The Use of Caries Preventive Agents Alone and in Regimen for Prevention of Enamel Demineralisation under Cariogenic Challenge *in situ*

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The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others.

Dedicated to my family

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ABSTRACT

Aim of the Research: To compare the efficacy of Casein Phosphopeptide-Stabilised Amorphous Calcium Fluoride Phosphate (CPP-ACFP) 900 ppm (MI Paste Plus™) combined with fluoride toothpaste 1450 ppm F as a regimen with Functionalised tri-calcium phosphate (f-TCP) 950 ppm (Clinpro tooth crème™) and fluoride toothpaste 1450 ppm F, on de/remineralisation dental enamel in an *in situ* randomised, cross-over design study.

Materials and Methods: Sterilised bovine enamel slabs, intact and with subsurface caries-like lesions, were prepared and worn by healthy volunteers (n=14). The study had four arms, 21-day duration each. Four pastes were tested (i) Placebo (0-ppm), (ii) 1450 ppm F, (iii) MI Paste Plus combined fluoride toothpaste 1450 ppm F, (iv) Clinpro tooth crème 950 ppm F. The pastes were randomly assigned and each subject crossed over to each paste after a seven-day washout period. Slabs were subjected to a five times/day cariogenic challenge (12% sucrose) with the subjects using one of the treatments. Volunteers dipped their appliances twice/day for 2 minutes, in a slurry of the toothpastes. For group 3, additionally, the appliance was dipped for 3 min in a slurry MI Paste Plus once/day immediately following the second exposure to the slurry of 1450 ppm F. Further de/remineralisation were assessed using Quantitative Light-Induced Fluorescence (QLF) and Surface Microhardness before and after the treatment period.

Results: QLF results for all test groups showed a significant reduction

(p< 0.05) in lesion volume **(**ΔQ) compared to the placebo. No statistically significant differences were found between the test groups. The Microhardness results for all test groups showed a significant increase (p<0.05) of enamel hardness compared with the placebo. No statistically significant differences were found between the test groups.

Conclusion: The use of MI Paste Plus in combination with 1450 ppm F toothpaste did not show a greater reduction of enamel subsurface lesions or decreased demineralisation of sound enamel in comparison to 1450 ppm F toothpaste and Clinpro tooth crème alone in our model.

TABLE OF CONTENTS

TABLE OF TABLES

TABLE OF FIGURES

1.0 INTRODUCTION

Dental caries can be defined as “a transmissible bacterial disease process caused by acids from bacterial metabolism diffusing into enamel and dentine and dissolving the mineral” (Featherstone, 2008). Despite massive prevention efforts, dental caries is still the most common disease in the United Kingdom and globally.

More conservative approaches are now advocated secondary to a better understanding of caries and oral environment. It is now well understood that caries is a dynamic process that results from the imbalance between demineralisation and remineralisation (Featherstone, 2008).

In recent years, several preventive agents have been introduced in conjunction with fluoride dentifrices for daily home use and deemed to be effective products to inhibit demineralisation and enhance remineralisation of the enamel. Two calcium and phosphate products have been introduced and claimed to be more effective than the routine use of fluoridated toothpaste; Casein phosphopeptide -amorphous calcium phosphate (CPP-ACP) and functionalised tricalcium phosphate (f-TCP) technologies.

The anti-caries effect of CPP-ACP has been reported in several studies (Reynolds, 2009; Reynolds, 2008). The anti-caries effect is believed to be due to the formation of stabilised amorphous calcium phosphate in the tooth surface and dental plaque (Reynolds, 2009; Reynolds, 2008).

The synergic effect of CPP-ACP and fluoride has also been investigated in the literature. Several studies have reported a positive synergic effect on inhibiting demineralisation and enhancing remineralisation of the enamel (Mendes et al., 2018; Altenburger et al., 2010; Reynolds et al., 2008). However, the benefits of using it as a regimen with fluoride over the use of fluoride products alone are investigated by a limited number of studies and is a matter of scholarly debate.

More recent, f-TCP technology has been developed by 3M ESPE Inc and claimed to be able to maintain high levels of calcium, phosphate and fluoride in the oral environment (Amaechi, 2015; Jo et al., 2014). This technology is now commercially available as Clinpro toothpaste™ with (5000 ppm NaF), Clinpro tooth crème™ with (950 ppm NaF) and fluoride varnish with (22600 ppm NaF). However, very limited clinical trials have investigated its remineralisation effect.

Therefore, the aim of this *in situ* study was to compare the efficacy of MI Paste Plus combined with fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride) as a regimen with Clinpro tooth crème and fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride), on remineralisation of the enamel subsurface caries like lesion in an *in situ* randomised, cross-over design study.

2.0 LITERATURE REVIEW

2.1 Dental caries

Dental caries has been discussed extensively in the literature. It is considered one of the most common causes of mineral loss. It has been defined *as "a transmissible bacterial disease process caused by acids from bacterial metabolism diffusing into enamel and dentine and dissolving the mineral"* (Featherstone, 2008).

Despite massive efforts in the prevention of dental caries, it is still one of the main issues in the United Kingdom and globally. Data obtained from the latest Child Dental Health Survey in England, Wales and Northern Ireland showed that in 2013, approximately half of 15-year-olds and almost one-third of 12-year-olds suffered from noticeable dental caries in their successor's teeth. In addition, nearly 33% of 5-year olds and about a half of 8-year olds had visible dental caries in their primary teeth. Although the survey stated that there was a reduction in the caries prevalence compared with the Child Dental Survey in 2003, this disease remains a significant problem in the United Kingdom (Health & Social Care Information Centre, 2015).

2.1.1 Pathogenesis of dental caries

The dynamic process of dental disease is now clear and well described in the literature. The dental caries process is affected by many pathological and protective factors, which include acid-producing bacteria, a fermentable

carbohydrate, saliva flow and the use of remineralisation products (Featherstone, 2008; Featherstone, 2004).

The bacteria responsible for dental caries (Streptococcus mutans, Streptococcus sobrinus and Lactobacillus Spp) mainly interact with fermentable substances and produce organic acids. The acids formed include lactic, acetic, formic and propionic, which then can penetrate and diffuse through enamel and dentine into underlying structures and start to dissolve acid-soluble minerals. This process, over time, can lead to dental cavitation (Featherstone, 2008).

2.1.2 Demineralisation and Remineralisation

As described above, dental caries is a dynamic process affected by pathological and protective factors, occurring over a period of time. In the mouth, as long as there are acid-producing bacteria, fermentable substances and saliva, cycles of demineralisation and remineralisation will continue to affect the tooth structure (Featherstone, 2008). Remineralisation can occur if there is an imbalance towards the protective factors. However, any shift in the balance towards the pathological factors will lead to demineralisation (Figure 2-1).

Therefore, demineralisation can be defined as *"the loss of calcified material from the structure of the tooth. This chemical process can be biofilm mediated (i.e. caries) or chemically mediated (i.e. erosion) from exogenous or endogenous sources of acid"* (Fontana et al., 2010).

Conversely, *remineralisation is "the net gain of calcified material within the tooth structure, replacing that which was previously lost through demineralisation"*(Fontana et al., 2010).

