Exploiting bioactive molecules in the dentine matrix for the treatment of apical periodontitis

By

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A thesis submitted to the University of Birmingham for the degree of MSc by Research

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1. Abstract

The main focus of endodontic treatment in to disinfect the root canal system with sodium hypochlorite (NaOCl) in order to facilitate periapical healing. Ethylenediaminetetraacetic acid (EDTA) has been shown to solubilise bioactive molecules sequestered in dentine, such as TGF-β1, which can modulate cell signalling pathways that enhance tissue healing. An *in-vitro* tooth model and sampling techniques were developed which investigated the ability of contemporary irrigant solutions to release TGF-β1 in order for it to be bioavailable at the interface between the root canal and periapical tissues. EDTA, chlorhexidine (CHX) and calcium hydroxide [$Ca(OH)_2$] all resulted in TGF-β1 release. EDTA released the highest concentration of TGF-β1, which increased in a time dependent manner. When a primary disinfectant stage of NaOCl was introduced, there were no detectable levels of TGF-β1 even after multiple rinses with EDTA. CHX released the lowest amounts of TGF-β1 but also attenuated the action of EDTA. When $Ca(OH)_2$ was introduced as an inter-appointment dressing between the disinfectant step of NaOCl and application of EDTA, TGF-β1 was released. Scanning electron microscopy showed the highest erosion of dentine occurred with a combination of CHX and EDTA. Clinically if both disinfection and growth factor release from dentine is to be achieved a two-visit endodontic procedure is supported using NaOCl at the first-visit, an inter-appointment dressing of $Ca(OH)_2$ and with EDTA being used alone at the second-visit in order to maximise the bioavailability of TGF-β1.

Acknowledgements

I could not have completed this project with the support and guidance of my two supervisors Professor Paul Cooper and Dr Phillip Tomson. Both these individuals have given me countless hours of their time and helped me tirelessly throughout this project. I wish to also acknowledge Professor Lumley, Dr Warren and Miss Blair who are always there to offer guidance and encouragement.

I would also like to thank the staff of the Laboratories, Gay Smith, Helen Wright, Michelle Holder and Jianguo Liu who has assisted me in my experiments over the last two years.

This work was supported by the following organisations:

The British Endodontic Society

The Royal College of Surgeons Edinburgh (Prime Pumping Grant)

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ADM	Adrenomedullin
$Ca(OH)_2$	Calcium hydroxide
CHX	Chlorhexidine gluconate
CSF	Colony stimulating growth factor
EDTA	Ethylenediaminetetraacetic acid EDTA
ELISA	Enzyme-linked immunosorbent assays
ESE	European society of endodontology
FGF	Fibroblast growth factor
HCL	Hydrogen chloride
IL-2	Interleukin-2
IGF	Insulin-like growth factor
INF- δ	Interferon-δ
$Na_2S_2O_2$	Sodium thiosulphate
NaOCl	Sodium hypochlorite
NaOH	Sodium hydroxide
TGF-β	Transforming growth factor-β
TGF-β1	Transforming growth factor-β1
TNF-α	Tumour necrosis factor -α
PTF	Periradicular tissue fluid
PUI	Passive ultrasonic irrigation
SE	Standard error
SEM	Scanning electron microscopy

2. Introduction

2.1. Conventional endodontic treatment

Apical periodontitis is a common disease which is reported to have a prevalence of 2.0 to 15.1% (Loftus et al., 2005); (Mukhaimer et al., 2012). Notably, if the tooth has been previously root filled then the prevalence is reported to be even higher and ranges from 29.7 to 71.9% (Boucher et al., 2002); (Al-Omari et al., 2011).

The primary aetiological factor behind the development of apical periodontitis is considered to be the microbial infection of the root canal system (Möller et al., 1981). Indeed work by Kakehashi et al. (1965) showed that bacteria were necessary to elicit periapical disease. With this basic understanding it has been established that the elimination of bacterial pathogens from the root canal system is understood to be the main factor in facilitating a return to periapical health. It has been shown that the presence of bacteria at the time of obturation has a significant effect on periapical healing, with bacteria-free tissues having a greater chance of periapical healing (Sjögren et al., 1991). Bacteria-free root canals in animal models also exhibit a positive influence on the resolution of periapical disease, with periapical healing more successful when there was a lack of detectable microorganisms (Fabricius et al., 2006). In the absence of bacteria, periapical healing occurred independently of the quality of root canal filling, even when the root canal obturation was suboptimal (Fabricius et al., 2006).

Contemporary endodontic strategies have thus focused on the ability of the operator to deliver antibacterial agents deep into the root canal system to reduce the microbial load (Endodontology, 2006). Sodium hypochlorite (NaOCl) has been the irrigant of choice, largely due to its ability to dissolve

organic matter and its efficacy against a broad range of microbiota. Clinical procedures are traditionally performed using hand/engine driven files and irrigating solutions that have high bactericidal efficacy, ability to inactivate endotoxin and dissolve organic tissue (Park et al., 2012). This approach is termed chemo-mechanical disinfection and is a process whereby mechanical instrumentation shapes the root canal for delivery of irrigants. These chemicals subsequently remove necrotic pulpal tissue, kill microorganisms and denature their endotoxins (Park et al., 2012).

Commonly, the main irrigants used in combination for contemporary root canal treatment (which are also discussed in section **2.7**) are NaOCl with an adjunct of Ethylenediaminetetraacetic acid (EDTA) (Basrani and Haapasalo, 2012, Haapasalo et al., 2010). NaOCl is applied at concentrations between 0.5-6% with both low and high concentrations shown to be equally effective in reducing bacterial load in the *in vivo* setting (Bystrom and Sundqvist, 1981). EDTA is used as a chelator to breakdown the inorganic tissue and remove the smear layer that remains in the root canal system after root canal preparation in order to facilitate the deeper penetration of NaOCl into dentine (Gulabivala et al., 2005).

Although the equipment available to perform root canal treatment has improved radically over the previous 20-30 years the fundamental philosophies of chemical disinfection and mechanical shaping remain the same (Tomson and Simon, 2016). Even with modern equipment, following such an ideology only produces complete healing of the periapical tissues in 83% of cases when primary root canal treatment is undertaken and in 80% of cases when teeth are retreated (Ng et al., 2011).

It is also important to state that the root canal system is not completely sterilised during this process of chemo-mechanical disinfestation (Nair et al., 2005) however the microbial load is reduced to a level which promotes

periapical healing (Haapasalo et al., 2012). Indeed the complexities of the root canal system including the presence of microorganisms deep within the dentinal tubules, lateral canals, accessory canals, fins and apical delta (Hess, 1925) may harbour residual pathogens which cannot be eliminated by chemo-disinfection alone (Peters et al., 1995). Additional steps in the process of root canal treatment such as intra-canal dressings, i.e. application of Calcium Hydroxide ($Ca(OH)_2$) (Athanassiadis et al., 2007), may reduce the pathogen numbers further as well entombing residual bacteria during the obturation process (Peters et al., 1995). The exact reduction in pathogen number that is required to facilitate periapical healing is not known but is usually titrated against the lack of signs and symptoms of a tooth, i.e. reduction in pain and resolution of a draining chronic sinus between visits (Haapasalo et al., 2010). This also presents an interesting strategy, if periapical healing can occur with residual bacteria present alternative strategies may also be present to modulate healing (this is discussed below in section **2.2**).

2.2. Alternative strategies in the treatment of periapical periodontitis

The healing and repair of a periapical lesion is modulated by autocrine and paracrine signalling by cytokines and growth factors produced during the inflammatory process. A fine balance of complex regulatory pathways exist that may favour progression of an inflammatory lesion, lead to a chronic equilibrium or, if favourable conditions exist, lead to repair and regeneration of healthy tissue architecture (Marton and Kiss, 2014). Notably, it is well established that microorganisms and their by-products are the driving force of this inflammatory process. From a biological standpoint, the singular focus of conventional treatment is to reduce the microbial load and its related by-products from within the root canal system. As our understanding improves of the regulatory processes that control inflammation and healing, opportunities exist to develop treatment strategies that could not only manage the microbiological component

causing the disease but also modulate inflammatory pathways and enhance tissue healing thus resulting in a better outcome for the patient.

The positive effect of using EDTA as an irrigant in the treatment of apical periodontitis may not only be restricted to merely removing the smear layer as thought. *In vitro*, EDTA has been previously shown by our group and others to solubilise a cocktail of bioactive molecules that are sequestered within the dentine matrix (Graham et al., 2006, Tomson et al., 2007, Tomson et al., 2013). These molecules are sequestered in the dentine matrix during dentinogenesis and are released from the extracellular matrix (Smith and Lesot, 2001) in response to caries (Dung et al., 1995) the application of biomaterials (Tomson et al., 2007, Smith et al., 2016), intra-canal dressings (Graham et al., 2006) and irrigation solutions (Tomson et al., 2013) (**Figure 1**).

More recently EDTA has been proposed for use as an irrigant to expose and release bioactive molecules from dentine for revitalisation procedures (Smith et al., 2016, Galler et al., 2015b). Numerous different cytokines have been identified including members of the transforming growth factor-β (TGF-β) family, angiogenic growth factor family, insulin-like growth factor (IGF) family, fibroblast growth factor (FGF) family, neurotrophic growth factor family and colony stimulating growth factor (CSF) family. Many of these cytokines and growth factors are multifunctional and are involved in wound healing processes such as cell growth and proliferation, chemotaxis, differentiation, angiogenesis and bone formation. Notably multipotent peptides, such as Adrenomedullin (ADM) and TGF-β1 (considered separately below) have been reported to be able to be released from the dentine matrix and these molecules can not only modulate inflammation and healing processes (Elsasser and Kahl, 2002) but in addition they have antibacterial action (Allaker et al., 1999).

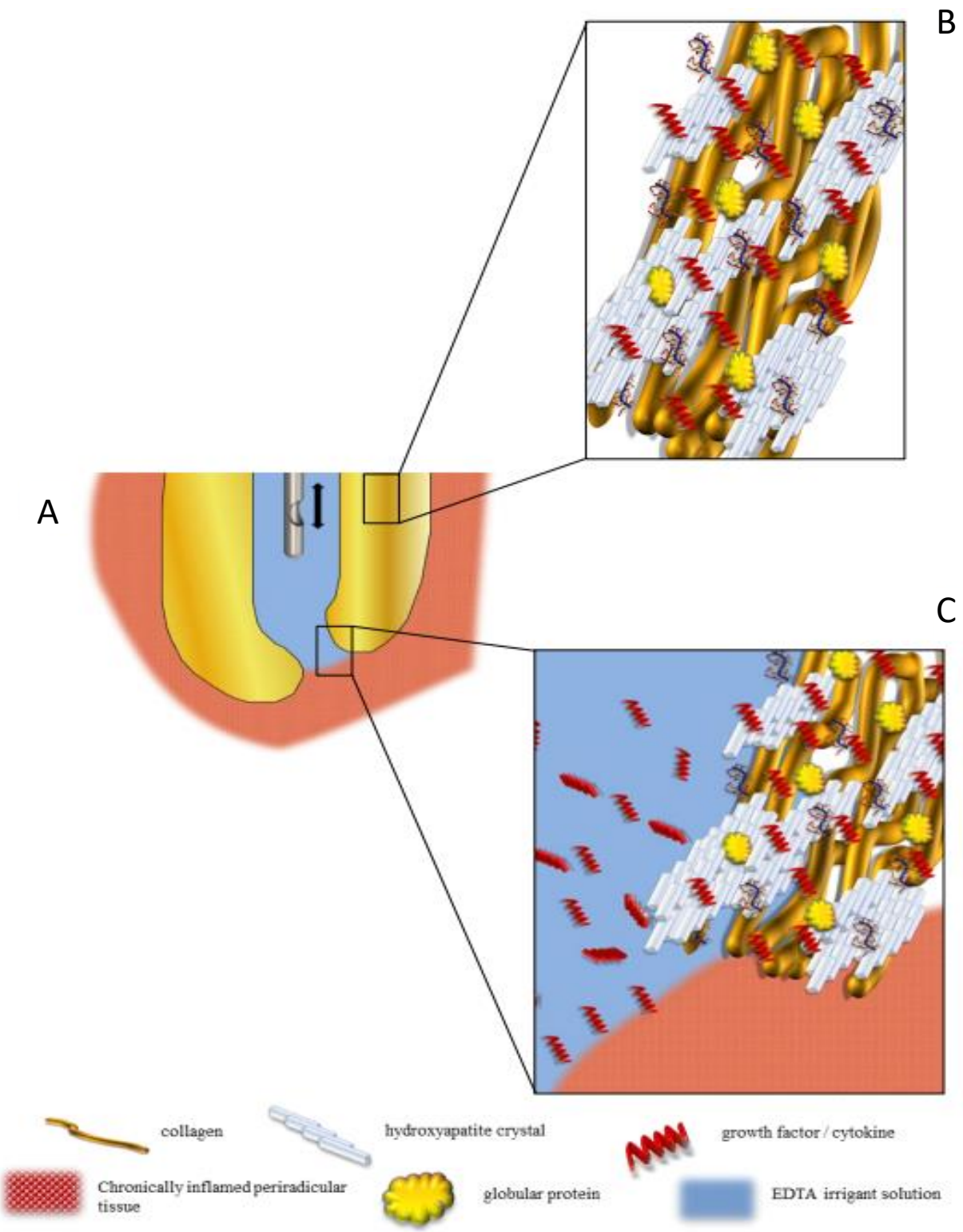

Figure 1. Illustration of the potential interaction of growth factors derived from dentine with the periapical tissues. Cross section of a root end with chronically inflamed periradicular tissue surrounding it (A). Irrigation is in process with the EDTA irrigant which will interact with the internal surface of the dentine walls of the canal and apical tissues. (B) Illustration of dentine structure at a molecular level. It is predominately comprised of collagen impregnated with hydroxyapatite crystals. Within these structures growth factors, cytokines and other bioactive molecules reside. (C) Illustrates growth factors and other cytokines being liberated from dentine matrix by EDTA irrigant making them available to interact with diseased apical tissues; these bioactive molecules can subsequently modulate the inflammatory process and promote wound healing signalling pathways leading to repair (Illustration and caption courtesy of Dr Phillip Tomson).

2.3. Transforming Growth Factor-β1

Members of the TGF-β family have received significant attention due to their role in in modulating processes involved in regenerative endodontics (Graham et al., 2006, Cassidy et al., 1997). TGF-β1 is released from dentine in relatively high concentrations by EDTA (Graham et al., 2006, Tomson et al., 2007, Tomson et al., 2016) and is a potent regulator of cell proliferation, differentiation, and formulation of the extracellular matrix. Interestingly, it is also an important anti-inflammatory cytokine as it suppresses the proliferation and differentiation of T- and B-cells and limits Interleukin-2 (IL-2), Interferon-δ (INF- δ), and Tumour Necrosis Factor -α (TNF-α) production. Furthermore severe and uncontrolled inflammatory reactions are observed in TGF-β1 knockout mouse (Shull et al., 1992). In addition the complex cocktail of molecules which can be released from the dentine matrix also has been shown to have significant antibacterial properties (Smith et al., 2012). Release of these naturally occurring cytokines during root canal treatment could therefore have a positive benefit in disinfecting the root canal system / periapical tissues, modulating the inflammation response and subsequently promoting local tissue healing. However, there is little evidence available to determine if this occurs clinically and can also be potentially harnessed for patient benefit with new treatment strategies.

2.4. Sampling methods

Sampling of dentine derived growth factors has been achieved *in vitro* be using powdered extracted teeth followed by EDTA solubilisation (Baker et al., 2009). Studies have investigated the release of dentine growth factors by applying irrigant solutions to uniform dentine discs (Widbiller et al., 2017, Galler et al., 2015a) or by using root segments without crowns (Zeng et al., 2016). It is important to note that these models do not replicate the complexities of tooth anatomy and do not replicate the *in vivo* application and retrieval of irrigants. Interestingly the quantitative analysis of dentine

growth factors using enzyme-linked immunosorbent assays (ELISA) and a quantitative multiplex ELISA method showed that these bioactive molecules are released and may also be released clinically (Tomson et al., 2013).

In vivo methods to sample bioactive molecules have included periradicular tissue fluid (PTF) / interstitial fluid sampling (Martinho et al., 2015, Martinho et al., 2016, Rechenberg et al., 2014) which have been used to try to determine the inflammatory state of apical tissues. Similar to gingival crevicular fluid, samples of periradicular tissue fluid allow biochemical and molecular analysis for potential assessment of the disease state of the apical tissues and following any responses to treatment intervention. A myriad of different markers can be assessed which include extracellular matrix proteins, host mediated enzymes, and inflammatory mediators. Sampling this fluid beyond the confines of the root canal may enable the determination of the health/disease state of the tissues at a molecular level rather than using clinical markers (such as radiographic healing) which are currently performed.

2.5 A surrogate marker - Transforming Growth Factor-β1

As there is a complex cocktail of bioactive molecules released from the dentine extracellular matrix; the sampling of all derived products is challenging. The use of a quantitative multiplex ELISA detects a range of molecules, however this approach is relatively expensive and time consuming. A different technique is to sample a well characterised dentine-derived cytokine or growth factor which then acts as surrogate marker for the release of the cocktail of molecules. As noted in section **2.3**, TGF-β1 is a well-studied dentine matrix derived molecule which is released in relatively high concentrations, with substantial evidence for its involvement in biological mechanisms such as modulating inflammation and signalling cascades (Graham et al., 2006, Tomson et al., 2007, Tomson et al., 2016).

This molecule therefore provides a useful target molecule which can act as a surrogate marker for an array of bioactive molecules.

2.6 Balancing disinfection with growth factor release – current concepts

As discussed previously, the current strategy of root canal treatment is solely focused on removing microorganisms within the root canal system. The literature is rich in content focused on the effects of disinfectant strategies but little attempt has been made at investigating other biologically based treatment based strategies even when success rates for treatment are approximately 80% (Ng et al., 2011). The effect of inducing or enhancing the release of naturally occurring bioactive molecules to treat apical periodontitis from dentine is largely unknown. Furthermore, bioavailability at the canal / periradicular tissue interface also remains unknown and therefore the development of a full tooth model would be of significant benefit to determine if such a strategy would be plausible.

Galler et al. (2015b) investigated the effects of common irrigant solutions on the release of dentine growth factors from dentine discs. This work focused on the use of NaOCl, EDTA, Chlorhexidine Gluconate (CHX) as well as intra-appointment dressings materials such as $Ca(OH)_2$. Studies were aimed at developing revitalization procedures and data generated supported previous findings that irrigants and inter-appointment dressings were effective at solubilising dentine growth factors (Graham et al., 2006, Tomson et al., 2007, Tomson et al., 2013). This study did not examine the influence of NaOCl in detail but did determine it has a deleterious effect on dentine growth factor release. Therefore, indicating that achieving a balance between disinfection and the release of bioactive molecules is challenging clinically.

Interestingly, the position statement published by the European Society of Endodontology (ESE) on revitalization procedures advises the use of NaOCl

at the first visit only to minimise the damage to growth factors and subsequently the use of EDTA 17% alone on the second visit with an inter-appointment dressing of $Ca(OH)_2$ (Galler et al., 2016). Although, this is concerned with pulp revitalization it provides a similar concept focussing on dentine growth factors release and how these may interact with the periapical tissues. In order to better understand irrigant interactions it is important to establish and describe the commonly occurring irrigants available to endodontics. The information below is not a comprehensive review but focuses on the disinfectant phase and smear removal phase.

2.7. Contemporary irrigants

The ideal properties of an endodontic irrigant are discussed extensively in the literature; these are mainly targeted at the elimination of microorganisms and their components, including bacterial endotoxin, tissue solubilisation capabilities and the ability to remove the smear layer (Zehnder, 2006). As there is no single irrigant which is capable of all these functions, a combination of irrigants is necessary; and this is further complicated by interactions between irrigants. There is also substantial variation in the irrigants used including the concentration and order they are applied, the delivery method, along with the adjuncts to treatment, e.g. heat and agitation techniques. Although there are proposals as to how and which irrigants should be used there are “no gold standard” techniques, although the most commonly used irrigant types are NaOCl, CHX and EDTA (Zehnder, 2006, Park et al., 2012). The most frequently reported application of an endodontic irrigant sequence uses a mixture of NaOCl and EDTA 17% (Dutner et al., 2012).

2.7.1. Primary irrigants- disinfection phase - Sodium Hypochlorite

Sodium hypochlorite (NaOCl) is the most accepted and used endodontic irrigant (Basrani and Haapasalo, 2012). This is due to its ability to predictably and successfully disinfect the root canal system of

microorganisms, remove their bacterial endotoxins, dissolve organic tissue including pulpal remnants and the organic components of the smear layer (Zehnder, 2006, Byström and Sundqvist, 1981). NaOCl has the further action of lubrication, flushing away debris and ensuring the root canal system remains patent. NaOCl application ranges from 0.5% to 6% and the concentration does not appear to have a significant effect on its efficacy (Moorer and Wesselink, 1982, Siqueira Jr et al., 2000). NaOCl application in higher concentrations exhibits improved tissue dissolving capabilities however as the concentration increases, so does the toxicity (Zehnder, 2006). As NaOCl has a relatively low surface tension, it is able to penetrate into the complexities of the root canal system and dentine (Palazzi et al., 2012). NaOCl can penetrate up to 0.3mm within dentine tubules, with the maximum penetration achieved due to time, increased concentration and heat (Zou et al., 2010). Furthermore, the efficacy of NaOCl as a disinfectant is increased with time, temperature, agitation and surfactants (Stojicic et al., 2010)

The literature is inconsistent in reporting the time necessary for the disinfection of root canals when using NaOCl, although its effectiveness is reduced on reaction with organic tissue and needs to be continuously replaced during endodontic procedures (Haapasalo et al., 2010). There remains no recommended optimal time period for the clinical application of NaOCl within the root canal system (Zehnder, 2006).

The mechanism of action of NaOCl is a two-fold process. On contact with organic tissue (such as remaining pulpal tissue), the NaOCl dissolves the proteins by severing the peptide links, this in turn lead to the formation of chloramines, which have significant antimicrobial effects (Basrani and Haapasalo, 2012). The antimicrobial effects also occurs due to the chemicals direct action on bacterial enzymes, degradation and hydrolysis of the bacteria protein structure and the high pH (>11) which interferes with cytoplasmic membrane integrity (Basrani and Haapasalo, 2012). This process is non-selective, with an effect on both host and bacterial organic tissues.

It is important to note that while NaOCl is considered the 'gold standard' of disinfection; full canal disinfection does not occur (Shuping et al., 2000), resulting in bacteria remaining present and be able to proliferate within the root canal system. Furthermore, NaOCl is unable to remove inorganic debris from the root canal system. Notably, the use of NaOCl has also been associated with deleterious effects including NaOCl accidents; which can result in significant soft tissue necrosis (Kleier et al., 2008), higher strength concentrations being damaging to healthy tissues (Pashley et al., 1985), reducing the mechanical properties of dentine (Sim et al., 2001) and attenuating growth factor release (Galler et al., 2015a). The interactions with NaOCl with other irrigants are considered below in section **2.7.4**).

2.7.2 Primary irrigants- disinfection phase - Chlorhexidine

Chlorhexidine gluconate (CHX) has a wide range of antimicrobial activity including a bacteriostatic action at relatively low concentrations to bactericidal action at higher concentrations. CHX is a detergent and interferes with the permeability of cell membranes in microorganisms and alters metabolic pathways in bacteria (Basrani and Haapasalo, 2012). The majority of endodontic solutions and studies reportedly use a formulation of 0.2% CHX. Microbiology studies have shown CHX to disinfect root canals effectively *in vivo* (Ercan et al., 2004), performing similarly to NaOCl (Jeansonne and White, 1994) and also having a synergistic effect when used in combination (Kuruvilla and Kamath, 1998). Conversely other studies have concluded that CHX has a reduced efficacy compared with NaOCl at disinfection (Ringel et al., 1982).

The additional benefits of CHX are reduced tissue toxicity compared with NaOCl, less damaging effects on the organic components of dentine and prolonged antimicrobial properties of up to 12 weeks within the root canals (Mohammadi and Abbott, 2009b). A disadvantages of CHX is its inability to remove and dissolve organic components of organic tissue including pulp

remnants (Vianna et al., 2004) and severe allergic reactions have also been reported (Pemberton and Gibson, 2012).

2.7.3. Secondary irrigants – smear layer removal phase - Ethylenediaminetetraacetic acid

The mechanical preparation of the root canal system facilitates the penetration of irrigants deep into the root canal system but also results in the formation of dentine debris, consisting of both organic and inorganic components, this is termed the 'smear layer' (McComb and Smith, 1975). The smear layer can be subdivided into two distinct layers, that of a relatively thin 1-2 microns thickness covering the canal walls and a second more extensive layer which penetrates into the dentinal tubules of up to 40 microns (Mader et al., 1984). The smear layer consists of small inorganic particles, residual organic material, remnants of odontoblastic processes and bacteria(McComb and Smith, 1975).

EDTA removes the inorganic components of the smear layer and enhances the direct action of our disinfecting solutions by engaging regions of the dentine which cannot be mechanically instrumented due to the complexity of the root canal system (McComb and Smith, 1975). Indeed, mechanical instrumentation during the chemo-mechanical process produces debris which can be further forced into the complexities of the root canal system (Gulabivala et al., 2005). Furthermore, by removing the smear layer the contact between the obturation materials and the dentine wall is improved, which enhances the penetration of clinically applied cements to form tags (Mader et al., 1984). EDTA also benefits from a relatively low toxicity and enhances the negotiation of narrow canals (Basrani and Haapasalo, 2012).

EDTA is a chelator and can effectively remove the inorganic component of dentine. The mechanism underlying this chelating effect is by binding to di- and tri- cationic metals i.e. Ca^{2+} and Fe^{3+} (Basrani and Haapasalo, 2012).

EDTA in the concentration of 15-17% has been suggested as an adjunctive to NaOCl use, either as a penultimate or final rinse. Indeed in root canal retreatment an additional rinse with EDTA improved the outcomes of treatment (Ng et al., 2011). The use of NaOCl and EDTA intermediately during root canal treatment has been proposed to enhance chemo-mechanical preparation (Soares et al., 2010) but the use of NaOCl following EDTA has been shown to increase dentine erosion (Qian et al., 2011, Mader et al., 1984). There are recommendations that EDTA irrigation should not be used for more than 1 minute (Calt and Serper, 2002) with other studies proposing EDTA takes a longer time period for smear layer removal (Goldberg and Spielberg, 1982).

EDTA has shown limited antimicrobial effects in a number of studies including reports of having limited or no activity against biofilms (Arias-Moliz et al., 2009, de Paz et al., 2010). Contrasting studies have shown antimicrobial activity against bacterial strains, such as *Enterococcus faecalis* (Baca et al., 2011, Zhang et al., 2015), as well as antifungal activity (Sen et al., 2000).

The mechanism for the antibacterial aspect of EDTA is a change of the permeability of the cell membranes in bacteria by chelating and removing ions from this structure (Zhang et al., 2015). The chelating effect of binding ions, i.e. calcium (George et al., 2009), antagonises the mineral uptake, subsequently inhibiting the metabolic pathways in the bacterial cell (Al-Bakri et al., 2009). Outside of dentistry EDTA has been shown to be an effective bacterial disinfectant for use in catheters (Venkatesh et al., 2009, Root et al., 1988).

The action of EDTA is enhanced by adjunctive treatments such as passive ultrasonic irrigation which has also been demonstrated to promote dentine growth factor release *in vitro* (Widbiller et al., 2017).

2.7.4. Interactions of irrigants

The reaction of NaOCl and CHX has been shown to form the molecule para-chloroaniline (Basrani et al., 2007), a toxin which forms methaemoglobin which is a potential carcinogen (Thomas and Sem, 2010). Notably however, Thomas and Sem (2010) showed the levels of para-chloroaniline were not produced at a measureable quantity. The interaction is also further confounded by formation of a brown precipitate which creates a chemical smear layer and may therefore decrease the efficacy of further irrigants used (Akisue et al., 2010). Notably a reaction between CHX and EDTA forms a white precipitate (Rasimick et al., 2008); occluding the dentine surface, which may limit the efficacy of any further irrigant.

NaOCl has limited or very slow action at attenuating EDTA (Grawehr et al., 2003, Rossi-Fedele et al., 2012, Grande et al., 2006) which is in contrast with EDTA which has a greater effect at reducing the efficacy of NaOCl when they are combined. The suggestion is therefore that a 'wash-out' where all the primary irrigant is removed step is used between irrigants to reduce potential interactions (**Figure 2**).

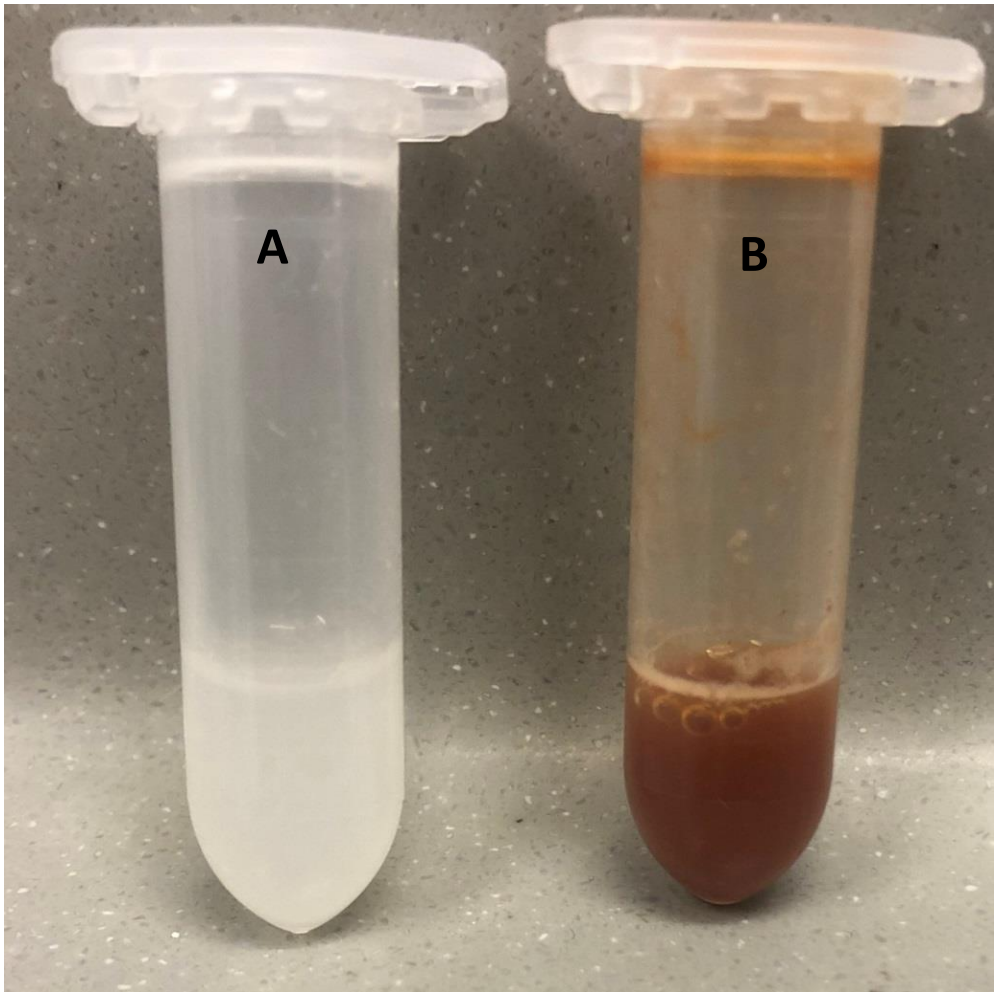

Figure 2 Image showing the formation of precipitate as a result of the interaction between irrigants. EDTA + CHX produce a white precipitate (A) and the NaOCl + CHX produce a brown precipitate (B). These may occluding the dentine surface, which may limit the efficacy of any further irrigant

2.8 Irrigation delivery methods

2.8.1 Positive pressure irrigation

The delivery of irrigant is achieved by positive pressure with a narrow endodontic needle and syringe administered by hand. The size of the needle is 27 gauge with a side-venting or closed-end which is freely applied within the root canal (unbound to the canal wall) to reduce the risk of extrusion into the periapical tissues (Park et al., 2012, Zehnder, 2006). This device should be placed in the apical third of the root canal or 1mm from the working length to ensure the irrigant is delivered to the apical aspect of the root canal system (Shen et al., 2010). Although this method is extensively used, the flow dynamics of irrigation can prevent adequate penetration. Interestingly, Boutsioukis et al. (2007) showed substantial variation in the flow rate and volume of irrigation delivered between operators. This method of delivery also has the disadvantage of air entrapment and vapour lock, which is defined as vapour trapped within the root canal system which prevents the exchange of an irrigant with the apical portions (Spoorthy et al., 2013). This can result in the failure of the irrigant to disinfectant the apical intricacies of the root canal system.

