

**Antibacterial activity of different Iodoform-based preparations used as
root filling materials in Paediatric Dentistry**



**A mini-thesis submitted in partial fulfilment of the requirements for the degree of MSc
in Paediatric Dentistry**

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KEYWORDS

Pulp therapy

Primary teeth

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ABSTRACT

Background: The primary goal of pulp therapy in the deciduous dentition is to keep the teeth fully functional in order to prevent arch space loss. A pulpectomy is a pulp therapy procedure indicated when an irreversibly inflamed or necrotic radicular pulp is encountered. ZOE and iodoform pastes (i.e. Kri 1 paste and Vitapex) have been recommended as root filling materials after pulpectomy. High clinical success rates have been reported with Vitapex and the fact that it resorbs readily when extruded beyond the apex is an added advantage. However, it has also been reported to resorb from within the root canals and even cause pathological root resorption in cases where the vital pulp is inflamed (Nurko *et al.*, 2000). Iodoform-based preparations are thought to be more appropriate because they fulfill nearly all the requirements of the ideal root filling materials in primary teeth. Despite this, there are limited studies comparing the antibacterial effect of iodoform-based preparations.

Aim: The aim of this study was to evaluate the antibacterial efficacy of Vitapex (V), Kri 1 paste (K) and pure iodoform (I) against *E. faecalis*.

Methods: The antibacterial activity of the three iodoform materials was evaluated against *E. faecalis* in an in vitro study by quantifying the bacterial metabolic activity using the Cell Proliferation Kit II (XTT). The bacterial suspension was treated with each of the investigated materials. A positive control served as a baseline background and a negative control (without treatment) served as an indicator of bacterial growth. Viable bacterial cells convert the XTT dyes into an orange coloured solution called formazan. The colour intensity of the resultant formazan was assessed by measuring the material absorbance (Ab_{450}) for all the study samples using a spectrophotometer at 0, 1, 2, 4 and 24-hour intervals. The antibacterial activity was determined by the change in the material absorbance (Ab_{450}) values. Higher absorbance values are indicative of a greater number of viable *E. faecalis* cells in the mixture and therefore a lower antibacterial efficacy of the investigated material. A statistical

comparison was carried out between the study groups using Two Way Mixed Measures ANOVA. A p-value of less than 0.05 was considered statistically significant.

Results: All of the investigated materials had an inhibitory effect on *E. faecalis* as they demonstrated a significantly superior antibacterial effect when compared with the negative control ($p < 0.001$). There was also a statistically significant difference in the antibacterial efficacy across the experimental groups (V, I, K) at some of the investigated time intervals. Overall, Kri 1 paste seemed to have a more potent antibacterial effect than Vitapex.

Conclusion: All the investigated materials showed an antibacterial action against *E. faecalis*. This makes all these materials suitable as root canal filling materials for primary teeth as they will be able to overcome the inadequacy of the root canal bio-disinfection.

The antibacterial action of the tested materials did not only depend on the effect of their different constituents against *E. faecalis* but also on their ratio and the ability of the active ingredients to dissociate and exert an effect on the bacteria. The addition of camphorated parachlorophenol seemed to make Iodoform more potent against *E. faecalis* while Ca(OH)_2 seemed to minimize the efficacy of Iodoform. More in-vivo studies are however required to confirm these findings.

DECLARATION

I declare that “*Antibacterial activity of different Iodoform-based preparations used as root filling materials in Paediatric Dentistry*” is my own work, that it has not been submitted for any degree or examination at any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Razan Azahry Abdelhalim Mohamed

November 2019

Signed:



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DEDICATION

To my parents

Azhari Mohamed & Ensaf Albakri

Whose many sacrifices made my education possible, for which I will forever be grateful.

To my siblings

Mohamed, Ahmed, Olla, Ali & Rawa

For their unfailing and selfless love and support.

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LIST OF ABBREVIATIONS

AAPD: American Association of Pediatric Dentistry

Ab: Material Absorbance

ADT: Agar Diffusion Test

ATCC: American Type Culture Collection

BHI: Brain Heart Infusion

Ca(OH)₂: Calcium Hydroxide

CLSI: Clinical and Laboratory Standards Institute

CPCP: Camphorated Parachlorophenol

DCT: Direct Contact Test

E. faecalis: *Enterococcus faecalis*

Freq.: frequency

Hrs: Hours

I: pure Iodoform

K: Kri 1 paste

NC: Negative Control

NCTC: The National Collections of Type Cultures

OD: Optical Density

PBS: Phosphate Buffered Saline

PC: Positive Control

Std. Dev. / SD: Standard Deviation

Std. err: Standard Error

V: Vitapex

VBNC: Viable but Non-cultivable

XTT: Cell Proliferation Kit II

ZOE: Zinc Oxide Eugenol

LIST OF ADDENDA

Appendix A: Ethics approval

CHAPTER 1

INTRODUCTION

The primary goal of pulp therapy in the deciduous dentition is to keep the teeth fully functional in the dental arch, while acting as space maintainers until the subsequent eruption of their permanent successors (Ounsi *et al.*, 2009). A pulpectomy is indicated as a conservative treatment modality when an irreversibly inflamed or necrotic radicular pulp is encountered. It preserves the primary teeth in order to prevent arch space loss, mesial tipping of adjacent molars and future permanent tooth impactions (Ingle and Bakland, 2002). A pulpectomy involves the removal of the inflamed tissues of the root canals and biomechanical cleaning to eliminate or minimize the bacterial load in order to obtain a healthy and clean root canal system before filling the canals with a resorbable paste (Rodd *et al.*, 2006).

In general, control of endodontic infection is a fundamental goal of pulp therapy due to the close proximity of the permanent tooth germs to the roots of primary teeth and the ample medullary bone spaces in children (Silva *et al.*, 2006). In primary teeth, optimum bio-disinfection of the root canals presents a real challenge because of the difficulty with patient management and isolation, the tortuous canal morphology and the presence of accessory canals (Byström and Sundqvist, 1981). Physiologic root resorption also increases the root surface permeability to microbial toxins, due to the associated structural changes and exposure of the dentinal tubules (Ahmed, 2013). All these factors contribute to persistent infection and re-infection, leading to endodontic treatment failures in primary teeth. Root filling materials should therefore have a bactericidal effect to inhibit re-invasion and proliferation of the remaining microorganisms in the root canals. This quality is acquired by incorporating antimicrobial components into these materials (Smadi *et al.*, 2008).

Other essential requisites of root canal filling materials for primary teeth are that they should be radiopaque with a similar resorption rate to the roots of deciduous teeth, and should be harmless to the periapical tissues and permanent tooth germs. These materials should also be able to adhere to the walls of the root canals but not shrink or discolour the tooth (Rifkin, 1980; Holan and Fuks, 1993; Machida, 1983).

The American Academy of Pediatric Dentistry (AAPD) guidelines (2019/2020) recommend the use of non-reinforced zinc-oxide eugenol (ZOE), iodoform pastes (Ki 1 paste and Vitapex) as root filling materials after pulpectomy. However, due to the limitations of some of the root filling materials for primary teeth, the search for new alternatives resulted in iodoform-based root filling materials being considered as more appropriate filling materials because they fulfill nearly all the requirements of the ideal root filling materials in primary teeth. Despite this, there are limited studies comparing the antibacterial effect of iodoform-based preparations and no specific indications for use have been advised.

CHAPTER 2

LITERATURE REVIEW

The most popularly used endodontic filling materials in primary teeth are Zinc Oxide Eugenol (ZOE), Calcium Hydroxide-based pastes and iodoform-based pastes (Dunston and Coll, 2008).

High clinical success rates of 82.5% (Barr *et al.*, 1991) and 86.1% (Gould, 1972) have been reported for Zinc Oxide Eugenol when used as a root canal filling material for primary teeth. Despite this, it has fallen out of favour due to certain disadvantages such as periapical tissue irritation and necrosis of cementum and bone (Ranly and Garcia-Godoy, 2000; Hashieh *et al.*, 1999). Additionally, it resorbs at a slower rate when compared to physiologic root resorption, leading to possible deflection of the succedaneous tooth (Coll and Sadrian, 1996). Najjar and colleagues (2019) concluded that ZOE is not preferred for teeth with root resorption and those which are about to exfoliate due to the slow resorbing nature of this material and the probability of periapical tissue irritation. Cox and colleagues (1978) demonstrated that Zinc Oxide powder had no antibacterial effect and the addition of eugenol only retarded the growth of the gram-positive organisms (Cox *et al.*, 1978). Combinations of Zinc Oxide Eugenol and Calcium Hydroxide (Ca(OH)₂) have been used but showed a reduced antibacterial effect (Cox *et al.*, 1978).

The antibacterial property of Calcium Hydroxide (Ca(OH)₂) is mainly attributed to the release of hydroxyl ions that is responsible for the basic nature of this substance, with a pH of up to 12.5 (Farhad and Mohammadi, 2005; Heithersay, 1975). This high alkalinity counteracts the inflammatory process and inhibits the bacteria via protein denaturation, thereby damaging the bacterial DNA and/ or the cytoplasmic membrane (Siqueira and Lopes, 1999; Halliwell, 1987; Estrela and Holland, 2003; Imlay and Linn, 1988). The antibacterial effect of Calcium Hydroxide however requires it to be in close proximity to the targeted bacteria (Byström *et al.*, 1985). Nevertheless, due to the “hollow tube effect” as a consequence of the quick resorption of Ca(OH)₂ from within the root canals (Goldman and Pearson, 1965) and the buffering effect of the bacterial acidic by-products (Sirén *et al.*, 2014),

poor antibacterial efficacy is associated with this material (Kim and Kim, 2014). Sathorn and colleagues (2007) in their literature review of Calcium Hydroxide as a root canal dressing, proposed that Calcium Hydroxide has a limited efficacy on microorganism eradication, even after irrigation and instrumentation of the infected root canals (Sathorn *et al.*, 2007).

Another disadvantage is the potential of Ca(OH)₂ to elicit a cascade of inflammatory root resorption when it comes into contact with pulp remnants as in cases where the vital pulp is inflamed (Nurko *et al.*, 2000). This resorption can also occur if Ca(OH)₂ comes into contact with the periapical tissues when it is inadvertently extruded beyond the apex, leading to the formation of a layer of necrosis that damages the predentine and exposes the dentine to further resorption (Ravi and Subramanyam, 2012).

When Calcium Hydroxide is mixed with other agents such as Chlorohexidine, the resorption slows down and it becomes a more potent inhibitor of microorganisms (Sinha *et al.*, 2013; Souza-Filho *et al.*, 2008; Dutta *et al.*, 2017). Additionally, several studies have proven enhanced antibacterial efficacy of Ca(OH)₂ when mixed with Iodoform (Queiroz *et al.*, 2009; Chawla *et al.*, 2001; Huang *et al.*, 2007).

Iodoform-based pastes showed superior disinfecting and resorbing abilities when compared with ZOE (Garcia-Godoy, 1986; Primosch *et al.*, 1997; Mortazavi and Mesbahi, 2004). Pure iodoform is not per se a root canal filling material for primary teeth, but rather used in combination with other materials such as Ca(OH)₂ and Camphorated parachlorophenol (CPCP) (Wright *et al.*, 1994; Thomas *et al.*, 1994). Different formulations of iodoform-containing pastes are produced by several manufacturers after the addition of other antimicrobial substances or vehicles (Barja-Fidalgo *et al.*, 2010) (Table 2.1).

Some of the most popular iodoform preparations are those mixed with Calcium Hydroxide and silicone oil which are commercially available as Vitapex and Metapex. These products resorb readily if they are accidentally extruded beyond the apex (Nurko and Garcia-Godoy, 1999). Similarly, the rate of intra-radicular resorption is also faster than the rate of physiologic root resorption, which may result in a lower success rate after a long follow-up period (Nurko *et al.*, 2000). A recent systematic review and meta-analysis stated that the clinical and radiographic success rates of the iodoform/ Ca(OH)₂ mixture is comparable to that of ZOE at 6- and 12-month follow-up periods. However, the success rate reported for ZOE at 18 months was statistically higher (Najjar *et al.*, 2019).

Table 2.1: Chemical composition of different Iodoform-based pastes (Barja-Fidalgo *et al.*, 2010).