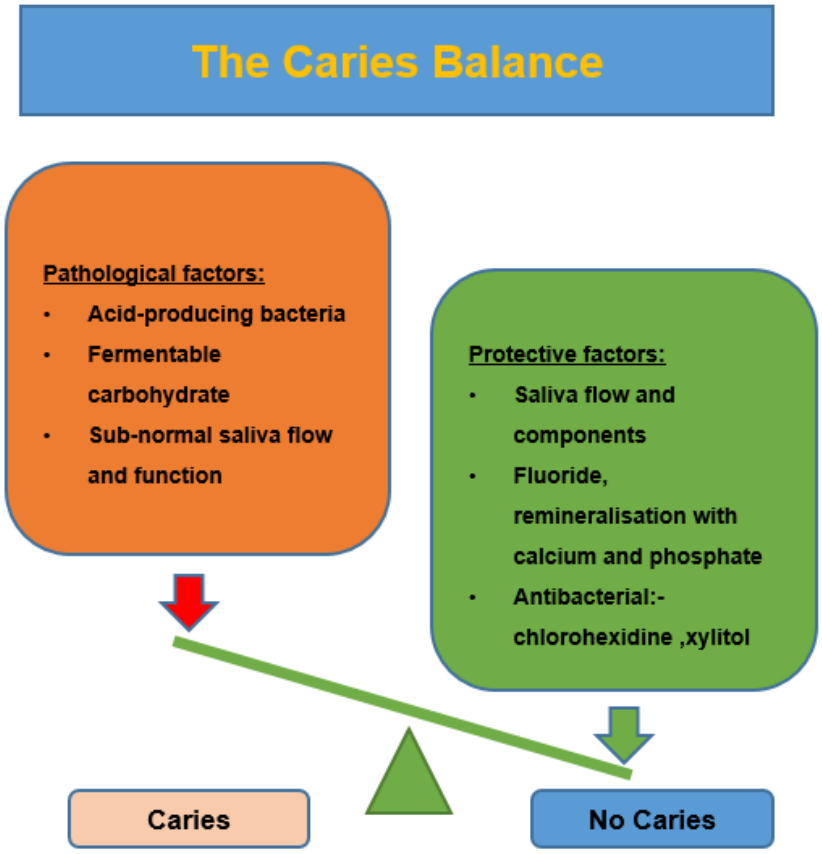

Figure 2-1 The caries balance concept based on (Featherstone, 2008)

2.1.3 Enamel caries

Darling (1956) histologically classified the enamel lesion under a polarised light microscope into four different zones to demonstrate the different phases of demineralisation and remineralisation:

1- The translucent zone: This is defined as at the deepest part of the enamel lesion with a mineral loss of about 1.2% and is more porous compared to the normal enamel structure (Hallsworth et al., 1973).

2- The dark zone: This is defined as the area located next to the translucent zone and is characterised by a mineral loss of approximately 5–10% (Hallsworth et al., 1973).The chemical analysis of the material from this zone revealed a tooth structure closer to the apatite (Robinson et al., 2000).

3- The body of the lesion: This accounts for the highest percentage of mineral loss with up to around 30% of mineral loss (Hallsworth et al., 1973). Robinson et al. (2000) stated that fewer soluble materials were found in this zone following chemical analysis of mineral loss.

4- The surface zone: Around 1-10% mineral loss is seen in this zone with a wide variation in mineral distribution (Hallsworth et al., 1973).

2.1.4 Role of saliva

Saliva is a diluted liquid comprising more than 99% water and is produced from the major salivary glands (parotid, submandibular and sublingual) and several minor salivary glands with a neutral pH level between 6 to 7 (Garcia-Godoy and Hicks, 2008).

The average salivary flow has considerable individual variation. However, the minimum unstimulated and stimulated salivary flow rates have been discussed in the literature as being more than (0.1 mL/min and 0.2 mL/min) respectively. Reduction in the quantity or quality of saliva can lead to dental caries (García-Godoy and Hicks, 2008).

Saliva plays a vital role in maintaining optimum oral health. Its functions can be classified into the following main rules (García-Godoy and Hicks, 2008):

- Lubrication of the oral environment and protection from damage to the soft tissue during mastication
- The saliva acid's buffering capacity and food clearance
- Preservation of tooth integrity
- Antibacterial ability against microorganisms due to the presence of proteins, mucins immunoglobulins, enzymes and nitrogenous products, such as urea and ammonia
- It has a role in helping with taste and digestion
- A calcium and phosphate-rich environment helping to enhance remineralisation

Many clinical studies have investigated the role of saliva in the prevention of dental caries. In a review that included seven clinical studies, the effect of stimulated saliva using sugar-free chewing gum was investigated. The result showed a significant reduction in caries' incidence due to the increase in salivary flow rather than the chewing gum (Kalin, 2011).

It has been concluded that saliva is considered the key biological factor to maintain oral health. This is not only for the prevention of dental caries but also the prevention of other diseases like erosion (Afonso et al., 2012).

2.2 Enamel Remineralisation Therapies

Chole et al. (2016) described the ideal requirements for enamel remineralisation therapies as follows: remineralisation therapy should be able to diffuse or deliver calcium and phosphate into the enamel subsurface. At the same time, it should not deliver excess calcium that could interfere with the fluoride. It should also be able to function in an acidic environment. Moreover, it should encourage the saliva function and be able to work in xerostomic oral conditions.

In this part, different enamel remineralisation agents will be discussed with the main focus on the use of fluoride, Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP) and functionalised tricalcium phosphate (f-TCP) therapies.

2.2.1 Fluoride

Fluoride plays an important role in the prevention of enamel demineralisation and in enhancing enamel remineralisation. The anti-cariogenic properties of fluoride have been one of dentistry's great discoveries (Fejerskov, 2004). The evidence behind the anti-caries ability is well-established. Many studies have shown that regular use of fluoridated toothpaste has a major impact on caries reduction (Marinho et al., 2016).

2.2.1.1 Mechanism of action of fluoride

Several mechanisms have been discussed in the literature to explain how fluoride's mechanism act, which includes the systemic and local effect on teeth. The systemic effect is during tooth formation and topically after the eruption of the teeth, however, numerous systematic reviews and studies have shown that the principle action of fluoride is through its topical rather than its systemic effect (Marinho et al., 2016).

The proposed mechanisms of action for fluoride systematically (pre-eruptive mechanism) (Cate and Cate, 1999; Featherstone, 1999) are:

(i) It has an effect during crown formation by creating shallow pits and fissures, which affects the crown.

(ii) It also plays an important role during tooth formation by stabilising the enamel crystals.

Topically, fluoride works directly on the surface of erupted teeth (post-eruptive mechanism) in three different ways (Cate and Cate, 1999; Featherstone, 1999):

(i) The first is that it inhibits demineralisation when in solution. Fluoride that comes from local sources like water and other fluoride products can impede enamel crystal dissolution when fluoride is present in a solution at a low level.

(ii) Secondly, it enhances remineralisation when in solution by forming fluorapatite. Fluoride from topical sources, when present in solution, improves remineralisation by attracting calcium and phosphate ions, thereby forming a new layer which is fluorapatite-like. Fluorapatite is more resistant at a lower pH than hydroxyapatite.

(iii) The third way is by inhibiting plaque bacteria. It has been shown that fluoride inhibits plaque bacteria by blocking the enzyme enolase during glycolysis.

2.2.1.2 Fluoride products and delivery systems

There are several ways to deliver fluoride supplements including fluoride dentifrices, varnishes, gels and mouth rinses. Fluoride can also be supplemented via systemic methods like water fluoridation, milk fluoridation and tablets.

2.2.1.2 a Fluoride toothpaste

The introduction of fluoridated toothpaste has been associated with the increased reduction of dental caries over the last four decades (European Academy of Paediatric Dentistry, 2009). A Cochrane review, published by Marinho et al. (2016), which included 42300 children, concluded that brushing with fluoridated toothpastes significantly reduces dental caries compared to non-fluoridated toothpastes. The authors also concluded that the anti-caries effectiveness increases with the frequency of tooth brushing, supervised brushing and increased DMFT baseline.

The effectiveness of different fluoride concentrations for toothpastes has also been extensively investigated. A Cochrane review of 75 studies, to investigate the anti-caries efficacy of different concentrations of fluoridated toothpastes, concluded that the caries reduction efficacy is dose-dependent. It also found that the anti-caries effect was only significant for 1000 ppm and above (Walsh et al., 2010). These results are in line with the current guidelines for the use of toothpaste (DOH Toolkit, 2017).

2.2.1.2 b Fluoride rinses

In addition to the use of toothpastes, fluoridated mouth rinses can be considered for patients at high risk of caries (Toolkit, 2017; European Academy of Paediatric Dentistry, 2009). Such mouth rinses are commercially available as daily (225 ppm F) and weekly (900 ppm F). The main advantages of using these mouth rinses are the short contact time and low fluoride concentration. However, patient compliance is crucial to increase its efficiency and avoid fluoride toxicity.

The anti-caries effectiveness of fluoridated mouth rinses has been investigated in the literature. A recent Cochrane review, which included 35 clinical trials with more than 15800 patients, found a significant caries reduction of 27% when compared with placebo mouth rinses (Marinho et al., 2015).