2.8.2 Negative pressure irrigation

Negative pressure irrigation aims to overcome the air entrapment issue, increasing the penetration into the apical anatomy and prevent extrusion injuries (Nielsen and Baumgartner, 2007). The market leader for negative pressure irrigation is the EndoVac® retrieval system, which utilises a negative irrigation approach for irrigant delivery along the entire length of the root canal system (Nielsen and Baumgartner, 2007). The EndoVac® retrieval system also has the benefit of ensuring the irrigant overcomes some of the limitations of a ‘closed root canal’ system; which is defined as

vapour trapped within the root canal system which prevents the exchange of an irrigant with the apical portions (Spoorthy et al., 2013). With the EndoVac® system large volumes of irrigant can be conveniently delivered in a relatively over a relatively short time frame of several minutes.

Notably, with both positive pressure and negative pressure irrigation systems there is little information available regarding the ability of the techniques to release dentine growth factors.

2.9. Adjunctive techniques to irrigation

To enhance the ability of irrigants to disinfect and chelate inorganic material a number of techniques are available, these include cone pumping, and sonic and ultrasonic irrigation (Van der Sluis et al., 2007, Stamos et al., 1987). Notably, the ultrasonic and sonic irrigation techniques producing significantly cleaner canals (Jensen et al., 1999). It is understood that the penetration of irrigants into dentine occurs due to the sonic and ultrasonic energy producing powerful acoustic micro-streaming (Ahmad et al., 1987, Verhaagen et al., 2014, Van der Sluis et al., 2007). This process is defined as the shear stress produced at the canal walls due to oscillation of the file which causes a mixture of pressure and irrigation, enhancing the action of the irrigant (Verhaagen et al., 2014). Recent literature has focused on Passive Ultrasonic Irrigation (PUI) as a predictable cleaning technique (Van der Sluis et al., 2007). PUI ultimately describes the oscillation of the file in the frequency of 25-30 kHz, which is passive, i.e. does not engage the canal wall, and is therefore not shaping the dentine (Van der Sluis et al., 2007). This techniques reportedly appears to have been well adopted by endodontic clinicians (Dutner et al., 2012). Widbiller et al. (2017) also encouraging showed ultrasonic activation of EDTA significantly enhanced the release of dentine growth factors.

2.10. Intra-canal dressing – Calcium Hydroxide

Calcium hydroxide [$Ca(OH)_2$] has been shown to successfully disinfect root canal systems (Allard et al., 1987, Sjögren et al., 1991, Ørstavik et al., 1991) and promote apical healing (Siqueira and Lopes, 1999). Although a number of intra-canal dressings are available, $Ca(OH)_2$ remains the most commonly used medicament in the world (Tang et al., 2004). It has low water solubility enabling it be present within root canals for prolonged periods of time and it has a pH of >11 which effectively kills bacteria on contact. This ultimately is due to the release of hydroxyl groups (OH^-), which leads to a highly alkaline environment, neutralising microorganisms (Athanassiadis et al., 2007). Additional benefits include the neutralisation of lipopolysaccharides (Safavi and Nichols, 1993), creating a physical barrier to microorganism re-infection and enhancing hard tissue formation (Holland and de Souza, 1985). It also has the ability to dissolve organic tissue, has a prolonged antimicrobial activity and shows little interaction with other irrigant solutions (Siqueira and Lopes, 1999). Previous research from our group has also shown the release of dentine growth factors using $Ca(OH)_2$ (Graham et al., 2006) indicating that a release of growth factors could occur between appointments. Indeed, as noted previously, the position statement issued by ESE on revitalization procedures promote the use of $Ca(OH)_2$ (Galler et al., 2016).

The reported disadvantages of using $Ca(OH)_2$ is the weakening of the dentine structure and the increased potential for tooth fracture with prolonged periods of use (White et al., 2002).

2.11. Single visit vs multiple visit endodontic treatments

For an intra-canal dressing such as $Ca(OH)_2$ to be used the endodontic treatment would require treatment over two visits. Others however have compared the outcomes of root canal treatment over single or multiple

visits (Penesis et al., 2008, Sathorn et al., 2005). Indeed, Figini et al. (2008), in a systematic review, have found minimal difference between the effectiveness of root canal treatment performed over a single or multiple visits. Furthermore, the findings of another systematic review and meta-analysis, specifically aimed at determining the efficacy of $Ca(OH)_2$ as an intra-canal dressing concluded that it had limited efficacy in eliminating bacteria from human root canals when assessed by culture techniques (Sathorn et al., 2007). However, the effect $Ca(OH)_2$ may have on the release of bioactive molecules is unreported and requires further investigation.

2.12. Developing a tooth model system

Several groups have investigated the release of dentine growth factors using various model systems (Widbiller et al., 2017, Galler et al., 2015a, Zeng et al., 2016, Smith et al., 2016). These studies have used dentine discs or root forms with the purpose of showing dentine growth factor release necessary for regenerative endodontics. These types of models are not clinically translatable and are less suited for the purpose of the investigations reported here as the focus is on the release of dentine growth factors and their potential for interactions with the periapical tissues.

There are several possible approaches for developing a model with the requirement of paralleling the findings of *in vivo* endodontic cases. However, the primary proposal for developing such as model involves the use of an anterior tooth with an immature apex; this would benefit from a large surface area at the apex in contact with the simulated periapical tissues and this would facilitate the development of a sampling technique. Teeth could also be standardised to a specific length and internal diameter through preparation methods. The delivery methods and irrigant regimes could be developed based on this model to guide future *in vivo* testing and thus the development of this tooth model is essential to this project. There

were no models reported in the literature which fitted this design brief, as such a novel model needed to be developed. The challenges are:

1. Developing a reproducible model that is able to simulated the interactions with the periapical tissues;
2. Developing a collection and sampling method.

2.13. Hypothesis

Irrigants such as EDTA which are used during orthograde root canal treatment liberate bioactive molecules from the dentine matrix are bioavailable to interact with periradicular tissues modulating inflammatory responses to promote wound healing leading to repair of the apical tissues in cases of apical periodontitis.

Developing a better understanding of how conventional endodontic procedures may release these bioactive molecules and their interactions with periapical tissues is essential to investigate this process. Indeed, the harnessing of the innate repair mechanisms of the dental and periradicular tissues may lead to novel biomimetic approaches for treating apical periodontitis which will lead to better patient outcomes. It is now timely for a paradigm shift in our biological treatment philosophy to not only manage microbial load within the root canal system but to use therapies that manipulate the regulatory pathways of an inflammatory lesion to favour healing and repair of the periradicular tissues in disease states such as apical periodontitis.

2.14. Aims and objectives

The aim is to determine the ability of contemporary root canal irrigants and intra-canals medicaments to release bioactive molecules from dentine matrix both within the retrieved irrigant and the simulated periapical tissue in a clinically translatable *in vitro* set-up.

The objectives are:

1. To develop a tooth model to replicate the clinical conditions of a tooth with incomplete root formation.
2. To develop irrigant delivery and retrieval methods in this *in vitro* model to guide future *in vivo* sampling.
3. Investigate the changes in the frequency, delivery method and time of irrigant delivery to enable release of dentine growth factors.
4. Optimise the irrigation process to deliver a primary irrigant aimed at disinfection with a secondary irrigant aimed at dentine growth factor release.

3. Materials and methods

3.1. Tooth preparation

Single rooted maxillary anterior non-carious teeth were obtained from the tooth bank at the School of Dentistry, University of Birmingham, consisting of upper central incisors and upper canines; the reason for extraction was not available. These were not disinfected and stored in sodium azide 15mM and stored at -80˚C. In order to be included in the study the teeth were not previously root canal treated and did not have extensive caries into the pulpal-root complex. These types of teeth were selected for their relatively straight canal configuration and length. Ethical approval for the use of these teeth was approved by NHS trust R&D, (referenceBCHCDent374.ToothBank) under generic ethics (14/EM/1128).

The teeth were cleaned to remove soft tissue, bone and calculus using a scalpel and a Cavitron ultrasonic scaler (Denstply Sirona, OK, USA). The teeth had conventional access cavities prepared to access the pulpal-root canal complex by one operator and the canal prepared using a sequential instrumentation technique (which is listed below) in a crown down fashion in accordance with the recommended technique by the manufacturer (Denstply Sirona, OK, USA). To minimise the potential for heat generation water coolant was used to lubricate the files however no irrigant was used in the preparation. The file sequence was as follows: size 8, 10, 15 and 20 K-Flex files (SybronEndo, Glendora, CA, USA), ProGlider file (Dentsply Sirona Endodontics, OK, USA), X1 to X5 ProTaper NEXT Files (Dentsply Sirona Endodontics, OK, USA) and finally to a 1.5mm ParaPost XT drill (Coltene Whaledent LTD, Burgess Hill, UK) to create a standardised the internal diameter of the root canal system to 1.5mm or ISO 150 for each of the teeth that mimics a incompletely developed root and is a canal with a large internal surface area.

The length of the teeth was also standardised to 17mm by measuring from the CEJ to the apex of the tooth, with a ruler. The apex of the root was flattened with a diamond polishing disc (Horico, Berlin, Germany) to facilitate a close fit against a PerioPaper strips (Oraflow, New York, USA) or a Microcon centrifugal filter (Merck Millapore, Watford, UK). The inside of the canal was cleaned of debris using a TePe interdental brush (TePe Oral Hygiene Products, Somerset, UK) and coated with clear nail polish (Rimmel, London) on the external surface to prevent the interaction of the irrigant with any exposed dentine present on the external surface of the tooth (Error! Reference source not found. **3**).

The crowns of the teeth were left *in situ* as preliminary experimentation showed less irrigant loss when using a passive ultrasonic agitation technique. This approach also replicated *in vivo* conditions and enabled the refinement of the technique prior to *in vivo* testing (**Figure 3**).

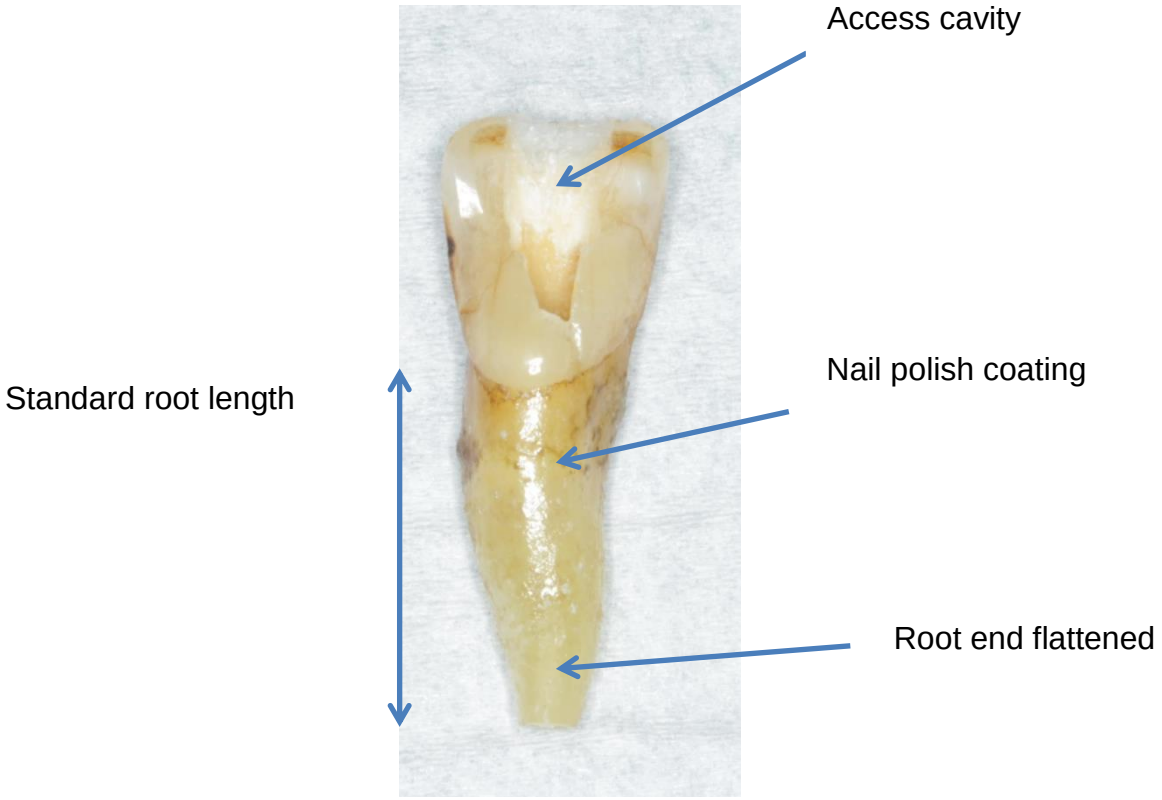

Figure 3. Representative image of a prepared tooth simulating a model of a wide open apex. The access cavity is prepared and the tooth length standardised with the apical portion flattened. The teeth were coated with clear nail varnish on their external surface.

3.2. Pilot study

Twenty randomly selected central incisor teeth were prepared in accordance to the protocol described in section **3.1** and randomly assigned to two separate groups (n=10). Teeth were then further subdivided into 5 teeth within each experimental group and 5 teeth within each control group (**Table 1**).

	Group 1 – immediate collection	Group 2 – 24 hour incubation followed by the collection
Experimental	EDTA 17% (n=5)	EDTA 17% (n=5)
Control	Saline (n=5)	Saline (n=5)

Table 1: Experimental groups used for the pilot study. Group 1 consisted of an experimental group treated with 17% ETDA compared with a control of saline treated teeth. Irrigants were administered in a simple rinse with 17% EDTA or saline and immediate sample collection procedure. Teeth in Group 2 were subjected to same experimental and control group irrigants however the irrigant was applied for 24 hours prior to the collection of the irrigant.

Teeth were mounted in a 10KDa Microcon centrifugal filer (Merck Millapore, Watford, UK), this size was chosen to ensure that the larger TGF-β1 molecules (25KDa) would not pass through the membrane and could be collected. The teeth were sealed with light bodied silicone (Henry Schein, NY, USA) (**Figure 4Error! Reference source not found.**) to hold the tooth in place and prevent any irrigant from contacting the external tooth surface. A standard 27 Gauge side venting endodontic syringe (Medtronic, Hertfordshire, UK) was used to apply irrigant until the tooth was filled to the level of the access cavity and the irrigant was subsequently retrieved. Teeth were divided into group 1 with the experimental group (n=5) receiving 17% EDTA (Cerkamed, Stalowa Wola, Poland) and the control group (n=5) receiving saline solution (Mölnlycke Health Care, Gothenburg, Sweden). The irrigant solution was syringed into the tooth construct until it filled the root

canal system. It was immediately retrieved, using a fresh 27 Gauge endodontic side venting syringe (Medtronic, Hertfordshire, UK) and stored on ice until further processing.

Group 2 utilised the same experimental procedure although the 17% EDTA (n=5) or saline (n=5) was sealed in the tooth for 24 hours at room temperature using a light bodied silicone (Henry Schein, NY, USA) (**Figure 4**) placed in the access cavity prior to irrigant removal.

The collected irrigant was subsequently filtered and washed through the Microcon column assembly in a centrifuge (Centrifuge 5415D, Eppendorf, Stevenage UK) at 6000rpm to remove EDTA and to concentrate the extracted irrigant. Care was taken not to desiccate the Microcon membrane by adding 60µl of ice-cold nuclease free water when the irrigant solution reached relatively low volumes. To capture the concentrated irrigant a further 100µl of ice-cold nuclease free water was added to the Microcon assembly, gently agitated and incubated at room temperature for 10 minutes. Finally the Microcon assembly was inverted in an Eppendorf tube and centrifuged at 1000rpm for 30 seconds to capture the concentrate (Centrifuge 5415D, Eppendorf, Stevenage UK). The filtered samples were then stored at -20˚C and thawed before being analysed using a TGF-β1 ELISA (R&D systems, Abingdon UK, see section **3.4.1** for analysis details. As this was an initial proof of concept study the PerioPaper mimicking the periapical tissues were not sampled in this experimental set up at this stage (**Figure 4**).

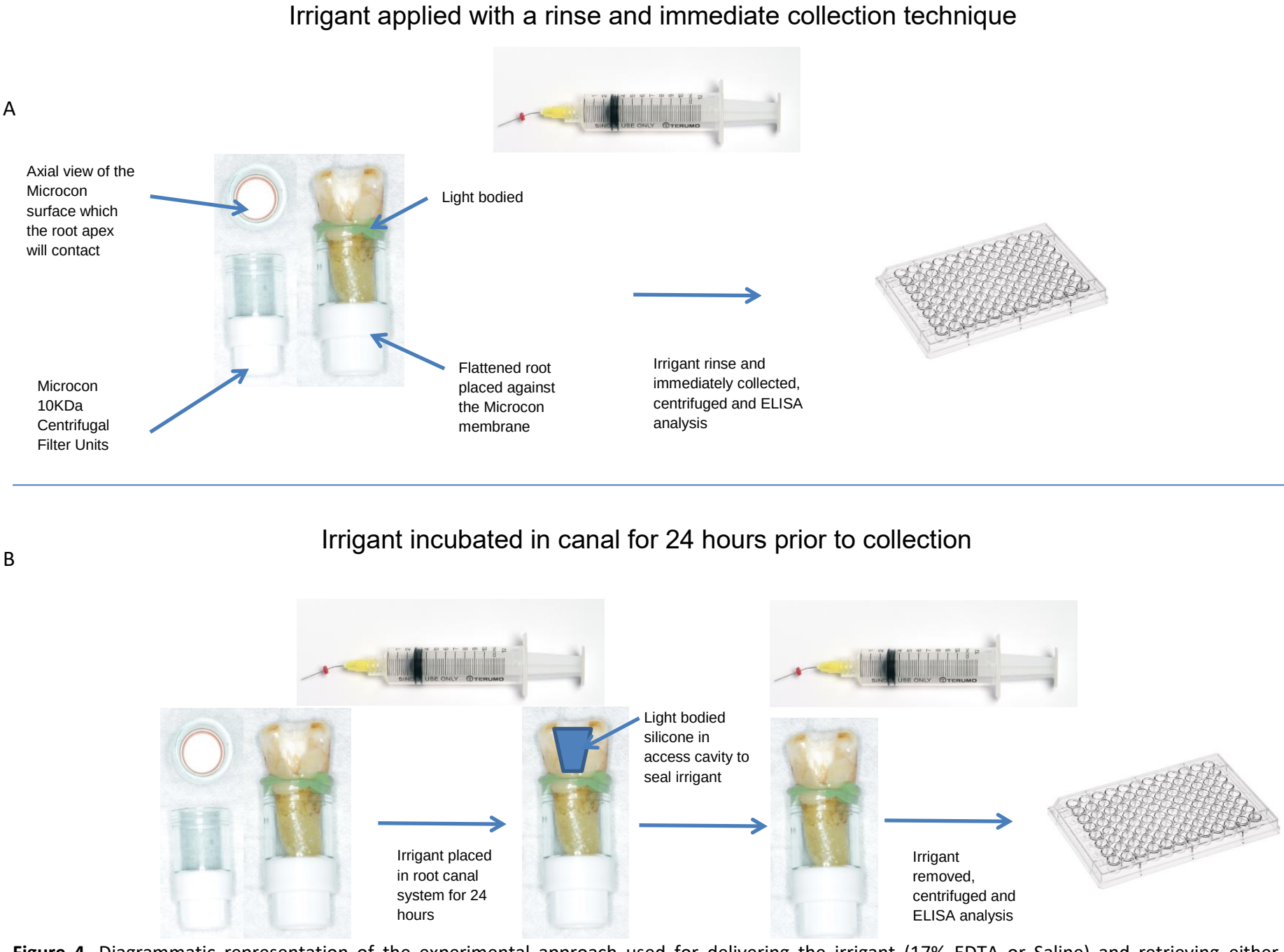

Figure 4. Diagrammatic representation of the experimental approach used for delivering the irrigant (17% EDTA or Saline) and retrieving either immediately (A) or following 24hrs incubation (B). Once the irrigant was retrieved it was then processed using a 10kDa Microcon (Merck Millapore, Watford, UK) and analysed using the TGF-β1 ELISA (R&D Systems, Minnesota, USA).

3.3. Sampling used irrigant with the EndoVac® retrieval system, a negative pressure irrigation system

In order to determine the effects of EDTA concentration on the liberation of TGF-β1 from dentine during irrigation, twenty teeth were prepared in accordance to the protocol described in section **3.1** and distributed to 4 separate experimental groups (n=5) as shown in **Table 2**:

Sampling method	Irrigant type			
	Saline	EDTA 5%	EDTA 10%	EDTA 17%
EndoVac retrieval	n=5	n=5	n=5	n=5
Periapical sampling				

Table 2: Experimental groups used for the EndoVac® retrieval system analysis. Groups consisted of saline, 5% EDTA, 10% EDTA and 17% EDTA. Each tooth was mounted (as in 3.2.) in the 10KDa Microcon centrifugal filter and the irrigant applied and retrieved using the modified EndoVac® system and the simulated periapical tissues sampled for each tooth by filtering the Microcon membrane when the tooth was removed.

The teeth were mounted in the 10KDa Microcon centrifugal filter (Merck Millipore, Watford, UK) and sealed with light bodied silicone (Henry Schein, NY, USA). Care was taken to position the root apex flat against the membrane of the Microcon. An EndoVac® system (Kerr, Orange CA) was customised to provide irrigant delivery but allowed retrieval of irrigant by attaching a customised universal container (Genta Medical, York, UK) (**Figure 5** & **Figure 6**).

Saline (Mölnlycke Health Care, Gothenburg, Sweden) and EDTA 17% (CERKAMED, Kwiatkowskiego 1, 37-450 Stalowa Wola, Poland) were obtained from the manufacturers. As 5% and 10% solutions could not be obtained from dental manufacturers the EDTA solutions were produced in the laboratory from a dry EDTA (Sigma, Pharmaceuticals, Watford, UK) compound mixed with water, ensuring that the pH was 7 and mixed thoroughly to allow complete dissolution.

Each solution was applied in 3ml aliquots to the tooth using the micro cannulas in the EndoVac® system and the irrigants retrieved and stored on ice. The teeth were removed from the Microcon filter assembly and the Microcon membrane (previously in contact with the root apex to simulate the periapical tissues) was rehydrated with 100µl nuclease free water.

The retrieved irrigant using the modified EndoVac® system were then centrifugally filtered at 6000 rpm through a Microcon assembly to remove the irrigant solution using the same protocol as **3.2**.

The Microcons which sampled the periapical tissues were gently agitated and allowed to stand for 10 minutes. This enabled the molecules contained on the membrane to diffuse into the solution. The microcon was then inverted at centrifuged at 1000rpm for 30 seconds to capture the concentrate in an Eppendorf (Centrifuge 5415D, Eppendorf, Stevenage UK).

The filtered samples were then stored at -80˚C and thawed before being analysed using the TGF-β1 ELISA (R&D systems, Abingdon UK), see section **3.4.1** for analysis details.

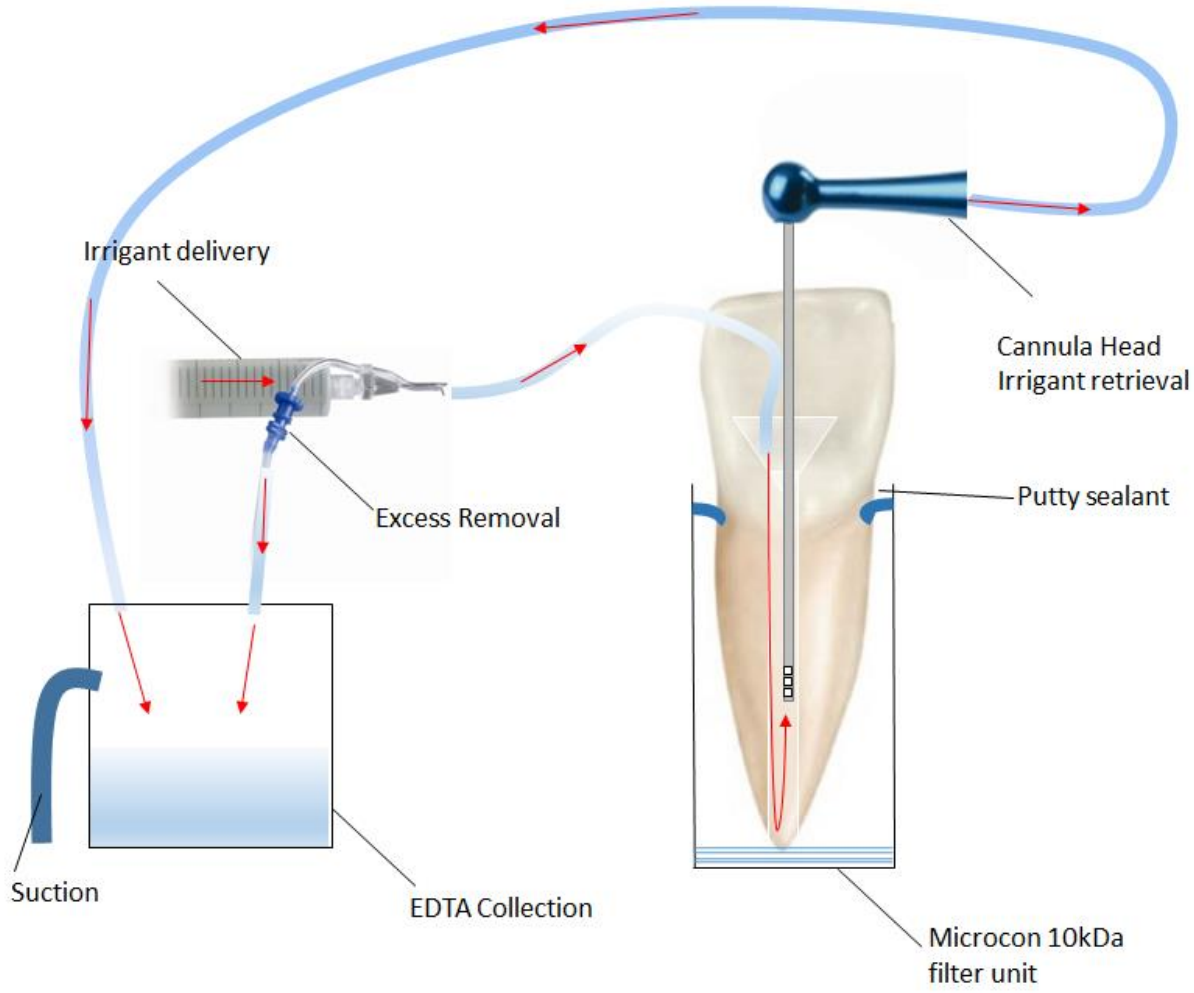

Figure 5: A diagrammatic representation of the *in-vitro model* system developed whereby a single rooted human extracted tooth was sealed into a Microcon-10kDa Centrifugal Filter Unit (Merk Millapore, Watford, UK). An EndoVac® negative pressure irrigation system (Kerr, Orange, CA) will be used to deliver and retrieve the used irrigant. The Microcon filter membrane acted as simulated apical tissue and will collect any dentine growth factors that have the potential to interact with such tissues.

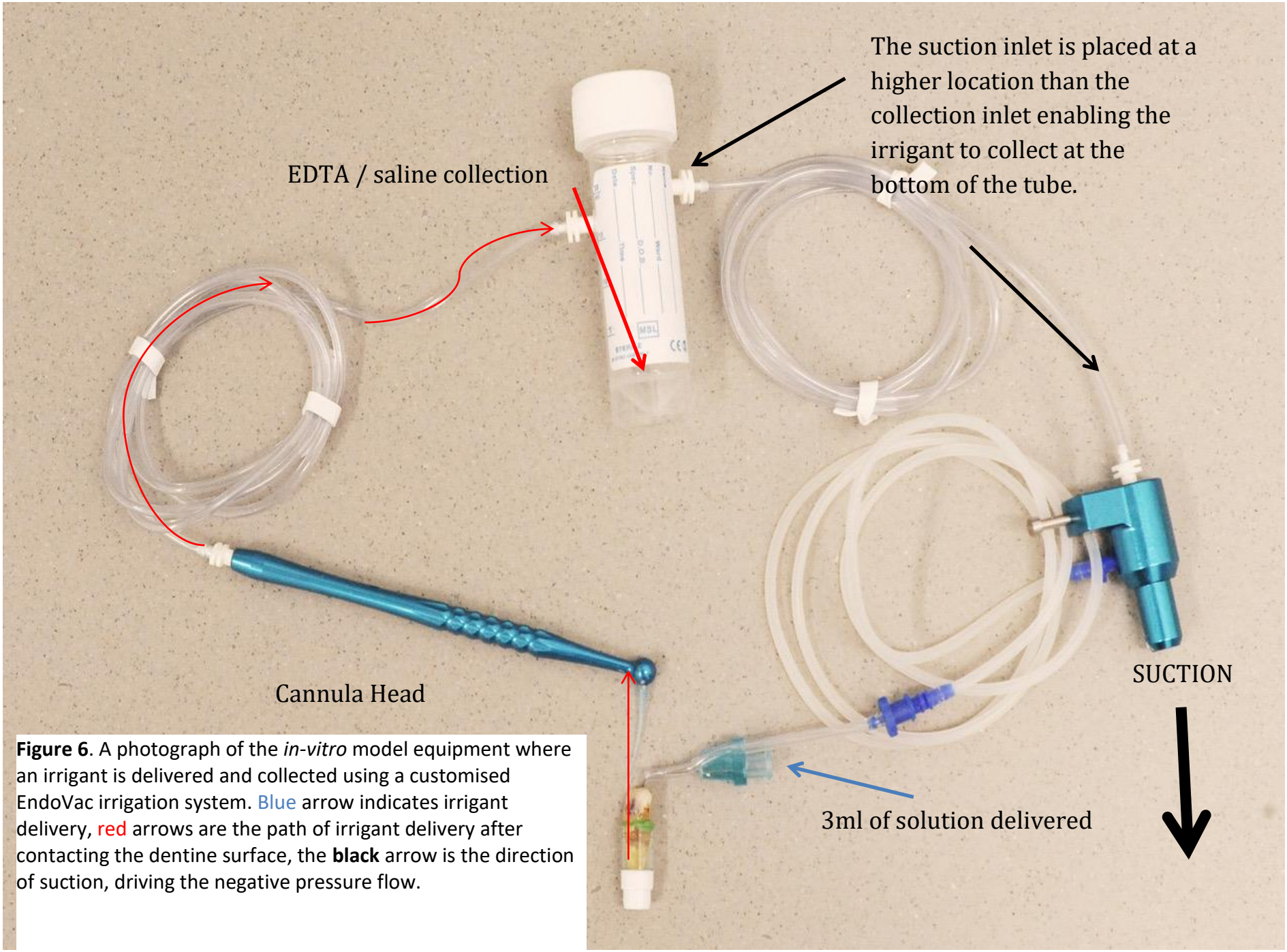

Figure 6. A photograph of the *in-vitro* model equipment where an irrigant is delivered and collected using a customised EndoVac irrigation system. Blue arrow indicates irrigant delivery, red arrows are the path of irrigant delivery after contacting the dentine surface, the **black** arrow is the direction of suction, driving the negative pressure flow.

3.4. Contemporary endodontic irrigation and their effect on dentine growth factor release

Sixty-five extracted non-carious, canine teeth were selected and prepared to the standard protocol in **3.1**.

The flattened apex was mounted against a PerioPaper strip (Oraflow, New York, USA) and encased in a putty silicone (Henry Schein, NY, USA) (**Figure 7**) (**a & b**)). An operating microscope (Global Microscopes, MO, USA) was used to confirm the PerioPaper had not moved during mounting.

The teeth were randomly allocated to 13 experimental groups (n=5) based on **(**
Table 3):

1. Exposure to a **primary irrigant** [2% Chlorhexidine (QED, Peterborough, UK) or NaOCl 2% (Cerkamed, Stalowa Wola, Poland) which simulated the disinfection phase and a control(nuclease free water)].

2. Treatment with an inter-appointment canal medication between the primary and secondary irrigant applications- $Ca(OH)_2$ paste (UltraCal, Ultradent, USA) or control (no paste).