Iodoform-based material	Composition
Vitapex	calcium hydroxide, iodoform and polysiloxane oil
Metapex	calcium hydroxide, iodoform and silicone oil
Endoflas	calcium hydroxide, zinc oxide, eugenol, barium sulfate, and paramonochlorophenol
Kri 1 paste	iodoform, camphor, menthol, and parachlorophenol
Guedes-pinto paste	iodoform, camphorated parachlorophenol, and Rifocort
Maisto paste	iodoform, camphor, menthol, parachlorophenol, zinc oxide, lanolin, thymol

Vitapex is an iodoform-based paste composed of a mixture of 40.4% iodoform and 30% Calcium Hydroxide. It is reported to be nearly ideal as a root filling material for primary teeth as it is easy to apply (Mortazavi and Mesbahi, 2004).

Ozalp *et al.* (2005), and Trairatvorakul and Chunlasikaiwan (2008) carried out in-vivo studies which compared the clinical effectiveness of Vitapex and Zinc Oxide Eugenol (ZOE). They reported 89% and 100% success rates for the ZOE group while that of Vitapex was 100% and 89% respectively (Ozalp *et al.*, 2005; Trairatvorakul and Chunlasikaiwan 2008). Only one study reported a significant difference in the frequency of successful cases between the compared groups, with Vitapex outperforming ZOE by 13% i.e. 100% versus 87% (Mortazavi and Mesbahi, 2004). The diverse success rates reported in these studies could be due to various treatment techniques as in the number of appointments used to complete the pulpectomy, the different root canal irrigants and the type of final restoration that was utilized. Additionally, the different follow-up periods and definition of criteria for radiographic success adopted by different authors could also result in diverse outcomes (Barja-Fidalgo *et al.*, 2010). For instance, Ozalp and colleagues (2005) implemented a longer

follow-up interval and more strict criteria for success than Mortazavi and Mesbahi (2004) and Trairatvorakul and Chunlasikaiwan (2008). For a case to be considered successful there should be no post-operative radiolucency at the follow-up appointment according to Ozalp *et al.*'s criteria of success (2005). On the other hand, the other two studies considered cases with reduced radiolucency as a success which also impacts on the interpretation of the results (Mortazavi and Mesbahi, 2004; Trairatvorakul and Chunlasikaiwan, 2008).

Endoflas is another iodoform and calcium hydroxide paste with the addition of zinc oxide, eugenol and barium sulfate. Studies reported favourable clinical and radiographic outcomes (Ramar and Mungara, 2010; Ozalp *et al.*, 2005). Unlike iodoform/ Ca(OH)₂ combinations, resorption of Endoflas is limited to the extra-radicularly extruded material and not from within the root canals (Dandotikar, 2014). Unfortunately, due to the eugenol content of this preparation, it can result in periapical tissue irritation (Fuks *et al.*, 2003). There is however no conclusive evidence on the efficacy of this material (Smaïl- Faugeron *et al.*, 2018).

Other antimicrobials such as camphorated parachlorophenol (CPCP) have also been mixed with iodoform (Rajsheker *et al.*, 2018). Kri 1 paste (Pharmachemie AG, Zurich, Switzerland) is another iodoform-based paste which consists mainly of iodoform (80.8%) in addition to camphor (4.8%), menthol and parachlorophenol (2.1%) (Barja-Fidalgo *et al.*, 2010). Camphor and parachlorophenol are widely used antimicrobial substances (Cerqueira *et al.*, 2007). According to Arújo *et al.* (2013), these substances can increase the bactericidal spectrum of iodoform and promote deeper paste penetration into the dentine (Arújo *et al.*, 2013). Kri 1 paste can be inserted and removed easily from the root canals (Garcia-Godoy, 1986).

One clinical study compared the clinical performance of Kri 1 paste and ZOE and reported that Kri 1 paste had a success rate of 84% as opposed to 65% for ZOE, and the difference was statistically significant (Holan and Fuks, 1993). Castagnola (1952) stated that only 20% of the potency of Kri 1 paste is lost in 10 years. Despite this, it resorbs from the periapical tissues within two weeks as it does not form a hard mass, thus making it superior to ZOE (Garcia-Godoy, 1986). Moreover, Kri 1 paste does not resorb readily from inside the root canals, thus outperforming Vitapex in this regard (Castagnola, 1952). The pursuit for a material which has the advantages of Vitapex and resorbs at a rate similar to that of physiologic root resorption, makes Kri 1 paste a possible suitable alternative; however, the antimicrobial efficacy of this paste needs to be explored further.

Numerous laboratory techniques have been used in the literature to establish the in-vitro antimicrobial activity of different materials. The most well-known and commonly used techniques are the agar diffusion (ADT) and broth or agar dilution methods as well as the direct contact test (DCT). For more in-depth evaluation of the antimicrobial effect, flow cytometric methods and time-kill tests are recommended (Jenkins and Schuetz, 2012). They are able to determine the nature of bacterial inhibition (bactericidal or bacteriostatic) as well as the effect of time or concentration of the antimicrobial agent on the tested microorganism (Balouiri *et al.*, 2016).

With agar diffusion methods as described by the Clinical and Laboratory Standards Institute i.e. CLSI (CLSI, 2012b), the standard tested microorganism is inoculated into the agar plate in a petri dish. A hole is then punched on the agar surface and filled with the experimental material (Agar well diffusion method) (Magaldi *et al.*, 2004). The material could also be impregnated into filter paper discs with a diameter of 6mm and placed on the agar plate surface (Agar disc diffusion method) (Hudzicki, 2009). Usually the experimental agent diffuses from this well or disc into the agar and inhibits the growth of the inoculated microorganism, which is demonstrated as a halo known as “the zone of inhibition” (CLSI, 2012b). After incubating the petri dish under suitable conditions for a certain period of time (as required by CLSI), the zone of inhibition around the well or the disc is measured (CLSI, 2012b). An antibiogram is used to provide qualitative results by classifying the microorganisms as resistant, intermediate or susceptible to the antimicrobial agent (Reller *et al.*, 2009).

The agar diffusion methods offer many advantages such as low cost, simplicity, the capacity to investigate large numbers of antimicrobial agents and microorganisms as well as the ease of interpretation of the acquired results (Balouiri *et al.*, 2016). Nevertheless, since microbial growth inhibition does not particularly mean microbial death, these methods cannot discriminate between a bacteriostatic or bactericidal effect (Balouiri *et al.*, 2016). Additionally, the resultant zone of inhibition largely depends on the solubility of the experimental material and its diffusibility in the agar medium which is dependent on the viscosity of the agar and the density of the inoculum (Estrela *et al.*, 1999; Estrela *et al.*, 2001a; Estrela *et al.*, 2001b)

On the contrary, the broth dilution method yields a quantitative result for the amount of antimicrobial agent that is required for the microbial growth inhibition but it can only be utilized for substances that are soluble in the culture medium (CLSI, 2012a). This test is performed with a series of plates or tubes that are prepared with agar or broth medium with the addition of different concentrations of the investigated material. Thereafter, a standardized inoculum of the tested microorganism is added. These plates or tubes are then incubated at $35 \pm 2^\circ\text{C}$, and examined to determine the minimum concentration of the tested material that is required to inhibit visible growth of the microorganism which is known as “the Minimal Inhibitory Concentration” (Ericsson and Sherris, 1971).

In the direct contact test the antimicrobial efficacy is evaluated by mixing a fixed volume of the material and the bacterial suspension. Thereafter, bacterial activity is evaluated by measuring the bacterial growth (Luddin and Ahmed, 2013). This method ensures close contact between the investigated material and the tested microorganisms. It can therefore be used for testing water-insoluble materials as it does not depend on the material solubility or diffusibility (Weiss *et al.*, 1996). It can also discriminate between a bacteriostatic and bactericidal effect (Eldeniz *et al.*, 2006) and can be used to evaluate the antimicrobial effect of the material at the different phases of setting. This is therefore of relevance in material maturation studies (Shalhav *et al.*, 1997)

The time-kill test as described in the M26-A document of the CLSI (CLSI, 1998), is the most suitable method for determining the bactericidal capacity of the investigated material. This test is very useful to assess the dynamic interaction between the tested microbial strain and the antimicrobial agent as it can be utilized to evaluate the required concentration of the material or the time required to kill the bacteria. It is performed using tubes containing broth medium in which the antimicrobial agent is added and another tube with no treatment which is regarded as the growth control. These tubes are incubated under suitable conditions for the tested bacteria for varied time periods (0, 4, 6, 8, 10, 12 and 24 hours) (Konaté *et al.*, 2012; Pfaller *et al.*, 2004). The percentage of dead cells is then calculated by quantifying the amount of viable cells relative to the growth control. The agent is described as bactericidal if a lethality of 99.9% is obtained after 24 hours (Konaté *et al.*, 2012).

Historically, the quantification of microbial cells used to be carried out using conventional plating which is a slow and labour-intensive procedure (Donlan and Costerton, 2002).

Recently, several surrogate assays were developed to assist in the quantification process. These assays are usually carried out in microtiter plates and can be categorized as viability assays (based on the quantification of viable cells), biofilm biomass assays (based on the quantification of living and dead cells as well as the matrix) and matrix quantification assays (based on the specific staining of matrix components) (Peeters *et al.*, 2008).

Viability stains such as tetrazolium salts can discriminate between viable and non-viable cells according to the metabolic activity of the living cells. Tetrazolium salts such as CTC XTT are composed of 5-cyano-2,3-ditolyl tetrazolium chloride and 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]- 2H-tetrazolium hydroxide respectively (Gabrielson *et al.*, 2002; McCluskey *et al.*, 2005). The XTT assay works by reducing the XTT dye into a water-soluble formazan when it encounters metabolically active cells (Roehm *et al.*, 1991). The number of the metabolically active microbial cells will therefore determine the material absorbance of the cell supernatant (Roehm *et al.*, 1991). According to the literature, XTT assays have been used in the quantification of the viable planktonic microbial cells (Gabrielson *et al.*, 2002) as well as biofilm cultures (Adam *et al.*, 2002; Pettit *et al.*, 2005; Honraet *et al.*, 2005).

Studies have proven that XTT-reduction assays can provide a semi-quantitative measurement of biofilm formation as it has shown an excellent correspondence between cellular density and metabolic activity (Hawser *et al.*, 1998; Honraet *et al.*, 2005; Jin *et al.*, 2005; Ramage *et al.*, 2001; Tellier *et al.*, 1992). The XTT-reduction assay is a non-destructive and non-invasive method of organism quantification. It has surpassed the conventional plating method in terms of time consumption as it requires minimal post-processing of samples (Pierce *et al.*, 2008).

Despite its popularity, Peeters and colleagues (2008) suggested that the outcomes of the XTT-reduction assay should be interpreted with caution as the results may vary when it is utilized to assess different microorganisms and different species of the same microorganism. Kuhn and colleagues (2003) reported that different fungal species and even strains from the same species show marked differences in their ability to metabolize the XTT substrate resulting in fluctuating results (Kuhn *et al.*, 2003). This finding is however almost always associated with fungi rather than bacteria, and several authors confirmed the reliability of the outcomes of the XTT assay when used for *E. faecalis* quantification. Comparable results

were found between the XTT assay and other bacterial quantification methods (Pourhajibagher *et al.*, 2016; Habib *et al.*, 2014).

Traditionally, the majority of the studies in the literature which evaluated the antimicrobial efficacy of iodoform-based materials, utilized the agar diffusion method and either investigated their action on microorganisms isolated from the root canals or freeze dried bacteria obtained from The National Collections of Type Cultures (NCTC) (De Paula *et al.*, 2015; Harini *et al.*, 2010; Jeeva and Retnakumari, 2014; Kriplani *et al.*, 2013; Navit *et al.*, 2016; Pabla *et al.*, 1997; Reddy and Ramakrishma, 2007; Sapna *et al.*, 2012; Wright *et al.*, 1994). A few authors employed the direct contact test (Pilownic *et al.*, 2017b; Estrela *et al.*, 2001a), while others implemented both ADT and DCT (Amorim *et al.*, 2006; Estrela *et al.*, 2001b).