2.2.1.2 c Fluoride varnishes

The effectiveness of fluoride varnish has been shown in several systematic reviews. A recent Cochrane review included 22 trials with approximately 12000 participants. It found that fluoride varnish significantly reduces dental caries in primary and permanent teeth (37% and 43%) respectively when compared with no treatment (Marinho et al., 2013).

Fluoride varnishes offer several advantages including being available in different flavours, and they do not have a bitter taste like acidulated phosphate fluoride gel. There are also easy and quick to apply and require minimal clinical settings. Another key advantage is that they dry immediately on contact with

saliva, which maximises the effect. Moreover, fluoride varnishes can be applied by dental nurses under a dentist's prescription, provided that the nurses have undertaken the required training (DOH Toolkit, 2017).

Fluoride varnishes are available in different types for example Duraphat™ with 2.2% NaF, Fluor Protector™ with 7% ammonium fluoride and Bifluoride™ with 5.63 % NaF. The current recommendation for the use of fluoride varnish is to apply the varnish (2.2% NaF) twice a year for all children aged three years and above (DOH Toolkit, 2017).

2.2.1.2 d Fluoride gel

Fluoride gels with low fluoride concentration can be used as self-application home products. They are also available for professional use at high concentrations (1.23%). Fluoride gel can be obtained from different fluoride formulations including acidulated phosphate fluoride, sodium fluoride and stannous fluoride.

In a recent Cochrane review that investigated the effectiveness and safety of fluoride gel, twenty-eight studies (9000 children) were combined. The results showed 27% caries reduction with fluoridated gel compare with placebos. It also found studies that reported unwanted adverse effect s were mainly associated with swallowing the gel (Marinho et al., 2015). Due to toxicity concerns, fluoride varnish has replaced gels in many countries.

2.2.1.3 Fluoride toxicity

The optimum daily intake of fluoride is between 0.05 -0.07 mg/kg. Excess consumption of fluoride may lead to fluoride toxicity. This can be classified as chronic or acute based on the duration and concentration of the consumed fluoride.

2.2.1.3.1 Acute fluoride toxicity

This is defined as the ingestion of a large dose of fluoride over a short period. Several signs and symptoms can be associated with acute fluoride toxicity. The severity of symptoms is dependent on the amount of ingested fluoride.

Signs and symptoms of acute fluoride toxicity (Shulman and Wells, 1997):

- “*Nausea, vomiting*
- *Abdominal pain*
- *increased salivation*
- *Generalized weakness, muscle spasm and pain*
- *Reduced plasma calcium level, increased plasma potassium level*
- *Weak pulse, low blood pressure*
- *Cardiac arrhythmia*
- *Cardiac failure*
- *Respiratory system failure*
- *Coma and death*”

A lethal fluoride dose can be defined as the ingested dose that would be lethal if not treated promptly. This has been estimated at between 32 and 64 mg/kg NaF (Whitford, 1989).

However, the toxic dose definition is, *“the minimum dose that could cause serious life-threatening systemic signs and symptoms and that should trigger immediate therapeutic intervention and hospitalisation”,* which was estimated to be 5.0 mg/kg NaF (Whitford, 1989).

Acute toxicity should be managed immediately by inducing vomiting to reduce the fluoride absorption. Acute fluoride toxicity is a life-threatening condition; therefore, the patient needs to be transported to the emergency department as soon as possible.

2.2.1.3.2 Chronic fluoride toxicity

This is defined as the ingestion of an excess amount of fluoride level over a prolonged period. Chronic toxicity depends on many factors, including fluoride level in the body, the stage of tooth development, the fluoride dose and the length of exposure to fluoride. Furthermore, renal function and interaction with other materials contribute to the toxicity level (DenBesten and Li, 2011; Ullah et al., 2017). Chronic toxicity from fluoride can have a wide range of effects including dental fluorosis, skeletal fluorosis, renal and gastrointestinal problems.

Dental fluorosis

The ingestion of excess fluoride during tooth formation can result in dental fluorosis, one of the most common problems associated with chronic toxicity from fluoride. Furthermore, it is an early sign of chronic fluoride toxicity. In the United States, according to a National Health and Nutrition Examination Survey, the dental fluorosis prevalence has increased in children aged 15-17-year-old. In the 1999-2004 study, 40.6% of children aged 15-17-year-old had dental fluorosis compared with the lower prevalence (22.6%) recorded in an earlier survey from 1986-1987 (DenBesten and Li, 2011).

Skeletal fluorosis

Skeletal fluorosis is one of the complications that result from fluoride toxicity. It is defined as an increased deposition of fluoride within the bone. Many symptoms can be seen with skeletal fluorosis, including joint rigidity, bone pain and difficulties with movement. Radiographic signs may act as osteosclerosis and calcification of ligaments (Ullah et al., 2017).

Renal effects

Approximately 60% of fluoride excretion occurs in the kidneys. The excess consumption of fluoride, over recommended doses, may cause renal structural changes due to fluoride toxicity. These can include swelling, degeneration of tubular epithelium, fibrosis, atrophy of glomeruli and tubular necrosis (Ullah et al., 2017).

Gastrointestinal tract (GIT) effects

In the gastrointestinal tract (GIT) system, high fluoride can irritate the gastric mucosa. This irritation is caused by the formation of hydrofluoric acid that results from the interaction between fluoride and hydrochloric acid (Ullah et al., 2017).

2.2.2 Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP)

The clinical application and remineralisation benefits of using calcium and phosphate were limited and ineffective in the past. This is secondary to the low solubility of these ions, especially with fluoride ions' presence. The low solubility of the ions complicates their localisation effectively at the tooth surface. In addition, acid is usually required to make these ions more soluble and able to diffuse into enamel subsurface lesions. Thus, the application of insoluble calcium phosphate is not easy (Reynolds, 2009).

The intrinsic insolubility of soluble calcium and phosphate ions, especially the calcium fluorophosphate, makes the use of these ions very limited, only at low concentration levels. Moreover, the ability of soluble calcium and phosphate ions to penetrate the subsurface enamel is reduced as they do not effectively bind to dental plaque (Reynolds, 2009).

To overcome the limitations of the bioavailability of calcium and phosphate ions and to enhance remineralisation, many calcium phosphate-based technologies have been introduced and discussed in the literature. One such system involves using CPP-ACP. In this technology, an increased level of calcium, phosphate and fluoride are maintained and stabilised, at the tooth structure by casein phosphopeptide (CPP). Casein phosphopeptide (CPP) binds these ions to pellicle and plaque. The bioavailability of the stabilised ions is claimed to have been improved, enabling the ions to penetrate and diffuse into the enamel subsurface (Reynolds 2009; Reynolds, 2008).

The anti-caries effect of dairy products has been shown in numerous *in vitro* and *in situ* animal and human models. The anticariogenic ability mainly comes from casein, calcium and phosphate. In a rat caries model, Reynolds and del Rio (1984) showed that the addition of soluble sodium caseinate, at 2%, to drinking water, reduced caries' activity in rats. In addition, Reynolds (1987) investigated the enamel demineralisation ability of sodium caseinate, alpha s1 tr-casein, and tryptic digest of alpha s1- casein using a modified human *in situ* caries model. This study concluded that using 2% sodium caseinate prevented the enamel demineralisation caused by the sugar challenge used in the study. It also showed that consumption of alpha s1- casein did not affect the proteins' anticariogenicity. In addition, the tryptic peptide of alpha s1- casein was found to be incorporated in the inter-enamel plaque, with high levels of calcium and phosphate in the plaque.

It has been shown that caseinate's anticariogenic activity is derived from the tryptic peptides that were the calcium-phosphate-stabilising casein phosphopeptides (Reynolds, 1998). CPP contains the group sequence of -Ser(P)-Ser(P)-Ser(P)-Glu-Glu-, which has the notable capability to stabilise ACP (Reynolds, 1997).

CPP-ACP is commercially available in different forms. The most common are topical crème; Tooth mousse ™ (TM; GC Crop, Tokyo, Japan) and MI Paste Plus™ (GC MI Paste Plus; GC Crop, Tokyo, Japan) which contains 900 ppm fluoride in addition to CPP-ACP. CPP-ACP is less commonly found as chewing gum, mouth rinse, milk, sealant and varnish.