3. Exposure to a **secondary irrigant** which simulated removal of the smear layer and potential growth factor release or a control- EDTA 17% (CERKAMED, Kwiatkowskiego 1, 37-450 Stalowa Wola, Poland) or nuclease free water which was delivered:

    a) In a single application for 15 minutes with a 30 second application of passive ultrasonic activation;

    Or,

    b) Three applications of 5 minutes with three 30 second separate applications of passive ultrasonic activation.

Group (each group n=5)	Primary irrigant with mechanical file debridement	Inter-appointment canal medication	Secondary irrigant with water, EDTA 17%. With ultrasonic activation. Applied in X 3 separate 5 min rinses or 15 mins.		
Group 1*	NaOCl 2%	Nil	Water		
Group 2**	NaOCl 2%	Nil	Water	Water	Water
Group 3*	NaOCl 2%	Nil	EDTA 17%		
Group 4 **	NaOCl 2%	Nil	EDTA 17%	EDTA 17%	EDTA 17%
Group 5*	Water	Nil	Water		
Group 6 **	Water	Nil	Water	Water	Water
Group 7*	Water	Nil	EDTA 17%		
Group 8 **	Water	Nil	EDTA 17%	EDTA 17%	EDTA 17%
Group 9*	CHX 2%	Nil	EDTA 17%		
Group 10*	CHX 2%	Nil	Water		
Group 11***	NaOCl	$Ca(OH)_2$ (2 weeks)	EDTA 17%		
Group 12***	CHX 2%	$Ca(OH)_2$ (2 weeks)	EDTA 17%		
Group 13***	Water	$Ca(OH)_2$ (2 weeks)	EDTA 17%		

Table 3. Composition of the 13 separate treatment protocol groups which are categorised based on the application of a primary irrigant (NaOCl 2% or CHX 2% or nuclease free water). Whether an inter-appointment canal medication of $Ca(OH)_2$ was used and finally the application of a secondary irrigant (EDTA 17% or nuclease free water); this was delivered as a single or multiple irrigant change within a 15 minute time frame. The diagrammatic summaries for (*), (**) and (***) are summarised in figures **11, 12** and **13** respectively.

During the administration of the primary irrigant a ProTaper Next X5 file was used for 30 seconds circumferentially around the internal aspect of the tooth. This approach was taken to simulate chemo-mechanical disinfection that would occur during root canal preparation clinically. The irrigant was then removed with a micropipette brand (Low retention, graduated 10µl tips, Appleton, Birmingham, UK). The teeth were dried with paper points and rinsed with 60µL nuclease free water and dried again.

In three groups, a calcium hydroxide ($Ca(OH)_2$) paste (UltraCal, Ultradent, USA) was placed in the root canal system; an endodontic sponge was placed coronally with a light bodied silicone (Henry Schein, NY, USA) which was used to seal the access. The teeth were incubated at 37°C in humidified conditions for two weeks (Hybridisation Oven / Shaker, S1 20H, Stuart Scientific Ltd, Staffordshire, UK) (**Figure 7** (**c & d**)) (**Figure 13**). The other 10 groups were treated immediately with the secondary irrigant (**Figure 11** & **Figure 12**).

The secondary irrigant rinse consisted of 60µL nuclease free water or 60µL 17% EDTA. This was applied in either one 15 minute application using 30 seconds of passive ultrasonic activation (irrisafe 25mm ISO 25 ultrasonic tip (QED Endo, Peterborough, UK) mounted in a satelec piezoelectric ultrasonic unit at power setting 6 (Acteon, St Neots, UK) (**Figure 11**). The alternative approach included three 5 minute applications of the 60µL irrigant with 30 seconds of passive ultrasonic activation in each of the irrigant applications (total of 180µL and 90 seconds of passive ultrasonic activation) (**Figure 12**).

Between each application the tooth was moved to a separate putty base containing PerioPaper which was pre-prepared (**Figure 7** (**e**)). The irrigant was extracted using micropipettes and placed in an Eppendorf tube; the PerioPaper was dissected out of the putty and placed in a separate Eppendorf tube. Both types of samples were weighed to determine the

volume of irrigant which was retrieved. This was calibrated against pre-determined weight/volume standard curves. The samples were stored at a temperature of -20˚C prior to use.

Figure 7: Photographs of the experimental set up used in experiment 3.4. (a & b) cross sectional views of the tooth apex in contact with the PerioPaper (OraFlow, New York, USA); which is marked with a black arrow and mounted in a putty base putty (Henry Schein, NY, USA) (c & d) groups with $Ca(OH)_2$ paste (UltraCal, Ultradent, USA) kept in a container with an incubator (not shown) and the access cavities sealed with light bodied silicone (Henry Schein, NY, USA) (e) multiple putty bases for the teeth to be moved during each 5 minute irrigation with the secondary irrigant.

3.4.1. ELISA analysis

The retrieved irrigants were equilibrated to reach room temperature prior to analysis. The samples were treated with the addition of 30 µL of 1 M hydrochloric acid (SigmaAldrich, UK) for 10 minutes; this activated the TGF-β1. The samples were then neutralised with 30 µL of 1.2 M sodium hydroxide (SigmaAldrich, UK) for a further 10 minutes.

50 µL of the resultant solution were assayed in duplicate using the Quantikine Human TGF-β1 ELISA kit (R & D Systems, UK), according to the manufacturer's instructions. A serial dilution of TGF-β1 was created from a stock solution of 2000 pg/mL with the following concentrations: 500, 250, 125, 62.5, 31.25, 15.625 and 7.81pg/ml to create a standard curve. 50µL of standard, sample or blank was added to a well of the prepared 96-well plate and 50µL of assay diluent making a total of 100 µL; this was then sealed and incubated at room temperature for 3 hours. After incubation, the wells were washed with wash buffer in an automated plate washer (Bio-Tek Instruments, USA). Visual inspection was used to ensure that the wash buffer was completely removed prior to advancing to the next step. 100 µL of TGF-β1 conjugate was added to each well, sealed and incubated for 3 hours at room temperature. The washing procedure was repeated and then 100 µL of substrate solution was added to each well and incubated for 30 minutes at room temperature in the dark. To terminate the reaction, 100 µL of the stop solution (R & D Systems, UK) (**Figure 8**) was added and the absorbance was read using an ELX800 Universal Microplate Reader (BioTek, Swindon, UK) at 450 nm and with correction set at 570 nm to reduce optical interference. Concentrations of TGF-β1 in the retrieved irrigant and periapical sampling were calculated from the standard curve produced (**Figure 9**).

To determine the volume of irrigant in the PerioPaper it was weighed prior to the experiment and an average weight calculated. PerioPaper containing a known volume of irrigant were also weighed using a 4 decimal point balance (Oxford Laboratory Balance, European Instruments, Headington, UK) which was used to make a weight / volume conversion chart (**Figure 10**).

The PerioPaper samples were equilibrated to room temperature and then 120µl 1 N of HCL placed in the Eppendorfs for 30 minutes to release bound TGF-β1 (a larger volume of HCL was used to ensure complete coverage of the PerioPaper facilitating diffusion of the EDTA from the strip). This was based on preliminary studies undertaken in the laboratory which showed a 95% retrieval or bound TGF-β1 (**see Appendix 1**). The HCL was neutralised after 30 mins with 120µl 1.2N NaOH for 10 minutes and the samples processed using the same TGF-β1 ELISA technique as above.

The actual concentration of TGF-β1 present in the PerioPaper volume could then be calculated by working out the dilution factor of the irrigant after application of the 120µl 1 N of HCL, 120µl 1.2N NaOH and the ELISA diluents. As the initial volume of EDTA was known, the final concentration of TGF-β1 could be worked backwards to determine the actual concentration of TGF-β1 in the PerioPaper (**Figure 10**).

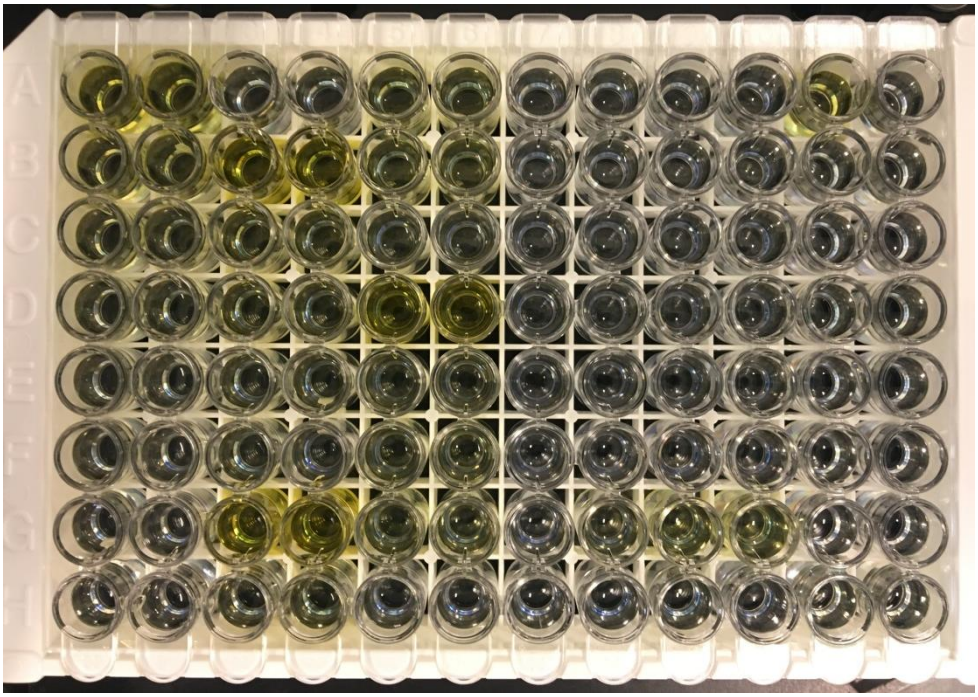

Figure 8: Showing the 96-well plate following termination of the reaction with the stop solution (R & D Systems, UK). The absorbance was then read using an ELX800 Universal Microplate Reader (BioTek, Swindon, UK).

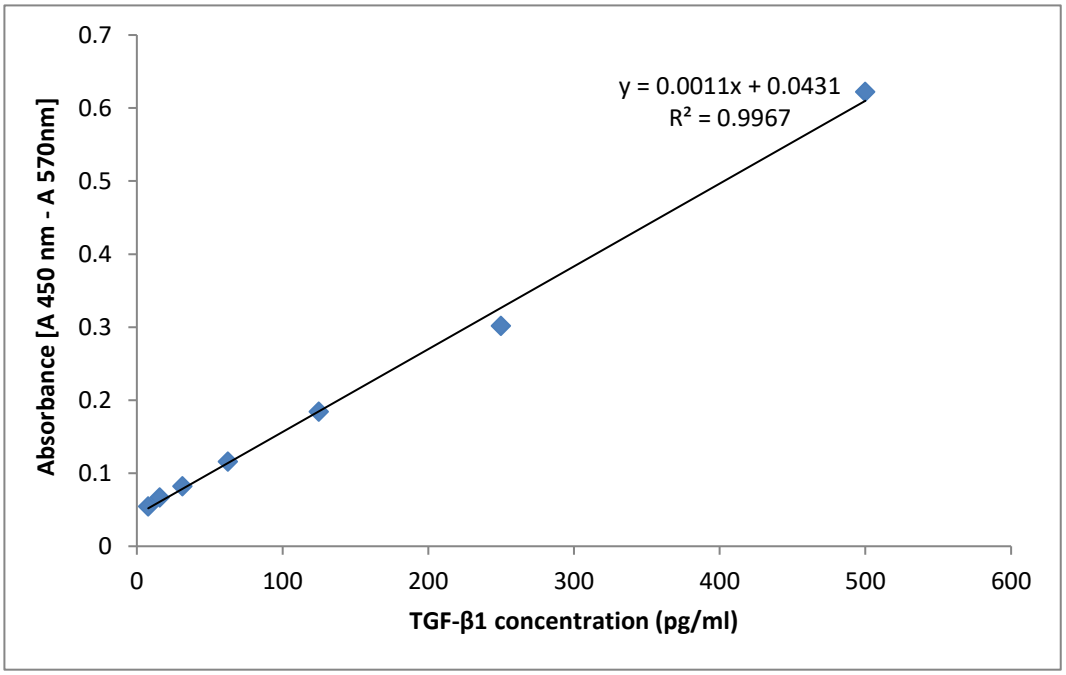

Figure 9: Representative example of a standard curve used to determine the concentration of TGF-β1 using ELISA analysis. The R^2 coefficient (>0.9) and linear regression equation are displayed next to the line of best fit.

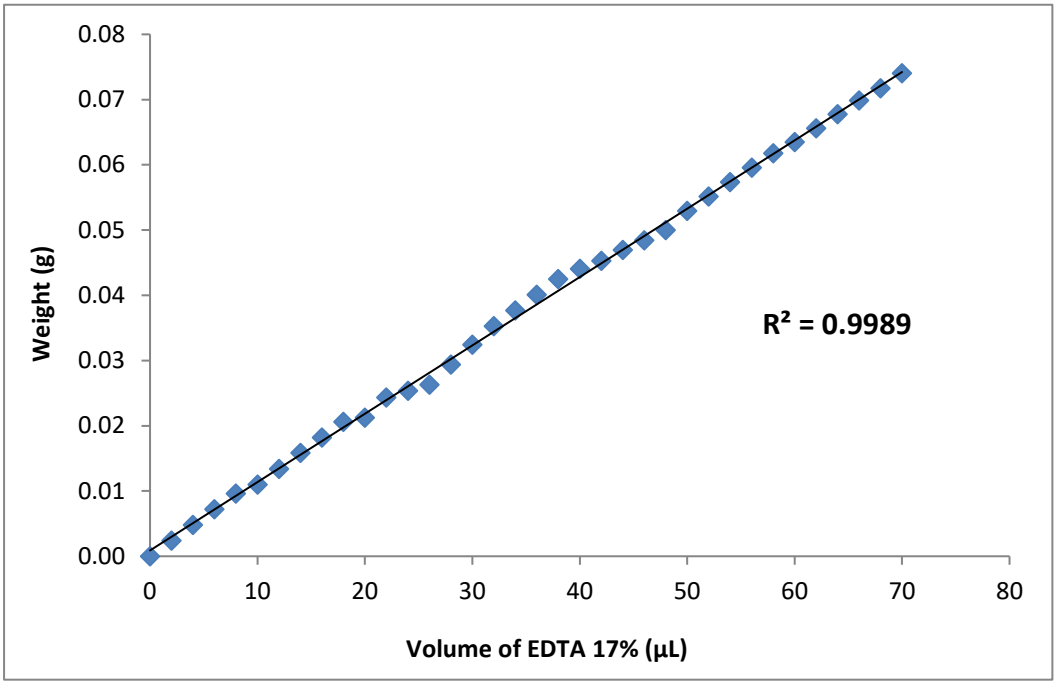

Figure 10: Representative example showing the conversion chart used to determine the volume of EDTA 17% (Oxford Laboratory Balance, European Instruments, Headington, UK). The PerioPaper (Oraflow, New York, USA) was removed after the experiment and weighed; the weight was then compared to this conversion chart which allowed the volume of irrigant retrieved from the periapical sampling to be known. This approach was then used to determine the exact concentration of TGF-β1 present following the TGF-β1 ELISA. The final concentration of TGF-β1 could then be adjusted to compensate for the dilution factor of the EDTA 17% during the PerioPaper extraction process and the reagents used in the ELISA analysis. The coefficient of determination (R^2) is 0.9989.

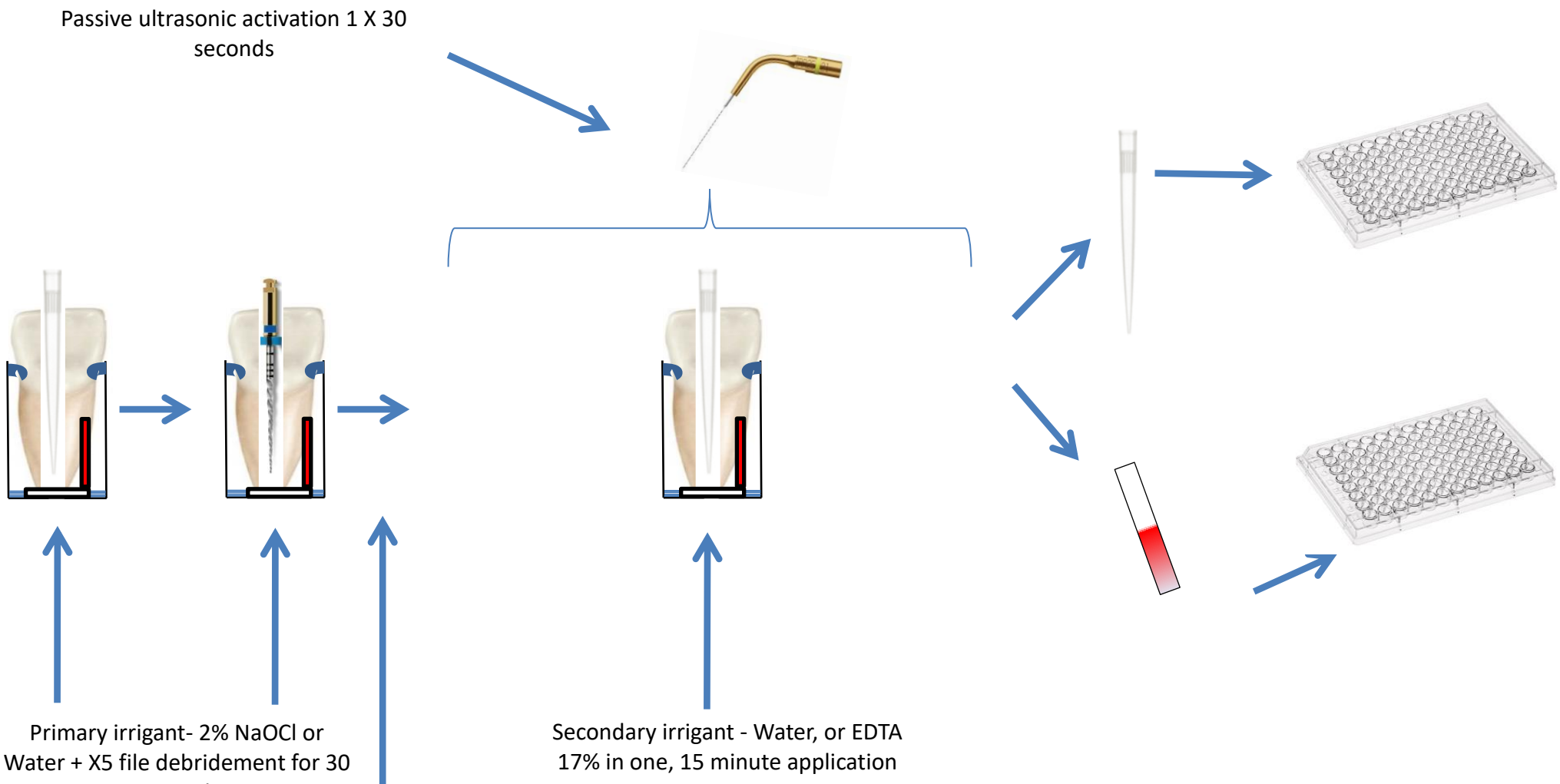

Figure 11: Diagrammatic representation of the protocol used for groups (*) **1, 3, 5, 7, 9** and **10** (**Table 3**) where the tooth is prepared with a primary irrigant and an X5 ProTaper file, followed by drying and rinsing with water, followed by the application of a secondary irrigant for 15 minutes with 30 seconds of ultrasonic activation. The sample of the irrigant and the periapical tissues were then processed in a TGF-β1 ELISA plate.

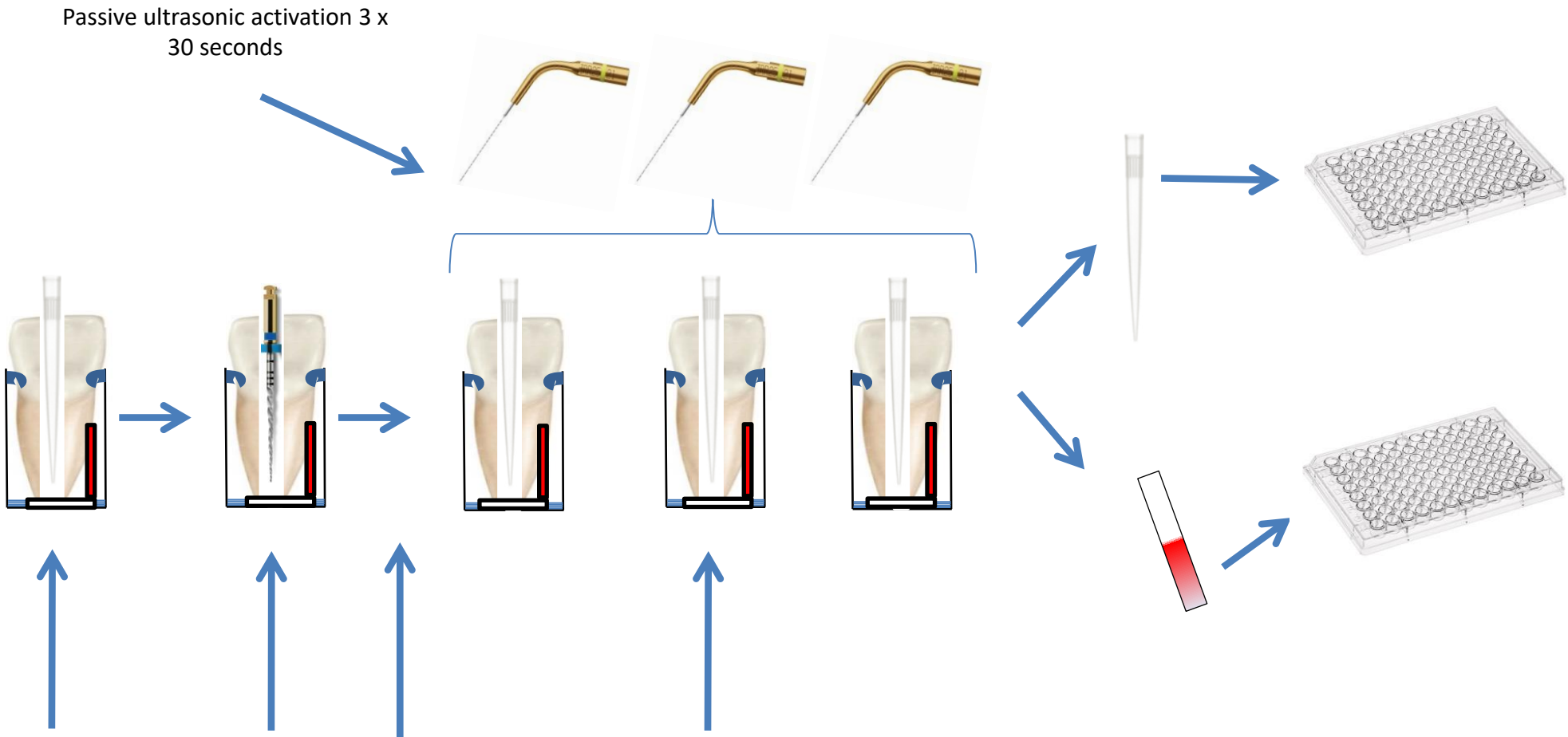

Figure 12: Diagrammatic representation of the protocol described for groups (**) **2, 4, 6** and **8** (**Table 3**) where the tooth is prepared with a primary irrigant and an X5 ProTaper file, followed by drying and rinsing with water, followed by the application of a secondary irrigant X 3 for 5 minutes each with 30 seconds of ultrasonic activation in each of the 3 applications. The sample of the irrigant and the periapical tissues were then processed in a TGF-β1 ELISA plate.

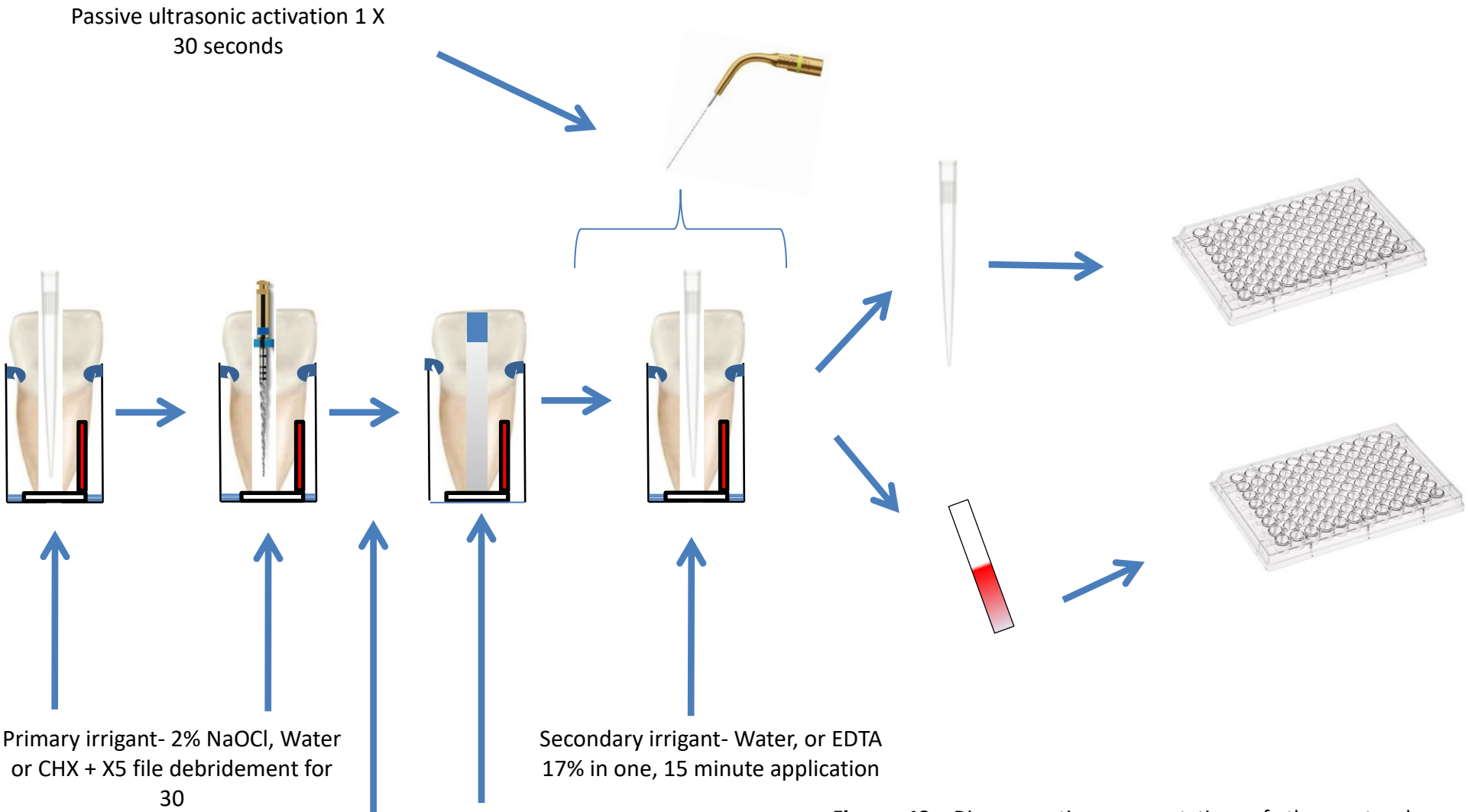

Figure 13: Diagrammatic representation of the protocol described in (***) **11,12** and **13** (**Table 3**) where the tooth is prepared with a primary irrigant and an X5 ProTaper file, followed by drying and rinsing with water then the tooth sealed with a $Ca(OH)_2$ dressing for two weeks. The tooth is then re-accessed, the $Ca(OH)_2$ removed and irrigant two applied for 15 minutes with 30 seconds of ultrasonic activation. The sample of the irrigant and the periapical tissues was then processed in a TGF-β1 ELISA plate.

3.4.2. Scanning electron microscopy

Following experimentation representative samples from each group were used, the samples are as follows (the group number for the irrigant regime was obtained from
Table 3):

1. Group 1, NaOCl 2% + Water
2. Group 3, NaOCl 2% + EDTA
3. Group 5, Water + Water
4. Group 7, Water + EDTA 17%
5. Group 9, CHX 2% + Water
6. Group 10, CHX 2% + EDTA 17%

The samples were sectioned using a diamond disc (Horico, Berlin, Germany) in a straight hand piece (Kavo, Uxbridge, UK) at the midpoint of the root on the buccal and palatal surfaces with care taken not to enter the lumen of the teeth. The teeth were then split down the long axis with a flat plastic instrument (Henry Schein, NY, USA), in order to minimise the potential for any debris to contaminate the internal dentine surface. Two representative teeth from each group described above in section **3.4** were processed. This involved dehydration in a vacuum extractor, gold labelling and analysis with a Zeiss electron microscope (EVO, MA10, Carl Zeiss Microscopy, Germany). Images at a range of magnifications were obtained in order to analyse the root surface.

3.5. Characterisation of the effect of NaOCl pre-treatment on TGF-β1 detection

In order to ascertain if the residual NaOCl was interfering with the ELISA plate and/or affecting the sampled TGF-β1 an experiment was devised. This involved using a known concentration of TGF-β1 (250pg/ml) was used to determine the effect of NaOCl pre-treatment on TGF-β detection by ELISA. The concentrations of NaOCl were in serial dilutions of 0.5%, 0.25%, 0.125%, 0.0625%, 0.0313%, 0.0156%, 0.0078% and 0%.

3.5.1. Pre-treatment of wells with NaOCl concentrations

To determine if the NaOCl was preventing the ELISA from working correctly, eight wells in duplicate were treated for 5 minutes with a range of concentrations of NaOCl from 0.5% to 0.0078%. Solutions were then removed using an automated plate washer (ELx50, BioTek, Swindon, UK) with washer buffer (R&D systems, Abingdon UK). A known concentration of 250pg/ml TGF-β1 was then added to a solution of RD1-73 diluent (R&D systems, Abingdon UK) in each of the ELISA wells (**Figure 14**).

3.5.2. Pre-treatment of TGF-β1 known concentrations

To determine if the NaOCl was denaturing the TGFβ1 solution before it was sampled within the ELISA plate a 25µl volume of 1000 pg/ml TGF-β1 was added to 8 eppendorfs in duplicate. Each sample was treated for 10 minutes with NaOCl solution at a range of concentrations as previously described in **3.5**. These were gently mixed and allowed to stand for 5 minutes. RD1-73 diluent (R&D systems, Abingdon UK) was then added to each Eppendorf tube together with the TGF-β1 / NaOCl solution, again as listed in **3.5**. After dilutions of the above reagents (75µl) and ensuring the pH in the range of

the ELISA kit, each well in total contained 100 µl 250 pg/ml of TGF-β1 in solution (**Figure**)

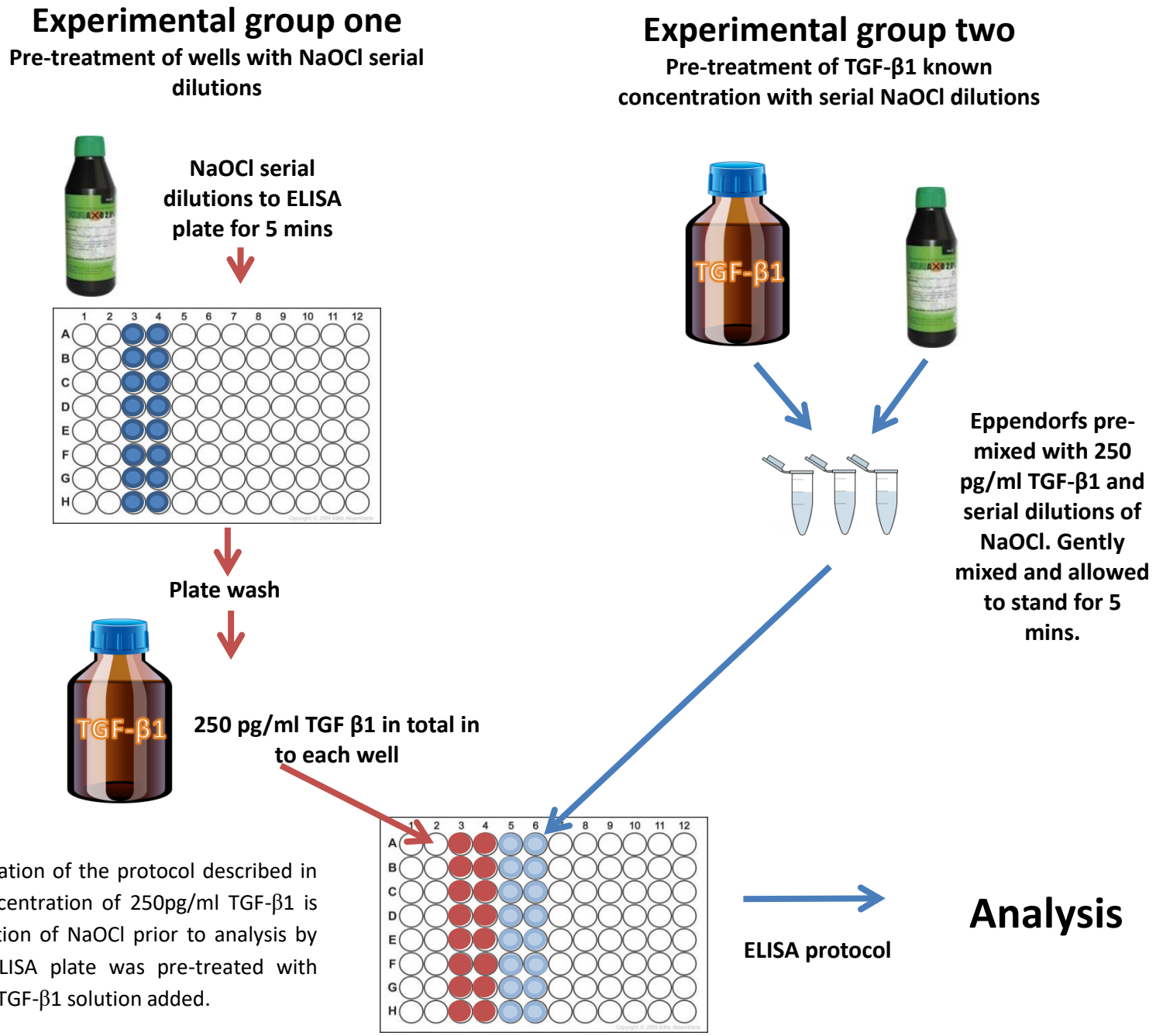

Figure 14: Diagrammatic representation of the protocol described in section **3.5** whereby a known concentration of 250pg/ml TGF-β1 is treated with a range of concentration of NaOCl prior to analysis by TGF-β1 ELISA. Alternatively the ELISA plate was pre-treated with NaOCl before the known 250pg/ml TGF-β1 solution added.