Recently, Ordinola- Zapata *et al.*, (2015) and Pilownic and colleagues (2017a) adopted a new method where they used the “live/dead dyes” to differentiate between viable and non-viable bacteria using the Confocal Laser Scanning Microscopy. Pilownic *et al.* (2017a), in their experiment evaluated the relative effect of pure iodoform on the anti-biofilm efficacy of Vitapex.

To establish a superiority of one root filling material over another, the previously mentioned requisites should be explored in conditions mimicking the clinical scenario. For instance, the antimicrobial efficacy of these materials should be evaluated against microorganisms which are commonly isolated from inflamed or infected root canals. According to Marsh and Largent (1967), there is no difference between the microorganisms isolated from the root canals of deciduous and permanent teeth which are predominantly facultative anaerobic gram positive bacteria, such as *Enterococcus faecalis* (Punathil *et al.*, 2014). *Enterococcus faecalis* (*E. faecalis*) is the most commonly isolated microorganism in teeth with failed root canal therapy, displaying a prevalence of 12–90% (Siqueira and Rôças, 2004). It has also been cultured from teeth with primary root canal infections, particularly those with coronal leakage (Gomes *et al.*, 2006).

E. faecalis has certain qualities which escalate their antimicrobial resistance such as the capacity to adhere, colonize and form biofilms, as well as favour associations between species (Guerreiro-Tanomaru *et al.*, 2013). Additionally, *E. faecalis* has the ability to deeply

invade the dentinal tubules, resist routine chemo-mechanical canal preparation, and solely survive in filled canals without the support of other bacteria (Sundqvist *et al.*, 1998). All these factors contribute to the high virulence and resistance of *E. faecalis* (Kayaoglu and Ørstavik, 2004) and the low clinical success rate of root canal treatment associated with the presence of *E. faecalis* in the root canals at the time of obturation (Kriplani *et al.*, 2013). Thus, *E. faecalis* has been considered to be a standard organism when evaluating the antibacterial effect of root canal filling materials (Marsh and Largent, 1967; Cohen *et al.*, 1960).

The antibacterial efficacy of Kri 1 paste is not well documented in the literature and has never been explored against *E. faecalis*. To the best of our knowledge there are only two studies that compared the antibacterial effect of Kri 1 paste and Vitapex (Tchaou *et al.*, 1996; Minah and Coil, 1995). Tchaou *et al.*, (1996) used standard bacterial strains and reported moderate to no effect of Kri 1 paste when tested against gram positive facultative anaerobes, however *E. faecalis* was not included. On the other hand, Minah and Coil (1995) utilized microbial specimens which were directly isolated from the root canals of primary teeth. They reported a stronger antibacterial effect for Kri 1 paste when compared to Vitapex, but they did not disclose the organism which was tested. A similar iodoform-based paste (Gudes Pinto paste), which contains Rifocort in addition to the constituents of Kri 1 paste, was found to be only bacteriostatic against *E. faecalis* (Praetzel *et al.*, 2008). When Ørstavik (1981) evaluated the antibacterial effect of Kri 1 paste against *E. faecalis*, it was found to be bacteriostatic with a zone of inhibition of 9.4mm in diameter. Both Praetzel *et al.*, (2008) and Ørstavik (1981) used the agar well diffusion method in their studies. This might have drastically affected their results, as this method cannot differentiate between bactericidal and bacteriostatic bacterial inhibition and depends mainly on the solubility and diffusibility of the tested material which may vary with the different consistencies of the investigated materials (Balouiri *et al.*, 2016).

This present study will explore the antibacterial effect of Kri 1 paste, Vitapex and pure iodoform, in order to understand the relative role of iodoform in these compounds, against *E. faecalis* using the XTT assay.

CHAPTER 3

METHODOLOGY

3.1. Aims and Objectives

3.1.1. Aim

The aim of this study was to investigate the antibacterial effect of different iodoform-based preparations (i.e. Vitapex, Kri 1 paste and pure Iodoform) against *E. faecalis*.

3.1.2. Objectives

1. To evaluate the antibacterial efficacy of Vitapex against *E. faecalis* using the XTT assay at 0, 1, 2, 4 and 24 hours.
2. To evaluate the antibacterial efficacy of pure Iodoform against *E. faecalis* using the XTT assay at 0, 1, 2, 4 and 24 hours.
3. To evaluate the antibacterial efficacy of Kri 1 paste against *E. faecalis* using the XTT assay at 0, 1, 2, 4 and 24 hours.
4. To compare the antibacterial efficacy of pure Iodoform, Vitapex and Kri 1 paste against *E. faecalis* using the XTT assay.

3.1.3. Null hypothesis

There is no difference in the antibacterial efficacy of Vitapex, Kri 1 paste and pure Iodoform at 0, 1, 2, 4 and 24 hours.

3.2. Study design

An in vitro laboratory study was conducted at the Institute of Oral and Dental Research (IORD) at the Faculty of Dentistry, University of the Western Cape, Tygerberg campus.

3.3. Sample size

A statistician was consulted to assist with the estimation of the sample size. After doing a power calculation, 45 samples were found to be representative. The sample consisted of 45 wells equally divided into 5 groups i.e. a negative control group, a positive control group (baseline background) and three experimental groups (Vitapex, pure Iodoform and Kri 1 paste). Three wells were allocated to each of the groups and the experiment was repeated three times i.e. a total of nine wells per material.

3.4. Ethical considerations

The protocol was submitted to the Senate Research Ethics Committee of the University of the Western Cape for approval and permission to carry out the study was obtained (Ethics approval reference number: BM17/7/13). See Appendix A.

3.5. Materials and methods

3.5.1. *Materials*

Vitapex (Vitapex; Neodental International Inc., Federal Way, Wash., USA) and Kri 1 paste (Kri I paste, Pharmachemie. AG, Zurich, Switzerland) are premixed pastes. The pure Iodoform paste was prepared in the same manner as reported by Pilownic *et al.* (2017a), by mixing 1.0 g iodoform with one ml sterile water.

3.5.2. *Methods*

The current experiment was carried out following the steps demonstrated in Figure 3.5.2.1.

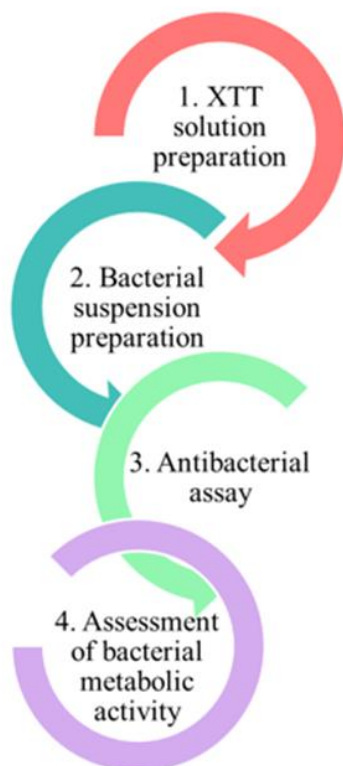


Figure 3.5.2.1: A diagram demonstrating the study methodology.

3.5.2.1. XTT preparation

As mentioned previously, the XTT assay provides an accurate and fast way of assessing cell viability and quantifies the number of cells as it measures the bacterial activity after each treatment. It produces a concentration dependent colour change according to the amount of viable cells. An increase in the number of living bacteria results in an increase in the overall activity of the enzymes in the respiratory chain of the bacteria in the sample, which directly correlates to the amount of orange formazan formed (Nordin *et al.*, 2015). The darker the colour, the greater the amount of viable bacteria present in the mixture.

The Cell Proliferation Kit II (XTT) is packaged in two bottles (i.e. XTT labeling reagent liquid and electron-coupling reagent liquid) which are stored at -80°C before use. After thawing in a waterbath at 37°C , the two components were reconstituted according to the manufacturer's instructions to obtain a clear solution. Thereafter, the vial was covered with aluminium foil to protect it from the light as the newly mixed solution is light sensitive (Pierce *et al.*, 2008).

3.5.2.2. Bacterial suspension preparation

The microorganisms used in this study were obtained from a standard stock culture collection stored at the Oral and Dental Research Institute, University of Western Cape. The National Collections of Type Cultures (NCTC) (Public Health England) of *E. faecalis* strains used was ATCC 29212.

E. faecalis (ATCC 29212) used in this study was freshly prepared from 18- to 24-hour cultures on an agar plate following the CLSI standards. This was done to be able to observe bacterial activity at its maximum potential during the log phase of growth rather than the lag and stationary phases. The lag phase is a growth phase in which the bacteria is unable to function and replicate at normal rates. This occurs when it is introduced to a new environment (Gardini *et al.*, 2001). Thereafter, the bacterial mitochondria and the respiratory chain enzymes re-establish their normal functions.

The microorganisms were suspended in a sterile brain heart infusion (BHI) (Oxoid, Basingstoke, UK) broth after which they were sub-cultured using the Streak Plate procedure, as described by Sanders (2012) to isolate bacterial colonies using the Quadrant Method. The purpose of this technique was to isolate pure bacterial culture from mixed populations in a petri plate by simple mechanical separation (Sanders, 2012). After preparing a safe and sterile workspace, a mixture of cells was spread over the surface of a semi-solid, agar-based nutrient medium in a petri dish using a heat sterilized metal loop, so that fewer and fewer bacterial cells were deposited at widely separated points on the surface of the medium (Figure 3.5.2.2). This was done to avoid the overlap of cells that may increase the probability of two or more bacteria giving rise to what appears to be a single colony (Sanders, 2012).

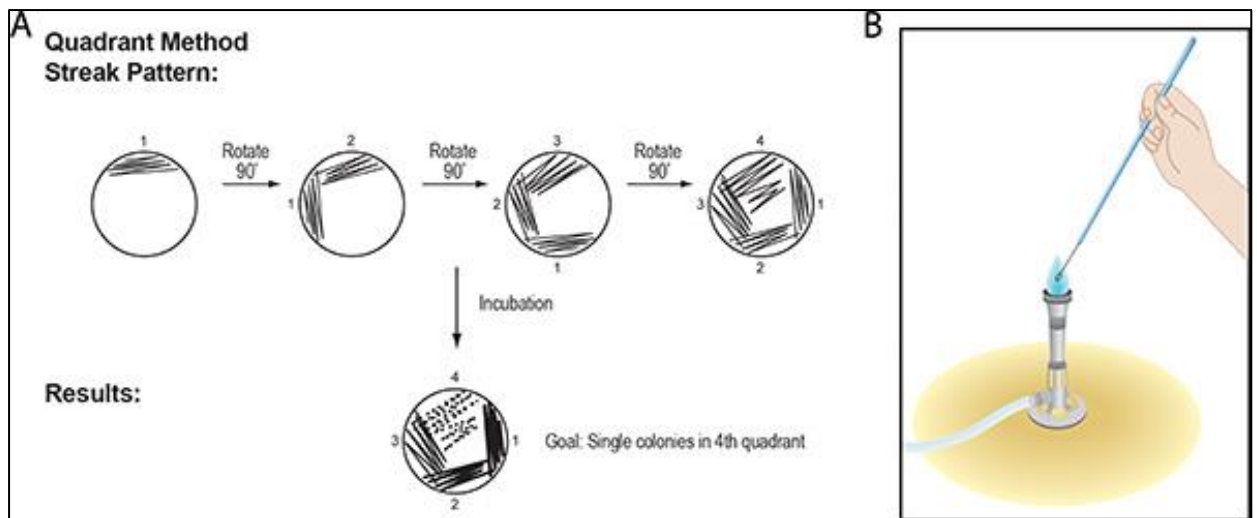


Figure 3.5.2.2: Streak plate procedure, Quadrant Method.

A: Quadrant method streak pattern; B: metal loop sterilization with Bunsen burner
(Courtesy: Sanders 2012)

Following the CLSI standards for dilution susceptibility tests, the previous step was followed by aerobic incubation of the plate at 37°C for 18 to 24 hours to ensure bacterial growth, allowing the streaked bacterial cells to develop into colonies. Under the same aseptic conditions, a well-isolated colony was transferred into phosphate buffered saline (PBS) in a glass tube. Using an isolated colony would result in uniformity of the sample and therefore, minimize the risk of bias. Theoretically, the cells of a single colony are genetically identical as they arise from a single cell undergoing binary fission (Sanders, 2012). The concentration of *E. faecalis* was then adjusted to 0.5 McFarland standard. McFarland Standards are used to gauge the approximate bacterial density in a liquid suspension by comparing the turbidity of the test suspension with that of the McFarland Standard either manually or electronically with devices such as the DensiCHEK Plus (BioMérieux, Inc Durham, USA), as in Figure 3.5.2.3.