2.2.2.1 CPP-ACP mechanism of action

CPP-ACP works effectively to stabilise and localise ACP in dental plaque and tooth surfaces. This function maintains a state of super-saturation, which inhibits demineralisation and enhances remineralisation of the enamel (Reynolds, 2008).

The ability of (CPP) to inhibit plaque formation has been recorded in the literature. Guggenheim et al. (1999) showed that *S. sobrinus* numbers are significantly reduced in rats, secondary to the consumption of a diet containing casein or CPP. Casein has also been shown to have the ability to inhibit adherence of *S. mutans* to a tooth's surface (Reynolds, 1987).

2.2.2.2 CPP-ACP Effect on Demineralisation

As discussed earlier, CPP can localise ACP in dental plaque as well as on the tooth surface. This will help to buffer the available calcium and phosphate and maintain a state of supersaturation, which inhibits demineralisation and encourages remineralisation (Reynolds, 1998). Several *in vitro* and *in situ* studies have been conducted to evaluate the effect of CPP-ACP to prevent enamel and dentine demineralisation.

Reynolds, (1987) conducted a 10 day *in situ* study using microradiography and microhardness tests to assess the ability of casein agents in preventing sub-surface enamel demineralisation and incorporation into the plaque. The study concluded that inhibition of enamel demineralisation was seen by a twice-daily application of sodium caseinate 2%, oxt1-casein, or TD-casein solution (pH 7.0) that was caused by a frequent sugar challenge.

Moreover, Yamaguchi et al. (2006) conducted an *in vitro* study to investigate the effect of CPP-ACP on demineralisation by using field emission-scanning electron microscopy. In this experiment, the morphological characteristics of enamel and dentine were examined and evaluated over a period of 28 days. It was noted that the specimens treated in CPP-ACP prior to the acid challenge showed relatively slight changes in the tooth surface.

Furthermore, Yamaguchi et al. (2007) evaluated the effect of CPP-ACP on inhibiting the dentine demineralisation. They used the ultrasound technique as an indicator for mineral loss in an *in vitro* study. No significant difference in the sonic velocity was found with the slabs of CPP-ACP paste used for 10 minutes before the acid challenge. They concluded that CPP-ACP has the ability to inhibit dentine demineralisation.

In addition, the effect of using CPP-ACP paste on demineralisation and remineralisation were evaluated *in vitro* by using Fourier infrared spectroscopy. In this experiment, forty sound human dentine slabs were randomly allocated into four groups. To evaluate the effect of CPP-ACP on dentine demineralisation, Group A was treated with Tooth Mousse™ (CPP-ACP), whereas no treatment was applied to Group B. Both groups A and B were subjected to an acid challenge for seven days before being analysed. Groups C and D were used to assess the ability of Tooth Mousse to enhance remineralisation. Both groups C and D were subjected to seven days demineralisation challenge with no treatment applied prior to the challenge. Group C then was treated with Tooth Mousse™ while Group D received no treatment. Groups C and D were kept in prepared artificial saliva for a week

before the analysis. The result indicated that the application of Tooth Mousse™ on the dentine surface inhibited demineralisation and enhanced remineralisation of the dentine surface in comparison to groups that received no agent (Rahiotis and Vougiouklakis, 2007).

In contrast to above studies, Pulido et al. (2008) reported that Tooth Mousse Paste™ had no effect on lowering enamel demineralisation *in vitro*. The researchers studied the effect of Tooth Mousse Paste™ on preventing enamel demineralisation using Polarized-Light-Microscopy (PLM). In this study, enamel specimens were randomly assigned into four groups (n=21): Artificial saliva as a control group, 5000 ppm NaF, Tooth Mousse Paste™, 1100 ppm NaF and a combination of 1100 ppm NaF and Tooth Mousse Paste™. The duration of pH cycling of this study was six days at 20c°. Enamel slabs were exposed to two acid challenges a day. The agents were applied twice a day for two minutes and washed immediately after application. The result showed that minimal changes in lesion size and depth were noted in the 5000 ppm NaF compared with other groups. In the study, the application time was short, and products were washed out immediately after application. In addition, the samples were cycled for six days only. In most studies, the normal cycling protocol ranges from 21-28 days.

2.2.2.3 CPP-ACP Effect on demineralisation around Orthodontic brackets

Enamel demineralisation and development of white spot lesion are considered common side effects of orthodontic treatment (Benson et al., 2013). The effects of using topical treatment with calcium phosphate technologies and white spot lesion have been widely investigated in the literature.

The effect of using MI Paste Plus ™ CPP-ACPF (900 ppm F) to prevent enamel decalcification around orthodontic brackets has also been studied. Robertson et al. (2011) carried out a double-blind, randomised clinical trial to evaluate the effectiveness of using MI Paste Plus ™ to inhibit enamel demineralisation in orthodontic patients. Fifty participants were randomly assigned into two groups: placebo (n=24) and treatment group (n=26). Participants were asked to use the pastes daily for 12 weeks, and the analysis was carried out using the enamel decalcification index (EDI) scores and the international caries detection and assessment system (ICDAS). It was found that the EDI score declined by 53.5% with the MI Paste Plus™ group but increased by 91% in the placebo group. The ICDAS results were in line with EDI scores. However, one of the limitations of this study was the use of subjective assessment systems, which can vary between examiners.

Uysal et al. (2010) used *in vitro* and *in vivo* models to compare the ability of CPP-ACP and a fluoride agent to inhibit enamel demineralisation around orthodontic brackets. In the *in vivo* experiment, orthodontic brackets were bonded for 60 days on first premolars that were scheduled for orthodontic extraction. The participants (n=21) were randomly allocated into three groups: Tooth Mousse™, sodium fluoride gel and control group (standard home care).

After 60 days, sixty-one teeth were extracted, and brackets were removed. Using cross-sectional microhardness, the efficacy of the test products on demineralisation was assessed i*n vivo*.

For the *in vitro* experiments, sixty extracted premolars were bonded and randomly divided into the same groups, as per the *in vivo* study. Teeth from treatment groups received a coat of prepared solution (0.5 mL) before the treatment in cycling solutions, while the control group received no agent before immersion in demineralisation and remineralisation solutions. Specimens underwent a pH cycling of 14 days. The findings of both experiments revealed that both agents have a definite benefit in lowering enamel demineralisation around orthodontic brackets compared with the control group. However, no statistically significant difference was detected between CPP-ACP and fluoride containing gels agents on minimising enamel demineralisation.

Bröchner et al. (2011) carried out a randomised, single-blind, controlled trial to study the effect of applying CPP-ACP paste to white spot lesions, over the use of standard fluoride. Sixty subjects, who had visible white spot lesions, were randomly allocated into two groups. The intervention group (n=22) was asked to apply Tooth Mousse™ once a day in addition to the standard toothpaste brushing. The control group (n=28) was advised to brush normally twice a day with a standard toothpaste 1100 ppm F. After 28 days of application; the lesions were evaluated using quantitative light-induced fluorescence (QLF). It was found that the area of the lesions and the fluorescence were significantly reduced for both groups compared with the baseline readings. No statistically significant difference was detected between the two experimental groups.

Furthermore, a recent evidence-based review was conducted to evaluate the effectiveness of CPP-ACP and CPP-ACPF to prevent enamel demineralisation and in enhancing enamel remineralisation on white spot lesions. It concluded that there is a tendency toward the benefit of using CPP-ACP to enhance enamel remineralisation for orthodontic patients. However, the quality of evidence is limited (Raphael and Blinkhorn, 2015).

2.2.2.4 CPP-ACP Effect on Remineralisation

The ability of CPP-ACP to enhance enamel remineralisation has been widely investigated using different study models. As discussed previously, CPP-ACP has a significant effect on increasing the level of calcium and phosphate in plaque, which enhances enamel remineralisation and inhibits plaque formation (Reynolds, 2009).

In vitro studies

Various *in vitro* studies have used different protocols to assess the ability of CPP-ACP products to enhance enamel remineralisation. Zhang et al. (2011) compared the remineralisation ability of CPP-ACP paste to 500 ppm NaF solution in an *in vitro* study. A total of ninety enamel slabs from primary teeth with enamel subsurface like lesions were randomly allocated into three groups. Group 1had deionised water as a negative control, Group2 used 500 ppm NaF solution as a positive control and Group 3 was 10% CPP-ACP paste. Remineralising agents were applied twice a day for thirty days. The enamel slabs were assessed at baseline before demineralisation and after thirty days of pH cycling, using surface a microhardness test. The study found a significant increase for the surface microhardness in the CPP-ACP group compared with others and concluded that CPP-ACP paste can potentially be used for the management of early childhood caries in primary teeth. These findings were in line with another *in vitro* study published by Zhou et al. in 2014.