3.6. Analysis of the effect of sodium thiosulphate has on TGF-β1 analysis.

The deleterious effects of NaOCl on both the free TGF-β1 and the antibody coating of the ELISA plate well were investigated by neutralising the effects of NaOCl with sodium thiosulphate ($Na_2S_2O_2$).

Sodium thiosulphate solution was synthesised by mixing dry crystals of 99.9% sodium thiosulphate (Fisher Chemicals, NH, USA) and de-ionised water. The protocol was repeated as described in section **3.5.2** where 250pg/ml of TGF-β1 was pre-treated with NaOCl. Following the application of NaOCl for 5 minutes sodium thiosulphate was added to the solution so the overall concentration of sodium thiosulphate was 5%. The solution was mixed and allowed to equilibriate for 5 minutes. The solutions were then added to the TGF-β1 ELISA kit and mixed with RD1-73 diluent (R&D systems, Abingdon UK) by adding a higher initial concentration of TGF-β1 of 1000pg/ml following dilution with the other reagents the overall concentration of TGF-β1 in each well was 250pg/ml (**Figure 15**).

3.6.1. The effect of sodium thiosulphate has on TGF-β1 analysis in the model of a wide open apex

The protocol in section **3.6** was applied to the tooth model to determine if a 5% $Na_2S_2O_2$ rinse after the administration of 2% NaOCl and prior to 17% EDTA rinse would promote growth factor release.

Five teeth were prepared using the standardised protocol described in section 3.1 and mounted in putty with Periopaper at the apex. The teeth were treated with 2% NaOCl delivered using a micropipette and prepared for 30 seconds using an X5 ProTaper Next file (Dentsply Sirona Endodontics, OK, USA). The teeth were then dried with paper points, and rinsed with water, dried again and 60µl of 5% sodium thiosulphate delivered using a micropipette, extracted and dried again. 17% EDTA was then delivered for 15

minutes with 30 seconds passive ultrasonic activation. The irrigant was then extracted and the Periopaper dissected and samples stored at -20°C until they were processed using the TGF-β1 ELISA (**Figure 16**).

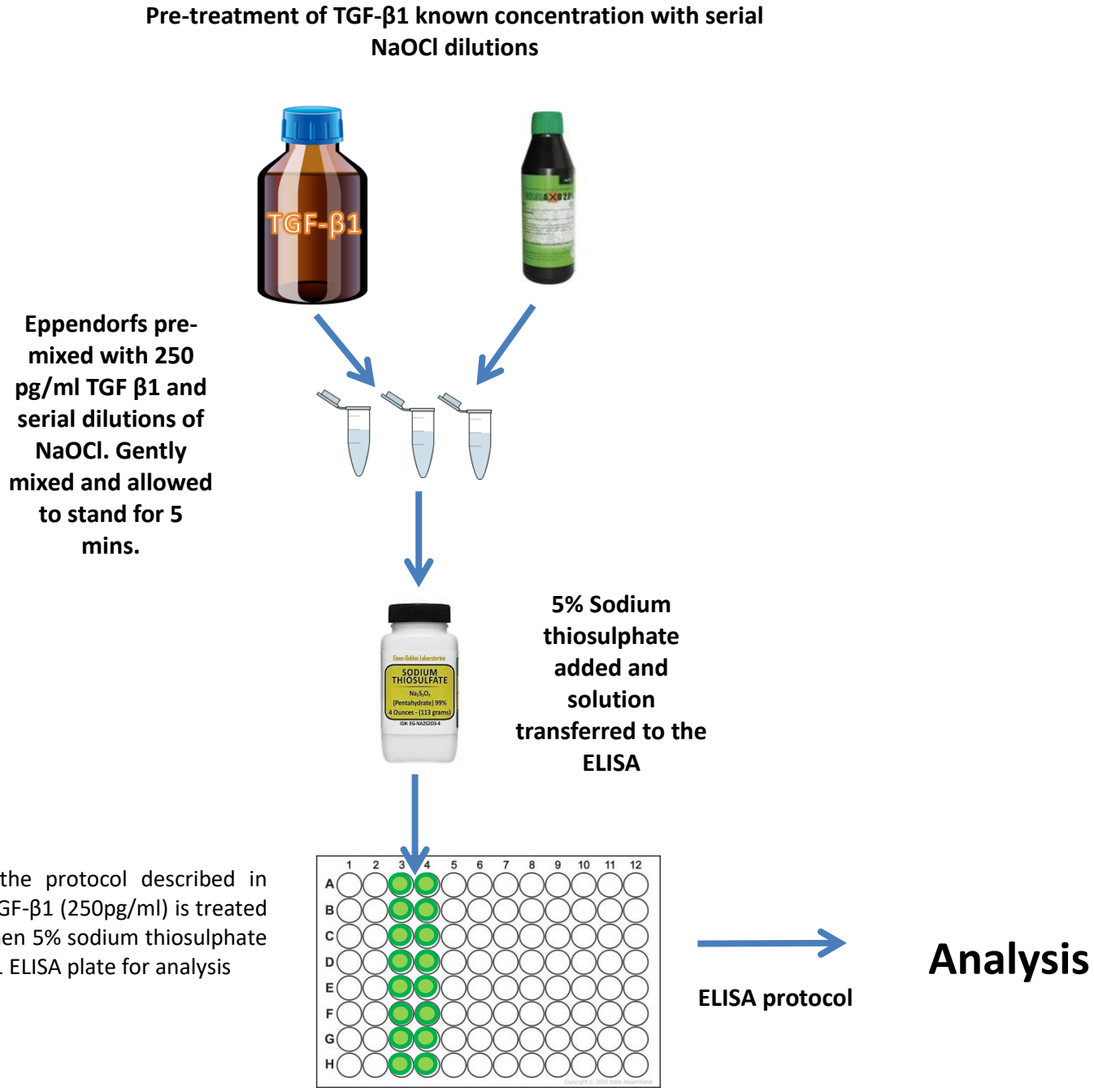

Figure 15: Diagrammatic representation of the protocol described in section **3.6** where a known concentration of TGF-β1 (250pg/ml) is treated with a range of concentrations of NaOCl and then 5% sodium thiosulphate is added before being transferred to the TGF-β1 ELISA plate for analysis

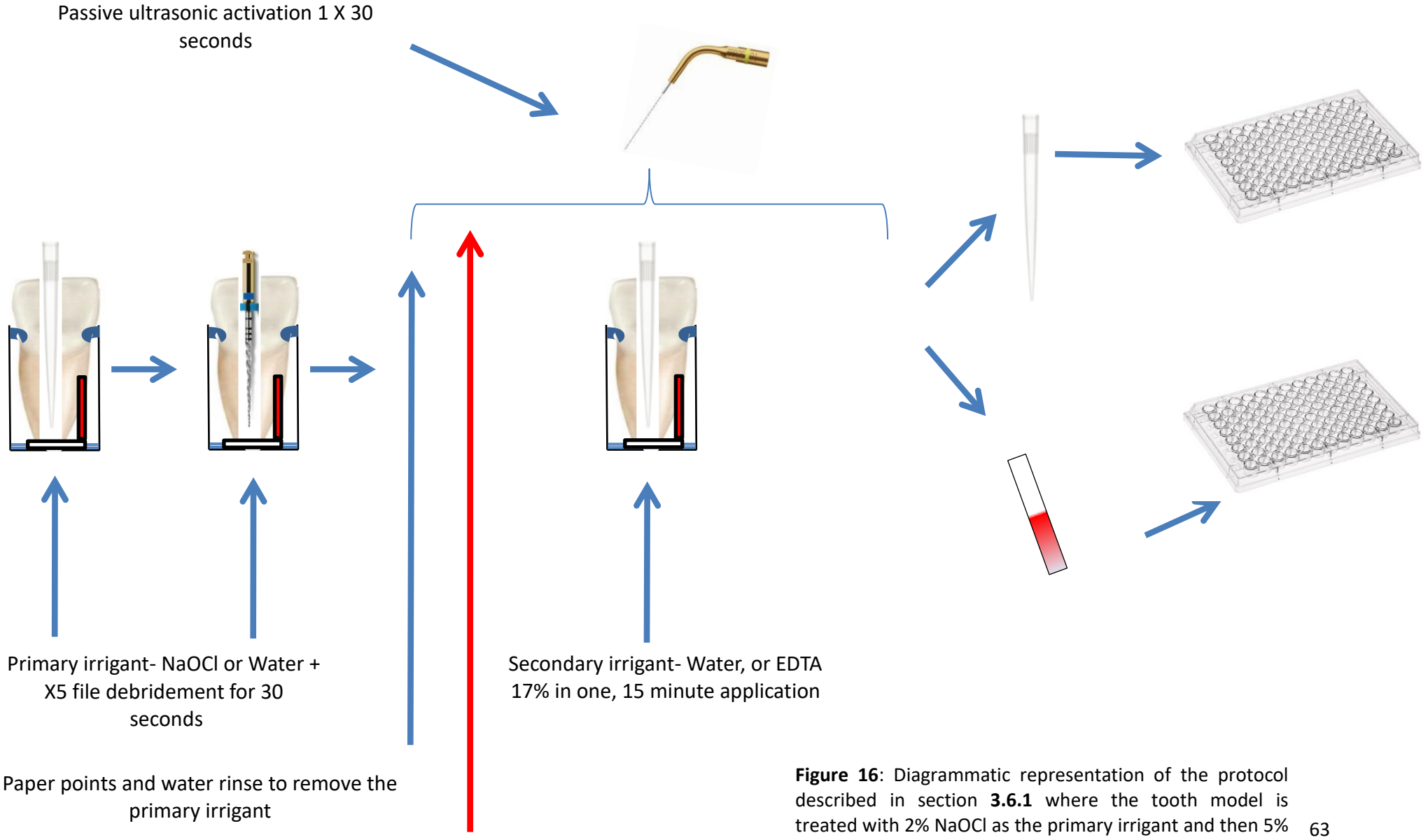

Figure 16: Diagrammatic representation of the protocol described in section **3.6.1** where the tooth model is treated with 2% NaOCl as the primary irrigant and then 5% sodium thiosulphate added in an attempt to neutralise the remaining NaOCl before the application of secondary irrigant, 17% EDTA.

3.7. Statistical tests

Statistical differences between the experimental and control groups were determined using one-way ANOVA (SPSS Inc, Chicago, IL, USA) with $P < 0.05$ (*) or $P < 0.001$ (**) deemed to be statistically significant.

4.0. Results

4.1. Pilot study

To initially assess growth factor release from dentine a pilot study was undertaken as described in section **3.2.** The experimental aims were:

1. To determine if TGF-β1 could be detected from the retrieved irrigant;

2. To determine the affect time of irrigant application had on the concentration of detected TGF-β1.

17% EDTA or saline irrigant was collected and sampled to determine the concentration of TGF-β1 present in the solution using a TGF-β1 human ELISA (R&D systems, Abingdon UK). The samples were subject to spectrophotometer (Universal Micropipette Reader, ELx800, BioTek, Swindon, UK) analysis at 450nm and 570nm with the wavelength concentrations subtracted and analysed against the standard concentration curve (see section **3.4.1**).

4.1.1. Rinse and immediate collection assay

The rinse with 17% EDTA and immediate collection detected TGF-β1 concentration of 13.55 ± 7.36 pg/ml^{-1} and the rinse with saline did not contain any detectable TGF-β1. Irrigation with 17% EDTA did not produce a significantly greater concentration of TGF-β1 release compared with saline irrigation (**Figure 17**).

4.1.2. Twenty-four hour incubation followed by collection assay

The 24 hour incubation of 17% EDTA within the model system resulted in the retrieval of TGF-β1 at 1008.40 ± 531.08 pg/ml^{-1} and the application saline

did not result in the detection of TGF-β1. The difference was not statistically significantly (P < 0.001) (**Figure 17**).

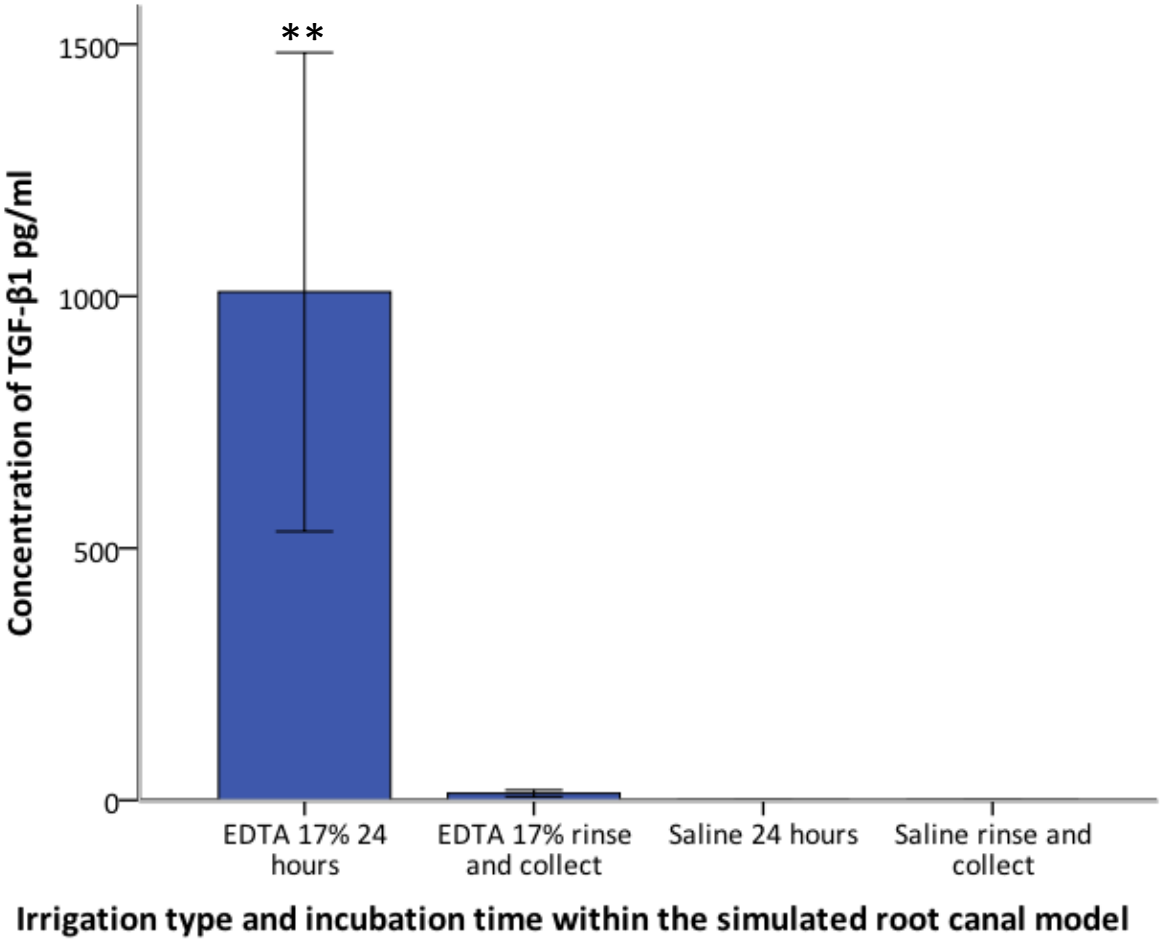

Figure 17: The data showing the mean concentration of TGF-β1 (pg/ml +/- SE (n=5)) when 17% EDTA or saline were applied to the simulated root canal model. This was delivered in either a rinse and immediate collection or incubation of the irrigant for 24 hours in the model followed by irrigant collection. When 17% EDTA was incubated for 24 hours in the model the statistical testing showed TGF-β1 concentration liberated were significantly greater (P < 0.001; **) than those achieved with EDTA 17% rinse and collect and both saline groups. EDTA 17% as a simple rinse and collect showed positive detection however this was not statistically significant compared with the saline control.

4.2. EndoVac® retrieval system, a negative pressure irrigation system

The pilot study showed that TGF-β1 could be retrieved and analysed in our model of a wide open apex. The experimental design used which is described in section **3.3**, utilised the clinically relevant EndoVac® retrieval system which aimed to:

1. Deliver high volumes of irrigants and to determine if retrieval and analysis were feasible;

2. Attempt to release and sample dentine growth factors in a time-scale which was more clinically feasible (<15 mins);

3. Determine the effects of different EDTA concentrations on the release of growth factors;

4. Attempt to sample TGF-β1 in a model of the periapical tissues.

The collection and processing of samples are described in **3.3** and follow the same analysis as **4.1** using the TGF-β1 human ELISA (R&D systems, Abingdon UK) and spectrophotometer readings (Universal Micropipette Reader, ELx800, BioTek, Swindon, UK) with additional analysis of the simulated periapical tissues.

4.2.1 Irrigant retrieval

The retrieval of TGF-β1 and analysis were deemed feasible using the EndoVac® protocol as there was positive detection of TGF-β1. This was achieved in a time-scale of less than 15 minutes and the data showed a difference between different EDTA concentrations used and their yield of TGF-β1.

However, the overall yields for TGF-β1 were lower than those retrieved from the pilot experiment described in section **4.1** although the volumes of irrigants were much higher. The mean yield of TGF-β1 for retrieved irrigants were: saline 0.28 ± 0.62 pg/ml^{-1}, 5% EDTA 5.60 ± 10.40, 10% EDTA 21.82 ± 16.93 pg/ml^{-1} and 17% EDTA 8.04 ± 6.61 pg/ml^{-1}. The highest irrigant yield was achieved using 10% EDTA (**Figure 18**).

The statistical analysis showed that the use of EDTA at 10% irrigation produced greater concentrations of TGF-β1 compared with all other groups ($P < 0.001$), other than 10% EDTA periapical sampling; which showed significance at $P < 0.05$.

4.2.2. Periapical sampling

The periapical sampling produced results which were positive for TGF-β1 retrieval and demonstrated that periapical sampling was feasible. The type and concentration of the irrigant used released different concentrations of TGF-β1 when applied to root dentine. These were: saline of 0.70 ± 1.4 pg/ml^{-1}, 5% EDTA 4.90 ± 7.1 pg/ml^{-1}, 10% EDTA 11.48 ± 4.93 pg/ml^{-1} and 17% 7.45 ± 3.06 pg/ml^{-1}.

EDTA at 10% periapical irrigation showed a statistically significant difference ($p < 0.05$), for TGF-β1 liberated compared with the other groups of saline irrigant, saline periapical and 10% EDTA irrigant. There were no other statistically significances detected between any of the TGF-β1 levels liberated from the irrigant compared with the periapical sampling. The highest concentration of TGF-β1 released from the periapical sampling was therefore from the 10% EDTA group (**Figure 18**).

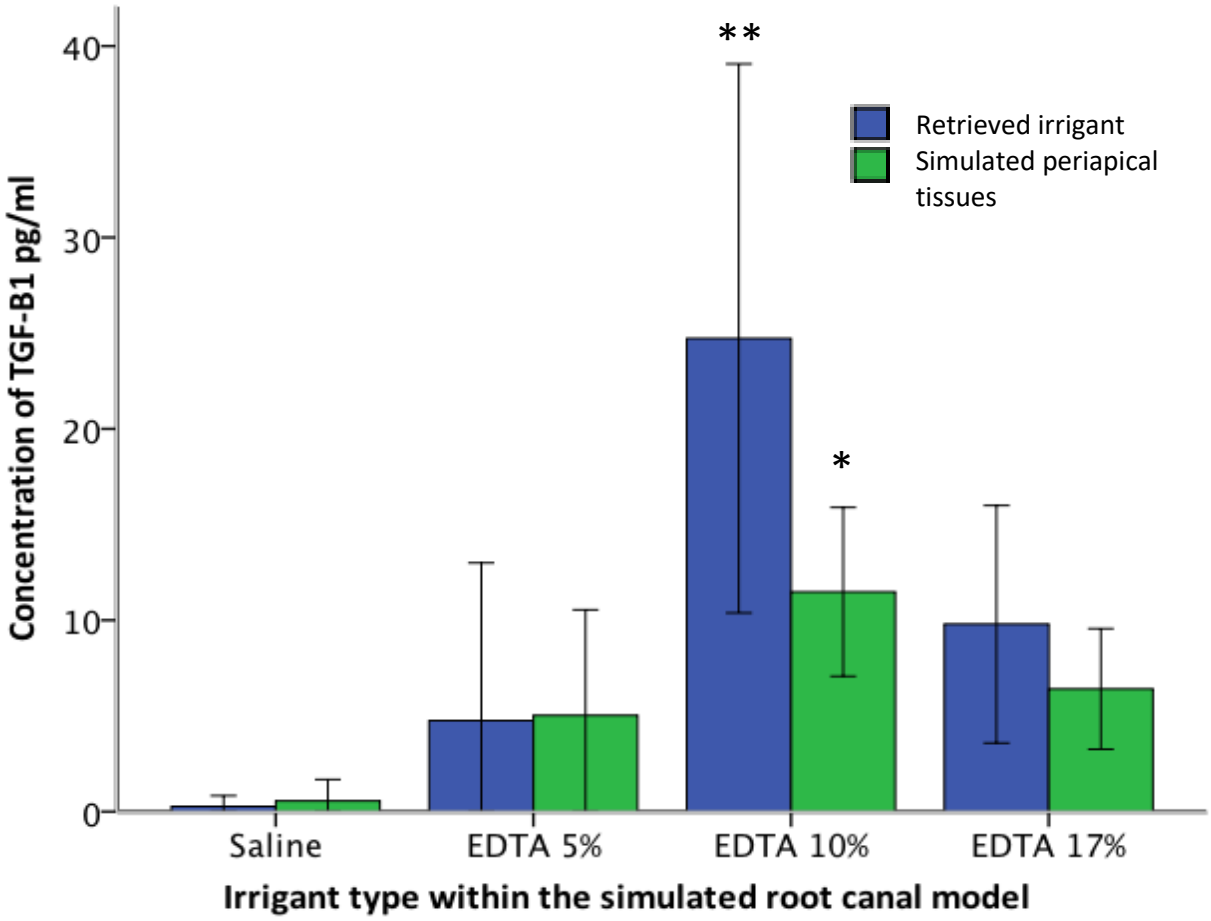

Figure 18: The data showing the mean concentration of TGF-β1 (pg/ml +/- SE (n=5)) when saline and different concentrations of EDTA were applied using the EndoVac® negative pressure irrigation system. The graph shows both the TGF-β1 retrieved from the irrigant and the TGF-β1 retrieved from the Microcon, representing the periapical tissues in the model. The application of 10% produced the highest concentration of TGF-β1 both in the retrieved irrigant and the periapical sampling in the model; this produced statistically higher concentrations of retrieved TGF-β1 ($p < 0.001$; **) compared to all groups other than EDTA 10% periapical which had a statistically significant of ($p < 0.05$; *). The EDTA 10% periapical in the model was statistically significant for liberating higher TGF-β1 than both saline groups ($p < 0.05$; *).

4.3. Contemporary endodontic irrigation and their effect on dentine growth factor release

The previous results presented in section **4.2** indicated that TGF-β1 could be detected in the retrieved irrigants and the simulated periapical tissues; however, the yield was significantly lower than what was detected in section **4.1.** The subsequent experiment aimed to:

1) Consolidate the technique to guide the *in-vivo* sampling approach;

2) Increase the yield of TGF-β1 within a timeframe which was in a clinically acceptable time period (<15mins);

3) Determine what effects contemporary irrigants have on the release of growth factors within this model of a wide open apex.

The protocol for chemo-mechanical treatment of the tooth model was standardised to test the effects of contemporary irrigants and is described in section **3.4.**

The irrigant analysis was undertaken using the same approach as that described in section **3.4.1** with PerioPaper (Oraflow, New York, USA) used to simulate the periapical tissues.

4.3.1. Multiple irrigant rinses

The concentration of TGF-β1 was analysed following multiple rinsing and retrieval of the irrigant from the root canal system, this approach is detailed in **Table 3** of section **3.4.** The groups analysed and data generated are presented in (**Figure 19**).

The highest concentration of TGF-β1 retrieved was with the 'Water + EDTA 17%' groups with mean values of TGF-β1 of 152.67 ± 55.58 pg/ml^{-1}, 196.7860 ± 77.51 pg/ml^{-1}, 138.72 ± 70.25 pg/ml^{-1} at 5, 10 and 15 minutes, respectively. The control group of the 'Water + Water' irrigants detected a small amount of TGF-β1 of 8.38 ± 4.10 pg/ml^{-1} at 5 minutes and 6.41 ± 6.08 pg/ml^{-1} at 10 minutes. In contrast both groups with the primary rinse of NaOCl 2% resulted in no growth factors being detected. The yields of retrieved irrigants in the 'Water + EDTA 17%' group were higher than were detected in the studies described in section **4.2** when the EndoVac® method was used, yielding more TGF-β1 in the same 15 minute period.

The analysis showed that the irrigant regime of 'Water + EDTA 17%' liberated statistically greater amounts of TGF-β1 than any other irrigant groups ($p < 0.001$). There was no statistical difference between the concentrations of TGF-β1 retrieved between the 5, 10 and 15 minute time-points.

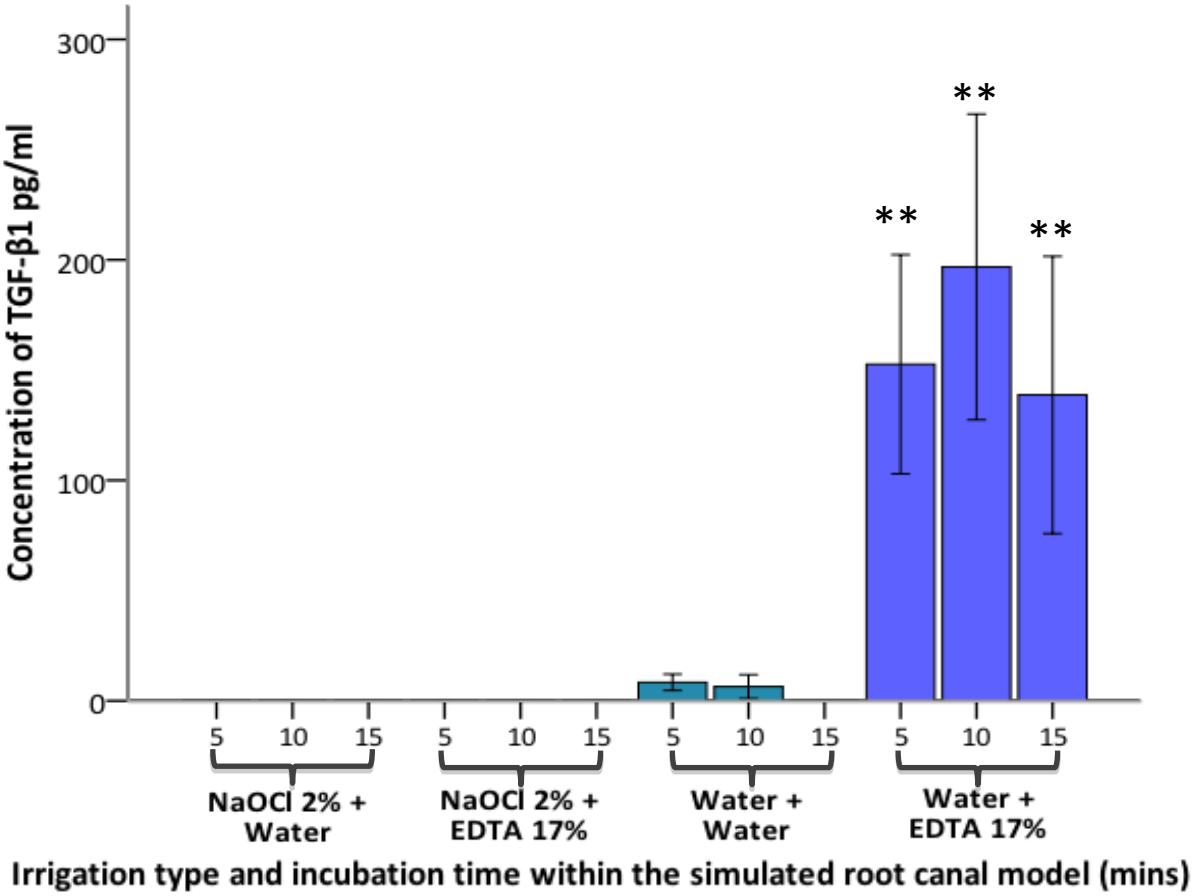

Figure 19: The data showing the mean concentration of TGF-β1 (pg/ml +/- SE (n=5)), with the standard error which was retrieved from the irrigant using a micropipette. The primary irrigant of NaOCl 2% or water and a secondary irrigant of water or EDTA 17% were used. These were administered in three separate five minute application with three bursts of 30 second passive ultrasonic irrigation. This corresponds to groups 2, 4, 6 and 8 in table 3. The graph shows that the group with a primary rinse of water and secondary rinses with EDTA 17% gave the highest release of TGF-β1 compared with all other irrigant groups. This was statistically significant ($p < 0.001$; **) compared to NaOCl 2% + water, NaOCl 2% + EDTA 17% and water + water. There was no statistical difference between the growth factors retrieved at 5, 10 or 15 minute intervals. When a primary rinse of NaOCl 2% was used there was no detectable amount of TGF-β1.

4.3.2. Multiple rinses of the irrigant with periapical sampling

The sampling of the simulated periapical tissues is described in section **3.4**. In order to determine the concentration of the irrigant within the PerioPaper® the exact volume of the irrigant contained within it needed to be determined. This was achieved by undertaking a conversion table where a known volume of 17 % EDTA was added to a PerioPaper strip and weighed at 2µl increments (**Figure 10**). When the PerioPaper was retrieved from the experimental model it was then weighed and the volume of irrigant retained and calculated from these tables. The analysis of the strip is described in section **3.4.1** and the final concentration of TGF-β1 was calculated based on the original volume.

The sampled periapical tissues showed similar findings to the irrigant retrieval as described in section **4.3.1**, in that the 'Water + EDTA 17%' group liberated TGF-β1 in the simulated periapical tissues. The other groups produced no detectable TGF-β1. There was also no significant difference between the PerioPaper sampled at 5, 10 or 15 minute intervals. The mean TGF-β1 sampled was 120.54 ± 35.72 pg/ml^{-1}, 119.62 ± 34.02 pg/ml^{-1} and 121.51 ± 27.86 pg/ml^{-1} for 5, 10 and 15 minutes, respectively (**Figure20**).