DensiCHEK Plus is a device used to measure the optical density or the material absorbance of a microorganism suspension. This device provides values in McFarland units, proportional to the microorganism concentrations. In this experiment, the DensiCHEK Plus was used to adjust the turbidity of the *E. faecalis* suspension in Phosphate Buffered Saline (PBS). A fresh bacterial suspension was prepared each time the experiment was carried out to ensure the maintenance of the same bacterial turbidity of 0.5 McFarland standard throughout all the tested samples.



Figure 3.5.2.3: DensiCHEK Plus (BioMérieux, Inc Durham, USA).

3.5.2.3. Antibacterial assay

The antibacterial effect of the three iodoform preparations on *E. faecalis* was investigated by quantifying the bacterial metabolic activity using the Cell Proliferation Kit II (XTT) (Roche Diagnostics GmbH, Sandhofer, Mannheim, Germany). A 12-well cell culture plate was used.

Each of the three wells in the first column of the cell culture plate was loaded with 0.05 ml of Vitapex, dispensed using a sterile plastic syringe. Thereafter, 1.5ml of BHI (nutrient broth prepared according to the manufacturer's instructions) and 0.5ml of the prepared bacterial suspension were added using a micropipette. This process was repeated with pure Iodoform and Kri 1 paste in the second and third columns respectively i.e. three wells in the cell culture plate for each material as illustrated in Figure 3.5.2.4.

In the last well column of the same cell culture plate, similar amounts of each material being tested were dispensed separately in each of the three wells. Identical amounts of BHI broth were added and instead of the bacterial suspension, 0.5ml of a sterile PBS was used. This served as a positive control (baseline background) groups to enable comparison with similar wells that contain the same material together with the bacteria, in order to quantify the staining effect of the nutrient medium as well as the tested materials.

At the same time, in another cell culture plate, identical amounts of BHI broth and XTT solution were added to the tested bacteria adjusted at 0.5 McFarland. No test material was added to these wells and they served as a negative control.

The cell culture plates were incubated in a shaker incubator to ensure continuous contact between the medium and the tested materials. All the study samples were coded to facilitate the data collection. Codes were as follows: Negative control: Group Nc; Vitapex: Group V; Pure Iodoform: Group I; Kri 1 paste: Group K; Positive control groups (baseline background): Group Vpc, Ipc and Kpc.

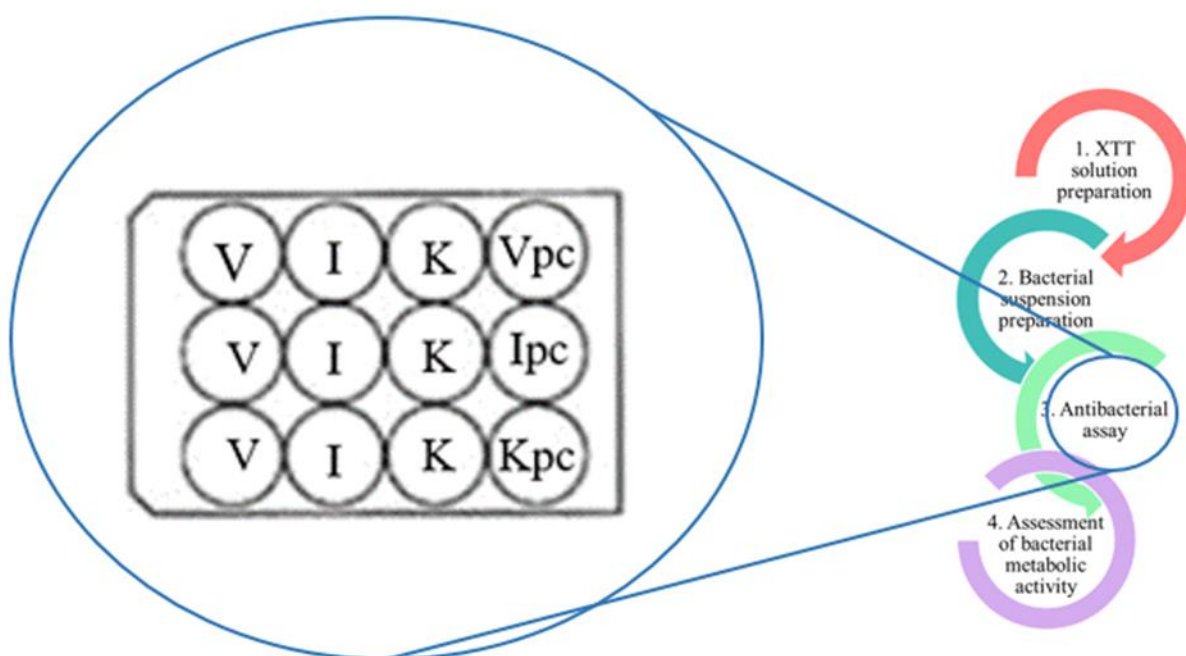


Figure 3.5.2.4: A diagram illustrating the antibacterial assay in the 12-well cell culture plate.

Different experimental groups (V, I and K) and positive control groups (Vpc, Ipc and Kpc).

3.5.2.4. Assessment of bacterial metabolic activity

The colour intensity of the orange formazan solution generated by the viable bacteria in all the study samples was quantified by a spectrophotometer and the cell proliferation kit II (XTT). The spectrophotometer is used to titrate the optical density (OD) or the absorbance of

material (Ab). It works by projecting a single wavelength light beam (450-600 nm) through a clear cuvette to measure the bacterial culture concentration within a suspension. The sample will absorb some of the light passing through from the beam depending on the degree of concentration of the solution (i.e. the more concentrated the solution, the more the light that is absorbed). The remaining light is quantified by a photometer which is inversely proportionate to the resultant material absorbance (Sutton, 2011).

From the mixture in the cell culture plate and using a micropipette, three aliquots of 100µl of the supernatant was transferred from each well and dispensed into three different wells in a pre-sterilized polystyrene flat-bottomed 96-well microtiter plate (Figure 3.5.2.5 and 3.5.2.6) at each time interval (0, 1, 2, 4 and 24 hours). 50 µl of XTT solution was added to each well with a multi-channel micropipette. After an incubation period of 4 hours at 37°C (as instructed by the manufacturer), the Ab values of these wells were scanned using the spectrophotometer at a wavelength of 450 nm. Fifteen minutes after adding the bacterial mixture in the cell culture plate, the first aliquots were transferred to be scanned to obtain the “zero hour” time interval reading. This allowed the bacteria to adjust to the new environment, escape the lag phase and secrete dehydrogenases that bind to the tetrazolium salt of the XTT to produce the colourful formazan (Berridge *et al.*, 1996).

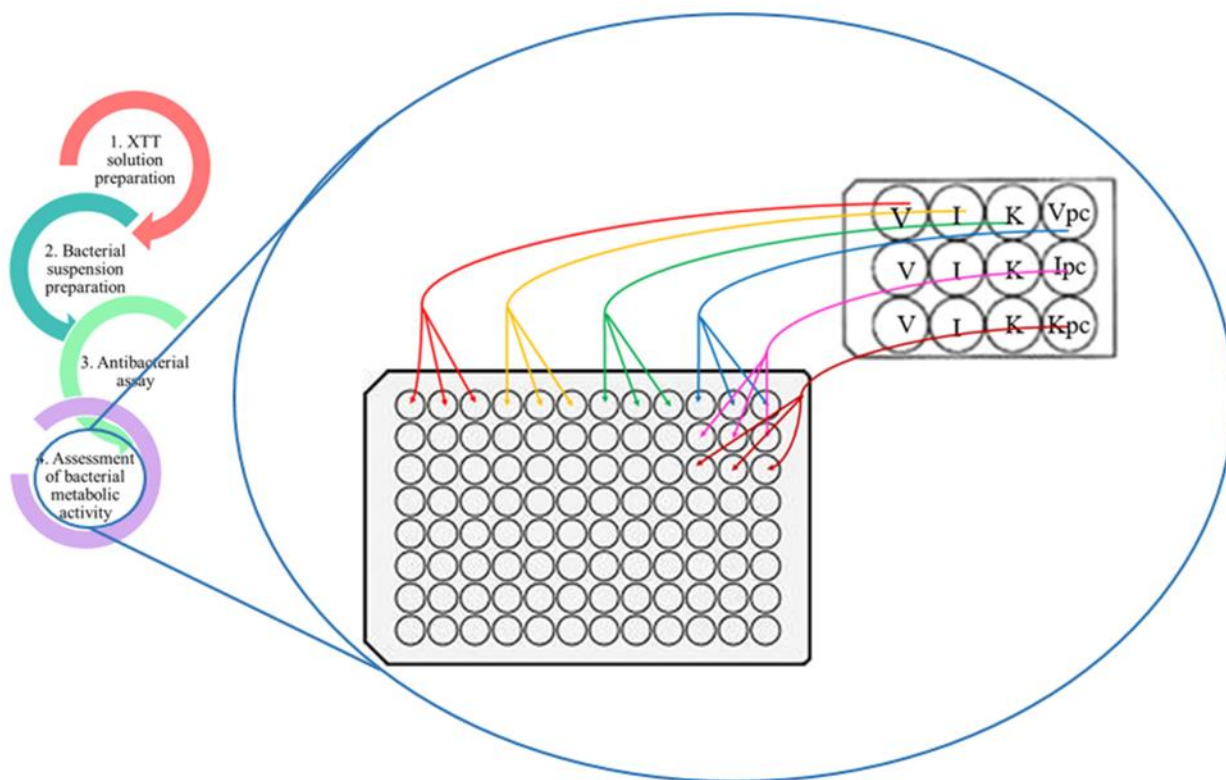


Figure 3.5.2.5: A diagram illustrating the assessment of bacterial metabolic activity in the 96-well microplate.

Three aliquots were taken at each time interval from each well in the cell culture plate and dispensed into different wells in the 96-well microplate where XTT was added..

As mentioned previously, 3 wells were allocated to each group (V, K, I, Nc and Pc). Thus a total of 9 readings (Figure 3.5.2.6) were taken from each of those wells at 5 different time intervals (0, 1, 2, 4 and 24 hours) i.e. a total of 45 readings form each group. The positive control wells in the cell culture plate were equally divided between the tested materials (Vpc, Kpc and Ipc) i.e. a total of three readings for the positive control of each material at each of the 5 investigated time intervals. The whole experiment was carried out in triplicate to ensure reproducibility of the results. A total of 135 readings were therefore taken for each of the 5 groups.