Moreover, the remineralising efficacy of CPP-ACPF (MI Paste™) pastes after short applications (60 seconds) were assessed using polarising light

microscopy and microradiography. Gopalakrishnan et al. (2017) used a 10-day pH cycling i*n vitro* model to assess the remineralising ability of CPP-ACPF and the benefit of using fluoridated toothpaste (1000 ppm F) prior to the application of the CPP-ACPF paste. The results showed that the short application of CPP-ACPF significantly remineralised the artificial enamel subsurface-like lesions. However no extra benefit was found in using fluoridated toothpastes prior to the CPP-ACPF application.

In contrast with previous findings, Vyavhare et al. (2015) conducted an *in vitro* study to assess whether CPP-ACP can be used as an alternative to fluoridated toothpaste. Enamel subsurface lesions were created on extracted flat labial surfaces of extracted human incisor teeth. Enamel slabs were treated with four different agents: (i) Nano-hydroxyapatite (10%), (ii) CPP-ACP (10%), (iii) NaF (1000 ppm) and (iv) deionized water. The enamel slabs were assessed at baseline, after demineralisation and after 3, 6, 9 and 12 days of *in vitro* pH cycling using surface microhardness. In their results, the authors reported that the percentage surface microhardness recovery (%SMHR) was significantly higher in groups (i) and (iii) and concluded that CPP-ACP is not an effective alternative to fluoride therapy.

In situ studies

Numerous *in situ* studies have been carried out to investigate the remineralisation efficacy of CPP-ACP agents.

Shen et al. (2011) conducted a 10-day double-blind, randomised, controlled cross-over *in situ* study to compare the remineralisation ability of different dental agents with added calcium and phosphate. In their study, enamel subsurface-like lesions were created on selected sound extracted human third molars. Enamel slabs were secured by sticky wax into upper removable appliances worn by five volunteers. Participants were instructed to rinse four times daily for 60 seconds using slurry of the assigned product. Volunteers were crossed over six products (i) placebo, (ii) 1000 ppm F, (iii) 5000 ppm F, (iv) Tooth Mousse™ (TM), (v) MI Paste™ (TMP) and (vi) Clinpro crème with 950 ppm F, with a wash-out period of seven days in between. Saliva samples were taken after each rinse to measure the calcium, phosphate and fluoride levels using chromatography. The mineral content was also assessed using transverse microradiography. The findings of this study showed that significant calcium and phosphate levels were seen in the TM and TMP groups compared with the other groups. Additionally, the highest remineralisation ability was noted in TMP and TM (29.4 % and 24.2% respectively). This study lacked any sample size calculation, and an additional weakness is the short study period (10 days), which could have influenced the results.

In addition, Walker et al. (2006) used an *in situ* model to study the effect of CPP-ACP added to bovine milk on enamel remineralisation. Ten participants wore upper removable appliances containing slabs with enamel subsurface-like

lesions. Once a day, the volunteers consumed 200ml of milk that contained either 2.0g or 5.0 g CPP-ACP/L or control bovine milk; this was over a three-week period. The subjects crossed over after each treatment period with a wash-out period of seven days. The study used microradiography and micro-densitometry tests to assess the mineral content. Significant enamel remineralisation in CPP-ACP groups was found compared with the control group. They also found that the CPP-ACP remineralisation effect was dose-dependent.

Moreover, Reynolds et al. (2003) compared the enamel remineralisation ability of CPP-ACP and different forms of calcium when delivered as a mouth rinse in a randomised double-blind crossover trial. Thirty volunteers were recruited and instructed to rinse for thirty seconds, three times daily, for five days. The participants crossed over to use each mouth rinse, which included: (i) oral rinse containing 2% CPP-ACP, (ii) oral rinse containing 6 % CPP-ACP, (iii) oral rinse containing un-stabilised slurry of $CaCl_2$ and sodium phosphate and (iv) control de-ionized water, with a wash-out period of 28 days between study arms. The study found that only the mouth rinse containing CPP-ACP significantly increased the calcium and phosphate levels in the plaque. They also found that the effect CPP-ACP had on calcium and phosphate levels was dose-dependent. This finding is consistent with a previous study carried out by Walker et al., (2006)

Furthermore, in a systematic review published by Yengopal and Mickenautsch (2009), five clinical trials were pooled for meta-analysis. The review concluded that a significant enamel remineralisation was seen in chewing gum that

contained CPP-ACP compared with chewing gum without CPP-ACP and control gum. However, this review only included clinical trials of a short-term period (7-21 days).

In vivo studies

In addition to the *in vitro* and *in situ* evidence, several *in vivo* experiments have been conducted to assess the ability of CPP-ACP technology in enhancing enamel remineralisation.

Bailey et al. (2009) conducted a clinical trial to evaluate the effect of using CPP-ACP crème on white spot lesions that resulted from orthodontic treatment. Forty-five subjects with more than four hundred white spot lesions were recruited for this study. The subjects were randomised into two groups: Group 1 received the CPP-ACP crème (n=23), Group 2 received a placebo crème (n=22). Participants were asked to apply the products twice a day after brushing with the fluoride toothpaste (1000ppm sodium fluoride) for twelve weeks. ICDASII Criteria were used to assess the progression and regression of the lesions. They found that more remineralised white spot lesions were significantly seen in the group treated with CPP-ACP crème compared with the placebo group.

In a recent systematic review, the long-term remineralisation effect of using CPP-ACP *in vivo* was investigated. Eight studies were included in the review with 2376 participants and an average follow-up of 3-24 months. Of these eight studies, six were randomised controlled trials, and two were controlled trials. The review concluded that CPP-ACP had significant long-term remineralisation benefits compared with the placebo. However, there was no significant

difference when compared with routine fluoride products. They also stated that the evidence to support the synergic effect between CPP-ACP and fluoride was not clear (Li et al., 2014).

2.2.2.5 Interaction of CPP-ACP with Fluoride

The combined effect of CPP-ACP and fluoride is believed to be due to the formation of CPP-stabilised amorphous calcium fluoride phosphate. This increases the concentration of bioavailable calcium and phosphate ions. It also enhances the integration of fluoride ions into the plaque (Cross et al., 2004).

The synergic effect of CPP-ACP and fluoride has been investigated in the literature. Although there is considerable research supporting the use of CPP-ACP as a remineralising agent, the benefits of using it as a regimen with fluoride over the use of fluoride products alone is a matter of scholarly debate.

2.2.2.5 a Studies reporting significant benefits of using CPP-ACP products over routine dental care on enhancing of enamel remineralisation

Mendes et al. (2018) tested *in vivo* the effect of four different products (placebo, 1.23% acidulated phosphate fluoride, CPP-ACP MI Paste™ and MI Paste Plus™) on the remineralisation of early enamel lesions on permanent anterior teeth. Thirty-six participants aged 5-13 years were divided randomly to receive one of the three products. All participants were instructed to brush with 1450 ppm F twice daily in addition to the product they used. Lesions were periodically observed and assessed at baseline, one month and three months using a DIAGNOdent Pen. The results of this study indicated a significant improvement in terms of enamel remineralisation with MI Paste™ and MI Paste Plus™ at 90 days, compared with other groups. One interesting finding was that the significant difference of lesion intensity was only noticed between the groups after day 30. The main shortcoming of this study was that there was no sample size calculation. Furthermore, it was not clear who assessed the lesions and whether the assessors were calibrated.

A further single-blind randomised controlled trial was carried out by (Altenburger et al., 2010). The study assessed the efficacy of a single daily application MI Paste™ on early carious pits and fissures. Thirty-two participants were randomised into control and test groups. The test group used MI Paste™ in addition to routine daily brushing with 1450 ppm F, whereas the control group had only the fluoridated toothpaste 1450 ppm F. DIAGNOdent. Visual assessments were used at baseline, day 8, day 14 and day 22 by two calibrated dentists. The results showed significantly lower fluorescence value

for the test group ($P < 0.001$) as compared with the control group. However, no significant difference was noted between the groups when using the visual assessment test.