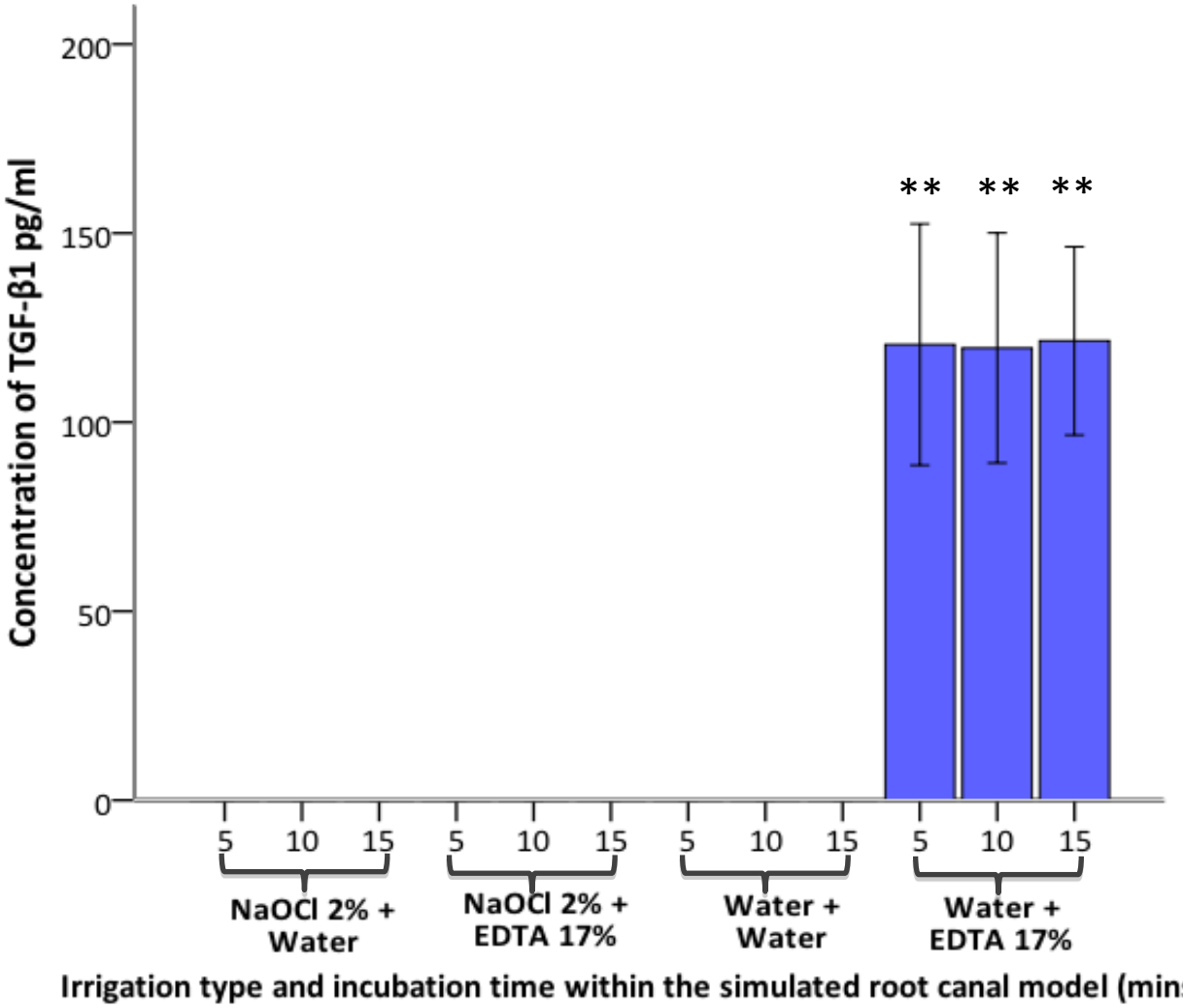

Figure 20: The data showing the mean concentration of TGF-β1 (pg/ml +/- SE (n=5)) in the retrieved PerioPaper® when using the different irrigant types and delivery time. The primary irrigants of NaOCl 2% or water and a secondary irrigant of water or EDTA 17% were administered in three separate five minute application with three bursts of 30 second passive ultrasonic agitation. This corresponds to groups 2, 4, 6 and 8 in Table 3. The graph shows that the group with a primary rinse of water and secondary rinses with EDTA 17% gave the only detectable concentration of TGF-β1. This was statistically significant ($p < 0.001$; **) compared with NaOCl 2% + water, NaOCl 2% + EDTA 17% and water + water. There was no statistical difference between the growth factors retrieved at 5, 10 or 15 minute intervals for the EDTA 17% irrigant.

4.3.3. Comparison of the TGF-β1 released between the irrigants and periapical sampling for Water + EDTA 17%

As there was no or limited release of TGF-β1 release between the 'NaOCl 2% + Water', 'NaOCl 2% + EDTA 17%' and 'Water + Water' groups these were excluded from further analysis. The 'Water + EDTA 17%' group was further analysed to determine if there were difference between the two sampling methods. 'Water + EDTA 17%' at 10 mins recorded a statistically significant difference ($p < 0.05$) for increased TGF-β1 recovered from the periapical sampling at 5, 10 and 15 minutes and TGF-β1 recovered from the irrigant retrieval at 5 mins (**Figure 21**).

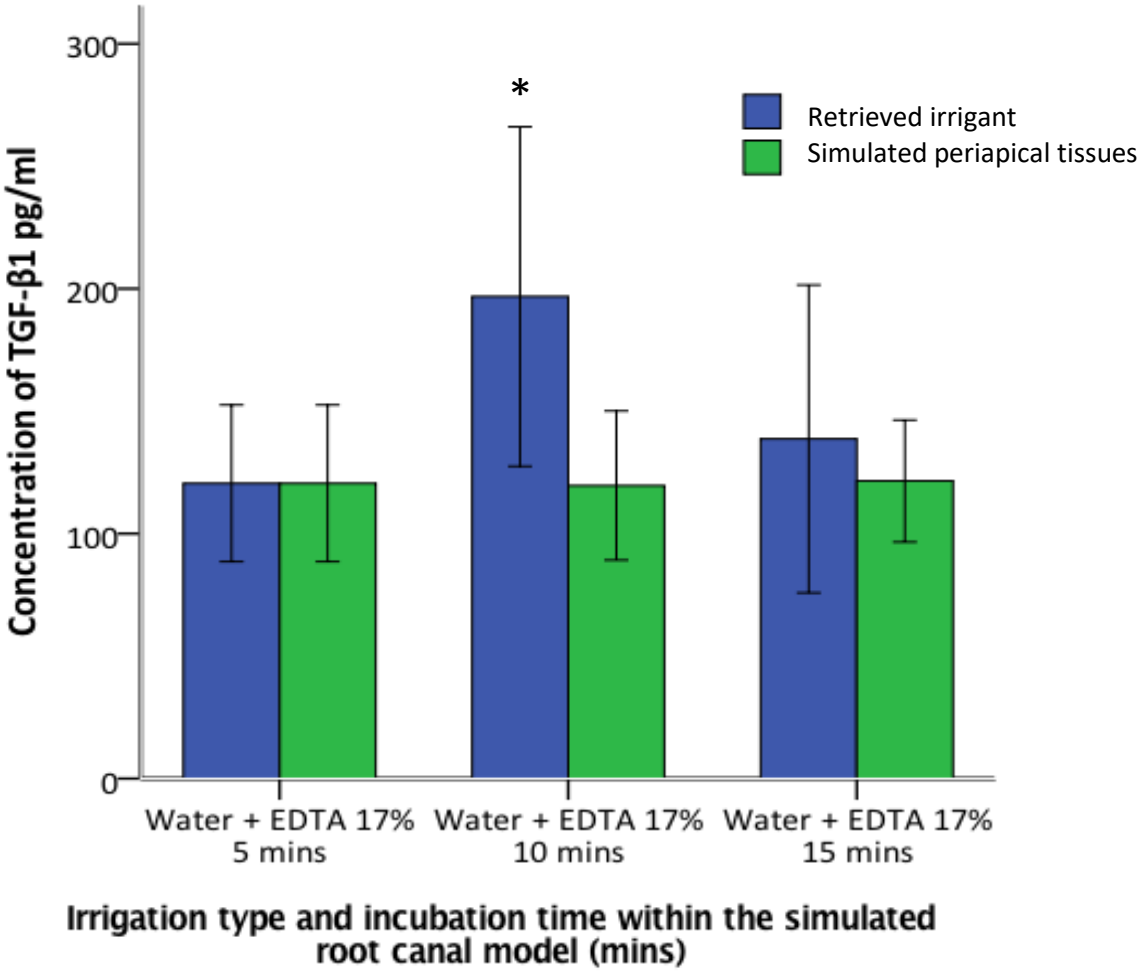

Figure 21: The data showing the mean concentration of TGF-β1 (pg/ml $^{+}$/- SE (n=5)) in the Water and + EDTA 17% group only at the time-points of 5, 10 and 15 minutes. The data shows the comparison between the concentration of TGF-β1 recovered for both the irrigant and periapical sampling methods demonstrating differences between the sampling techniques. There was a statistical difference ($p < 0.05$; *) between the Water + EDTA 17% group at 10 mins for the irrigant retrieval compared with the periapical sampling of the same group at 10 mins. The Water + EDTA 17% group at 10 minutes was also significantly greater ($p < 0.05$; *) than in both the irrigant and periapical sampling at 5 mins and the periapical sampling at 15 mins. There was no statistical difference between the Water + EDTA 17% irrigant and periapical groups at 5 mins and 15 mins.

4.3.4. Analysis of differences in the yield of TGF-β1 between multiple rinses or a single rinse application

The results presented in sections **4.3.1** and **4.3.2** were combined for the samples at 5, 10 and 15 minutes and compared with the data for the same irrigant groups which were treated with one single application of a 15 minute irrigant as opposed to three, 5 minute applications. This approach was used to ascertain if by changing the irrigant in multiple uses there was a significantly higher yield of dentine growth factors released as assayed by TGF-β1 as a surrogate marker.

The materials and methods, section **3.4, Table 3** illustrates the different groups applied. The groups compared are therefore the cumulative sum of all the TGF-β1 recovered from groups 2, 4, 6 and 8 compared with the results from groups 1, 3, 5 and 7.

Irrigation retrieval group

The yield of TGF-β1 was greatest in the 'Water + EDTA 17%' irrigant group for both the accumulative samples obtained at 5, 10 and 15 minute time-points and the single sample taken at 15 minutes. The accumulative samples produced a mean TGF-β1 concentration of 557.14 ± 316.42 pg/ml^{-1} and the single sample at 15 minutes produced a mean of 455.30 ± 309.24 pg/ml^{-1}. These results were not significantly different to each other but were statistically significant ($p < 0.001$) to all other groups. This indicated that multiple rinses and multiple ultrasonic irrigation techniques were not beneficial for growth factor release compared with the time importance of the irrigant incubation within the canal (**Figure 22**).

PerioPaper® sampling group

The yield of TGF-β1 was greater in the 'Water + EDTA 17%' periapical sampling group for both the accumulative samples obtained at 5, 10 and 15 minute time-points and the single sample obtained at 15 minutes. The

accumulative sample of the periapical sampling produced a mean TGF-β1 concentration of 361.47 ± 46.97 pg/ml^{-1} compared with 353.67 ± 225.02 pg/ml^{-1} for the single sample taken at 15 minutes (**Figure 23**).

The results mirrored the irrigant retrieval in that there was no statistical significant difference between the 3 time-points however the Water + EDTA 7% group was statistically significant ($p < 0.001$) compared with all other irrigant groups. These data indicated that multiple rinses and multiple ultrasonic irrigation techniques did not significantly impact growth factor release compared with the time of the irrigant application.

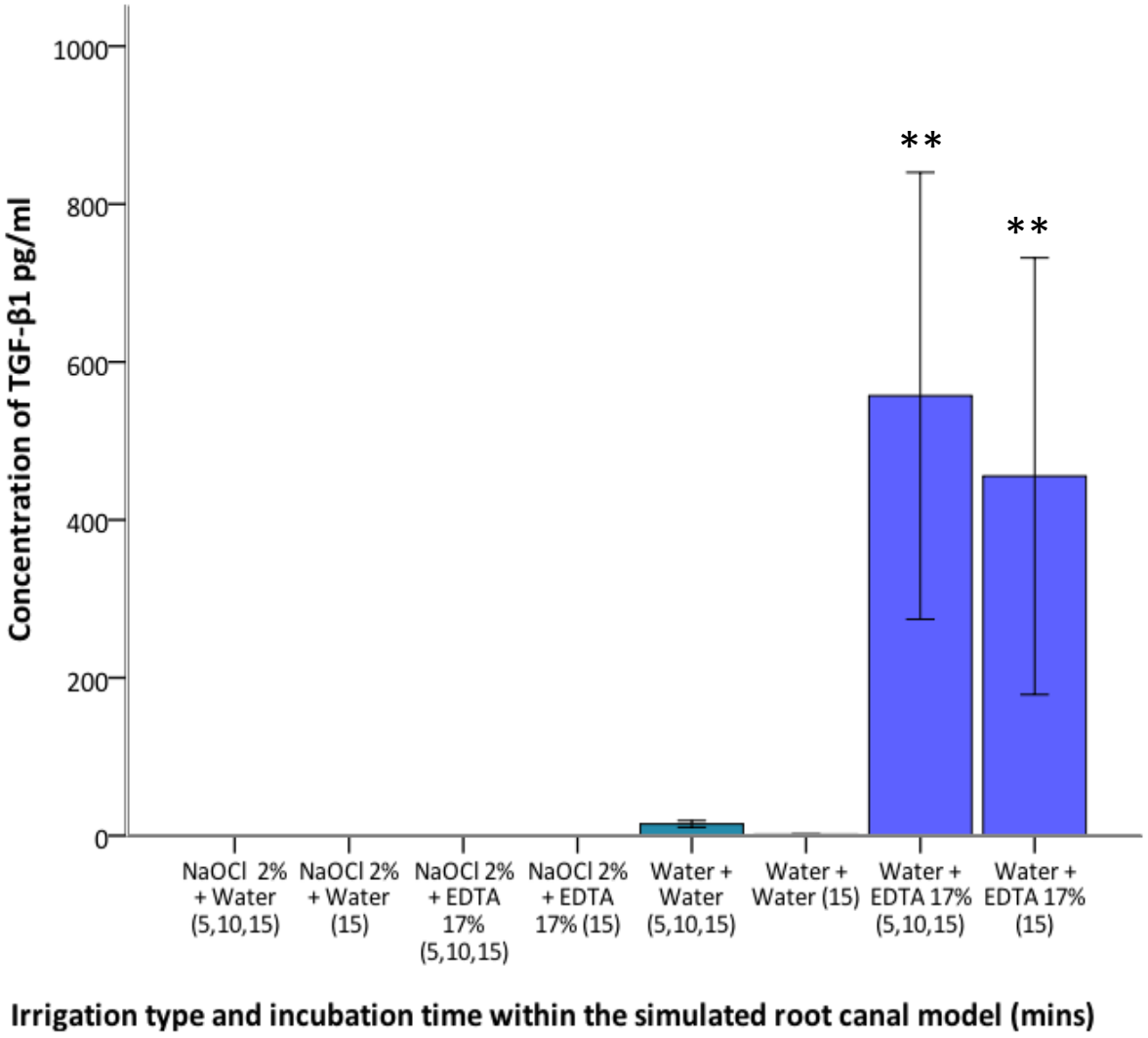

Figure 22: The data showing the mean concentration of TGF-β1 (pg/ml $^{+}$/- SE (n=5)) for the irrigant retrieval using the micropipette technique for the 4 groups: NaOCl 2% + Water, NaOCl 2% + EDTA 17%, Water + Water, Water + EDTA 17%. This includes the accumulative totals of 5, 10 and 15 minutes against the 15 minute time point. The data shows no statistical significant differences between the Water + EDTA 17% groups when multiple EDTA 17% irrigants were applied compared with one 15 minute irrigant of EDTA 17%. The Water + EDTA 17% groups produced statistically significantly greater concentrations of TGF-β1 ($p < 0.001$; **) compared with all other irrigant groups. Teeth initially treated with NaOCl 2% failed to yield any detectable TGF-β1, even when treated with EDTA 17% as a second irrigant rinse.

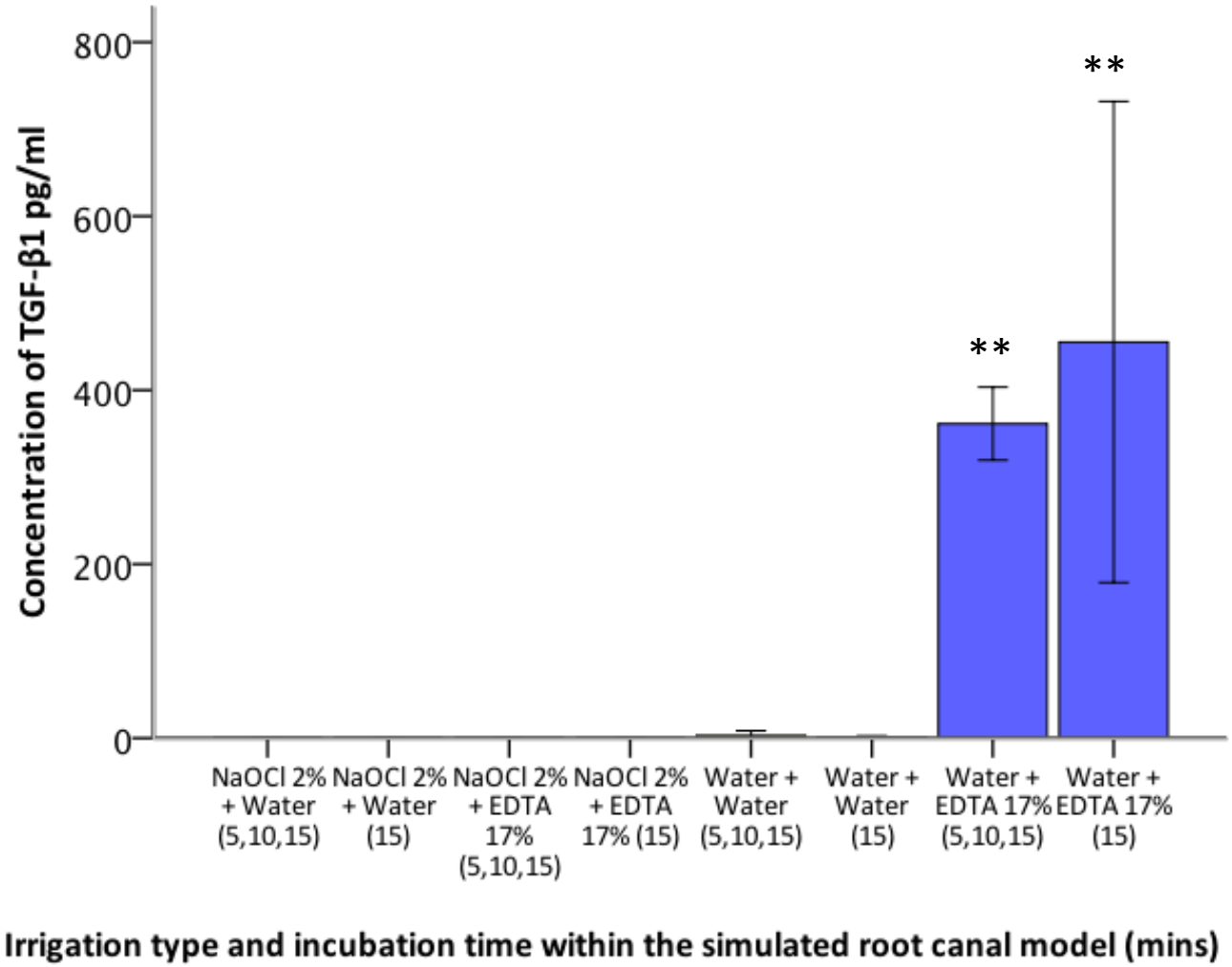

Figure 23: The data showing the mean concentration of TGF-β1 (pg/ml +/- SE (n=5)) retrieved with the PerioPaper® technique to simulate the periapical tissues, for the 4 groups: NaOCl 2% + Water, NaOCl 2% + EDTA 17%, Water + Water, Water + EDTA 17%. This includes the accumulative totals of 5, 10 and 15 minutes against the 15 minute time point. The data shows no statistical significant difference of TGF-β1 in the group for Water + EDTA 17% when used for 15 minutes compared with the accumulative total. The Water + EDTA 17% groups produced statistically significantly greater concentrations ($p < 0.001$. **) of TGF-β1 compared with all other irrigant groups. Teeth initially treated with NaOCl 2% did not yield any detectable TGF-β1, even when treated with EDTA 17% as a second irrigant rinse.

4.3.5. Comparison of the yields of TGF-β1 between accumulative water + EDTA 17% for all irrigant groups and sampling methods

Data were analysed to compare if there were any difference between all the results obtained in 'Water + EDTA 17%' for the retrieved irrigant and periapical sampling for both the accumulative application (5, 10 and 15 minutes) compared with the 15 minute application.

There were no statistical significant differences detected between any of the groups as illustrated in **Figure 24.** The results indicate that the release of TGF-β1 was not affected by the frequency of irrigation nor the sampling technique (irrigant retrieval vs periapical sampling).

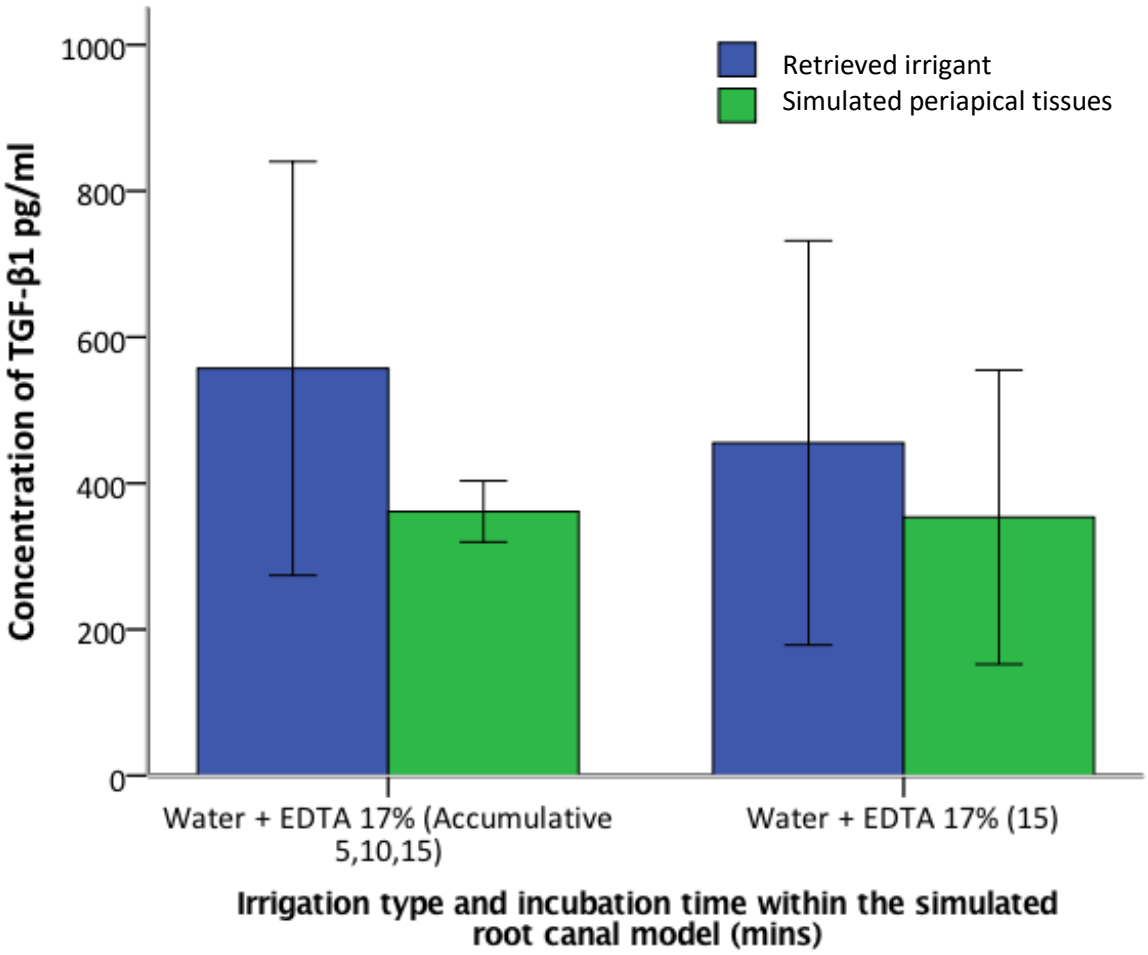

Figure 24: The data showing the mean concentration of TGF-β1 (pg/ml +/- SE (n=5)) for the Water + EDTA 17% group. The graph compares the concentration of TGF-β1, which was liberated in both the accumulative (5, 10 and 15 min) and 15 minute groups. This was for both the irrigant retrieval using a micropipette and periapical sampling using Periopaper®. There was no statistical significance between any of the delivery methods for the samples.

4.4. Attenuating the effects of NaOCl

The previous results presented in section **4.3** indicated that when NaOCl 2% was used as a primary irrigant there was no TGF-β1 release detected, even when 'Water + EDTA 17%' was subsequently applied. Strategies were explored to ascertain the effects NaOCl had on the experimental protocol, to minimise these effects and explore alternatives to NaOCl as a primary irrigant. The results of the strategies are detailed below:

4.4.1. Determining the effect that NaOCl treatment has on the assays of known concentrations of TGF-β1

The failure to detect TGF- β1 when NaOCl is applied poses the question: what effect does NaOCl have on the ELISA detection techniques applied? Potentially either the NaOCl interfered with the TGF-β1 ELISA (R&D systems, Abingdon UK) or denatured the TGF-β1 protein in either its bound or free form.

An experiment was therefore proposed with the aim of determining the effect on NaOCl on a known concentration of TGF-β1; the method is described in section **3.5** and **Figure 14**.

The results, which are presented in **Figure** indicates that NaOCl appears to attenuate the ELISA from detecting TGF-β1 when added to the ELISA wells prior to TGF-β1 delivery. The NaOCl applied to the TGF-β1 sample before being transferred to the ELISA wells also attenuated the detection of TGF-β1.

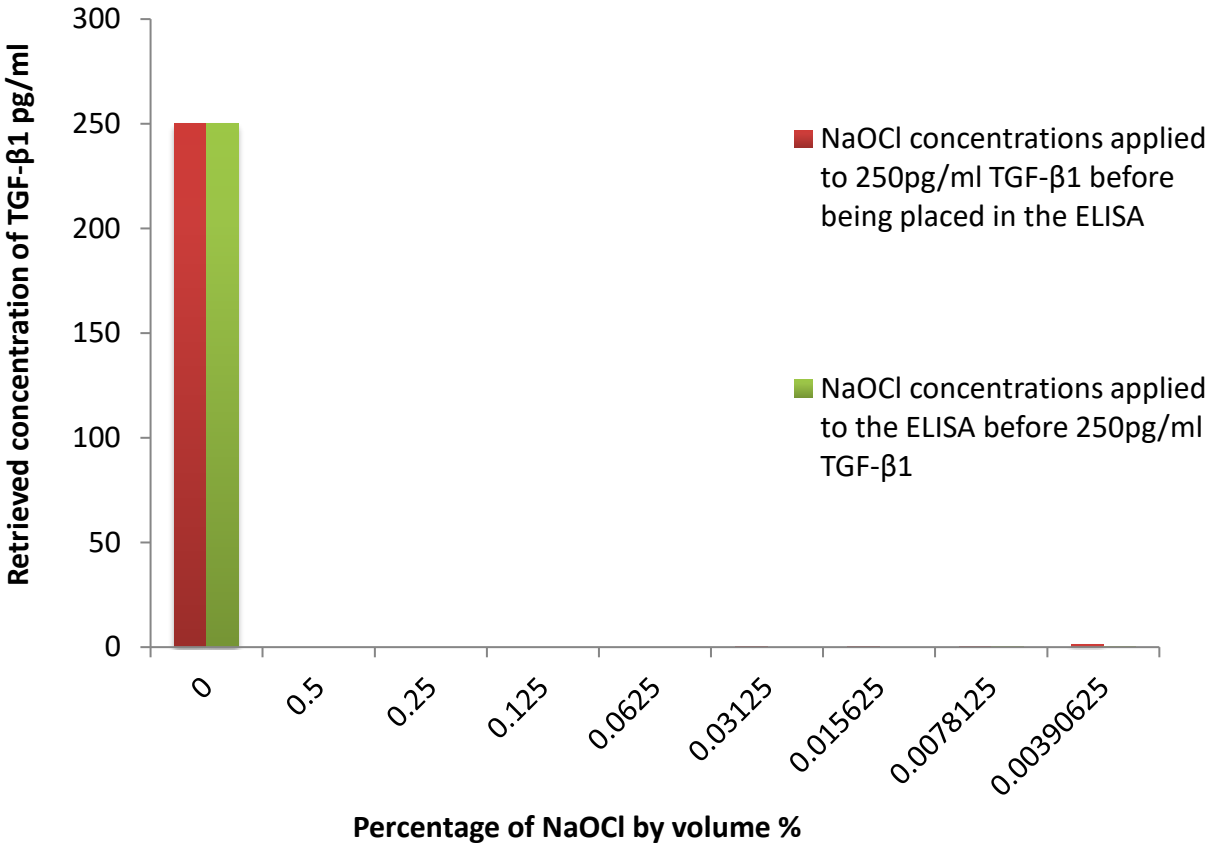

Figure 25: Bar chart illustrating the effect of NaOCl on both 250pg/ml TGF-β1 (R&D systems, Abingdon UK) before being analysed in the TGF-β1 ELISA and the effect on the TGF-β1 ELISA before the 250pg/ml TGF-β1 is incorporated. In both groups the addition of NaOCl at a range of concentrations attenuated the detection of TGF-β1. The value assayed when no NaOCl was present was 250pg/ml which was predicted.

4.5. Determining the effect chlorhexidine gluconate 2% has on TGF-β1 release in an *in-vitro* model as a primary irrigant to replace NaOCl 2%.

A further strategy investigated the effect of replacing the NaOCl with an alternative primary irrigant in the form of chlorhexidine gluconate 2% (CHX). The aim of this study was to determine the effect CHX 2% had on the release and detection of TGF-β1.

The experimental protocol is described in section **3.4**, **Figure 11**. The protocol was adjusted so that the secondary irrigant was only applied for 15 minutes. This approach was taken as data in **3.4** indicated no significant difference between the TGF-β1 liberated in one or three irrigant changes.

The results, which are shown in **Figure 26** show that in the 'CHX 2% + Water' group had a mean concentration of TGF-β1 20.19 ± 16.87 and 24.62 ± 20.18 pg/ml^{-1} attained for the irrigant and periapical retrieval approaches, respectively. The 'CHX 2% + EDTA 17%' group exhibited a mean concentration of TGF-β1 of 34.75 ± 21.01 and 33.98 ± 17.63 pg/ml^{-1} for the irrigant and periapical retrieval systems, respectively.

The statistical analysis showed there was no significant difference between any of the irrigant groups or sampling methods used.

A comparison of the CHX 2% groups and the 'Water + EDTA 17%' groups showed that there was a significant difference ($p < 0.001$), indicating that statistically significantly more TGF-β1 was released when 'Water + EDTA 17%' was applied in comparison with the 'CHX 2% + Water' or 'CHX + EDTA 17%' group. The CHX groups did not release statistically more TGF-β than the NaOCl or 'Water + Water' groups (**Figure 27** **Figure 27:**).