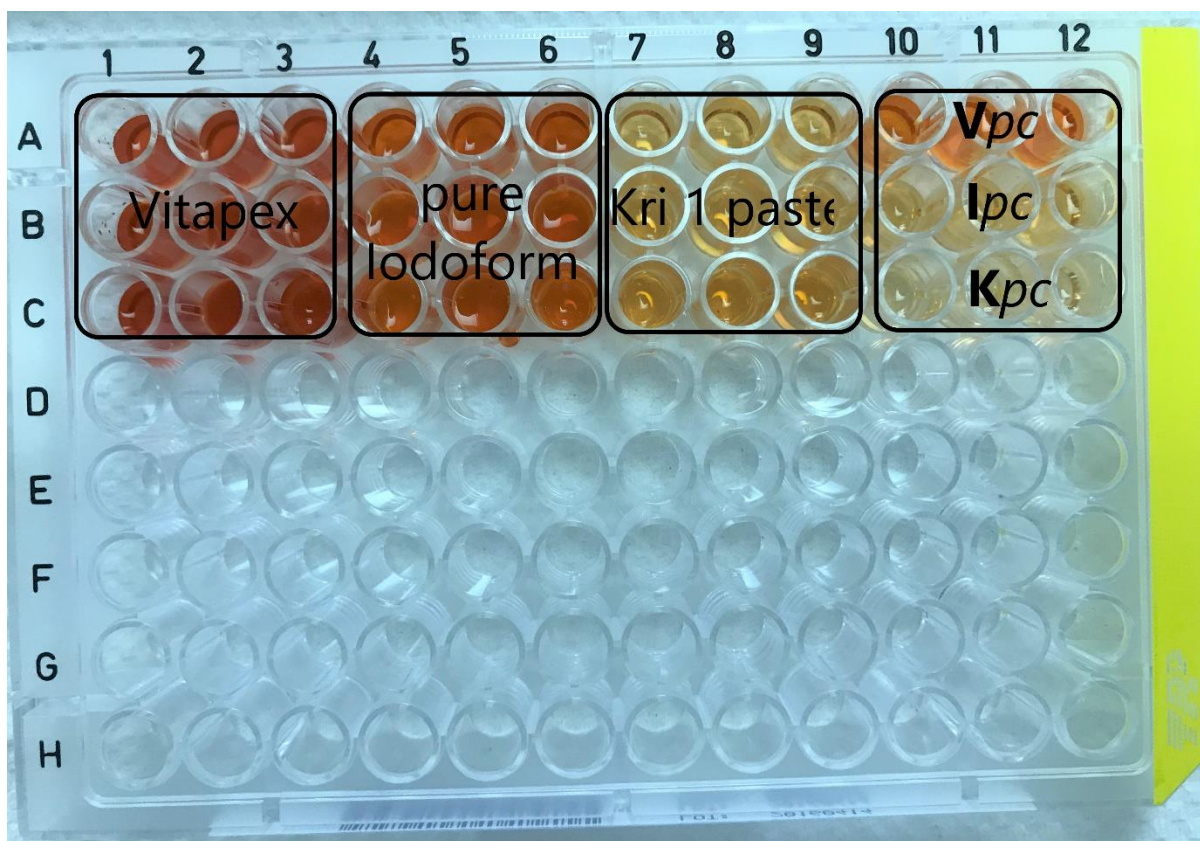


Figure 3.5.2.6: Coded bacterial metabolic activity assessment of the tested materials in a 96-well microplate.

From left to right (V: Vitapex; I: pure Iodoform; K: Kri 1 paste). Last three columns on the right were the baseline background reading (positive control) of each material as represented by different letters (Vpc, Ipc, Kpc).

3.5.3. Antibacterial activity evaluation

The antibacterial activity in this experiment was measured by the change in the material absorbance (Ab_{450}) values. An increase in the Ab in the scanned wells was either due to colour staining from the tested material, or increased bacterial metabolic activity. To eliminate the background effect, the final material absorbance (Ab) values were calculated by subtracting the mean material absorbance values of the baseline background (Ab_{450}) at each time interval from that of the corresponding experimental group.

An increase in the final Ab value indicated that the tested material failed to inhibit the bacteria and therefore had a reduced antibacterial effect. A reduction in this value is considered an indication of increased antibacterial effect of the tested material.

The results can be analyzed another way by calculating the percentage of remaining viable *E. faecalis* cells from the (Ab_{450}) mean value of the negative control at the corresponding time interval, as modified from Pettit *et al.*, (2005) according to the equation below:

$$\text{Percentage of viable cells} = \frac{\text{Ab of the material}}{\text{Ab of the negative control}} \times 100$$

If <50% of cells remain viable, the tested material is described as having an antibacterial effect. With $\geq 50\%$ cells remaining viable, little or no antibacterial activity is evident (Pettit *et al.*, 2005).

3.5.4. Pilot Study

A pilot study was carried out to measure the material absorbance of a serially diluted *E. faecalis* suspension with the aid of the XTT. The purpose of this process was to calibrate *E. faecalis* and generate a standard growth curve. It was also carried out to optimize the method so as to control any variable that might affect the results in the actual experiment such as the condition of the spectrophotometer. This session also provided the estimated time that is required to carry out the data collection as well as assessing the adequacy of the data capture sheet.

3.6. Validity and Reliability

The experiment was carried out in triplicate under aseptic conditions in a laminar flow hood to lessen contamination of the experimental materials with airborne contaminants, thereby minimizing the errors and ensuring reproducibility of the results (Coriell and McGarrity, 1968). Checks for contamination with gram staining were carried out at the end of each experiment for all the wells in the cell culture plate following a protocol described by Claus (1992). This was done to confirm the purity of the tested samples and to rule out any contaminant that may affect the results (Bartholomew and Mittwer, 1952).

3.7. Data analysis

Results were captured on an excel spreadsheet. Codes for each material (as previously described) were recorded.

3.7.1. *Statistical analysis*

The mean and standard deviation of the Ab values of all the samples from different investigated groups at each time interval were calculated. Statistical comparisons were carried out between each group and the negative control as well as across the different groups at each time interval, using the Two Way Mixed Measures ANOVA. A p-value of less than 0.05 was considered statistically significant.

CHAPTER 4

RESULTS

One of the wells of the cell culture plate in which the antibacterial activity of the pure Iodoform was evaluated, was found to be contaminated with another bacterial strain. This contamination was confirmed with gram staining, thus the Ab readings of the corresponding three wells in the flat-bottomed 96-well microtiter plate were omitted from the results. Additionally, to ensure an equal sample size across the experimental groups, the same well was omitted from the other experimental groups along with their corresponding Ab readings. The final sample size from the cell culture plate was therefore 8 wells as opposed to 9 (for each material).

The final Ab values of the experimental groups were calculated by subtracting the Ab means of the positive control (baseline background) readings (Tables 4.1.1, 4.2.1 and 4.3.1) from the corresponding group Ab mean value at each time interval. The final Ab mean values were compared with each other and with the negative control (without test material) (Tables 4.1.2, 4.2.2 and 4.3.2).

4.1. Antibacterial efficacy of Vitapex

Table 4.1.1: Mean material absorbance (Ab_{450}) of Vitapex and Vitapex positive control (baseline background) (V_{pc}) at each time interval and the final (Ab_{450}) Vitapex.

Time (hours)	0	1	2	4	24
(Ab ₄₅₀) Group V	0.043333	0.128667	0.397667	0.868333	1.165667
	0.082	0.085	0.350667	0.840333	1.113333
	0.103333	0.174667	0.343667	1.005333	1.150333
	0.011	0.114	0.102	1.203667	1.542333
	0	0.005333	0.147333	1.301667	1.597
	0.035	0.022667	0.106667	1.219667	1.483667
	0.047	0.163667	0.196	0.894667	1.419333
	0.062	0.154	0.184667	0.812667	1.462
Average (Group V)	0.047958	0.106	0.228583	1.018292	1.366708
V_{pc}	0.129	0.150167	0.259417	0.294167	1.121667
Final (Ab ₄₅₀) V	-0.08104	-0.04417	-0.03083	0.724125	0.245042

Table 4.1.2: Comparison of the final mean (Ab_{450}) values of Vitapex and the negative control (bacteria only) at each time interval.

Time Hours (hrs)	Vitapex		Negative Control		Contrast	Std. err	P-value	Confidence interval
	Mean	SD	Mean	Std. Dev.				
0	-0.08104	0.044708	0.08225	0.057783	-0.16329	0.01402	<0.001	-0.190770 to -0.135812
1	-0.04417	0.075173	0.241375	0.028871	-0.28555	0.016431	<0.001	-0.317746 to -0.2533372
2	-0.03083	0.126086	0.6125	0.035344	-0.64333	0.031688	<0.001	-0.7054414 to -0.5812253
4	0.724125	0.193695	0.892542	0.063093	-0.16842	0.049082	0.001	-0.2646159 to -0.0722174
24	0.245042	0.190238	1.164375	0.048767	-0.91933	0.057313	<0.001	-1.031665 to -0.8070015

4.2. Antibacterial efficacy of Kri 1 paste

Table 4.2.1: Mean material absorbance (Ab_{450}) of Kri 1 paste and Kri 1 paste positive control (baseline background) (Vpc) at each time interval and the final (Ab_{450}) Kri 1 paste.

Time (Hours)	0	1	2	4	24
(Ab ₄₅₀) Group K	0.106667	0.04	0.140667	0.152667	0.305
	0.088333	0.094	0.104667	0.133	0.323
	0.049333	0.078667	0.126333	0.174	0.407
	0.005667	0.210667	0.495667	0.330333	0.540667
	0.006	0.128667	0.355333	0.252333	0.485667
	0.021	0.114333	0.399667	0.293333	0.591667
	0.024667	0.139667	0.11	0.365	0.553
	0.002333	0.094667	0.078667	0.389333	0.843
Average (Group K)	0.038	0.112583	0.226375	0.26125	0.506125
<i>Kpc</i>	0.022917	0.124167	0.240833	0.152583	0.3755
Final (Ab ₄₅₀) K	0.015083	-0.01158	-0.01446	0.108667	0.130625

Table 4.2.2: Comparison of the mean (Ab_{450}) values of Kri 1 paste and the negative control (bacteria only) at each time interval.

Time hrs	Kri 1 paste		Negative Control		Contrast	Std. err	P-value	confidence interval
	Mean	SD	Mean	Std. Dev.				
0	0.015083	0.046438	0.08225	0.057783	-0.06717	0.01402	<0.001	-0.0946456 to -0.0396877
1	-0.01158	0.054466	0.241375	0.028871	-0.25296	0.016431	<0.001	-0.2851628 to -0.2207539
2	-0.01446	0.16728	0.6125	0.035344	-0.62696	0.031688	<0.001	-1.146082 to -0.9214181
4	0.108667	0.095998	0.892542	0.06093	-0.78388	0.049082	<0.001	-0.8800742 to -0.6876758
24	0.130625	0.165751	1.164375	0.048767	-1.03375	0.057313	<0.001	-1.146082 to -0.9214181

4.3. Antibacterial efficacy of pure Iodoform

Table 4.3.1: Mean material absorbance (Ab_{450}) of Iodoform and Iodoform positive control (baseline background) (V_{pc}) at each time interval and the final (Ab_{450}) Iodoform.

Time (Hours)	0	1	2	4	24
(Ab ₄₅₀) Group I	0.027	0.119	0.169333	0.445	0.737
	0.074667	0.15	0.068667	0.451333	0.602333
	0.032333	0.180333	0.09	0.430333	0.535
	0.002333	0.086	0.173667	1.025	1.095
	0	0.071333	0.141333	1.003333	1.062
	0.026	0.077867	0.176667	0.972667	1.073667
	0.059667	0.129	0.148333	0.846	1.353667
	0.034667	0.060333	0.171667	0.776667	1.327333
Average (Group I)	0.032083	0.109233	0.142458	0.743792	0.97325
I_{pc}	0.034667	0.098583	0.215083	0.205167	0.804917
Final (Ab ₄₅₀) I	-0.00258	0.01065	-0.07263	0.538625	0.168333

Table 4.3.2: Comparison of the (Ab_{450}) values of pure Iodoform and the negative control (bacteria only) at each time interval

Time Hrs	Iodoform		Negative Control		Contrast	Std. err	P-value	Confidence interval
	Mean	SD	Mean	Std. Dev.				
0	-0.00258	0.044055	0.08225	0.057783	-0.08483	0.01402	<0.001	-0.1123123 to -0.0573544
1	0.01065	0.05923	0.241375	0.028871	-0.23073	0.016431	<0.001	-0.2629295 to -0.1985205
2	-0.07263	0.055404	0.6125	0.035344	-0.68513	0.031688	<0.001	-0.747233 to -0.623017
4	0.538625	0.254795	0.892542	0.063093	-0.35392	0.049082	<0.001	-0.4501159 to -0.2577174
24	0.168333	0.302702	1.164375	0.048767	-0.99604	0.057313	<0.001	-1.108374 to -0.8837098

Results were analyzed according to the change in the Ab values which was proportionate to the percentage of remaining viable *E. faecalis* cells and inversely related to the antibacterial effect of the tested material. A higher Ab value or increased percentage of viable cells indicates a reduction in the antibacterial efficacy of the investigated material and vice versa.

4.4. Inter-group comparison

Table 4.4.1: Average of (Ab₄₅₀) values at all investigated time intervals for Vitapex, pure Iodoform, Kri 1 paste and the negative control groups

Group	Summary of total Ab		
	Mean	Std. Dev.	*Freq.
Negative control	0.598608	0.473162	120
Vitapex	0.162625	0.334516	120
pure Iodoform	0.12848	0.283852	120
Kri 1 paste	0.045667	0.131623	120

*Freq. (frequency) refers to the number of observations (scanned wells).