Reynolds et al. (2008) undertook a randomised, double-blind cross-over *in situ* study to assess the enamel remineralisation ability of different toothpaste slurries using microradiography and microdensitometry. Enamel sub-surface lesions were created and inserted into upper removable appliances worn by fourteen volunteers. Five products were tested (i) placebo, (ii) 1100 ppm NaF, (iii) 2800 ppm NaF, (iv) 2% CPP-ACP and (v) 2% CPP-ACP Plus 1100 ppm NaF. Participants were asked to rinse for one minute four times daily for two weeks. The results of the study showed significant residual remineralisation for the 2% CPP-ACP Plus 1100 ppm NaF group compared to other toothpastes.

Adding to the evidence, a recent meta-analysis (Tao et al., 2018) compared the efficacy of fluoride alone to a combination of CPP-ACP and fluoride on early carious lesions. Ten randomised controlled trials with 559 patients were included in the study. Its outcomes were measured using four methods: laser fluorescence, quantitative light-induced fluorescence (QLF), lesion area and visual assessment scores.

The study concluded that the combination therapy (CPP-ACP and Fluoride) is more effective than the monotherapy with fluoride only on early occlusal lesions. However, no significant difference was noted on the early proximal lesions. The main weakness of this study was that they defined the fluoride as any type of fluoride product, for example, fluoride mouth rinse, varnish or toothpaste with any concentration.

2.2.2.5 b Studies reporting no significant benefits of using CPP-ACP products over routine dental care on enhancing enamel remineralisation

A randomised double-blind controlled trial was carried out to assess the advantage of using CPP-ACP-containing paste over the use of daily 1000 ppm fluoridated toothpaste in preventing caries progression and enhancing enamel remineralisation. In this study 296 high caries healthy pre-school children were randomised and assigned to experimental and control groups. The former received a daily application of 10% w/v CPP-ACP paste whereas the control group used a placebo paste. Both groups were brushing with fluoridated toothpaste (1000 ppm F). The (ICDAS) score and DMFS were assessed by a single assessor (a paediatric dentist) at baseline, six months, and one year. The results showed there was no significant difference between the groups and no added benefits of the CPP-ACP-containing paste over the use of daily 1000 ppm F in the prevention of caries in primary teeth (Sitthisettapong et al., 2012). One of the main shortcomings of this trial was a high dropout rate with no intention to treat analysis performed. Furthermore, the pastes were only applied on school days, (the teachers applied the pastes), thus excluded weekends.

Huang et al. (2013) published randomised single-blind study to assess the added benefit of using either MI Paste Plus™ or 22600 ppm fluoride varnish over the use of routine oral hygiene at home using fluoridated toothpaste (1100 ppm F). Subjects (n=115) aged between 12 -20 years, with at least one white spot lesion, were randomly assigned to three groups. Group 1 received a twice-daily application of MI Paste Plus™ for eight weeks and routine home care. Group 2 had a single application of 22600 ppm fluoride varnish and routine

home care. Group 3 received only routine home care. Photographs were taken at baseline and after treatment (8 weeks) and assessed by a group of dental health professionals and laypersons. The results showed no significant difference between the control and test groups.

Furthermore, a double-blind randomised controlled trial (Beerens et al., 2010) evaluated the CPP-ACP effect on lesion regression and plaque composition. Fifty-four patients, diagnosed with white spot lesions following their orthodontic treatment, were randomly assigned to test and control groups. The former was instructed to use MI Paste Plus™ whereas the latter used a placebo paste. Both groups used the pastes as a supplement to their normal fluoridated toothpaste. Quantitative light-induced fluorescence (QLF) images and bacteria count (Streptococcus mutans, and Lactobacillus spp) were measured at baseline, six weeks and 12 weeks. The study concluded that there was a significant decrease in fluorescence loss and bacteria count in both groups following the three months of application. However, no statistically significant difference was detected between the test and control group. In this study, the concentration of the normal toothpaste used by both groups as the routine toothpaste was unspecified.

A further single-blind randomised control trial was published by Bröchner et al. (2011). It assessed the efficacy of a daily application of CPP-ACP paste compared with the control group. Both groups brushed with fluoridated toothpaste (1100 ppm F) and received standard oral hygiene instructions. Three hundred twenty-seven white spot lesions were assessed using QLF and visual inspection at baseline and after four weeks from application. The results

were consistent with the previous study Beerens et al. (2010) as both groups showed a significant reduction in fluorescence loss and lesion area compared to the baseline readings and no significant difference was found between the groups.

In addition, a recent single-blind randomised control trial was carried out by Karabekiroğlu et al. (2017). They investigated the efficacy of the topical application of CPP-ACP paste for management of white spot lesions resulting from orthodontic treatment. In this study, 41 participants with white spot lesions following orthodontic treatment were randomly allocated to either experimental (CPP-ACP paste + 1450 ppm) or control groups (1450 ppm F only). The labial surface of the lesions was assessed at baseline and after three years of using the pastes using a DIAGNOdent pen, Gorelick Index and ICDAS II criteria. The study concluded that there was no extra benefit in using CPP-ACP over the use of routine fluoridated toothpastes (1450 ppm F).

Moreover, Bataineh et al. (2017) conducted an *in vitro* study to investigate the remineralisation efficacy of CPP-ACP paste, CPP-ACP with 950 ppm F and 2800 ppm F toothpaste, to compare these with 1450 ppm (positive control) and non-fluoridated toothpaste (negative control). The study used QLF as the method of assessment. QLF images were taken and analysed at baseline and after 21 days of pH cycling. The results showed that ΔQ (percentage fluorescence loss times the area of the lesion) of the negative control group was significantly lower compared to other groups. However, no significant difference was detected between other groups.

Furthermore, Raphael and Blinkhorn (2015) carried out a systematic literature review to evaluate the effectiveness of Tooth Mousse™ (MI Paste™) and MI Paste™ (MI Paste Plus™) to prevent enamel demineralisation and enhance enamel remineralisation on white spot lesions. The study included 12 clinical trials that met the inclusion criteria. The results of the review stated that there were no significant benefits of using CPP-ACP products over routine brushing with fluoridated toothpaste for the prevention of early enamel caries.

2.2.3 Functionalised tricalcium phosphate (f-TCP)

Recent technology containing f-TCP has been produced by 3M ESPE Inc. In this technology, the remineralisation of enamel lesions results from milling TCP with sodium lauryl sulphate, which helps to inhibit unwanted premature interaction between calcium and fluoride. In addition, f-TCP maintains high levels of calcium, phosphate and fluoride, which enhances enamel remineralisation and inhibition of enamel demineralisation (Amaechi, 2015; Jo et al., 2014). Currently, f-TCP is commercially available as Clinpro toothpaste™ with (5000 ppm NaF), Clinpro tooth crème™ with (950 ppm NaF) and fluoride varnish with (22600 ppm NaF). In the literature, several number of *in vitro* studies have investigated the remineralisation ability of f-TCP. However, few clinical trials have investigated its remineralisation effect.

In vitro studies

A recent QLF *in vitro* study was conducted to evaluate the remineralisation efficacy of Clinpro tooth crème™ (950 ppm NaF) and compare it with CPP-ACP crème, fluoridated toothpaste (1000 ppm NaF) and a negative control group (deionised water). Forty-five enamel specimens from twelve extracted premolars with artificial subsurface-like lesions were randomly allocated to each group. The agents were applied twice a day for two weeks. Baseline and after treatment readings were taken using QLF. The results showed a significant fluorescence per cent increase in f-TCP and CPP-ACP groups (1.446% and 0.940%) respectively compared with (0.500% and 0.480%) in the

fluoridated and negative control groups respectively. This indicated more enamel remineralisation in the former groups (Jo et al., 2014).