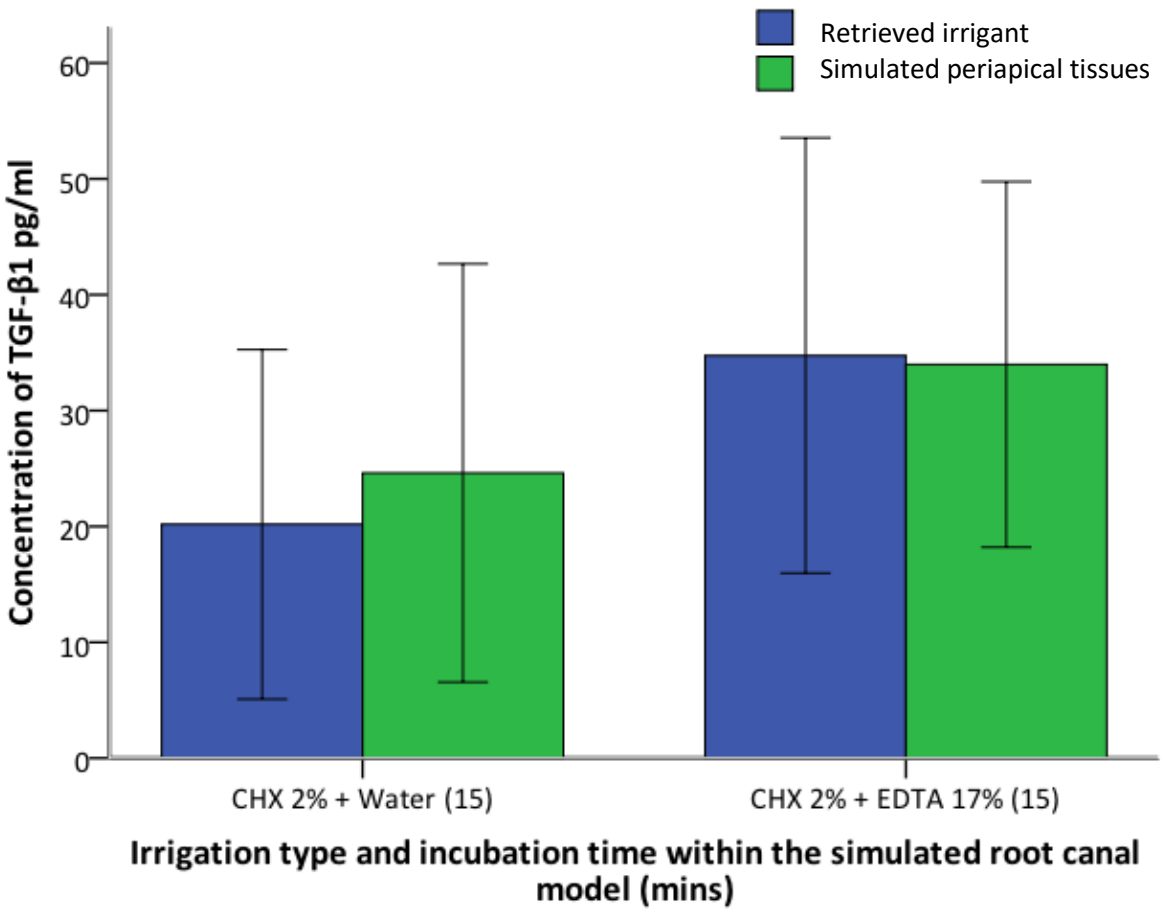

Figure 26: The data showing the mean concentration of (TGF-β1 pg/ml ⁺/- SE (n=5)) for groups CHX 2% + water and CHX 2% + EDTA 17% group. The data presented shows the concentration of TGF-β1 retrieved using both the irrigant retrieval using a micropipette and periapical sampling using Periopaper® approaches. There was no statistical significance between any of the data presented. There was however a positive detection of TGF-β1 for all groups.

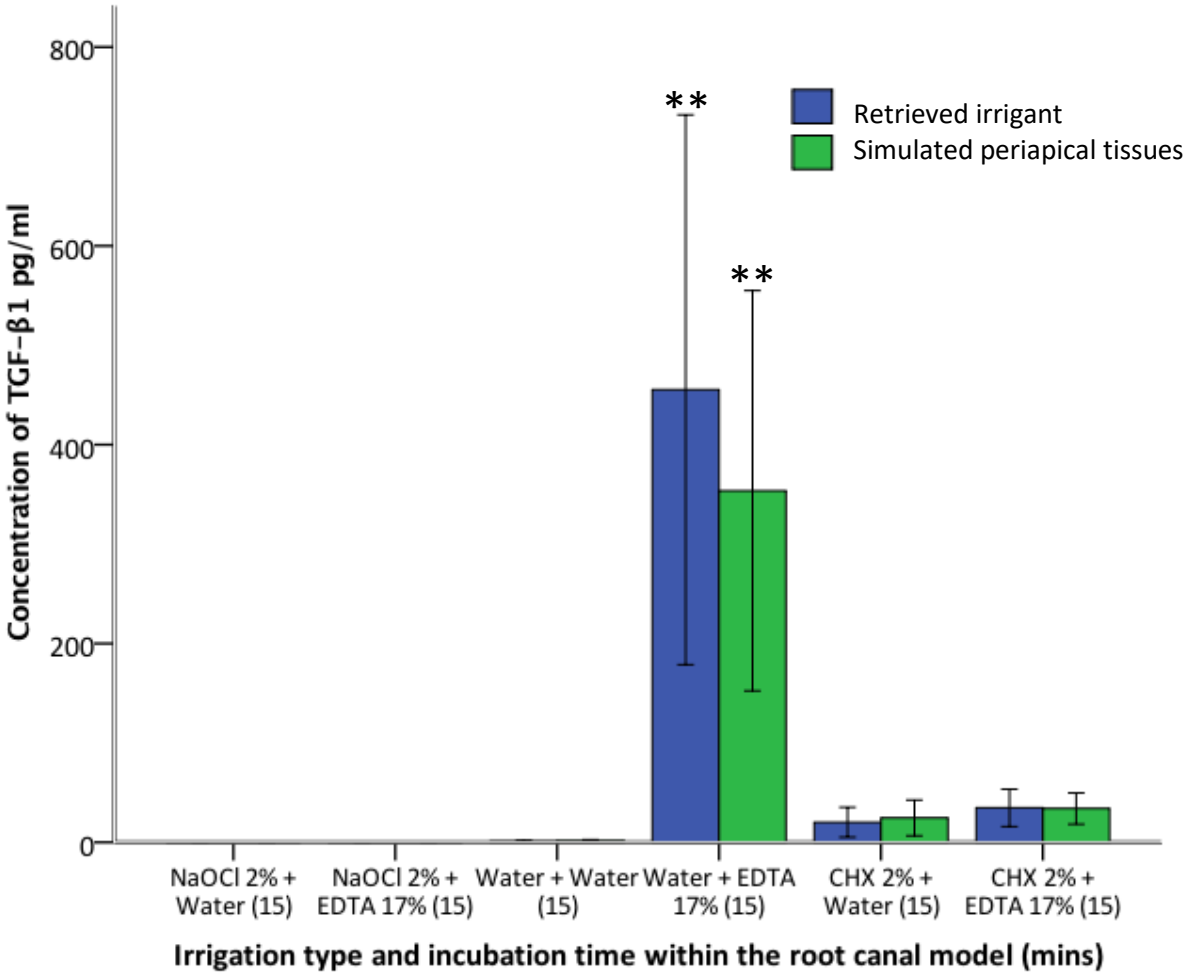

Figure 27: The data showing the mean concentration of TGF-β1 (pg/ml ⁺/- SE (n=5)) two was administered for 15 minutes. The graph shows the concentrations of TGF-β1 obtained using both the irrigant retrieval systems using a micropipette and periapical sampling approach using Periopaper®. There was a statistically significant difference (p < 0.001, **) between the Water + EDTA 17% group and all other groups. There was no significant difference between CHX 2% groups and NaOCl 2% or the water / water group.

4.5.1. TGF-β1 release using CHX 2% alone

CHX 2% was tested alone to determine what effect it had on the concentration of TGF-β1 released. Data show that use of CHX 2% alone released a mean concentration TGF-β1 of 120.73 ± 102.65 pg/ml^{-1} and 2.22 ± 1.56 pg/ml^{-1} from the retrieved irrigant and periapical tissue sampling approaches, respectively.

The irrigant retrieval of TGF-β1 concentrations were statistically significantly higher ($p < 0.05$) in comparison with the periapical sampling approach. This indicates that more TGF-β1 was retrieved from the irrigant approach as compared with what could be available to interact with the periapical tissues (**Figure 28**).

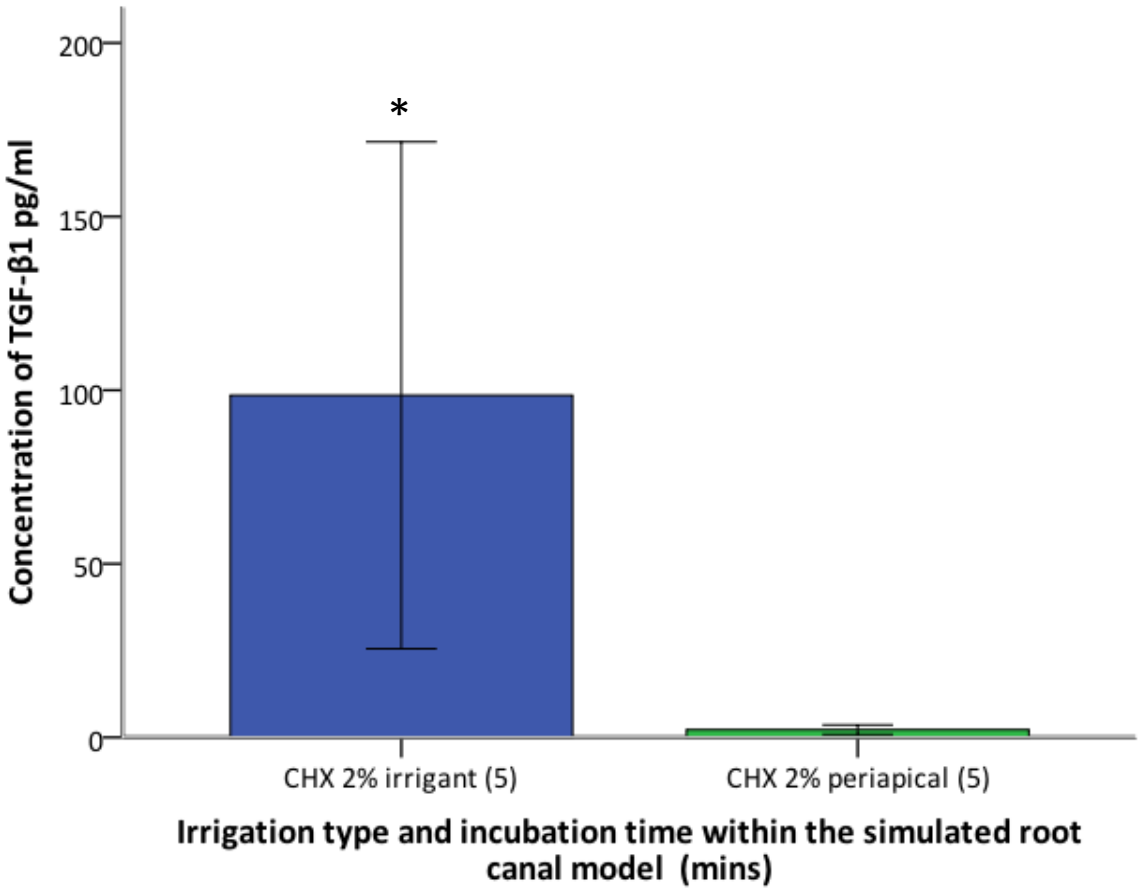

Figure 28 . The data showing the mean concentration of TGF-β1 (pg/ml +/- SE (n=5)) when the CHX 2% alone was retrieved in section 4.5 prior to Water or EDTA 17% being applied as the second irrigant. The results show a greater concentration released for the extracted irrigant than when water or EDTA 17% was subsequently applied. The CHX 2% irrigant retrieved TGF-β1 levels were statistically significantly higher p < 0.05 (*) than for the periapical sampling technique.

4.5.2. Application of calcium hydroxide as an inter-appointment dressing in the model system

The ability to neutralise the effects of NaOCl were investigated using an inter-appointment medicament. The method used is described in section **3.4** and **Figure 13**.

The previous results presented in **Figures 19**, **20**, **22** and **23** show a failure to detect TGF-β1 following the application of NaOCl 2% in the model system of a wide open apex in a single visit technique, i.e. primary and secondary irrigants delivered following on from each other on the same day. Subsequently, the effect an inter-appointment dressing has on this ability to yield TGF-β1 was investigated.

The results show a positive release of TGF-β1 with a mean value of TGF-β1 for the 'NaOCl 2% + $Ca(OH)_2$ + EDTA 17%' group of 11.10 ± 9.73 pg/ml^{-1} and 4.08 ± 2.53 pg/ml^{-1} for the irrigant and periapical sampling, respectively. The 'CHX 2% + $Ca(OH)_2$ + EDTA 17%' group of 5.62 ± 6.5 and 8.33 ± 11.49 pg/ml^{-1} for the irrigant and periapical sampling. The 'Water + $Ca(OH)_2$ + EDTA 17%' group yielded a mean TGF-β1 ± standard error for the irrigant retrieval of 8.35 ± 6.05 and the simulated periapical tissues of 14.48 ± 12.94 pg/ml^{-1}.

There was no statistically significant difference detected between the three groups as is presented in **Figure 29,** indicating that the extracted irrigant solution containing $Ca(OH)_2$ could be a key factor or the time-period the teeth were incubated in it for could also be important.

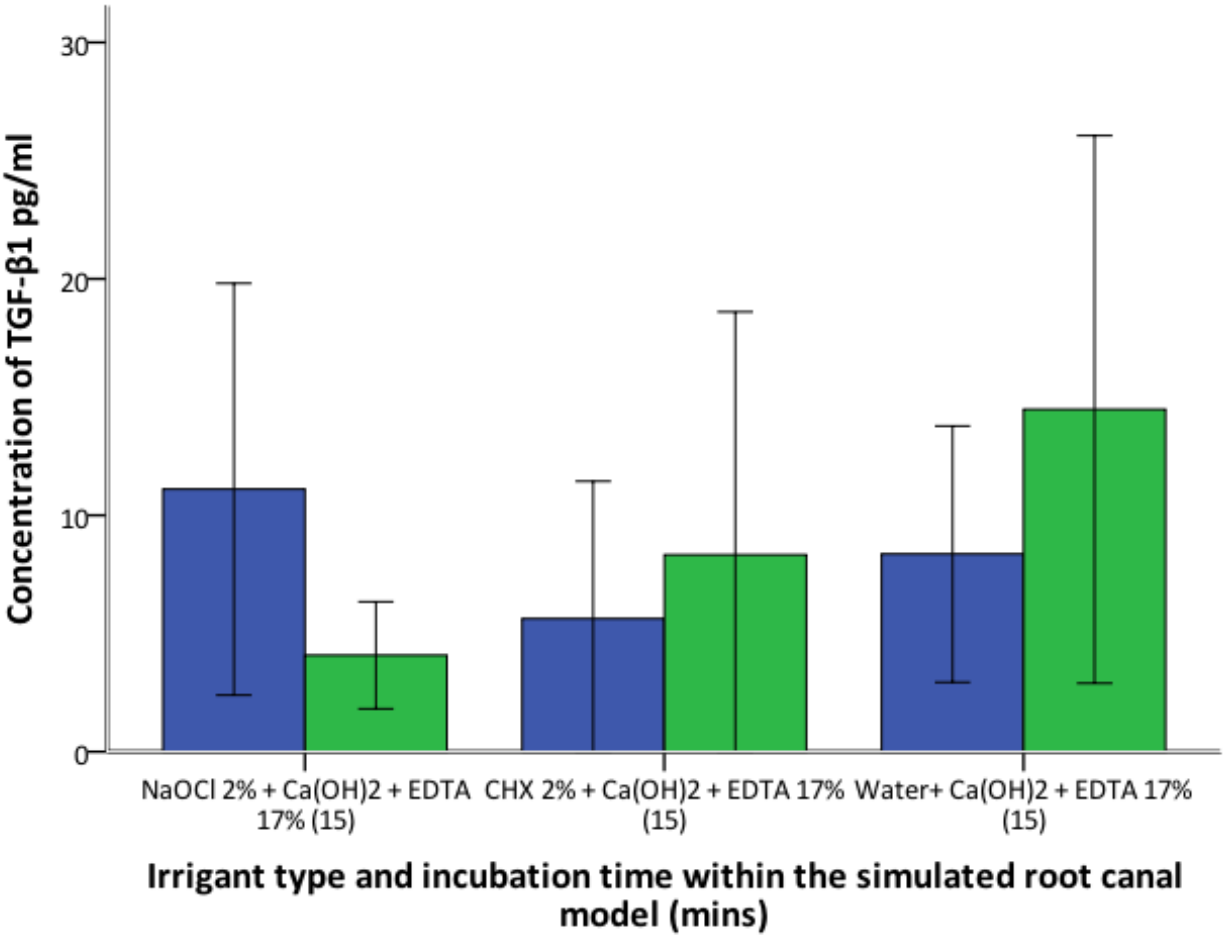

Figure 29: The data showing the mean concentration of TGF-β1 (pg/ml $^{+}$/- SE (n=5)) released for three experimental groups all containing $Ca(OH)_2$ as used as an inter-appointment dressing and a secondary rinse of 17% EDTA but differing in the primary rinse used (NaOCl 2%, CHX 2% or Water). The results show a positive release of TGF-β1 for all groups but there was no statistical significance in the yielded amount of TGF-β1 between groups.

4.5.3. Effects of NaOCl 2% + EDTA 17% treatment with and without calcium hydroxide

The original NaOCl 2% + EDTA 17% treatment resulted in no detectable TGF-β1 release however, when $Ca(OH)_2$ was introduced into the system there was detectable release of TGF-β1 (**Figure 29**). The statistical analysis showed that the irrigant solution of 'NaOCl 2% + $Ca(OH)_2$ + EDTA 17%' produced a significantly ($p < 0.05$) higher yield of TGF-β1 compared with both the irrigant and periapical sampling with 'NaOCl 2% + EDTA 17%' group alone. The periapical sampling with $Ca(OH)_2$ was not significantly different to the irrigant within the $Ca(OH)_2$ group or the 'NaOCl 2% + EDTA 17%' sampling methods used alone.

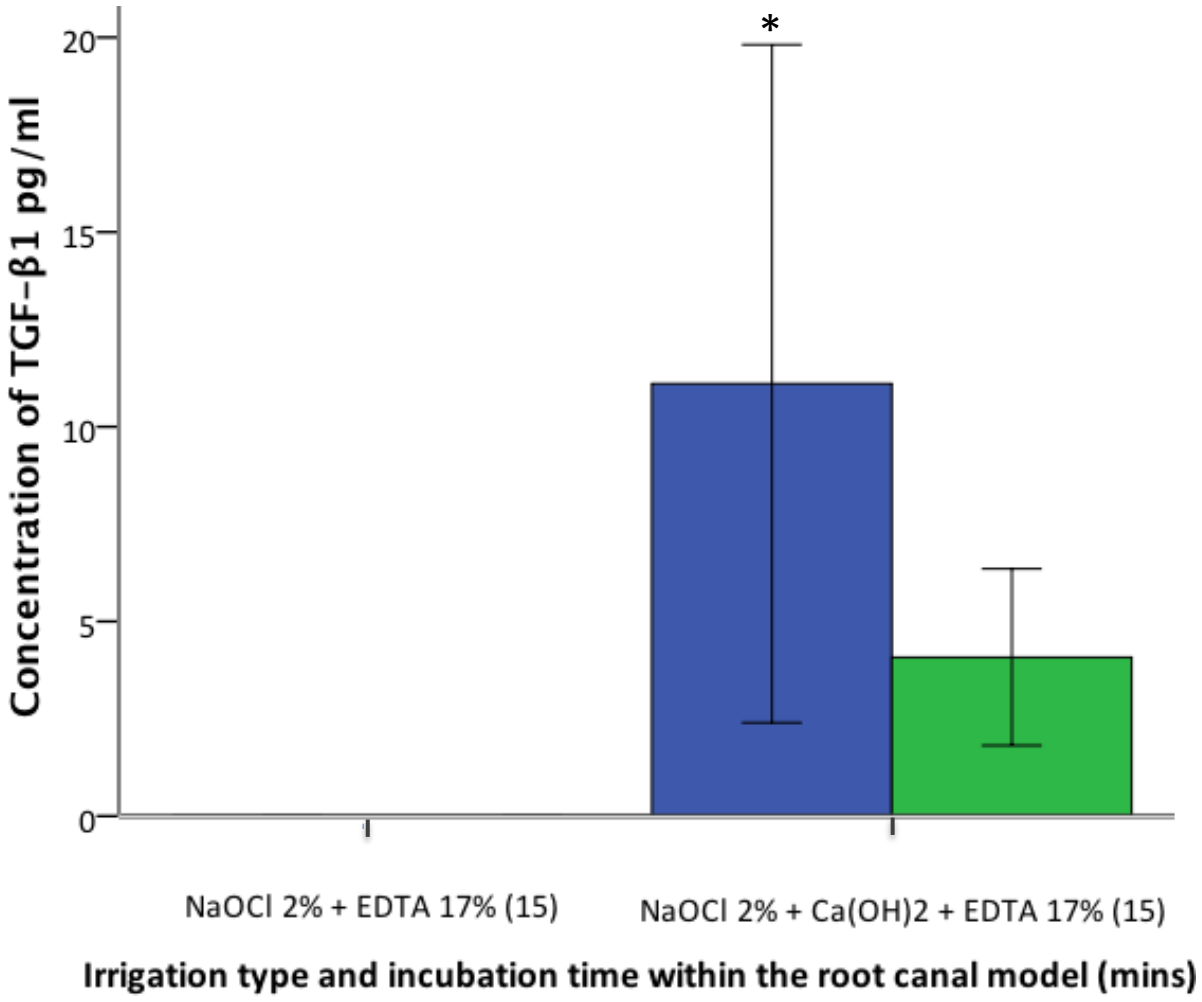

Figure 30: The data showing the mean concentration of TGF-β1 (pg/ml +/- SE (n=5)) released by the three experimental groups of NaOCl + EDTA 17% with and without a clinically relevant inter-appointment dressing of $Ca(OH)_2$. The irrigant application of NaOCl 2% + $Ca(OH)_2$ + EDTA 17% group yielded significantly greater ($p < 0.05$, *) concentrations of TGF-β1 compared with the NaOCl 2% + EDTA 17% group alone. The periapical sampling within the $Ca(OH)_2$ did not yield a significantly different result from the other groups analysed.

4.5.4. The effect of sodium thiosulphate treatment on TGF-β1 analysis following NaOCl application

The deleterious effects of NaOCl on both the free TGF-β1 and ELISA were investigated by neutralising its effects using sodium thiosulphate ($Na_2S_2O_2$) as described in section **3.6**.

A solution of 5% $Na_2S_2O_2$ was added to the ELISA and data demonstrated that it did not interfere with a known 250pg/ml^{-1} concentration of TGF-β1. This showed that when 5% $Na_2S_2O_2$ was added to a known 250pg/ml^{-1} the mean concentration following the ELISA was not significantly different from the positive control of TGF-β1 250pg/ml^{-1} alone ($p < 0.001$).

The known concentration of 250 pg/ml^{-1} of TGF-β1 was attenuated in all groups as shown in **Figure 25**. At lower percentage concentrations of NaOCl, i.e. 0.016 and 0.0078 %, the effects of the NaOCl were counteracted with $Na_2S_2O_2$and the TGF-β1 concentration increased as the concentration of NaOCl decreased (**Figure 31**). The TGF-β1 detected at 0.016 and 0.0078 % NaOCl were statistically higher ($p <0.05$) when $Na_2S_2O_2$ 5% was used.

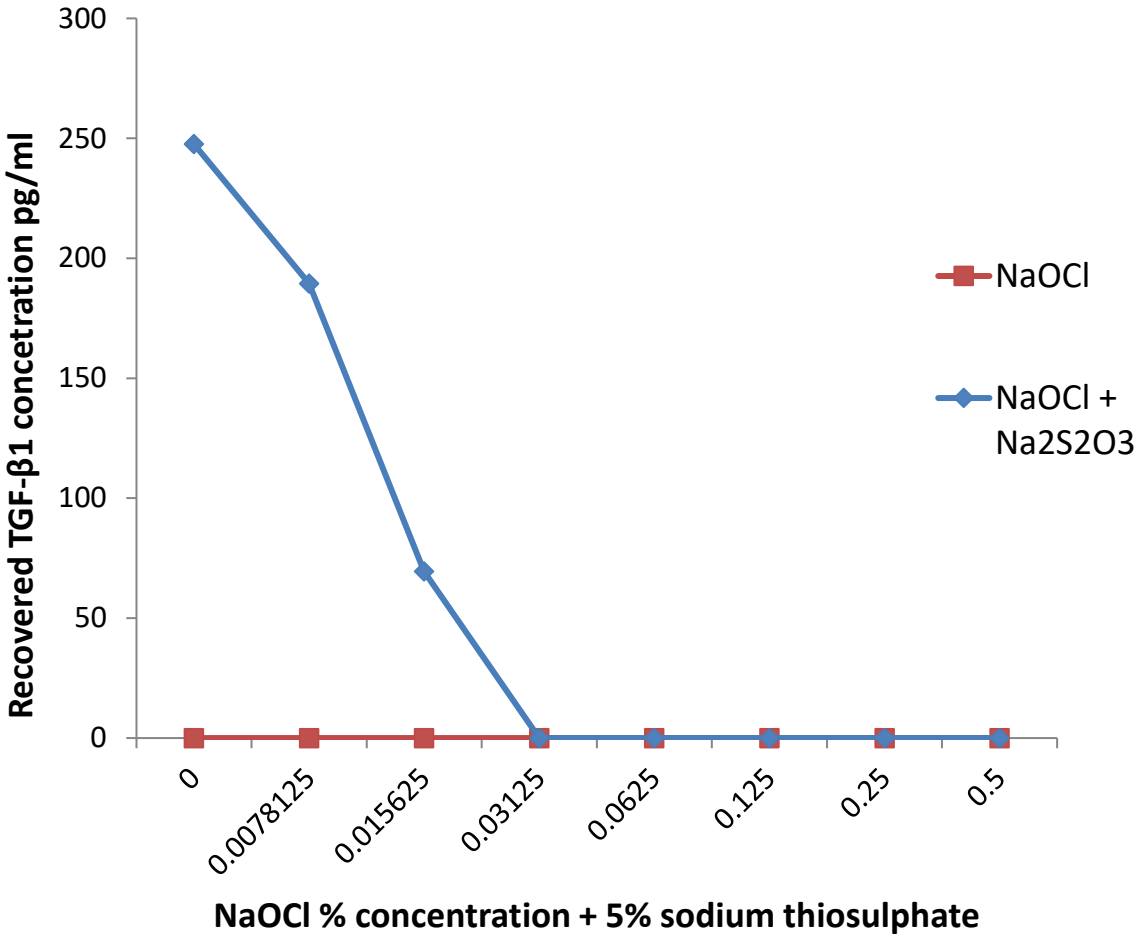

Figure 31: Data demonstrating the effect NaOCl had on 250pg/ml TGF-β1 (R&D systems, Abingdon UK) detection applied prior to being included in the TGF-β1 ELISA with 5% sodium thiosulphate addition. At the lower concentrations of NaOCl applied the results were reversed when 5% sodium thiosulphate was added, with the concentrations of TGF-β1 approaching the actual value of 250pg/ml which was initially spiked into the assay. At 0.016 and 0.0078 % NaOCl the concentration of TGF-β1 was significantly higher when 5% sodium thiosulphate was added.

4.5.5. The effect of sodium thiosulphate on TGF-β1 retrieval in the model of the wide open apex

The conditions used demonstrating the detection of TGF-β1 as described in section **4.5.4** were subsequently applied to the model of a wide open apex and the method used is as described in section **3.6.1**. The aim of this experiment was to ascertain if $Na_2S_2O_2$ delivered after NaOCl 2% application and before EDTA 17% treatment in the model of a wide open apex could release detectable TGF-β1.

The results showed that this approach was not successful using this protocol as no TGF-β1 was detected.

4.6. Scanning electron microscopy analysis of the dentine surface which were treated with different irrigant types

To visualise the effects of the irrigants on the dentine structure, scanning electron microscopy (SEM) analysis was undertaken. The previous data showed there was no difference in the concentration of TGF-β1 retrieved when an irrigant was applied 3 times in a 15 min period compared to once in the a 15 min period. As such the SEM analysis was undertaken of those irrigants applied for 15 minutes. This was described in section **3.4** of the methodology.

The experimental aim was therefore to describe the effects on dentine structure of the irrigant regimes:

a) Water + Water
b) Water + 17% EDTA
c) NaOCl 2% + Water
d) NaOCl 2% + EDTA 17%
e) CHX 2% + Water
f) CHX 2% + EDTA 17%

The results of the scanning electron microscopy at varying magnifications are detailed below in **Figures 32, 33** and **34**.

The images are representational samples of the dentine structure when viewed under the SEM. Each sample is described as a letter and relates to the three images of increasing magnification **Figures 32, 33** and **34.**

Water + Water images

The sample of 'Water + Water' group shows the smear layer is still present on the dentine surface; consisting of loose inorganic and organic debris. When the magnification is increased, there are sporadic dentinal tubules

openings which can be seen intermediately over the surface but are occluded with material. The surface is heterogeneous in nature with the underlying dentine structure barely visible.

Water + 17% EDTA images

The sample of 'Water + 17% EDTA' shows a homogenous, uniform dentine structure with waves of dark and light bands, indicating the surface is not completely flat but has raised and non-raised sections. There is no debris present as the smear layer has been removed by the 17% EDTA. The dentine tubules are open and patent; with increasing magnification there has been a loss of intra-tubular structure indicating penetration of the irrigant into the tubules and exposure of inter-tubular surface dentine.

NaOCl 2% + Water

The sample of '2% NaOCl + Water' shows a rough surface but with regular dentine tubular structure present across the surface. There are still remnants of the smear layer present with an overlying rough irregular surface. The tubules have smaller diameter lumens than the tubules in Figure B images indicating less intra-tubular dentine removal and less patency of the dentinal tubules.

NaOCl 2% + EDTA 17%

The sample of '2% NaOCl + 17% EDTA' shows a homogenous dentine structure of open and patent dentinal tubules. There are no remnants of the smear layer and there has been removal of intra-tubular dentine to a greater degree and are uniformly opened with greater diameter lumens than the Group B images.

CHX 2% + Water

The sample of '2% CHX + Water' shows a heterogeneous dentine structure with destruction of the surface tomography. The dentinal tubules are

generally exposed but are non-uniform in appearance; there are areas of patent tubules and areas where the inter-tubular structure has been eroded to the same level as the tubule openings.

CHX 2% + EDTA 17%

The sample of '2% CHX + 17% EDTA' shows a severely heterogeneous dentine structure with a loss of dentinal architecture; tubules are present but there is an uneven loss of dentine structure across the surface. At higher magnifications there is sporadic patency of the dentinal tubules and loss of a significant amount of the inter-tubular dentine. The dentine surface is heavily eroded and greater removal of the surface is present that all other groups.