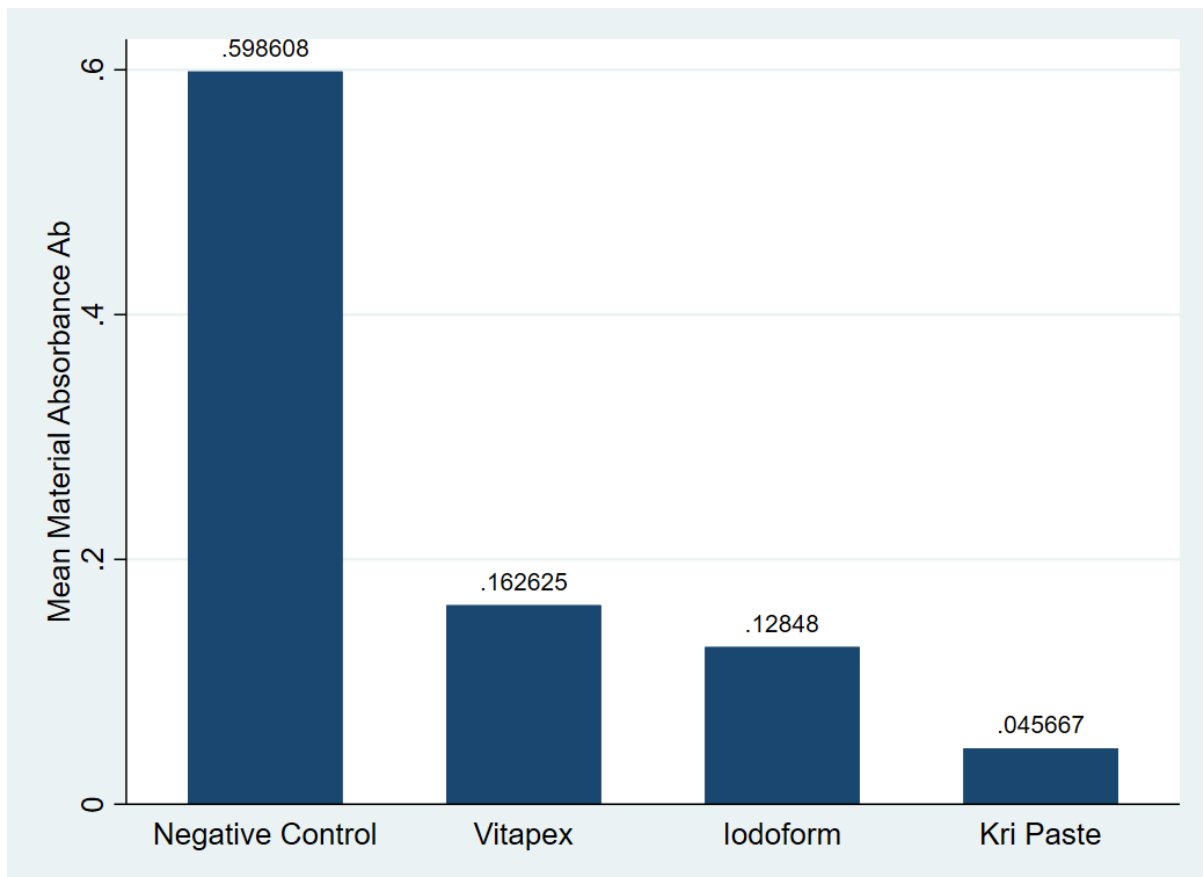


Figure 4.4.1: Average of material absorbance (Ab_{450}) values at all investigated time intervals for Vitapex, pure Iodoform, Kri 1 paste and negative control

As seen in tables 4.1.2, 4.2.2 and 4.3.2, the Ab values of the negative control (without test material) increased throughout the investigated time periods and were significantly larger (p -value < 0.001) than all the other experimental groups (V, I, K).

The results of the Two Way Mixed Measures ANOVA showed that all the investigated materials had a significant antibacterial effect on *E. faecalis* when compared with the negative control ($p < 0.001$).

Overall, *E. faecalis* levels (as represented by the average Ab value among all the investigated time intervals) displayed the highest mean value for the negative control, 0.599 (SD 0.40), followed by Vitapex 0.163 (SD 0.33); Iodoform 0.128 (SD 0.28) and Kri 1 Paste 0.046 (SD 0.13) (Table 4.4.1 and Figure 4.4.1).

Table 4.4.2: Pairwise comparison between the Ab values of pure Iodoform and Vitapex at each time interval.

Time	Contrast pure Iodoform Vs Vitapex	Std. err	Confidence interval	P-value
0	0.0784583	0.01402	0.0509794 to 0.1059373	<0.001
1	0.0548157	0.016431	0.226122 to 0.0870211	0.001
2	-0.0417917	0.031688	-0.1038997 to 0.0203164	0.187
4	-0.1855	0.049082	-0.2816992 to -0.0893008	<0.001
24	-0.0767083	0.057313	-0.1890402 to 0.0356235	0.181

Table 4.4.3: Pairwise comparison between the Ab values of Kri 1 paste and Vitapex at each time interval.

Time	Contrast Kri 1 paste Vs Vitapex	Std. err	Confidence interval	P-value
0	0.096125	0.01402	0.068646 to 0.123604	<0.001
1	0.0325833	0.016431	0.0003789 to 0.064787	0.047
2	0.016375	0.031688	-0.045733 to 0.078483	0.605
4	-0.6154583	0.049082	-0.7116576 to -0.5192591	<0.001
24	-0.1144167	0.057313	-0.2267485 to -0.0020848	0.046

Table 4.4.4: Pairwise comparison between the Ab values of Kri 1 paste and pure Iodoform at each time interval.

Time	Contrast Kri 1 paste Vs pure Iodoform	Std. err	confidence interval	P-value
0	0.0176667	0.01402	-0.0098123 to 0.0451456	0.208
1	-0.0222333	0.016431	-0.0544378 to 0.0099711	0.176
2	0.0581667	0.031688	-0.0039414 to 0.1202747	0.066
4	-0.4299583	0.049082	-0.5261576 to -0.3337591	<0.001
24	-0.0377083	0.057313	-0.1500402 to 0.0746235	0.511

Table 4.4.5: Percentage of remaining viable cells in Vitapex, pure Iodoform and Kri 1 paste groups relative to the negative control at each time interval

Time Hrs	Percentage of viable cells		
	Vitapex	Iodoform	Kri 1 paste
0	-68.49%	-3.14%	24.11%
1	11.23%	18.28%	-5.66%
2	36.31%	-11.86%	-7.57%
4	88.15%	60.35%	11.81%
24	44.54%	14.46%	12.03%

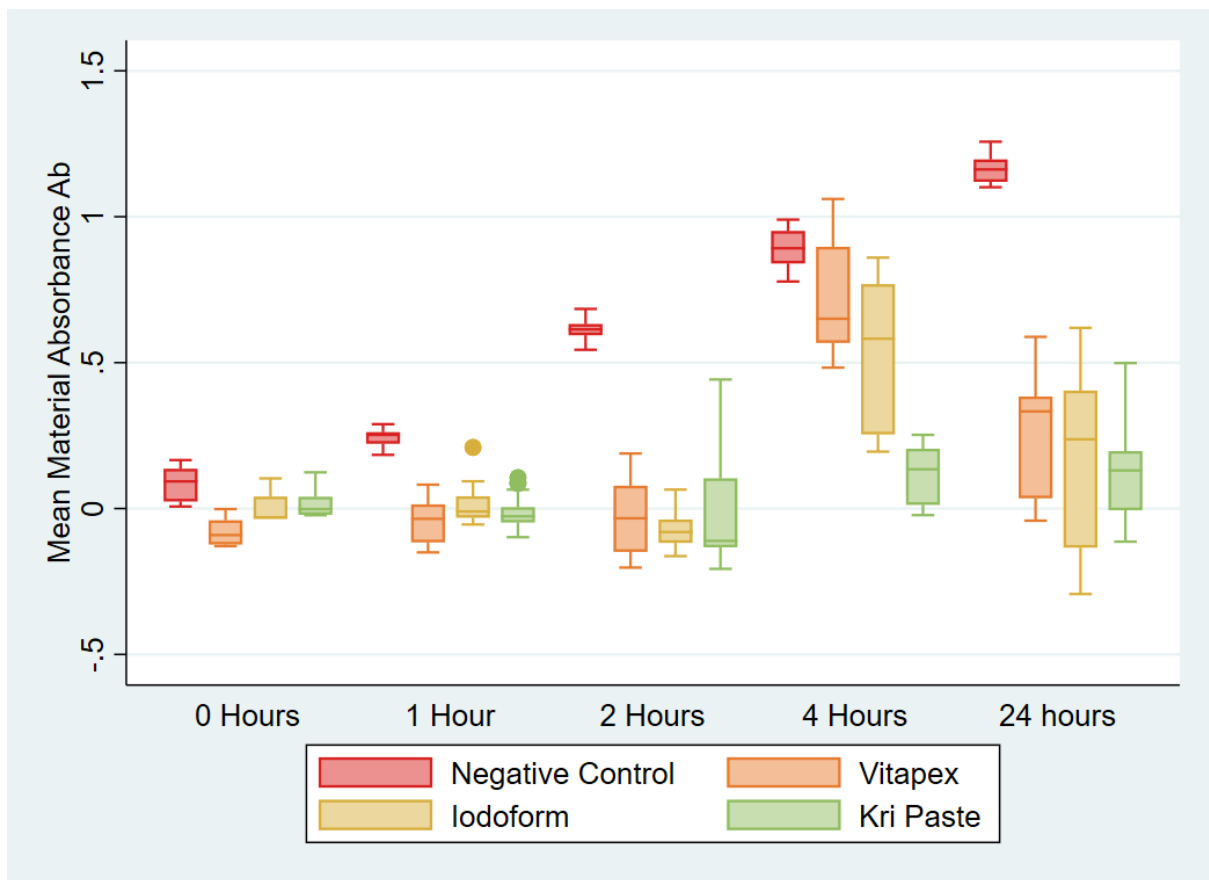


Figure 4.4.2: Comparison of Ab values of Vitapex, Pure Iodoform, Kri 1 paste and negative control at time 0, 1, 2, 4 and 24 hours

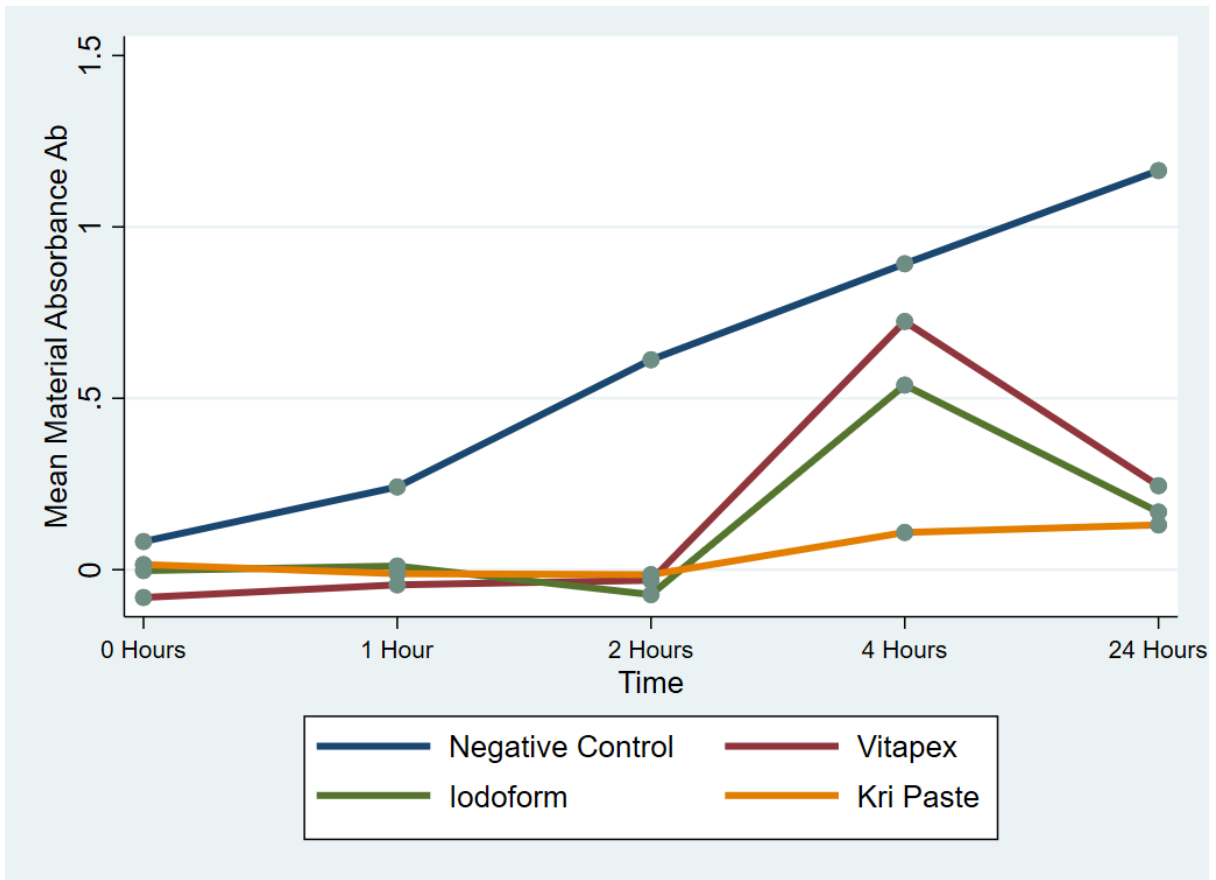


Figure 4.4.3: Comparison of the mean Ab values of Vitapex, pure Iodoform, Kri 1 paste and negative control over the investigated time period