Using a scanning electron microscope (SEM) and DIAGNOdent tests, Joshi et al. (2013) compared the enamel remineralisation efficacy of f-TCP with 900 ppm NaF, (CPP-ACP) and (CPP-ACPF) agents in an *in vitro* model. A total of 76 permanent posterior teeth with artificial subsurface enamel-like lesions were randomly assigned to four groups: (i) CPP-ACP, (ii) CPP-ACPF, (iii) f-TCP (900 ppm NaF) and artificial saliva. Samples were subjected to seven-day pH cycling with the agent being applied once a day for 4 minutes. All experimental groups showed a significant amount of remineralisation compared to the artificial saliva. The highest remineralisation amount was found in f-TCP followed by CPP-ACPF. However, no statistically significant difference was detected between the tested groups. One of the main shortcomings of this study was the short period of cycling, which might have affected the amount of remineralisation. In addition, the agent's application did not follow the manufacturer's recommendations.

In a further *in vitro* study, Makoto Asaizumi et al. (2014) used X-ray microtomography (micro-CT) to assess the density of white spot lesions (WSLs) exposed to three different toothpastes: (i) fluoride-free toothpaste, (ii) Clinpro crème™ with (950 ppm NaF) and Clinpro toothpaste™ (5000 ppm NaF). Thirty bovine enamel slabs with artificial subsurface-like lesions were randomly assigned to three groups. Each group was subjected to one acid challenge and four applications of one of the experimental toothpastes for ten days. Micro-CT assessment was undertaken before demineralisation, at

baseline and after treatment of ten days. At the end of the study, the Micro-CT findings showed an increased density of WSLs associated with teeth treated with toothpastes containing f-TCP compared with fluoride-free toothpaste. These results are consistent with another *in vitro* study that compared anti-caries ability of Clinpro™ toothpaste (5000 ppm) with NaF toothpaste (5000 ppm) and fluoride-free toothpaste using a microhardness test (Karlinsey et al., 2010).

Clinical studies

In line with previous *in vitro* studies, Amaechi et al. (2012) conducted a randomised, crossover, double-blind *in situ* study to assess the enamel remineralisation ability of high fluoride toothpaste containing f-TCP technology. Thirty participants were recruited and wore appliances with six enamel slabs for each phase (three demineralised slabs and three healthy slabs). The study included three arms and participants were crossed over., Each arm is lasting 28 days with a wash-out period of one week. The three arms were: (i) 5000 ppm F with f-TCP (Clinpro 5000™), (ii) 5000 ppm NaF and (iii) non- fluoridated toothpastes. The subjects were instructed to brush twice a day using the assigned toothpaste for each period. Using surface microhardness and transverse microradiography, lesions were assessed at baseline and after treatment. It was found that both fluoridated toothpastes significantly reduced enamel demineralisation and enhanced enamel remineralisation compared with the non-fluoridated toothpaste. It also indicated that of the three toothpastes,

Clinpro 5000™ had resulted in greater remineralisation compared with the 5000 ppm F toothpaste, 114% and 86.5% respectively.

Moreover, Vanichvatana and Auychai (2013) carried out a 14-day *in situ*, randomised controlled double-blind trial to assess and investigate the remineralisation efficacy of Clinpro Tooth Crème™ (900 ppm F) and CPP-ACPF (950 ppm F) and compare these to that of fluoridated toothpaste (1000 ppm NaF). The study had three different arms and participants (n=9) were crossed over using each toothpaste with a wash-out period of eight days. The CPP-ACPF (MI Paste™) in this study was used as a regimen after brushing with fluoridated toothpaste. A polarised-light microscope was used to assess the lesion area at baseline and at the end of day 14 of each arm. The study showed that there was a significant reduction in lesion areas in all tested groups. The authors concluded that Clinpro Tooth Crème™ produced a similar remineralisation effect to the fluoridated toothpaste and no extra benefits were noticed when using CPP-ACPF as a regimen with fluoridated toothpaste. One of the limitations of this study was that the participants did not wear the appliances all the time. They were advised to wear them at least 12 hours per day. The other limitation was the short period of the study. In addition, there was no clear sample size calculation.

2.3 Methods of study design used in caries research

Enamel demineralisation and remineralisation can be assessed using different models which include *in vitro*, *in situ* and *in vivo* models. Well-designed randomised controlled trials (RCTs) are considered the gold standard method. However, such clinical studies are time-consuming and costly (White, 1995).

2.3.1 In vitro

This type of study simulates the oral environment effects in laboratory settings. It can give meaningful information about enamel status under controlled conditions. The *in vitro* model is the most common model used for caries' research. In a recent systematic review that investigated the common models used for recent cariology research, *in vitro* models were the most frequent (84%) followed by *in situ* and *in vivo* model (15% and 1%) respectively (Yu et al., 2017).

Its advantages are that it can be a good alternative system as it is simple and reproducible. In addition, it generally requires less time and is relatively inexpensive compared to the RCT clinical studies. Another advantage is that the *in vitro* model offers more control over the experimental conditions compared to other systems. Furthermore, there are less restrictive ethical requirements required (Yu et al., 2017; White, 1995).

However, there are several limitations associated with *in vitro* models. The main disadvantage is the difficulty to mimic the complex oral environment completely. In addition, results can be significantly affected based on the

design of the model used. Furthermore, artificial caries' characteristics can be different from actual caries (Yu et al., 2017; White, 1995).

2.3.2 *In situ*

Zero, (1995) defined the *in situ* caries model as "the use of appliances or other devices which create defined conditions in the human mouth that simulate the process of dental caries". The *in situ* model can be described as a bridge between *in vitro* and *in vivo* models. The main components of *in situ* caries are tooth substance (enamel or dentine), plaque formation, caries challenge; obtained from participants' diet or controlled and time determined by the investigator. Unlike epidemiological studies, *in situ* studies require relatively fewer volunteers and a shorter period of time to avoid irreversible damage to the tissue (Zero, 1995).

The *in situ* system offers several advantages over other models. The main one is that it takes place inside the oral environment unlike *in vitro* experiments. This allows for several important biological factors to be considered like the role of saliva and plaque. Another advantage is that it provides more control over the experimental variables compared to clinical trials. Its short-term feature is another merit since this can help to overcome ethical challenges (Zero, 1995).

Nevertheless, the *in situ* model has several disadvantages. The foremost limitation is that due to the nature of the model, the sample size is usually small. This may raise concerns about applying the results to wider populations. Another key drawback of this model is that it is highly dependent on participants' compliance. It requires highly motivated participants as lack of

compliance might have a major impact on the outcome. In addition, this technique needs more ethical requirements compared to *in vitro* models. The technique is also demanding, which requires extensive resources and clinical expertise (Zero, 1995).

2.3.2.1 *In situ* study design

Zero (1995) noted several factors that need to be considered when designing an *in situ* model, which are set out in the following paragraphs.

Number of participants:

As discussed previously, the sample size is usually small due to the nature of the *in situ* model (up to 40 participants). It is important to obtain a sample size based on a validated sample size calculation.

The use of control:

Based on the research aims, appropriate positive and negative control should be considered. This can be, for example, using fluoride-free toothpaste as a negative control in fluoride studies.

The cross-over or parallel designs:

This needs to be determined based on the research type, resources and study aim. The advantages of using a cross-over design are that it requires a relatively small sample size. However, the residual effect may have an impact

on the outcomes. Therefore, a wash-out period needs to be considered when choosing this design. The wash-out period can be one week, two weeks or even longer(Zero,1995; Stephen et al. 1992).

Length of experimental period:

Based on the type of *in situ* model and research question, the study period can range from as short as 45 minutes up to six months.

The caries challenge:

Several methods can be used as a dietary challenge. The most common way is to use the individual's normal diet (Zero 1995). Modifying a participant's diet is another method utilised (Hall et al., 1995).

Standardisation:

It is important to standardise the oral condition as much as possible. The aim is to minimise the variations between participants. An example of how to do this is to standardise the oral hygiene practice. This was achieved in our model by asking participants to use only the assigned toothpaste for each arm. No other fluoride-containing food or products were used during the study. Other factors like saliva and medical history were carefully considered in our inclusion and exclusion criteria.

The compliance assessment:

As mentioned earlier, the *in situ* model is largely dependent on participants' compliance. Therefore, compliance needs to be assessed, and several methods have been suggested to evaluate this. A common way is to use a diary in which volunteers complete a daily form. Another way is to measure the dispensed products before and after. It is also important to realise that this type of design is dealing with human volunteers. Thus a realistic protocol is necessary to avoid poor compliance.