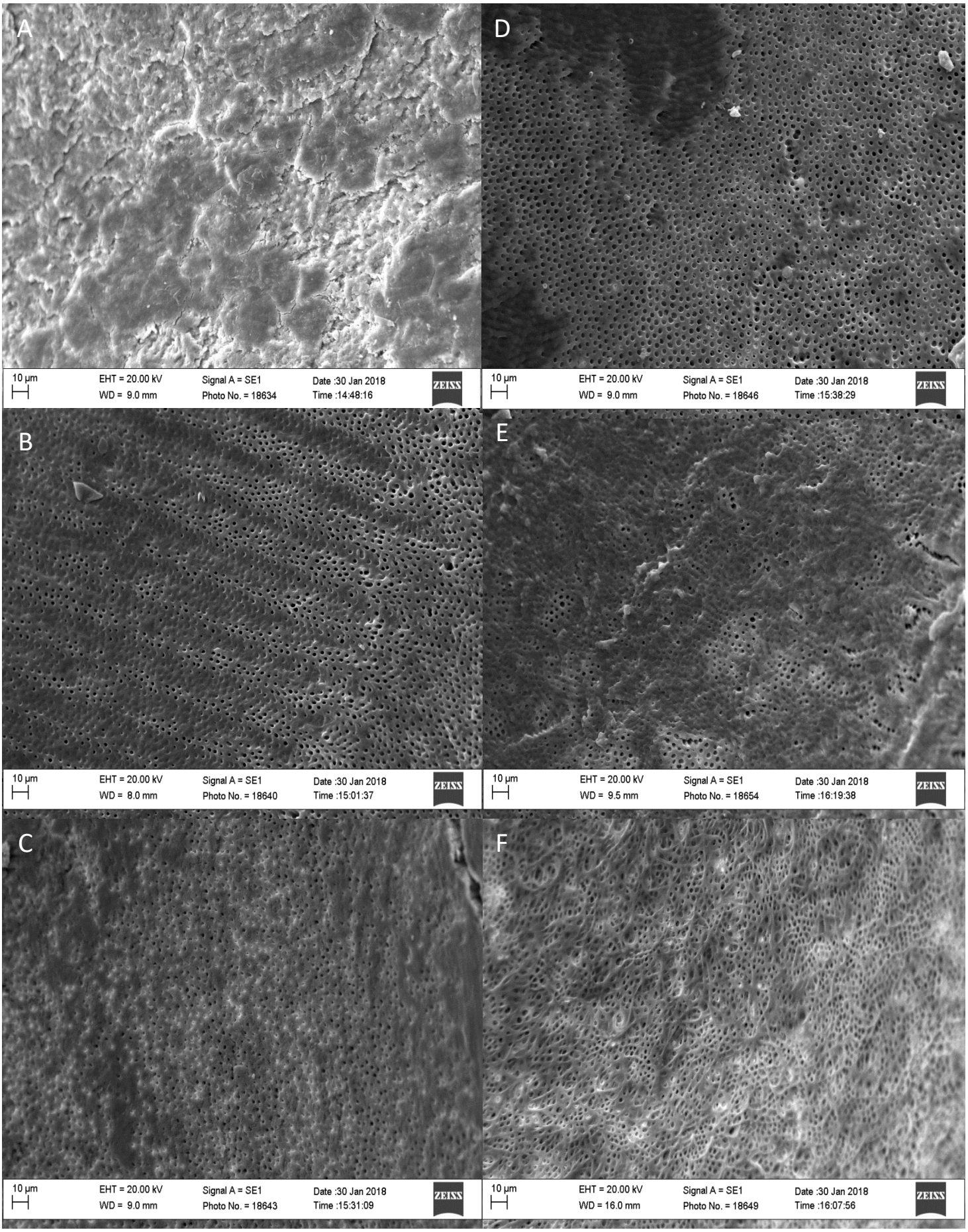

Figure 32: Representative images (n=2) from the scanning electron microscope analysis at magnification X1000. This shows the internal root dentine sectioned post preparation and using the irrigation regimes of a) saline followed by saline, b) saline followed by 17% EDTA, c) 2% NaOCl followed by saline, d) 2% NaOCl followed by 17% EDTA, e) 2% CHX followed by saline, and f) 2% CHX followed by 17% EDTA. Scale bars are shown (10µm).

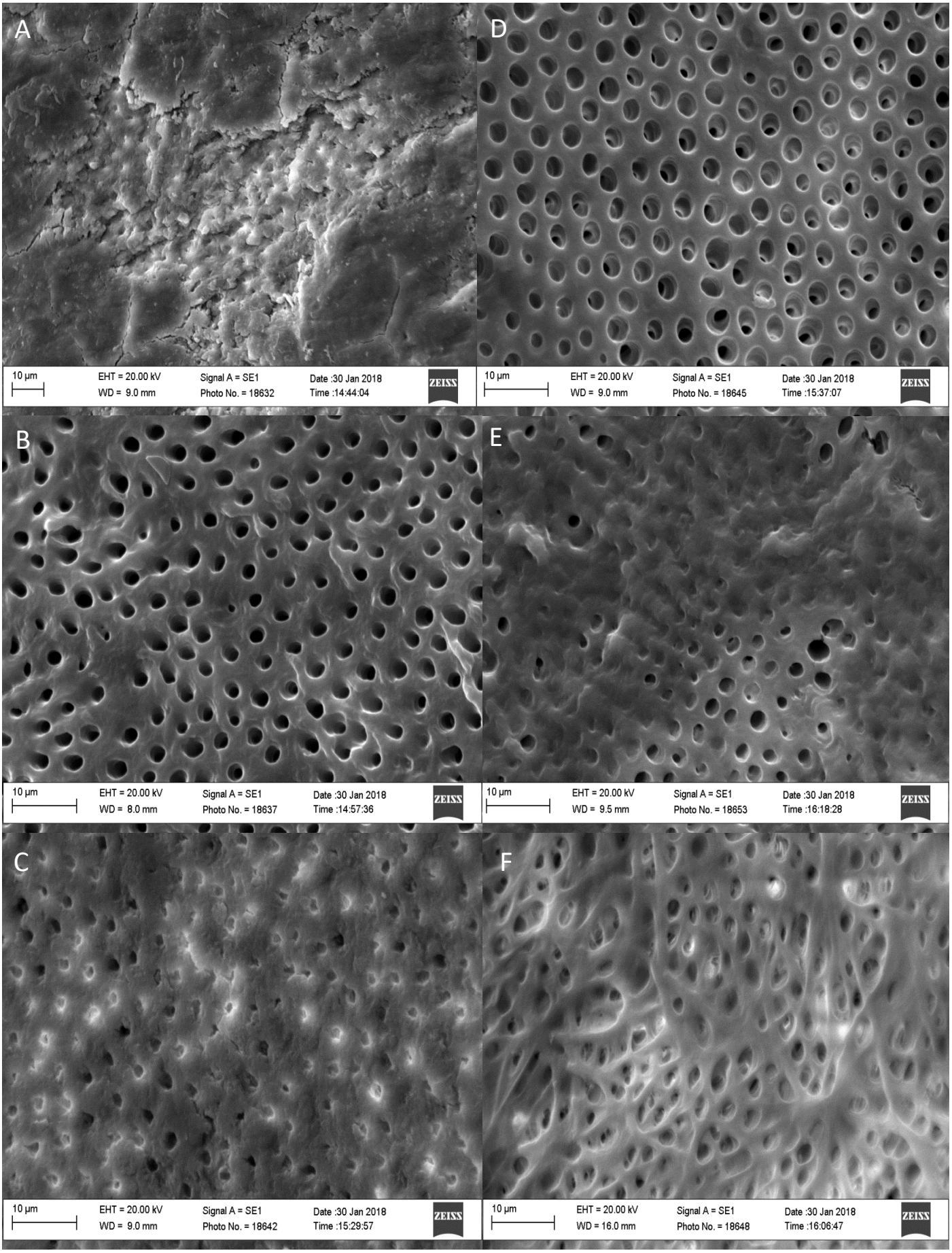

Figure 33: Representative images (n=2) from the scanning electron microscope analysis at magnification X4000. This shows the internal root dentine sectioned post preparation and using the irrigation regimes of a) saline followed by saline, b) saline followed by 17% EDTA, c) 2% NaOCl followed by saline, d) 2% NaOCl followed by 17% EDTA, e) 2% CHX followed by saline, and f) 2% CHX followed by 17% EDTA. Scale bars are shown (10µm).

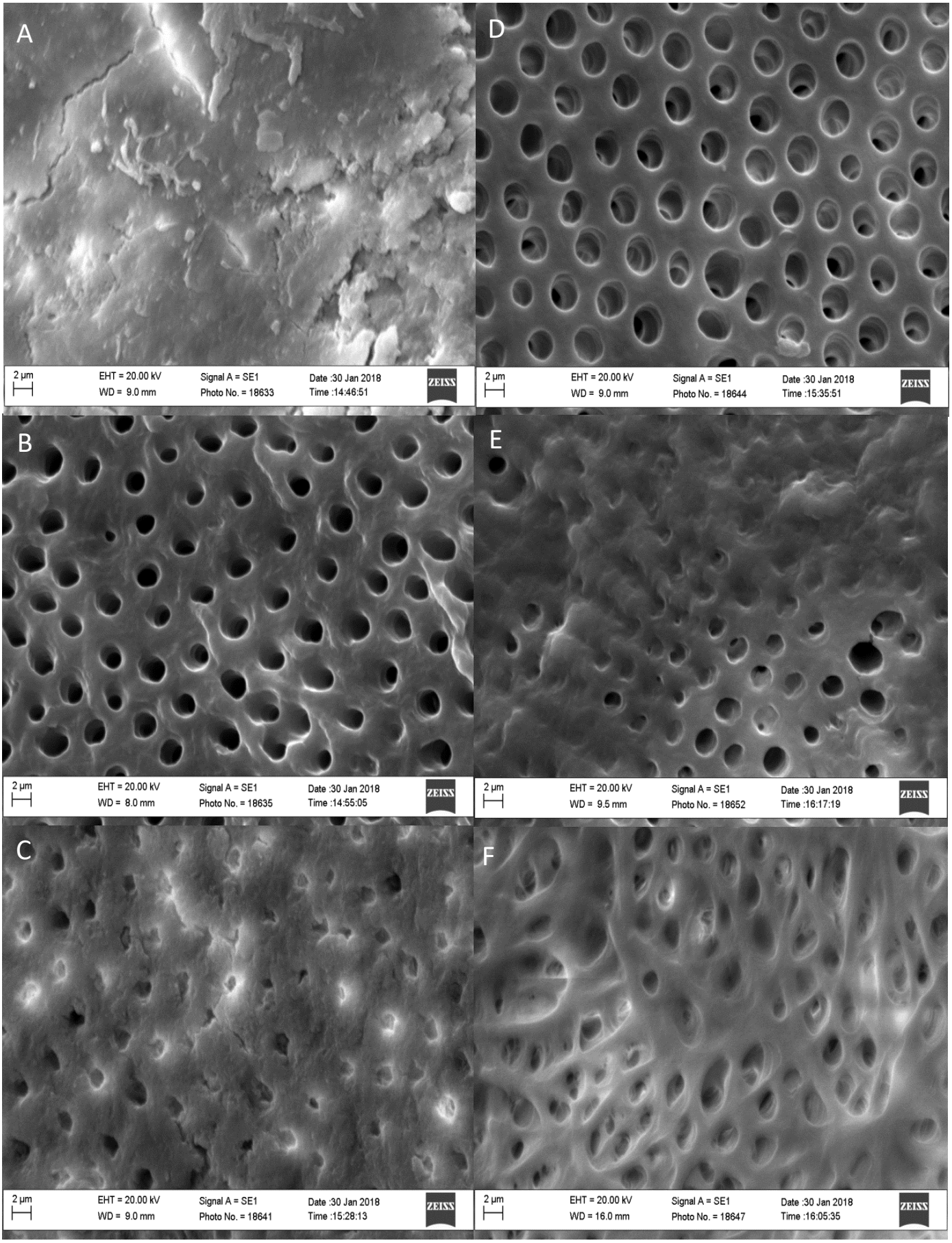

Figure 34: Representative images (n=2) from scanning electron microscope analysis at magnification X6000. This shows the internal root dentine sectioned post preparation and using the irrigation regimes of a) saline followed by saline, b) saline followed by 17% EDTA, c) 2% NaOCl followed by saline, d) 2% NaOCl followed by 17% EDTA, e) 2% CHX followed by saline, and f) 2% CHX followed by 17% EDTA. Scale bars are shown (10µm).

5. Discussion

5.1. Tooth selection and preparation

The purpose of this research was to investigate the ability of conventional irrigant regimes to release growth factors from dentine and if these dentine growth factors could be detected in the simulated periapical tissues. The importance of this is to determine if these biological molecules would be able to interact with the periapical tissues to manipulate the regulatory pathways of an inflammatory lesion to favour healing and repair of the periradicular tissues following apical periodontitis clinically. This would mean root canal treatment moves away from simply reducing the bacterial load but favours a shift towards harnessing endogenous healing processes.

This project therefore needed to develop an *in vitro* tooth model to replicate the clinical conditions of a tooth with incomplete root formation. *In-vitro* irrigant delivery and retrieval techniques could then be refined using this model to guide future clinical *in vivo* sampling. An endodontic case with a wide open apex was chosen as it maximised the surface area in contact with the simulated periapical tissues and aided in the sampling process.

The tooth selection process first considered using extracted teeth with incomplete root formation, such as teeth extracted prior to orthodontic treatment; commonly premolar teeth (McCaul et al., 2001). This approach would have the benefit of utilising teeth which required no additional preparation and the dentine profile would be that of a tooth with incomplete apex formation. However, the most likely tooth to possess a wide open apex due to trauma clinically is reportedly an upper anterior tooth (Andreasen, 1970). As the clinical arm of the study would focus on sampling this tooth type (due to more frequent exposure to dental trauma), it was considered necessary to develop an irrigant delivery and retrieval regime using this tooth in the laboratory so that it could be translated

clinically. The selection of extracted upper anterior with incomplete root formation would not be feasible as they would be root treated as the first line of treatment.

In order to replicate a tooth with a wide open apex, the root canals of upper anterior teeth were thus prepared using a standardised diameter of ISO 150 or 1.5mm. This approach was taken as it replicated the experience of treating this type of patient clinically and the literature defining an immature apex measurement of ≥ 1.2mm (Andreasen et al., 1986). This preparation was achieved using the 'crown down' technique, which is an established and well used method of tooth preparation (Williamson et al., 2009, Sonntag et al., 2007). This technique also benefited from minimising the heat generated to the dentine by using incrementally increasing the instrument size prior to post-drill preparation (Saunders and Saunders, 1989). In developing this technique alternative methods such as using implant drills (Straumann UK, Crawley, UK) or using post-drills without any crown down preparation were excluded as they produced physical heating of the teeth. This was due to inefficient cutting as the tips became rapidly blunt resulting in slow tooth preparation and excess heat due to friction. The heat generated may also denature the bioactive molecules and lead to false negative results. The tooth length was also standardised and the apex flattened to ensure a good seal against the periapical sampling methods as described in **3.1**.

The tooth type was changed during this project from maxillary incisors to maxillary canines. This was undertaken due to the challenges in obtaining sufficient numbers of maxillary central incisors which met our inclusion and exclusion criteria. Different tooth types are known to have different dentine profiles; indeed Rivera and Yamauchi (1993) have demonstrated a trend in the variation of cross-links from different teeth and at different tooth locations and thus subtle variations in the heterogeneity of dentine. The authors hypothesised that this could be due to different functional stresses and forces placed on teeth. The effect this subtle difference in dentine

composition has on the amount and type of sequestered growth factors present is also uncertain. To account for this, statistical analysis was only performed between the same tooth type i.e. canine versus canine and not canine versus incisor.

There would also be biological variation between the amounts of growth factors sequestered in teeth between patients, explaining the relatively large standard error in the groups. Indeed the exposure of the tooth to caries or restorations may also influence the amount of sequestered TGF-β1 released (Smith et al., 2003, Smith et al., 2001). To minimise these confounding factors, the teeth were randomised with the aim of evenly distributing these variations.

An observation during the preparation process was that over 75% of the teeth had residual pulps indicating that they were vital when extracted. The root calculus also showed periodontal attachment loss prior to the teeth being extracted, indicating that these teeth had a higher probability of being from an older cohort of patient (Löe et al., 1986). The applicability of how the sampled growth factors in this study would apply to a population of young patients with open apices is unclear and is poorly documented in the literature.

Previous studies investigating growth factor release have used tooth component tissue such as uniform dentine discs (Widbiller et al., 2017, Galler et al., 2015a) or by using root segments without crowns (Zeng et al., 2016). These models would not replicate the complexities of tooth anatomy and the results would arguably have less validity and transferability to clinical practice which involves root canal treatment.

Therefore due to the limitations of the age of the teeth, the reason for extraction and the need to prepare a tooth for a wide open apex the author feels the model used here is valid and simulates a clinical model of a wide open apex.

4.2. The Pilot study

The pilot study, which was conducted to determine the feasibility of growth factor release, as described in section **3.2** and the results reported in section **4.1** demonstrated that TGF-β1 could be retrieved from the root canal system of treated teeth with the endodontic irrigant EDTA 17%.

The retrieval and detection of TGF-β1 was time dependent with a significantly higher concentration of TGF-β1 being retrieved when the EDTA 17% irrigant was incubated within the root canal system for 24 hours. This data agreed with that of (Galler et al., 2015a) who showed a time-dependent release of dentine growth factors. Indeed the concentrations of TGF-β1 detected after 24 hour was comparable with the concentrations detected by Galler et al, (2015) who used a dentine disc model. Encouragingly, the data showed that the TGF-β1 could be detected after a prolonged period of time (24 hours) indicating stability in the 17%EDTA solution.

A difficulty with this developed model included challenges in recovering the relatively small volume of irrigant initially applied using a side venting endodontic needle. Another limitation of this model was that the filtration technique required utilisation of a Microcon column assembly with centrifugation to concentrate the sample. Although all efforts were made during the centrifuge process to keep the temperature from rising using ice-cold nuclease free water, the process took over an hour and likely resulted in some heat generation which may potentially denature TGF-β1.

EDTA 17% is used clinically as an irrigant during root canal treatment and is not incubated *in situ* for 24 hours as was undertaken in this model. As there were minimal levels of dentine growth factor released with the simple rinse and collect technique the experimental process was modified to investigate the effect of increasing the volume of irrigant on the release of dentine growth factors. This approached explored the levels of dentine growth

factors released within a <15 minute period, as this is more representative of how the irrigant is used clinically (see below for further discussion).

5.3. EndoVac® retrieval system, a negative pressure irrigation system

The methodology and results obtained when using the EndoVac® retrieval system, a negative pressure irrigation system, are described in sections **3.3** and **4.2**, respectively.

As noted in the previous section, the EndoVac® retrieval system provided a clinically relevant approach to enable delivery of increased volumes of irrigants into the entire length of the root canal system (Nielsen and Baumgartner, 2007). The EndoVac® retrieval system also had the benefit of ensuring the irrigant overcame some of the limitations of a 'closed root canal' system; which is defined as vapour trapped within the root canal system which prevents the exchange of an irrigant to the apical portions (Spoorthy et al., 2013). Indeed there are several strategies used to overcome these limitations including passive ultrasonic irrigation as well as the EndoVac® system (Parente et al., 2010, Spoorthy et al., 2013). The increased debris removal also enabled the sampling of more of the inorganic material removed by the irrigant compared with conventional irrigation (Siu and Baumgartner, 2010).

The EndoVac® system was modified to allow collection of the retrieved irrigant with a retrieval volume of greater than 2.9ml. The advantage of this method was that the irrigant was delivered and sampled within a time period more applicable to clinical treatment time (less than 15 minutes). The literature was initially searched for recommendations on the volume of irrigant to use; in particular for the recommended volume of EDTA. There were several surveys which reported the proportion of clinicians who used which types of irrigant but little information was evident regarding the average volume of irrigants used (Dutner et al., 2012). The volume of 3ml

was therefore chosen as this was our locally agreed amount used by clinicians in the Birmingham Dental Hospital, UK.

The EDTA irrigants were further divided based on their concentrations as other studies had reported that the concentration of EDTA was important in growth factor release. The pH for all the solutions were adjusted to pH 7 as this also reportedly proved important in maximising the growth factor yield (Galler et al., 2015a).

The data obtained showed a release of TGF-β1 in all groups for irrigant retrieval and periapical sampling. The EDTA 10% irrigant group resulted in statistically significantly higher concentrations of TGF-β1 being released compared with all other groups. The EDTA 10% periapical sampling showed a statistically significant release of TGF-β1 in comparison with the saline control. However, as the concentrations were relatively small the clinical significance of approximately 10-15pg/ml^{-1} more TGF-β1 is speculated to be less important.

In this study the yields of TGF-β1 were significantly smaller than those detected in the previous experiment reported in section **4.1**, even though much higher volumes of irrigants were used (3ml compared to 60µl). A mechanism for the reduced amount of growth factors could be contributed to the refinement process which was described in section **5.2.** Indeed as the volume of solution increased so did the time necessary to centrifuge and filter the EDTA and saline solution through the Microcon assemblies. Alternatively the lower amounts detected could be due to the small contact time the EDTA had with the dentine walls. This could indicate that even though the volume of EDTA was increased the most important variable was how long the EDTA was actually in contact with the dentine. The results reported in section **4.1** support this by showing that the application of EDTA for 24 hours resulted in a greater release of TGF-β1, with a much smaller volume of EDTA.

The detection of dentine growth factors in the simulated periapical tissues supports a potential release of dentine growth factors *in situ* occurring at the interface with the periapical structures. This is a positive finding and raises the potential for this to occur clinically; with the presence of dentine growth factors having the ability to elicit local biological responses such as periapical angiogenesis (Zhang et al., 2011). However, the yields of growth factors released were relatively low and only just within the limits of detection with the TGF-β1 ELISA kit; which are listed at $7pg/ml^{-1}$ in the supporting manual (R&D systems, Abingdon UK). At such relatively low concentrations there will be more chance of systematic error and less reliability of the concentrations detections.

EDTA 10% had a higher yield compared with the EDTA 17% which suggests that the EDTA 10% treatment leads to a more stable TGF-β1 in concentration. Other groups have found EDTA 10% also releases higher yields of TGF-β1 (Galler et al., 2015a). An alternative is that the EDTA 17% releases more TGF-β1 but this is less stable in solution compared with TGF-β1 released by EDTA 10%. This finding is not supported by data presented in section **5.2** where EDTA 17% shows a preservation of TGF-β1 molecules for 24 hours; but unfortunately no comparison with EDTA 10% was performed in this current study.

This may present challenges if these protocols were to be transferred to a clinical study as EDTA 15-17% is the only commercially available product which is CE (European Conformity) marked. The manufactures were contacted to determine if a 10% EDTA medicament could be obtained however this was not possible (**see Appendix 2**).

The original studies which used EDTA 15% (Byström and Sunvqvist, 1985) and EDTA 17%; indicate that this concentration was selected due to its effectiveness in removing the smear layer and due to the manufacturing procedure available (Aktener and Bilkay, 1993). The product was therefore not developed to optimise dentine growth factor release and this finding

now represents an additional action of EDTA. Clinically, titrating this to 15% would be problematic, especially when the pH would also have to be adjusted. Notably, such a change could result in a reduction in the efficacy of smear layer removal and stability of the compound over time. Subsequently EDTA 17% was selected as the concentration for use in the next phase of the study, as this would be the only available relevant commercial product that could be used clinically. Furthermore, data presented in section **4.1** showed that TGF-β1 could be successfully released with the immediate rinse and collect method using EDTA 17%. The findings of dentine growth factor release with EDTA 10% do however support potential future product development in this area.

In view of the original experimental aims in **3.3**, the studies performed showed that high volumes of irrigant could be delivered and sampled; however, the protocol for centrifuging may have interfered with the final assay yield of TGF-β1.

The results also showed the feasibility that the release and sampling of dentine growth factors in a time scale which was clinically feasible (<15 mins) albeit with a lower yield. The effects of different EDTA concentrations on the release of growth factors was also achieved but the relatively low yield towards the sensitivity levels of the ELISA kit made interpretation difficult.

An additional reflection was that the Microcon was an inert material with no ability to absorb dentine growth factors interacting with the simulated periapical tissues. The dentine growth factors would therefore not be absorbed but would be relatively free to diffuse back into solution and be retrieved with the irrigant. *In vivo,* the periapical tissues are a complex biological system which will interact with tissue fluid, vascular supply and immunological mechanisms. All of these systems would have some biological absorption and interaction and the model would need to be refined in order to reflect this.

The experimental procedure was therefore further developed to overcome some of the limitations of the EndoVac® system; aiming to replicate the clinical use of multiple different irrigants and refine the sampling techniques. As saline showed some biological ability to release growth factors it was replaced with water to provide a more relevant control in the downstream experiments.

5.4. Contemporary endodontic irrigation and their effect on dentine growth factor release

The technique reported in section **5.3** was further developed to reflect a clinical endodontic treatment protocol used for contemporary root canal preparation. This involved the addition of multiple steps as described in section **3.4**.

Conventional root canal treatment involves a disinfection stage where a primary irrigant, most commonly NaOCl is used to disinfect the root canal system and dissolve organic pulpal tissue. A mechanical debridement is also undertaken where the tooth is prepared to allow further penetration of the root canal complex using an irrigant. The use of a second irrigant such as EDTA removes the smear layer and the release of dentine growth factors sequestered within the dentine walls (Tomson et al., 2007). This approach is supplemented by the use of adjunctive treatments such as passive ultrasonic irrigation which has also been demonstrated to promote growth factor release *in vitro* (Widbiller et al., 2017).

The methodology used here was also refined by using a PerioPaper sample collection approach which provided the additional feature of absorbing growth factors and preventing them re-solubilising into the irrigant solution. Instead of centrifuging the retrieved irrigant, it was added directly into the ELISA well plates following preparation as described in the TGF-β1 ELISA kit by the manufacturers.

The results demonstrated that the technique was refined to deliver and retrieve irrigant using the micropipette method as discussed and Periopaper sampling in section **3.4.** In both the retrieved irrigant and periapical sampling groups, TGF-β1 was detected and the yield with the 'Water and EDTA 17%' group increased significantly within the 15 minute time frame used.

5.4.1. Multi rinses of irrigant and irrigant retrieval

In order to determine the effect of more clinically realistic irrigant sequence the use of three irrigants changes within a 15 minute time period compared with the use of one irrigant delivered in a 15 minute time period was investigated. The previous experimentation showed that the release of TGF-β1 was time-dependent but the effect of changing the irrigant multiple times for fresh solution was previously unknown.

The results presented in section **4.3.1** and **Figure 19** show that TGF-β1 was retrieved in the 'Water + EDTA 17%' group at all-time points of 5, 10 and 15 minutes. This supported previous findings that EDTA 17% mediated a sustained release of TGF-β1 which could be sampled (Galler et al., 2015a). Within the groups containing NaOCl 2% there was no TGF-β1 was detected. The most frequently reported application of an endodontic irrigant sequence uses a mixture of NaOCl and EDTA 17% (Dutner et al., 2012) however when applied here this protocol failed to elicit any TGF-β1 for detection.

A mechanism for the failure to detect TGF-β1 may be that residual NaOCl could be interfering with the action of EDTA. However, NaOCl has limited or very slow action at attenuating EDTA (Grawehr et al., 2003, Rossi-Fedele et al., 2012, Grande et al., 2006) although EDTA has a greater effect at reducing the efficacy of NaOCl when they are combined. Alternatively, the NaOCl could be potentially denaturing the growth factors released before the EDTA can release them or potentially any residual NaOCl carry over could interfere with the ELISA.

It was proposed in previous models (Galler et al., 2015a) that the failure of EDTA to release dentine growth factors when NaOCl is applied as a first irrigant could be overcome by the application of EDTA contacting fresh dentine, previously untreated with NaOCl. Indeed in this model the repeated replacement of EDTA 17% failed to elicit any release of TGF-β1 when NaOCl 2% was used as primary irrigant. This inhibitory mechanism may be due to the NaOCl being able to penetrate deeper into the dentine further than three changes of EDTA 17% can penetrate. As such the growth factors may already be denatured when the EDTA 17% contacts the deeper dentine.

NaOCl has excellent tissue denaturing capabilities and a low surface tension as it is able to penetrate into the complexities of the root canal system and dentine (Palazzi et al., 2012). NaOCl can penetrate up to a depth of 0.3mm within dentine tubules, with the maximum penetration achieved due to time, increased concentration and heat (Zou et al., 2010). EDTA has a higher surface tension (Giardino et al., 2006, Taşman et al., 2000) than NaOCl but its penetration can also be increased with the use of ultrasonic irrigation (Lui et al., 2007) and the use of surfactants and heat to decrease the intramolecular attractions within the liquid (Abou-Rass and Patonai, 1982). In microbiological studies EDTA 17% showed relatively high surface tension, high wettability and relatively low penetration into dentinal tubules (Zhang et al., 2015). Thus, it could be surmised that the NaOCl 2% with a lower surface tension can penetrate into the dentine structure far more readily than EDTA 17%, effectively neutralising dentine growth factor activity. Arguably if more EDTA 17% cycles were applied over a longer time scale and further mechanical debridement was undertaken, unaltered dentine and sequestered TGF-β1 could theoretically be reached. However clinically this may over prepare the dentine and potentially weaken the tooth structure.

Furthermore any residual NaOCl 2% carryover in the EDTA 17% irrigant may also prevent the ELISA from working despite the experimental protocol involved rinsing the root canal system with water and drying prior to EDTA 17% being applied. Any remaining NaOCl 2% would therefore be diluted to a

relatively small concentration (see section **5.6** for more specific details on the effect of NaOCl and the TGF-β1 ELISA) or attenuated by the high EDTA volume.

The data obtained therefore indicated the only predictable irrigant regime for the release and sampling of TGF-β1 was the 'Water + EDTA group'. There was no statistical difference between the groups sampled at 5, 10 and 15 minute time-points. This was supported by data presented in section **5.4.2** where the results from the periapical tissue samples were similar.

5.4.2. Multi rinses of the irrigant, simulated periapical tissue sampling

Section **4.3.2** and **Figure 20** present data showing a similar finding to the retrieved irrigant in Section **5.4.1** in that TGF-β1 was detected in the 'Water + EDTA' groups but not the NaOCl groups. The concentration of dentine growth factors was similar to those with the retrieved irrigant. This indicates that the method for calculating the concentration of TGF-β1 involving the Periopaper was feasible when combined with the volume and weight calculation method described in section **3.4.1**.

This presence of growth factors in the simulated periapical tissues again indicated that approach could be feasible clinically; where their presence may interact with endogenous biological mechanisms in the periapical tissues. Again, there were no sampled dentine growth factors in the simulated periapical tissues when NaOCl 2% was used. This implied that the irrigant regime of 'NaOCl 2% + EDTA 17%', which is the most readily used irrigation combination, is not conducive to harnessing the potentially positive biological properties of dentine growth factors.

5.4.3. Comparing TGF-β1 release between the irrigant and periapical sampling using Water + EDTA 17%

The results obtained from multiple rinses for irrigant retrieval and periapical sampling were combined and analysed for 'Water + EDTA 17%'. This was to determine if there was a difference between the irrigant retrieval, periapical sampling and time points used.

Figure 21 shows the combined data and indicates a statistically higher amount of TGF-β1 obtained using the irrigant retrieval approach compared with periapical sampling in the 'Water + EDTA 17%' at 10 minutes. Given the irrigant solution with TGF-β1 in solution should be the same; the disparity may be due to error in the volume calculations of the smaller concentration in the periapical group. However, the remaining groups at 5 minutes and 15 minutes contained similar concentrations of TGF-β1 between the irrigant and periapical retrieval indicating valid calculations.

The data also indicates that the second rinse of 10 minutes has the ability to penetrate further into the already demineralised dentine structure; with the majority of the smear layer removed it may expose fresh dentine containing sequestered growth factors. This is supported by data (Calt and Serper, 2002) exploring the effect time had on smear layer removal and noted that at 1 minute the majority of the smear layer was removed and at 10 minutes there was extensive peri-tubular and inter-tubular dentine removal. This could indicate why the third irrigant applied at 15 minutes did not generate significant different results.as it is slower to reach the deeper layer of the fresh dentine. The first rinse at 5 minutes may therefore simply remove the highly soluble smear layer and have insufficient time to penetrate the underlying dentine. The reaction of EDTA with the dentine is therefore not simply a linear relationship but is relatively slower in the first instance, increasing rapidly and then stabilising at a steady rate over time.

5.5. Analysis of differences in the yield of TGF-β1 between multiple rinses or a single rinse application

The results presented in section **4.3.4** show that multiple changes of EDTA do not release significantly more TGF-β1 than a single rinse approach. Importantly the presence of NaOCl 2% again had a negative impact of TGF-β1 growth factor detection. This was indicated to previously as NaOCl carryover may prevent TGF-β1 from being detected. If this is the case, clinically, it may mean than the contemporary irrigant regime of NaOCl followed by EDTA may disinfect the root canal but that growth factor release and the potential interaction with the periapical tissues would be inhibited. The reason for this occurring is therefore important to ascertain and could be due to:

1. Residual NaOCl denaturing the activity of the EDTA;
2. Residual NaOCl in the intricate dentine structure is denaturing the growth factor release after EDTA liberates it from the dentine but before it has time to be released into suspension;
3. NaOCl is able to penetrate much further than the EDTA and therefore the superficial growth factors are denatured before EDTA is applied;
4. Residual NaOCl may denature the dentine growth factor in solution or prevent the ELISA through interference with the detection process.

5.5.1. A comparison of the yields of TGF-β1 between accumulative water + EDTA 17% for all groups and sampling methods

The comparison between cumulative irrigant changes and a single irrigant administration showed that there was no statistical difference in the water + EDTA 17% group (**Figure 24**). This indicates that changing the irrigant and repeated ultrasonic irrigation techniques does not increase the amount of TGF-β1 released. However the important factor for these studies was in the time that the irrigant had to interact with the root canal system. The data did not show a saturation point, but it is reasonable to postulate that at a

time point the EDTA will be bound with the calcium ions within the dentine and there will be no further release of TGF-β1.