CHAPTER 5

DISCUSSION

The baseline background groups (positive control) showed that the material absorbance (Ab) values of the mixture of the different tested materials within the nutrient medium and the XTT changed over time (Tables 4.1.1, 4.2.1 and 4.3.1). This confirmed that the final Ab values can be affected by bacterial growth as well as the stains generated by the investigated materials and the nutrient medium (Sutton, 2011).

In the negative control group (without experimental materials), *E. faecalis* was allowed to proliferate freely in the BHI broth medium, and was not subjected to any treatment that might hamper their normal growth. The material absorbance (Ab) of aliquots from the mixture were then measured using the spectrophotometer after the addition of the XTT at each time interval (0, 1, 2, 4 and 24 hrs). An incremental increase was registered for the Ab mean values of the negative control samples over time (Tables 4.1.2, 4.2.2, 4.3.2 and Figure 4.4.3). The longer *E. faecalis* was left in the nutrient medium to proliferate, the greater the amount of viable bacterial cells in the mixture which were able to reduce tetrazolium salts into the orange formazan and hence the larger the Ab value.

In general, the final material absorbance values were either increased due to bacterial growth or colour staining, or decreased as a result of bacterial inhibition. Where the final Ab values are negative, the overall increase in the Ab values in the samples of the corresponding experimental group could be attributed to the staining effect rather than the bacterial growth as seen in some of the final mean Ab values of the experimental groups. This was confirmed by the increased Ab values of baseline background readings that exceeded the readings in the corresponding experimental group and hence the negative final Ab values (Tables 4.1.1, 4.2.1 and 4.3.1).

5.1. Antibacterial efficacy of Vitapex

In the current experiment, the antibacterial potential of Vitapex against *E. faecalis* was analysed (Tables 4.1.1, 4.1.2 and Figure 4.4.3). The bacterial metabolic activity, as measured by the final Ab value, increased slightly during the first two hours of incubation. Then it surged to reach a mean value of 0.72 (SD 0.193695) at 4 hours before it dropped to 0.25 (SD 0.190238) after 24 hours (Table 4.1.1). When compared with the negative control (Table 4.1.2), the difference was statistically significant ($p < 0.001$) at all the investigated time intervals. This implies an inhibitory effect of Vitapex on *E. faecalis*. This was validated by the results of various studies which verified the antibacterial efficacy of Vitapex on *E. faecalis*, either as a separate strain (Pilownic *et al.*, 2017b; De Paula *et al.*, 2015; Harini *et al.*, 2010) or as part of a bacterial biofilm (Pilownic *et al.*, 2017a).

Only one study in the literature by Pilownic *et al.*, (2017b) investigated the antibacterial effect of Vitapex against *E. faecalis* at 1 hour and 4 hour intervals. The Direct Contact Test (DCT) was utilised and the Colony Forming Units were calculated to measure the survival rates of the bacteria. They found that the remaining viable bacterial cells were 62% and 48% after 1 and 4 hours respectively. The results of the present study showed that the survival rates of *E. faecalis* after the same time intervals was 11.23% and 88.15% respectively (Table 4.4.5). The discrepancy between their results and those of the present study could be due to the different methodologies used.

The fluctuation in the survival rates of *E. faecalis* across the different time intervals in the Vitapex group could be explained by the limited bacteriostatic effect of Ca(OH)_2 which is a major constituent of Vitapex. As reported by various studies, the antibacterial effect of Ca(OH)_2 is derived from the instantaneous basic environment ($\text{pH}=12.5$) generated by the liberation of the hydroxyl ions (Estrela and Holland 2003; Siqueira and Lopes, 1999; Kontakiotis *et al.*, 1995). This alkalinity injures the bacterial cytoplasmic membrane and thus reduces bacterial metabolic activity (Estrela and Holland 2003; Kontakiotis *et al.*, 1995). As advocated by Evans and colleagues (2002), the proton pump activity of *E. faecalis* can act as a protective mechanism against the high alkalinity generated by the Ca(OH)_2 . At the same time, *E. faecalis* escalates the production of the extracellular polymeric substance which protects the cell wall from the alkaline environment (Chávez de Paz *et al.*, 2007). Additionally, *E. faecalis* may enter the viable but non-cultivable (VBNC) state (a survival mechanism adopted by many bacterial strains when exposed to environmental stress), and

resuscitate upon returning to favourable conditions (Lleo *et al.*, 2001). Thus, by the time the high alkalinity generated by the Ca(OH)₂ was buffered by the surrounding environment, the metabolism of *E. faecalis* returned to normal rates. All of these reasons may explain the surged Ab value at 4 hours of incubation.

The results of the present study revealed a significant reduction in the survival rate of *E. faecalis* when it was subjected to Vitapex. After 24 hours, the survival rate was 44.54% relative to the negative control (Table 4.4.5). Pilownic *et al.*, (2017b) used the DCT to show that Vitapex was able to eliminate more than 98% of *E. faecalis* after 24 hours. However this contradicts what was proven by De Paula *et al.*, (2015) who reported a minimal inhibitory efficacy of Vitapex on *E. faecalis* using the Agar diffusion Test (ADT). These variations could be due to the diverse methodologies, experimental conditions, inoculum size and age, culture medium, and the variation in the species of the bacterial strains used. The varied results obtained when different methodologies were used was further confirmed by Amorim and colleagues (2006) in their experiment. They found that *E. faecalis* was inhibited by Vitapex when the Direct Contact Test was used while no inhibitory zone was apparent when the Agar Diffusion method was employed. Since the current study was carried out using broth mediums, the inhibitory effect of Vitapex on *E. faecalis* was not affected by the confounding factors of the Agar diffusion method. This is because the material solubility and diffusibility issues were diminished by incorporating a shaker incubator which ensures a direct and continuous contact between the investigated material and the suspended *E. faecalis*.

5.2. Antibacterial efficacy of Kri 1 paste

The overall antibacterial effect of Kri 1 paste was superior to that of the other investigated materials (Figure 4.4.1). There was a superior inhibitory effect on *E. faecalis* during the first two hours when compared to the rest of the investigated time periods. This was followed by a steady elevation in the bacterial metabolic rate as represented by an Ab value reaching (0.13₄₅₀) (SD 0.165751) by 24 hours (Table 4.2.2 and Figure 4.4.3). However, despite this elevation, the final Ab value was still significantly lower than that of the negative control, which confirms the inhibitory capacity of Kri 1 paste on *E. faecalis* (Table 4.2.2).

The inhibitory effect during the first two hours (Table 4.2.2), is most likely attributed to the antimicrobial potential of parachlorophenol and camphor on *E. faecalis*. According to Cerqueira *et al.*, (2007) camphor and parachlorophenol are potent antimicrobial substances. Their efficacy on *E. faecalis* can be gauged by the results of various studies. For instance, Menezes *et al.*, (2004) reported that a mixture of Calcium Hydroxide and camphorated paramonochlorophenol (CPCP) paste was the most effective for the elimination of *E. faecalis* when compared to other commonly used intra-canal medicaments (Menezes *et al.*, 2004). Since Ca(OH)₂ is reported to have a weak bacteriostatic or limited effect on *E. faecalis* (Kim and Kim, 2014; Gomes *et al.*, 2009; Lee *et al.*, 2013; Estrela *et al.*, 1999), the efficacy of Calcium Hydroxide and camphorated paramonochlorophenol compound is most likely attributed to the CPCP portion. Harrison and Madonia (1970) found that *E. faecalis* was successfully inhibited with a very small concentration (less than 1%) of CPCP. This concurs with the current study which showed that Kri 1 paste (which contains approximately 7% CPCP) is a potent inhibitor of *E. faecalis*.

5.3. Antibacterial efficacy of pure Iodoform

Pure Iodoform is not used as a root canal filling material in clinical settings. It was only tested in this experiment to evaluate the relative role in the antibacterial efficacy of the other two investigated materials. The antibacterial effect of Iodoform is related to the dissociation of the iodine ions. These ions are responsible for precipitating proteins and oxidizing enzymes that are essential for bacterial metabolism (Thomas *et al.*, 1994).

The final Ab values of the pure Iodoform group, as seen in Table 4.3.2 and Figure 4.4.3, fluctuated during the first two hours of incubation which was followed by a marked increase to reach 0.54 (SD 0.254795) after 4 hours (Table 4.3.2). This reduced antibacterial efficacy of pure Iodoform could be due to the development of the protective mechanism of *E. faecalis* and the production of the extracellular polymeric substance. This mechanism protects the *E. faecalis* cell wall from the inhibitory effect of Iodoform in the surrounding environment. After 24 hours of incubation, pure Iodoform drastically inhibited *E. faecalis* activity where an Ab value of 0.17 was registered (Table 4.3.2). This can be attributed to the antibacterial effect of the diffusing iodine ions which possibly overcame the bacterial protective mechanism (Thomas *et al.*, 1994). The survival rate of *E. faecalis* was 14.46% after 24 hours of being exposed to pure Iodoform (Table 4.4.5). This is in agreement with the results of Pilownic *et*

al., (2017a) who adopted the Direct Contact Test along with the laser scanning microscopy to evaluate the effect of pure Iodoform against a multispecies biofilm including *E. faecalis* in anaerobic conditions over a 7- and 30-day period. They implemented certain dyes i.e. the live/dead dye, to differentiate between viable and non-viable bacteria. They found that 41% and 69% of the bacteria were killed at 7 and 30 days respectively (Pilownic *et al.*, 2017a). Thus, in both studies, pure Iodoform had an inhibitory effect on *E. faecalis* and the low survival rates in the present study could be because planktonic bacteria was evaluated instead of the more resistant bacterial biofilm assessed by Pilownic and his colleagues (2017a).

5.4. Inter-group comparison

In the current experiment, the results of the Two Way Mixed Measures ANOVA (Tables 4.1.2, 4.2.2 and 4.3.2) showed that there was a statistically significant difference in the final Ab mean values across the experimental and the negative control groups ($p < 0.001$). This was confirmed by the confidence interval which did not include the value of zero effect, indicating that the investigated preparations already started their effect on *E. faecalis*. The Ab mean values of the negative control were significantly larger than those of the other experimental groups at all the investigated time intervals (Tables 4.1.2, 4.2.2 and 4.3.2). This showed that all the researched materials had an antibacterial activity against *E. faecalis* which is on par with studies that have proven that iodoform-based preparations have an inhibitory effect against *E. faecalis* (Pilownic *et al.*, 2017b; Navit *et al.*, 2016). This result however contradicts what was found by several studies which reported a poor effect (Kriplani *et al.*, 2013) or even no antibacterial activity (Reddy and Ramakrishma, 2007; Jeeva and Retnakumari, 2014) of iodoform-based products like Vitapex and Metapex on *E. faecalis*. This contradiction arises from the use of the Agar Diffusion Test (ADT) in those studies which most likely reflected the material solubility and diffusibility on the solid agar mediums. Contrary to this, the methodology used in the current study overcame this major confounding factor by utilizing a shaker incubator and broth mediums instead of the solid agar mediums. On the other hand, the variable results reported by studies that utilized ADT could be explained by the diverse species of *E. faecalis* that was tested (Siqueira and Lopes, 1999).

