2013. Development of an Ex Vivo Coculture System to Model Pulpal Infection by Streptococcus anginosus Group Bacteria. *Journal of Endodontics* 39(1), pp. 49-56. doi: 10.1016/j.joen.2012.09.005

Rosen, E. et al. 2018. Challenges in the Eradication of Enterococcus faecalis and its Implications on Health. *Current Oral Health Reports* 5(1), pp. 70-77. doi: 10.1007/s40496-018-0172-4

Ruch, J. V. et al. 1995. Odontoblast differentiation. *International Journal of Developmental Biology* 39(1), pp. 51-68. doi: 10.1387/ijdb.7626422

Sadaghiani, L. et al. 2016. Growth Factor Liberation and DPSC Response Following Dentine Conditioning. *Journal of Dental Research* 95(11), pp. 1298-1307. doi: 10.1177/0022034516653568

Schnichels, S. et al. 2016. A novel ex-vivo retina model for oxidative stress - chances for the replacement of animal experiments. *Invest. Ophthalmol. Vis. Sci.* 57(12),

Schulte, J. M. and Van Waes, H. 2007. Dentinogenesis imperfecta. *Dentinogenesis imperfecta* 19(4), pp. 418-421. doi: 10.1007/s11825-007-0048-9

Shi, S. and Gronthos, S. 2003. Perivascular Niche of Postnatal Mesenchymal Stem Cells in Human Bone Marrow and Dental Pulp. *Journal of Bone and Mineral Research* 18(4), pp. 696-704. doi: 10.1359/jbmr.2003.18.4.696

Simon, S. et al. 2010. The MAP Kinase Pathway Is Involved in Odontoblast Stimulation via p38 Phosphorylation. *Journal of Endodontics* 36(2), pp. 256-259. doi: 10.1016/j.joen.2009.09.019

Sloan, A. and Smith, A. 2007. Stem cells and the dental pulp: potential roles in dentine regeneration and repair. Oxford, UK.

Sloan, A. J. and Lynch, C. D. 2012. Dental tissue repair: Novel models for tissue regeneration strategies. *Open Dentistry Journal* 6(1), pp. 214-219. doi: 10.2174/1874210601206010214

Sloan, A. J. et al. 2000. Stimulation of the rat dentine–pulp complex by bone morphogenetic protein-7 in vitro. *Archives of Oral Biology* 45(2), pp. 173-177. doi: 10.1016/S0003-9969(99)00131-4

Sloan, A. J. et al. 1998. An in vitro approach for the study of dentinogenesis by organ culture of the dentine–pulp complex from rat incisor teeth. *Archives of Oral Biology* 43(6), pp. 421-430. doi: 10.1016/S0003-9969(98)00029-6

Sloan, A. J. and Smith, A. J. 1999. Stimulation of the dentine–pulp complex of rat incisor teeth by transforming growth factor- β isoforms 1–3 in vitro. *Archives of Oral Biology* 44(2), pp. 149-156. doi: 10.1016/S0003-9969(98)00106-X

Smith, A. and Lesot, H. 2001. Induction and regulation of crown dentinogenesis: Embryonic events as a template for dental tissue repair? *Crit. Rev. Oral Biol. Med.*

Smith, A. J. et al. 1995. Reactionary dentinogenesis. *International Journal of Developmental Biology* 39(1), pp. 273-280. doi: 10.1387/ijdb.7626417

Smith, A. J. and Cooper, P. R. 2017. Regenerative Endodontics: Burning Questions. *Journal of Endodontics* 43(9), pp. S1-S6. doi: 10.1016/j.joen.2017.06.002

Smith, A. J. et al. 2016. Exploiting the Bioactive Properties of the Dentin-Pulp Complex in Regenerative Endodontics. *Journal of Endodontics* 42(1), pp. 47-56. doi: 10.1016/j.joen.2015.10.019

Smith, A. J. et al. 2001. Trans-dentinal Stimulation of Tertiary Dentinogenesis. *Advances in Dental Research* 15(1), pp. 51-54. doi: 10.1177/08959374010150011301

Smith, A. J. et al. 2012. Harnessing the Natural Regenerative Potential of the Dental Pulp. *Dental Clinics of North America* 56(3), pp. 589-601. doi: 10.1016/j.cden.2012.05.011

Soares, D. G. et al. 2015. Effect of hydrogen-peroxide-mediated oxidative stress on human dental pulp cells. *Journal of Dentistry* 43(6), pp. 750-756. doi: 10.1016/j.jdent.2014.12.006

Soares, D. G. et al. 2016. Indirect cytocompatibility of a low‐concentration hydrogen peroxide bleaching gel to odontoblast‐like cells. *International Endodontic Journal* 49(1), pp. 26-36. doi: 10.1111/iej.12426

Tani‐Ishii, N. et al. 1995. Immunolocalization of bone‐resorptive cytokines in rat pulp and periapical lesions following surgical pulp exposure. *Oral Microbiology and Immunology* 10(4), pp. 213-219. doi: 10.1111/j.1399-302X.1995.tb00145.x

Thannickal, V. and Fanburg, B. 2000. Reactive oxygen species in cell signaling. *Am. J. Physiol.-Lung Cell. Mol. Physiol.*

Thesleff, I. and Sharpe, P. 1997. Signalling networks regulating dental development. *Mechanisms of Development* 67(2), pp. 111-123. doi: 10.1016/S0925-4773(97)00115-9

Trowbridge, H. O. and Stevens, B. H. Microbiologic and pathologic aspects of pulpal and periapical disease. (1046-0764 (Print)),

van Rijkom, H. M. et al. 1996. A Meta-analysis of Clinical Studies on the Caries-inhibiting Effect of Chlorhexidine Treatment. *Journal of Dental Research* 75(2), pp. 790-795. doi: 10.1177/00220345960750020901

Waddington, R. J. et al. 2000. Periodontal disease mechanisms - Reactive oxygen species: a potential role in the pathogenesis of periodontal diseases.

Wataha, J. C. 2012. Predicting clinical biological responses to dental materials. *Dental Materials* 28(1), pp. 23-40. doi: 10.1016/j.dental.2011.08.595

Whiley, R. A. et al. 1992. Streptococcus intermedius, Streptococcus constellatus, and Streptococcus anginosus (the Streptococcus milleri group): Association with different body sites and clinical infections. *Journal of Clinical Microbiology* 30(1), pp. 243-244.

Yoshiba, K. et al. 2003. Class II Antigen-presenting Dendritic Cell and Nerve Fiber Responses to Cavities, Caries, or Caries Treatment in Human Teeth. *Journal of Dental Research* 82(6), pp. 422-427. doi: 10.1177/154405910308200604

Zander, H. A. 1939. Reaction of the Pulp to Calcium Hydroxide. *Journal of Dental Research* 18(4), pp. 373-379. doi: 10.1177/00220345390180040601

Zanini, M. et al. 2017. Pulp Inflammation Diagnosis from Clinical to Inflammatory Mediators: A Systematic Review. *Journal of endodontics.* 43(7), pp. 1033-1051. doi: 10.1016/j.joen.2017.02.009

Zehnder, M. et al. 2003. Cytokine gene expression—part of host defence in pulpitis. *Cytokine* 22(3), pp. 84-88. doi: 10.1016/S1043-4666(03)00116-9