Weinreb and Meier (1965) investigated the use of multiple changes of EDTA (5 changes in a 15 minute period) and noted that twice as much mineral salts were removed from the dentine than for one continuous application of EDTA for 15 minutes. Notably this study was not undertaken using our model but utilised dentine powder extracts (Weinreb and Meier, 1965). This approach would explain the elevated TGF-β1 released with cumulative rinses compared with the single 15 minute application.

It is suggested that once all the available chelating ions within the EDTA have reacted with calcium ions there will be no further dissolution and the EDTA solution will reach an equilibrium (Frithjof and Östby, 1963). Seidberg and Schilder determined that the rate of reaction between an EDTA solution and dentine was most rapid in the first hour of application and approached an equilibrium at 7 hours, regardless of the surface area investigated with only a proporionate amount of the actual dentine being chealted (Seidberg and Schilder, 1974).

The mechanism behind the minimal increase of TGF-β1 with multiple irrigant changes compared with one irrigant application is potentially due to the EDTA 17% not reaching the saturation point and that there is only a certain amount of calcium ions that can be bound to at any one time. This suggests that a lengthy application of EDTA 17% would theoretically release more dentine growth factors. It is difficult to ascertain when this equilibrium would be reached in this model and only retrieving irrigant at increased time points would determine this.

The aim of this work was to determine a protocol to obtain optimal TGF-β1 yields within a clinically acceptable time period of less than 15 minutes.

Subsequently it can be surmised that the most important factor for release is the time the irrigant has in contact with the dentine and that the use of passive ultrasonic irrigation will increase the wettability of the EDTA 17% at the dentine surface.

5.6. Attenuating the effects of NaOCl

In the previous section the effect NaOCl had on the dentine and/or the TGF-β1 detected was highlighted. Indeed there was insignificant TGF-β1 release detected when NaOCl was used as a primary irrigant.

5.6.1. Determining the effect that NaOCl has on a known concentration of TGF-β1

The methodology applied is described in section **3.5** and the results are presented in section **4.4.1**. Findings indicated that NaOCl even at very low concentrations had a negative impact on the TGF-β1 ELISA. Furthermore this was supported by the claims from the manufacturer (**see Appendix 3**).

Notably any degree of contact with small concentrations of NaOCl is reported to prevent the ELISA from functioning at its intended capacity, this is most likely due to its' considerable ability to denature proteins (Naenni et al., 2004). This effect was investigated by using the manufacturer's recommendations of serial dilutions (**see Appendix 3**). The findings demonstrated that both pre-treated solutions containing 250pg/ml^{-1} TGF-β1 and pre- rinsing the ELISA plates with NaOCl both resulted in no detection of TGF-β1. This suggests that the ELISA is more sensitive to NaOCl than the free TGF-β1. In this scenario, even though the NaOCl may not denature the TGF-β1 a small amount of residual NaOCl is transferred to the ELISA and may prevent it from detecting the TGF-β1. Alternatively, the NaOCl may interfere with both the pre-treatment TGF-β1 and the ELISA.

Indeed it appears that NaOCl, even at very small concentrations, prevents the ELISA from working. An alternative strategy may be to consider gold labelling the TGF-β1 within treated dentine and visualising this using scanning electron microscope as used by Zhao et al. (2000). However, this approach was not taken here as there was sufficient evidence indicating that NaOCl would denature the immunologically labelled proteins also (Naenni et al., 2004).

5.7. Determining the effect chlorhexidine gluconate 2% has on TGF-β1 release in an *in vitro* model as a primary irrigant to replace NaOCl 2%.

An alternative strategy for canal irrigation would be to replace the NaOCl 2% primary irrigant with CHX 2% which also has the ability to disinfect the root canal system and to determine if this was conducive to growth factor release. Indeed it is reported that the antimicrobial effect of CHX 2% is similar to that of NaOCl 2% (Wang et al., 2012) although other studies suggest it is less antimicrobial than NaOCl and lacks tissue dissolving capabilities (Mohammadi and Abbott, 2009a).

As data the discussed in section **5.5** showed no significant difference between the use of multiple irrigants and single irrigant applications the subsequent experimental protocol applied only used a single 15 minute application.

The CHX 2% application demonstrated the release of TGF-β1 was achieved when both water and EDTA 17% were used as a secondary irrigant. The release of TGF-β1 was not significantly different with EDTA 17% compared with water, which was in contrast to the previous data presented in section **5.4** where EDTA 17% released a considerable amount of TGF-β1 (**Figure 26**). However the yield was significantly smaller and ~20 pg/ml^{-1} compared with 600 pg/ml^{-1} (**Figure 27**). This indicated a separate mechanism was occurring between 'CHX 2% + EDTA 17%' than 'Water + EDTA 17%'.

The mechanism for this interference could be attributed to the interaction of CHX 2% and EDTA 17%, which forms a white precipitate; effectively restricting the action of EDTA 17% on the dentine (Rasimick et al., 2008, Basrani, 2005). The CHX 2% did not affect the ELISA readings as with NaOCl and positive controls showed it had no effect on a known concentration of TGF-β1 which eliminated ELISA interference.

The SEM images presented in section **4.6** are representative and derived from the teeth used in **Figure 27** and enable qualitative visualisation of the effects of the irrigant regimes on the dentine.

When 'Water + Water' was applied there was no growth factor detected, the likely reason for this was that the smear layer remained intact and this irrigant regime does not have the ability to dissolve the smear layer. Thus during preparation the debris occluded access to the underlying dentine, this likely consisted of a layer of 1-2µM of organic and dentine particles which can form smear plugs by extending into the tubules up to 40 µM (Mader et al., 1984). This was in contrast to 'Water + EDTA 17%' which resulted in the removal of the smear layer and the demineralisation of the dentine surface releasing the sequestrated growth factors into solution. The chelating effects of EDTA 17% were demonstrated visually explaining the significant amount of dentine growth factor retrieved from the demineralised dentine.

Interestingly, the effect of dentine demineralisation was not correlated with the concentration of TGF-β1. When 'NaOCl 2% + ETDA 17%' was applied there appeared more "opening" of the dentinal tubules compared with the 'Water + EDTA 17%' regime with greater intra-tubular demineralisation and patency of the tubules. This is supported in the literature as the combination of NaOCl and EDTA resulted in more dentine erosion (Nogueira et al., 2018, Qian et al., 2011) however this did not lead to more TGF-β1 detection.

When ‘NaOCl 2% + Water’ was applied only remnants of the smear layer remained, and these data are supported by other studies (Garberoglio and Becce, 1994). Although, the actual depth of penetration for NaOCl 2% cannot be calculated, it is clear that ‘NaOCl 2% + EDTA 17%’ exposes significantly more untreated dentine beneath the surface than NaOCl 2% application alone. These data would indicate that bioactive growth factors could be released however no dentine growth factors were detected. As previously discussed the low surface tension of NaOCl means it is extremely difficult to remove from the dentine surface. Galler et al. (2015a) also agreed with this finding that NaOCl 2% resulted in significantly less growth factor release although they still detected a small concentration when followed with a rinse of EDTA 17%. However, in that model dentine discs were used which do not have the same fluid dynamics of an enclosed root canal system as was undertaken here with this whole tooth model. As such the remaining NaOCl 2% may be extremely difficult to remove immediately and could affect subsequent dentine growth factor release or activity when the EDTA 17% was used.

The use of ‘CHX 2% + Water’ and ‘CHX 2% + Water’ resulted in significant demineralisation and erosion of the dentine surface (**Section 4.6**). The proposed mechanism underlying this effect is due to the lower pH of CHX. The corresponding dentine destruction did not result in more dentine growth factor release than the ‘Water + EDTA 17%’ groups and again indicates the contact of the irrigant to fresh dentine is not the important factor. The loss of dentine structure with CHX groups would result in a weakened surface structure and potential difficulties in achieving affective sealant penetration. Other groups have reported contrasting findings (Moreira et al., 2009) where CHX was not as erosive as EDTA, however their protocol did not involve the use of passive ultrasonic agitation and different time periods of application. Although no precipitate could be actively seen on the dentine walls when CHX and EDTA were applied it remains possible that this chemical interaction may inhibit the release TGF-β1 (**Figure 2**).

Furthermore, the demineralisation of the dentine surface could explain why EDTA 17% was less effective at removing dentine growth factors as the surface had been significantly damaged. The erosive potential of CHX 2% and the potential for the acidity to interfere with TGF-β1 stability in solution is also a potential reason why the clinical use of CHX 2% in this model should be extrapolated with caution.

The complex dynamic between irrigant types indicates that 'Water + EDTA 17%' is again the most appropriate for TGF-β1 release; however this combination would not result in a thorough disinfection of the root canal system. Subsequently the 'NaOCl 2% + EDTA 17%' protocol combination required modification to balance growth factor release with disinfection of the root canal system.

5.7.1. TGF-β1 release when CHX 2% was used alone

The CHX was also used as a primary irrigant, due to its acidic pH of 5 and previous literature indicating dentine growth factor release (Galler et al., 2015a). **Figure 28** shows the spike in TGF-β1 release when CHX 2% was applied for 5 minutes with the concentration approaching 100 pg/ml^{-1}. This amount was larger than the TGF-β1 retrieved when EDTA 17% was used as a second irrigant after CHX application. The periapical sampling was also significantly different to that of the retrieved irrigant; the mechanism underlying this is suggested to be due a much longer processing time with the growth factors to acidic solution, which may attenuate the protein stability. Indeed the findings of Galler et al. (2015) support the fact that TGF-β1 is more stable in a neutral pH.

It is reasonable to conclude therefore that CHX 2% is capable of growth factor release in contrast to NaOCl but alters the effectiveness of EDTA 17% and is associated with significantly more dentine erosion.

5.8. The potential effect of a calcium hydroxide inter-appointment dressing

The effects of $Ca(OH)_2$ on TGF-β1 release were investigated as it reported to stimulate growth factor release (Graham et al., 2006).

The Position Statement issued by European Society of Endodontology (ESE) on revitalization procedures advises the use of NaOCl at the first visit only to minimise the damage to growth factors and EDTA 17% used alone on the second visit with an inter-appointment dressing of $Ca(OH)_2$ (Galler et al., 2016). Although in this study we are not necessarily focused on revascularisation, the ESE highlight the disinfection step followed by a step which will release dentine growth factors; which is a comparable approach to what is being aimed at here. A potential difference is that this study aims to maximise a protocol which enables the interactions of these growth factors with the periapical tissues to facilitate healing as opposed to creating an environment to re-vascularise the pulp.

It was therefore proposed that an intermediate step may allow a primary disinfectant stage with NaOCl 2%, followed by stage which was conducive to growth factor release using a dressing of $Ca(OH)_2$ followed by ETDA 17%. Thus this may facilitate the release and sampling of dentine growth factors and would be similar to conventional root canal treatment undertaken over 2 hospital visits.

The *in vitro* conditions were replicated to match the clinical conditions when $Ca(OH)_2$ is used as an inter-appointment dressing and the recommended minimal time period for the use of $Ca(OH)_2$ is 7 days (Sjögren et al., 1991). Within this study fourteen days were chosen as this was locally agreed protocol at Birmingham Dental Hospital, UK. In addition it is important to state the effects of $Ca(OH)_2$ can be active for up to 3 months (Siqueira and Lopes, 1999).

The results obtained were promising in that all the groups, including the use of the primary irrigation of NaOCl 2%, generated detectable concentrations of TGF-β1 release (**Section 4.5.2, Figure 29**). This satisfied the primary aim of this study which was to use a primary irrigant for disinfection followed by an irrigant to promote growth factor release. However, the findings showed that the concentrations of TGF-β1 obtained were much lower than when 'Water + EDTA 17%' were used (**Figure 27** and **Figure 29**). In the simulated periapical tissues there was TGF-β1 detected but this was not statistically different from the controls. Furthermore, for both groups the relative yields of TGF-β1 were both relatively low compared with previous experiments. The effect this reduced concentration of TGF-β1 may have on the simulated periapical tissues is also unknown (as is also the case for the previous studies).

The effects of EDTA 17% were also attenuated as it did not result in significantly more TGF-β1 detection compared with water was when used as a secondary irrigant. The secondary irrigation may therefore only be solubilising the TGF-β1 present in the $Ca(OH)_2$ paste. This supports a prolonged action of $Ca(OH)_2$ at releasing dentine growth factors present in the root canal system (Graham et al., 2006, Tomson et al., 2007, Ferracane et al., 2013) as well as in disinfection. The remaining $Ca(OH)_2$ located on the walls of the dentine may also prevent the action of EDTA 17% by creating a physical barrier to the penetration of EDTA 17% (McCaul et al., 2001, Lambrianidis et al., 1999, Lambrianidis et al., 2006) and more mechanical debridement or application of fresh irrigant be necessary. The NaOCl 2% may also have been completed neutralised by the $Ca(OH)_2$ after the fourteen day period or by reacting with organic tissue. It would therefore not have the opportunity to react with the released TGF-β1 or be carried-over into the ELISA.

The translation of these findings indicate that a two visit endodontic treatment would be necessary in order to initially successfully disinfect on the first visit using NaOCl 2%, then dress with $Ca(OH)_2$, and then on the second visit use EDTA 17% to release dentine growth factors top stimulate tissue healing. The physical barrier of any residual $Ca(OH)_2$ could potentially be overcome any physical removal using mechanical debridement approaches (Chockattu et al., 2017). As this is a common combination of clinical irrigants used in endodontics, the only difference would be not using NaOCl at the second visit, which could harness the potential of dentine growth factors to stimulate the biological processes in the periapical tissues.

To determine if there were any potential solutions to enable a one visit endodontic procedure the attenuation of any remaining NaOCl were investigated and this is discussed in section **5.6**.

5.9. The effect of sodium thiosulphate on TGF-β1

The potential to neutralise the effects of NaOCl prior to ELISA using 5% $Na_2S_2O_2$ were investigated. The method used is described in sections **3.5** and **3.6** and the results are presented in sections **4.5.4** and **4.5.5**.

Sodium thiosulphate has been used previously to deactivate and neutralise the effects of NaOCl and this is reported in the endodontic literature (Byström and Sundqvist, 1983, Siqueira Jr et al., 2000, Radcliffe et al., 2004, Gomes et al., 2001). The reaction involves the neutralisation of the NaOCl, preventing the production of reactive oxidising agents which would denature proteins including TGF-β1 (Boal and Patsalis, 2017).

When 5% $Na_2S_2O_2$ was applied into a known concentration of TGF-β1 it attenuated the action of NaOCl at lower concentrations which enabled the detection of TGF-β1 but at a reduced level i.e. less than the actual concentration of 250 pg/ml[1] TGF-β1 initially applied. This outcome was

potentially due to the NaOCl still exerting an influence on the ELISA. **Figure 31** shows the two lowest concentrations of sodium thiosulphate applied which neutralised the action of NaOCl and allowed TGF-B1 to be detected.

This approach would not translate when used on the clinical model as described in section **4.5.5** indicates the failure to detect results is not the NaOCl polluting the ELISA. Indeed, the first irrigant applied was the 2% NaOCl which was subsequently removed using micropipettes, and any non-retrieved NaOCl may have been further removed by using the rotary instruments with some absorbed in the PerioPaper and a small volume likely retained within the root canal. The next stage involved a water rinse, which would dilute the residual NaOCl further; NaOCl would also be deactivated by reacting with any remaining organic tissue. Following this $Na_2S_2O_2$ was applied to neutralise any remaining NaOCl and finally a 15 minute rinse with EDTA 17% would react with any remaining NaOCl and further neutralise it.

The study described in section **4.5.4** shows that the ELISA does detect TGF-β1 levels above the 0.03% NaOCl concentration when $Na_2S_2O_2$ was used before being analysed in the ELISA. This amount equates to approximately 0.1µl of the 2% NaOCl solution and is also without the application of water and EDTA 17%. The probability of this amount of active NaOCl being transferred to the ELISA is therefore likely to be extremely low. The more probable explanation for this outcome is that the sequestered TGF-β1 had already been neutralised in the dentine and this was therefore unable to be released with the subsequent EDTA 17% solutions application If TGF-β1 were present it would be released into suspension and the irrigant combination would likely neutralise the residual NaOCl.

The negative results therefore indicate the NaOCl itself as a primary irrigant is so successful at dissolving organic tissue it attenuates the sequestrated dentine growth factors. For this issue to be overcome, it appears that a

simple neutralisation of the NaOCl is not sufficient to facilitate dentine growth factor release as the damage to the tissues and molecules has already occurred. There are a number of options for this which are discussed within the '**Future Work'** section.

6. Conclusions

In a one-step procedure (mimicking a single-visit endodontic procedure), the irrigants EDTA 17% and CHX 2% facilitated the significant release of the dentine-derived growth factor, TGF-β1. This was detected in both the retrieved irrigants from the simulated periapical tissues.

Interestingly the irrigant EDTA 17% released the highest concentrations of TGF-β1. The amount detected increased in a time dependent manner, with the highest concentration of TGF-β1 detected after 24 hours. Multiple changes of EDTA 17% did not significantly increase the amount of TGF-β1 released over a 15 minute time period.

The use of NaOCl 2% in a one-step procedure prevented the detection of TGF-β1, even after multiple rinses using EDTA 17% were undertaken. CHX 2% produced the highest amount of dentine erosion when viewed under scanning electron microscopy.

In a two-step irrigation approach (mimicking a two-visit endodontic procedure), where an inter-appointment dressing of $Ca(OH)_2$ was placed for two weeks, there was a detectable measurement of TGF-β1. This appeared to overcome the application of NaOCl 2% applied at the first visit.

Clinically the findings described above suggest a two-visit endodontic procedure should be undertaken if a disinfectant procedure along with dentine growth factor release is to be achieved.

7. Future work

The ideal irrigant would possess the ability to disinfect the root canal system, dissolve the remaining soft tissue, remove the smear layer and facilitate the release of dentine growth factors. Unfortunately, no single irrigant has the ability to do this and it is apparent that a combination of irrigant types is necessary. However, the exact types, timings and order of irrigant applications are not entirely clear and they remain challenging to balance to obtain the optimum outcome. Indeed this is notable with NaOCl application which has excellent disinfectant properties and the ability to penetrate into the complexities of the root canal anatomy but appears to attenuate the release of dentine growth factors. In contrast EDTA 17% has an excellent ability to remove the smear layer and promote dentine growth factor release but possesses sub-optimal disinfectant properties. Notably when these two irrigants are combined, the equilibrium shifts towards disinfection and away from the release of dentine growth factors. When EDTA 17% is used alone the equilibrium is shifted towards dentine growth factor release but away from disinfection.

The use of an inter-appointment dressing of $Ca(OH)_2$ appears to surmount the action of NaOCl 2% to a degree but decreased the action of EDTA 17% resulting in substantially lower concentrations of growth factor release. The underlying mechanism for this appears to be due to the ability of NaOCl 2% to penetrate into dentine and denature the dentine growth factors. Furthermore EDTA 17% requires a longer incubation period to chelate and react with the dentine and has a higher surface tension, being less able to penetrate into the dentine. Negating a disinfectant step would mean a higher microbial load within the root canal system and may lead to premature failure of the root canal treatment; therefore striking a balance between disinfection and releasing growth factors is essential.

7.1. Microbiology studies

7.1.1. Modifications to the order of the current irrigation regime – EDTA delivered before NaOCl application

Conventional techniques use a disinfectant and tissue dissolving step prior to smear layer removal. One feasible alternative would be to deliver EDTA 17% first, which would facilitate the release of dentine growth factors. An inter-appointment dressing could then be applied and at the second stage a disinfectant step could be undertaken, at this point the smear layer would be removed and the NaOCl could reduce the load of micro-organisms further. As discussed earlier, EDTA 17% does possess antimicrobial properties (Torabinejad et al., 2003) and this presents an interesting strategy for dentine growth factor release at the first stage of application whilst providing some degree of disinfection and a definitive disinfectant step is then applied at the second visit.

To better characterise these approaches the tooth model developed in this study could be used to investigate how the change in irrigant regime affects the bacterial load. This could be undertaken *in vitro* where the same tooth model is exposed to a known endodontic pathogen such as *Enterococcus faecalis* and NaOCl 2% is delivered first compared with the EDTA 17% effect. Subsequently, following a period of incubation the second irrigant would be applied, consisting of NaOCl 2% or EDTA 17%. The bacterial counts would then be compared, if there was no difference this would suggest the irrigants could be used in either order.

7.1.2. Modifications to the type of irrigant used – omission of NaOCl

There is evidence suggesting a reduction in bacterial load is achieved with instrumentation with saline alone (Ørstavik et al., 1991, BYSTRÖM and SUNDQVIST, 1981). The mechanism reported for this effect is the removal of

infected dentine along with the antimicrobial action of saline and a shift in the ecology of the root canal, introducing oxygen which attenuates the anaerobic bacteria (Trope et al., 1999). However, reportedly there was a lack of disinfection at the first visit and the bacteria multiplied between visits, with the conclusion being that this was not a predictable way of bacterial infection elimination (Trope et al., 1999). The effect of not using NaOCl at the first stage may also promote bacterial proliferation by residual pulpal tissue acting as a nutrient supply (Sundqvist, 1976) and microorganisms remaining present in the root's anatomical complexities which are not instrumented (Peters et al., 2001).

It is important to note that although NaOCl is considered the 'gold standard' for disinfection; full canal disinfectant does not occur using this irrigant (Shuping et al., 2000) and it is associated with deleterious effects on dentine. Reducing the concentration of NaOCl applied may reduce these negative effects, i.e. reducing the weakening of the dentine structure (Sim et al., 2001) whilst reducing the penetration of the irrigant into the dentine tubules. However, as EDTA 17% is slower at penetrating (Siqueira Jr et al., 2000) it is unlikely to enable the EDTA in reaching growth factors present in fresh and untreated dentine.

Using EDTA 17% alone provides a feasible treatment solution as it does possess disinfectant properties (Torabinejad et al., 2003) however this is likely to be significantly lower than NaOCl 2% activity. CHX 2% may also be proposed as a substitute for NaOCl however this has shown to be relatively erosive to dentine and possesses no tissue dissolving properties.

To investigate these proposed changes to the irrigant regime it is prudent to ascertain the efficacy of EDTA 17% as a disinfectant. The proposed studies would be as described above in section **7.1.1**, but instead of multiple changes of irrigants, the effects of NaOCl 2% would be directly compared with EDTA 17% without a second irrigant used. Data generated would

establish the efficacy in a tooth model at eliminating *Enterococcus faecalis* infection.

The primary hypothesis would be that in a tooth model cultured with *Enterococcus faecalis*, NaOCl 2% would reduce the bacterial load greater than EDTA 17% in a one-step procedure. The secondary hypothesis would be that in a two-step procedure, the order in which EDTA 17% and NaOCl 2% are applied would have no effect on *Enterococcus faecalis* viability.

7.2. Irrigant penetration studies

7.2.1. Modification to the properties of irrigant used in the treatment – SmearClear™

The inability of EDTA 17% to penetrate into fresh dentine is proposed to be due to its' relative high surface tension. The addition of a surfactant to the EDTA 17%, such as the commercially available SmearClear™ (Kerr, Orange CA), has the potential to increase the penetration of the EDTA to deeper areas of dentine, containing fresh growth factors, untouched by NaOCl 2%.

The evidence for this is suggestive but not conclusive. An experiment is therefore advised to investigate the contact surface angles of the irrigants water, NaOCl 2%, EDTA 17% and SmearClear™ with dentine. Giardino et al. (2006) utilised a contact surface angle technique previously and this would be relatively straightforward to replicate.

A further investigation is suggested to determine the penetration into dentine, this would be relatively straightforward to achieve for NaOCl 2% as it exerts a bleaching effect on dentine. ETDA 17% would likely require an associated marker to determine the penetration. This could then be used to determine if the NaOCl 2% is penetrating further than the EDTA 17% by visualising the depth of penetration (Zou et al., 2010).

The hypothesis for this further investigation is therefore that the NaOCl is penetrating deeper into the dentine and denaturing the dentine growth factors prior to application of the EDTA having the ability to penetrate to that level. The secondary hypothesis is that this is overcome with different EDTA preparations.

7.3. Investigating the biological effect of dentine growth factors – an angiogenic model

Throughout this study the released yields of TGF-B1 were investigated for different irrigation types and delivery methods. It is a reasonable assumption that the higher the concentrations of dentine growth factors released the more effect they will have on the periapical tissues. However, the effect is not likely simply due to dose but more so a complex interplay of biological mechanisms where the same type of dentine growth factors at different concentrations may elicit antagonistic biological outcomes. Indeed Zhang et al. (2011) showed that lower concentrations of dentine matrix components exerted a stimulatory effect on endothelial cell proliferation and at higher concentrations the same components had a inhibitory effect. This study was concerned with angiogenesis and indicates further work is necessary to understand the effect the differing concentrations of TGF-B1 and the dentine released growth factor cocktails may have on the periapical tissues.

The hypothesis for further investigation is therefore that the dentine growth factor released in this model have a positive effect on endothelial cell proliferation in a model of angiogenesis.

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9. Appendix

9.1. Appendix One

Methods

2.8.3.8: Reproducibility study:

A serum sample was diluted 1 in 200, six times. Samples were activated and assayed in six wells. This was repeated on three separate occasions.

2.8.3.9: Elution studies with the chemiluminescent ELISA:

2.8.3.9.1: Dry strips versus immediate elution at 4°C

A serum sample was diluted 1 in 10 in dilution buffer. 1µl of this was micropipetted onto a periopaper strip and a periotron reading taken. Twenty-five strips were dropped into dry cryovials and immediately stored in liquid nitrogen. Another 25 strips were placed into cryovials containing 200µl of dilution buffer and 5 of the 25 strips were eluted in each of the following ways:

- overnight, 4°C
- 30 mins, 4°C
- 60mins, 4°C
- 120mins, 4°C
- 60mins, 4°C, agitated every 5 mins of second 30 mins.

After elution the papers were removed with clean forceps and the samples stored in liquid nitrogen. The dry strips were eluted as described above, just prior to activation. In total 5 'dry stored' and 5 'immediate-eluted' samples were assayed for each elution method. Controls were prepared by micropipetting 1µl of the 1 in 10 serum in to 200µl of dilution buffer and storing immediately in liquid nitrogen.

2.8.3.9.2: Dry strips versus immediate storage and immediate elution on ice

Samples were prepared as described above. Twenty strips were stored dry, 20 strips were eluted immediately and 20 strips were placed into 200µl of dilution buffer and stored immediately. The strips that were eluted immediately, were placed onto melting ice as they would be in an experimental situation. The dry strips and those frozen in buffer were eluted just prior to activation. Strips were eluted in the following ways:

- overnight, 4°C
- 30 mins, 4°C
- 60mins, 4°C
- 60mins, 4°C, agitated every 5 mins of second 30 mins

2.8.3.9.3: Effect of activation on elution

Dry stored strips were prepared as described in section 2.7.3.9.1, and eluted in 2 ways, either 60mins at 4°C or 60mins at 4°C, agitated every 5 minutes of the second 30 minutes. 10 strips for each method were either:

- removed just prior to activation
- left in tube during activation
- eluted and activated at the same time

Results

5.3.2: In-house chemiluminescent ELISA

Elution experiments were repeated with the in-house assay. As this assay had a lower detection limit a smaller amount of serum was used in these experiments. One-microlitre aliquots of a single serum sample (~60ng/ml), diluted 1 in 10 in TBST 0.05% BSA 1%, were applied to periopaper strips and eluted as described in the Materials and Methods section 2.8.3.9. Non-eluted controls consisted of 1µl aliquots of the diluted serum added directly to 100µl of dilution buffer.

The experiment showed that immediate elution at 4°C followed by storage in liquid nitrogen generally produced higher percent recoveries than storing strips in dry tubes in liquid nitrogen and

elution just prior to assay. Repetition of the elutions under conditions which would be used when collecting samples on clinic produced different results. The strips eluted immediately were placed into buffer filled tubes and placed on ice for their allotted elution time. Dry strips were treated in the same way as the previous experiment and a third set of strips were placed into buffer, frozen in liquid nitrogen and eluted just prior to assay. This experiment indicated that storing the strips dry and elution immediately before assay produced a higher percent recovery (42-70%) than those eluted on ice (45-58%) or frozen in buffer (41-57%). It was decided to store all strips dry and all further elution experiments used the dry stored strips. 1 hour and 30 minutes followed by 30 minutes with agitation every 5 minutes were determined to produce the highest percent recovery of TGFβ1 from strips and both methods of elution were used for further elution experiments.

Most samples for TGFβ1 analysis by ELISA must be activated prior to assay (Materials and methods section 2.8.3.2). To investigate whether strips must be removed from the eluate prior to activation and to determine if activation and elution could be performed at the same time a third elution experiment was performed. The two elution techniques with the highest recovery in the previous experiment were used (1 hour at 4°C and 30mins at 4°C with 30 mins agitation). This experiment showed that activation without removing the periopaper strip produced a higher percent recovery than removal of the strip (82.5% & 64.4% respectively; 30mins at 4°C with 30 mins agitation). It also indicated that activation and elution at the same time was possible and that 30mins at 4°C with 30 mins agitation produced the highest percent recovery (91%). This method, activation whilst eluting for 30mins at 4°C with 30 mins agitation, was used for all the results described in chapter 6 except for the pilot study on TGFβ1 levels in gingival overgrowth.

Discussion

Elution:

Elution studies were performed using both assays. Pilot studies using the Genzyme kit showed that approximately 50% of the TGFβ1 could be recovered from the periopaper strips. However, there was a lot of variability, which appeared to be mainly due to the elution procedure rather than the assay per se. Attempts to increase the amount eluted and to improve reproducibility by changing the elution technique increased the yield to approximately 60%.

Elution studies with the 'in-house' assay initially repeated the Genzyme experiments but with lower serum volumes and different elution buffers. These demonstrated that approximately 57% of TGFβ1 was recovered from the periopaper strips (range 29-75%) when the 'Genzyme' method was used. Interestingly strips, could be stored dry and eluted later using the 'new' buffer (TBST BSA 0.1%) to give a similar percentage recovery (64%). Further experiments demonstrated that optimum recovery of TGFβ1 from periopaper strips was attained when they were stored dry and subsequently eluted and activated (acidified) at the same time. This method resulted in a mean recovery of 91% (72.5-111%). However, the large variation inherent in the procedure was still present as demonstrated by the recovery ranges.

The majority of previous work on elution of GCF from periopaper strips has been done using a saline based buffer, average pH 7.4, with or without a detergent (Tween 20 or Triton x100). Reported elution times vary between 1 minute and 24 hours and sometimes centrifugation has been used. TGFβ1 has been detected in GCF eluates previously but no mention of the efficiency of the elution method was mentioned (strips placed in 50μl PBS at 4°C for 24 hours; samples stored at –20°C). Work with other cytokines (e.g. IL-1) has been done using strips which were stored dry (-30 to -80°C). Good serum protein (e.g. α_2 macroglobulin, α_1 anti-trypsin) recovery rates have been reported (81-108%) using a technique similar to the one used in this study, 30 minutes static then 30 minutes with agitation.

This assay was developed to detect TGFβ1 in GCF samples as the commercial assay tested was neither sensitive enough nor reproducible enough to achieve this with the required accuracy. The assay that has been developed, in combination with the 'new' elution technique, is sensitive enough to detect TGFβ1 in GCF samples and for all samples to be run in duplicate. The reproducibility of the assay is similar to many commercial assays and greater than that found with the Genzyme kit.

9.2. Appendix Two

Dear Dr. Green:

Thank you for your message. Seventeen per cent is a nominal amount for the EDTA solution. The manufacturing procedure begins with a 17% EDTAsolution, and then it is buffered to a neutral pH. The end result is less than 17%.

The research on the percentage to use was done in the early 1990s. We do not have access to that data now. Endo companies for whom we manufacture have found our material superior to that of other manufacturers. Perhaps it's the process. There may be more to it than just percentage.

Best regards,

Fred

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9.3. Appendix Three

"Dear Mr Green,

Thank you for using our ELISA,

I believe you are probably using cat # DB100B, which is the format we validate to use with many sample types such as Cell Culture Supernates, Serum, EDTA Plasma, Urine.

We have not tested the effect of NaOCl on the kit as this is a chemical that would not be normally present in the sample types we validate.

I would imagine that it either have an effect of denaturation on the antibody pair or it is causing interference.

I am afraid I do not have recommendation to share a part from trying linear dilutions of the samples in the assay diluent to try and determine the effect of NaOCl, you will be of course limited depending on the levels of TGF-beta 1 in your samples and I am afraid this is something that cannot be changed.

I am sorry that I do not have a precise resolution, in this case the intrinsic nature of the sample it's the limiting factor and there is little we can do about it.

I remain at your disposal for any further questions,

Kind regards,

Manu"