The overall effectiveness of pure Iodoform 0.12848 (SD 0.283852) on the inhibition of *E. faecalis* metabolism was superior to that of Vitapex 0.162625 (SD 0.334516) and less than Kri 1 paste 0.045667 (SD 0.131623) (Table 4.4.1 and Figure 4.4.1). The disparity in their effectiveness could be attributed to the antibacterial efficacy of their basic constituents (Iodoform, Ca(OH)₂, CPCP). For instance, all the investigated materials share the presence of Iodoform in their chemical composition but in different quantities or ratios relative to the rest of the contents. This might be one of the reasons behind the discrepancy in the antibacterial effect of the tested materials.

Additionally, those basic constituents may have a varied inhibitory effect on *E. faecalis*. As reported by various authors, Ca(OH)₂ has a weak bacteriostatic or no inhibitory effect on *E. faecalis* (Farhad and Mohammadi, 2005; Sathorn *et al.*, 2007; Mohammadi *et al.*, 2012), unlike camphorated parachlorophenol which is reported to be potent against the same bacteria (Harrison and Madonia, 1970). Thus, the unique presence of CPCP in Kri 1 paste may have contributed to the superior antibacterial effect of this agent on *E. faecalis* when compared to pure Iodoform and Vitapex. On the contrary, the depleted effect of Vitapex on *E. faecalis* could be due to the presence of Ca(OH)₂ (one of the major constituents of Vitapex) in this paste instead of CPCP.

Hence, Kri 1 paste outperformed pure Iodoform due to the additive effect of CPCP, and both materials had a better inhibitory effect than Vitapex as the ratio of iodoform was larger relative to the other contents. The results of the current study corroborate the superiority of camphorated parachlorophenol to Ca(OH)₂ in inhibiting *E. faecalis*. This has been proven by many authors (Sukawat and Srisuwan, 2002; Gomes *et al.* 2002; De Almeida *et al.* 2002; Siqueira and de Uzeda, 1996; Haapasalo and Ørstavik 1987).

The significantly superior effect of Kri 1 paste compared to Vitapex seems to contradict the results of Byström *et al.*, (1985) who concluded that Calcium Hydroxide had a higher antibacterial effect than camphorated parachlorophenol when they were studied in-vivo. They found that more bacteria, (including *E. faecalis*), was isolated from the root canals dressed with camphorated phenol and parachlorophenol than those treated with Ca(OH)₂. However, their results might be biased because the teeth from the Ca(OH)₂ group were treated differently from those of the camphorated phenol and parachlorophenol groups. For instance, they used a higher concentration of the irrigant solution (Sodium Hypochlorite) in the Ca(OH)₂ group which might have resulted in their superior potency on bacterial inhibition.

They also implemented an uneven evaluation period. Samples from the CPCP group were evaluated after 2 weeks for bacterial sampling and detection while those of the Ca(OH)₂ group were re-evaluated after 1 month. Thus more bacteria might have been inhibited by Ca(OH)₂ as it was left inside the root canals for twice as long as CPCP.

At zero hours (i.e. 15 minutes of incubation), Vitapex outperformed pure Iodoform which was in turn superior to Kri 1 paste in inhibiting *E. faecalis* (Tables 4.4.5 and Figure 4.4.2). This difference could be due to the instant action of Ca(OH)₂ which is uniquely present in Vitapex (Estrela and Holland 2003; Kontakiotis *et al.*, 1995; Siqueira and Lopes, 1999). Furthermore, *E. faecalis* might have been in the exponential phase, during which time bacteria are more sensitive and prone to being killed within 3 to 10 minutes (Portenier *et al.*, 2005). To our knowledge, no study in the literature has reported the effect of the tested materials against *E. faecalis* after 15 minutes.

After one hour of incubation, intergroup comparison from the most to the least effective against *E. faecalis* according to the percentage of remaining viable cells was as follows: Kri 1 paste > Vitapex > pure Iodoform (Tables 4.4.5 and Figure 4.4.2). There was statistically significant difference between the Ab values of Vitapex and both pure Iodoform and Kri 1 paste at this time interval the p-values were 0.001 and 0.047 respectively and the confidence interval did not include the value of zero effect (Tables 4.4.2 and 4.4.3). The difference between pure Iodoform and Kri 1 paste was not statistically significant (Table 4.4.4; p= 0.176) after one hour. At this point in time in the study, disparity in the antibacterial effect was most probably due to the action of the other ingredients in the iodoform-based preparations (i.e. Calcium Hydroxide and camphorated parachlorophenol in Vitapex and Kri 1 paste respectively).

By two hours, the difference in the antibacterial effect across all the investigated materials was not statistically significant; Vitapex and pure Iodoform (p= 0.187), Vitapex and Kri 1 paste (p= 0.605) and Kri 1 paste and pure Iodoform (p= 0.066) (Tables 4.4.2 to 4.4.4). According to their efficacy on *E. faecalis*, the investigated materials were ranked in a descending order as follows: pure Iodoform, Kri 1 paste, Vitapex (Table 4.4.5 and Figure 4.4.2). At this time interval (2 hours), the antibacterial effect of all three experimental materials was most likely attributed to the iodoform content within each of the materials. This explains the superiority of the pure Iodoform to the other materials.

On the other hand, after four hours of incubation, Kri 1 paste outperformed pure Iodoform, which was in turn, superior to Vitapex with regard to inhibiting *E. faecalis* (Table 4.4.5 and Figure 4.4.2). At this time interval (4 hours) there were statistically significant differences between the antibacterial efficacy of Vitapex and pure Iodoform, Vitapex and Kri 1 paste and pure Iodoform and Kri 1 paste (Tables 4.4.2 to 4.4.4; $p < 0.001$). This contrast could possibly be due to the amount of the iodoform and the antibacterial effect of the other constituents within each material.

Similarly, after 24 hours of incubation, the antibacterial efficacy of the different investigated materials on *E. faecalis* was also recorded in a descending order as follows: Kri 1 paste, pure Iodoform, Vitapex (Table 4.4.5 and Figure 4.4.2). However, the difference was only statistically significant between Kri 1 paste and Vitapex (Table 4.4.3; $p = 0.046$). Paradoxically, when the antibacterial activity of Vitapex and Kri 1 paste was compared from the 4-hour to 24-hour time intervals, the inhibitory effect of Vitapex improved while that of Kri 1 paste deteriorated slightly by the end of the experiment (Figure 4.4.3). This can lead to the speculation that Vitapex might outperform Kri 1 paste over time.

All the above-mentioned results reiterate to the assumption that the efficacy of Vitapex on *E. faecalis* is mainly derived from the iodoform content of this material. This is in an agreement with Pilownic (2017a) and Ordinola- Zapata *et al.*, (2015) who suggested that the outstanding performance of Vitapex was attributable to the iodoform content and in fact, Calcium Hydroxide interfered with the antibacterial efficacy of iodoform. It was found that iodoform alone resulted in killing more of the biofilm bacteria than Vitapex (Ordinola- Zapata *et al.*, 2015; Pilownic *et al.*, 2017a).

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

The aim of this study was to evaluate the antibacterial effect of different iodoform-based preparations (i.e. Vitapex, Kri 1 paste and pure Iodoform) against *E. faecalis*. All of the investigated materials had an inhibitory effect on *E. faecalis* as they had a significantly superior antibacterial effect when compared with the negative control.

The results of this study partly rejected the null hypothesis which stated that there is no difference in the antibacterial efficacy of Vitapex, Kri 1 paste and pure Iodoform at 0, 1, 2, 4 and 24 hours. The present study demonstrated that there was a statistically significant difference in the antibacterial efficacy across the experimental groups (V, I, K) at some of the investigated time intervals.

Kri 1 paste had a superior efficacy to Vitapex and pure Iodoform against *E. faecalis* by the end of the investigated time period (24 hours). However, the difference was only statistically significant between Kri 1 paste and Vitapex. The antibacterial action of the tested materials did not only depend on the effect of their different constituents against *E. faecalis* but also on their ratio and the ability of the active ingredients to dissociate and exert their effect on the bacteria. The addition of camphorated parachlorophenol seemed to make Iodoform more potent against *E. faecalis* while Ca(OH)_2 minimized the efficacy of Iodoform.

Even though Kri 1 paste outperformed Vitapex and pure Iodoform, the action of Kri 1 paste at 24 hours, unlike that of pure Iodoform and Vitapex, seemed to deteriorate when compared with the preceding time period. However to reach a more meaningful conclusion, the antibacterial effect of Kri 1 paste and Vitapex have to be investigated over a longer period of time.

The results of this experiment suggests that the antibacterial effect of the investigated filling materials fluctuated and were susceptible to wear off over time as a result of material degradation and/or bacterial resistance. Hence, other factors such as the capacity of the material to fill the entire canal and be able to readily adhere to the dentinal walls without

shrinking, are essential (Rifkin, 1980). These features allow the material to act as a physical barrier against the ingress of nutrients from outside the root canals, thus depriving the remaining trapped bacteria and hampering their replication (Holan and Fuks, 1993).

6.1. Limitations of the study

Just like with other in-vitro studies, simulating the real clinical situation is almost impossible. Results therefore need to be interpreted with caution. For instance, in the current study, a single bacterial strain was used to evaluate the antibacterial susceptibility. The rationale was that *E. faecalis* is considered to be one of the most resistant organisms and is often associated with failed endodontic treatment. It was therefore considered as an indicator of microbial susceptibility. However, according to Svensäter and Bergenholtz (2004), endodontic infections are mixed with complex floral interactions. The effect of certain materials against a single strain may not be the same as that against a mixed microbial biofilm. Additionally, many other factors inside the root canal can complicate and modify the interaction between the microbes and the antimicrobial agent such as the presence of dentine, periapical tissue fluid and inflammatory exudate (Haapasalo *et al.*, 2007).

The time period over which the antibacterial effect of the tested materials was evaluated was relatively short. This became obvious when the Ab values were not stable between the last two time intervals. This was manifested by the improvement of the antibacterial activity of Vitapex and pure Iodoform and the deteriorating efficacy of Kri 1 paste towards the end of the experiment.

6.2. Recommendations

Based on the results and taking into consideration the limitations of this study, the following is recommended:

- All the investigated materials showed an antibacterial action against *E. faecalis*. This makes all these materials suitable as root canal filling materials of primary teeth as they will be able to overcome the inadequacy of the root canal bio-disinfection.

- Kri 1 paste showed a superior antibacterial activity to Vitapex against *E. faecalis* during the investigated time period, thus it can be suggested as an alternative when Vitapex is not appropriate as in the case of teeth with vital, inflamed radicular pulp. However these results appeared to reverse after 24 hours. Thus, investigating the antibacterial effect of these two root filling materials over a longer period of time is recommended.
- Other factors such as the cytotoxicity of these two materials should be investigated to rule out the potential for periapical tissue irritation using the XTT (which has proven to be accurate in several previous studies).
- For more representative and reliable in-vitro results which simulate the oral environment, the antibacterial efficacy of the investigated materials should be evaluated using the same in-vitro model but with a multi-species biofilm, instead of a single planktonic bacteria. Where possible, this model should also incorporate dentine, tissue fluids and inflammatory exudate to evaluate their possible effect on microbial action.
- More in-vivo studies should be carried out before reaching a consensus on material superiority as it is difficult to simulate the oral environment in in-vitro studies. These studies should particularly evaluate whether Kri 1 paste could cause pathological root resorption (as in the case of Ca(OH)₂-containing Vitapex) when used in teeth with vital inflamed pulp.

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APPENDICES

Appendix A

Ethics approval

1.1. Dr R Mohamed (Dentistry)

Study project: Antibacterial activity of different iodoform-based preparations used as root filling materials in paediatric dentistry

Registration no: BM17/7/13

Ethics: *Approved*