2.3.2.2 *In situ* models used for demineralisation and remineralisation studies

Various *in situ* methods and designs have been used for demineralisation and remineralisation studies. Three main designs have been discussed in the literature: the banding model, crown single-section model and the removable appliances. Of these, the removable appliance technique is the most commonly used method (Øgaard, 1990).

The removable appliances design has the advantage of allowing normal oral hygiene with minimal disturbance to the samples. However, this technique relies on patient compliance. Several designs have been used for *in situ* removable appliances. For example, Shen et al. (2001) used an upper removable acrylic appliance that covered a palate of the posterior teeth with two troughs to house the slabs. This technique was used by Vanichvatana and Auychai (2013) with a slight modification (Figure 2-2). The upper removable appliance design is used in 68% of *in situ* cariology research studies (Hollanders et al., 2018).

The removal appliance introduced by Koulourides et al. (1974) has a labial arch with two acrylic buccal posterior flanges and metal clasps to aid retention. The substrates are attached to the appliances and secured with sticky wax. In addition, a gauze or mesh can be used to accumulate plaque (Figure 2-3)

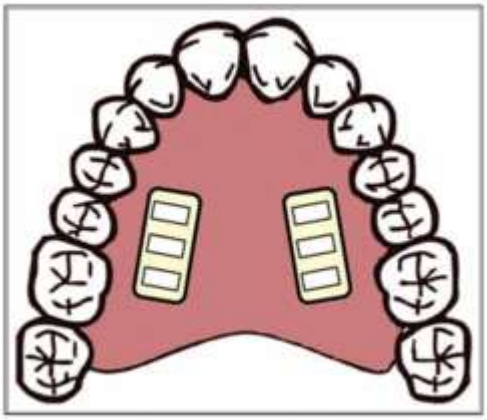

Figure 2-2 Upper removable acrylic appliance

(Vanichvatana and Auychai, 2013)

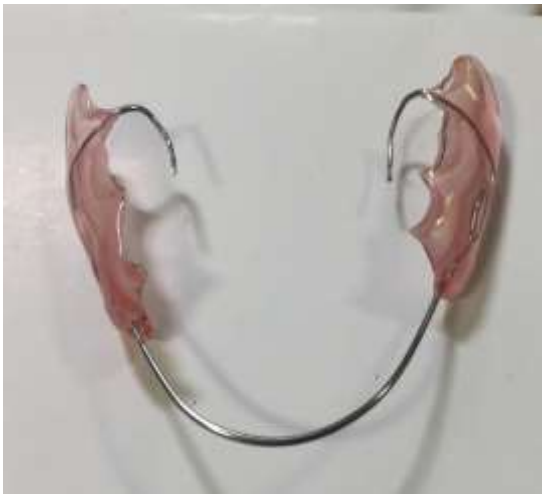

Figure 2-3: Lower removable appliance with two buccal acrylic flanges

2.3.2.3 Substrates used for *in situ* models

Different types of substrates have been used in different cariology *in situ* models. The most common substrate is the slabs derived from human enamel and dentine tissues. Other materials have also been used, including shark enamel, human root tissue and bovine enamel. Bovine enamel is good and the most common alternative for human enamel. (Table 2-1) summarises the main variations between human and bovine dental tissue (Hollanders et al., 2018; Sønju Clasen and ØGaard, 1999).

Table 2-1: Main variations between human and bovine dental tissues

Characteristic	Human dental tissue	Bovine dental tissue
Dentinal tubules	More dentinal tubules	Fewer dentinal tubules
Lesion progression	Slower	Faster
Tissue density	Less compared to bovine	Higher compared to human
Chemical compositions	More variation compared to bovine	Less variation compared to human
Fluoride concentration	Higher than bovine	Less than human

2.3.3 In vivo

In vivo models are experiments carried out in living organisms, on living tissues. This takes into account different biological and chemical factors occurring inside the living organism. In dental caries' research, such models are less common compared to others, as discussed previously (Hollanders et al., 2018). The most common example of this system is using teeth planned for orthodontic extraction in dental studies (Featherstone, 1996). The main advantages of this model for cariology are that it uses the natural oral environment like saliva, plaque and the teeth. It also considers the normal diet of the organism, which makes the results more relevant (Featherstone, 1996).

However, several limitations are associated with this system. It is time-consuming and expensive to undertake. In addition, as with the *in situ* model, it relies mainly on patient compliance. Furthermore, it is difficult to run over a long period due using vital tissues and participant compliance. Another challenge is the complexity of the ethical requirements (Featherstone, 1996).

2.4 Methods of mineral assessment

As described earlier, the enamel demineralisation and remineralisation are related to the process of losing or gaining minerals. Several methods have been used to assess the enamel de/remineralisation(Arends and Ten Bosch, 1992). This part will discuss the most common techniques used to evaluate the change in mineral content.

2.4.1 Microhardness

Microhardness technique measures the resistance of the dental hard tissue against the load produced from indention process. The indenter can be done using either Knoop or Vickers diamond which is placed on the dental hard tissue for a specified amount of time. The indentation-length left can then be measured in micromillimeter using a microscope. The microhardness technique is considered as an indirect method to assess mineral loss and gain (White et al., 1992; Arends and Ten Bosch, 1992).

Microhardness techniques are classified into two methods, Surface microhardness (SMH) and Cross-sectional microhardness (CSMH). In SMH the indenter is placed perpendicular on a highly polished flat dental sample. The advantages of this method include that it is an easy and inexpensive method. It is also non-destructive which allows for multiple assessments for the same sample. However, a flat highly polished surface is required in order to obtain a reliable result. In addition, it does not give information about mineral distribution and lesion shape. One more disadvantage of this technique was

described by Zero et al. (1990) is that this technique is effective up to limited level of lesion depth (Arends and Ten Bosch, 1992).

Whereas in CSMH the indenter is used parallel to the dental tissue; using this method offers several advantages include; being able to obtain more details about the mineral profile. Also, this technique provides quantitative information about mineral loss or gain. However, (CSMH) measurement excludes the outer 25 micromillimeters of the specimen (Arends and Ten Bosch, 1992).

Lippert and Lynch (2014) carried out an *in vitro* study to compare the ability of surface microhardness (SMH) using Knoop or Vickers diamond with Transverse Microradiography (TMR) to assess early enamel lesion formation. Bovine enamel (n=90) and human enamel (n=90) were subjected acid challenge to create artificial enamel subsurface lesions and divided into six treatment groups. Each group received a further demineralisation challenge with different times. SMH assessments were obtained at baseline and after demineralisation for each group. Lesions were also assessed using Transverse Microradiography (TMR). The results showed that there was a positive correlation between indentation length and demineralisation times. However, a state of plateau was noticed after 40 hours demineralisation time. The SMH's results were relatively consistent with TMR's findings. The study also found a significant positive relation between Knoop or Vickers diamond.

2.4.2 Quantitative light-induced fluorescence (QLF)

Since 1994, QLF has been widely used as a method of assessment for de and remineralisation. There are several parameters that can be obtained from QLF. The main three parameters that are used for assessment of remineralisation and demineralisation are summarised in Table 2-2 (Lippert and Lynch, 2014)

Table 2-2: Main QLF parameters that are used for caries research

Parameter	Unit	Description
ΔF	%	Percentage fluorescence loss with respect to the fluorescence of sound tooth tissue. Related to lesion depth.
ΔQ	$\%px^2$	Percentage fluorescence loss with respect to the fluorescence of sound tissue times the area. Related to lesion volume
Lesion area	px^2	Area with ΔF equal to or smaller than a specific threshold value of ΔF

The QLF principal based on the concept that change in mineral content of the tooth affects its autofluorescence. This means dental hard tissue with

demineralisation absorbs less light secondary to increase in its porosity (Karlsson, 2010).

Several studies have been conducted to assess the QLF validity. An *in vitro* study compared the ability of QLF and digital photography to measure remineralisation with the gold standard Transverse Microradiography (TMR). Forty slabs with artificial enamel subsurface like lesions were obtained from human enamel. The specimens were treated with remineralising agents for ten days. Baseline and after treatment readings were carried out using QLF and digital photography and compared with TMR readings. The results revealed a statistical positive correlation between the three methods; gold standard and QLF ($r = 0.63$), the gold standard and digital photography ($r = 0.59$), and digital photography and QLF ($r = 0.64$). The authors concluded that QLF and digital photography can give valuable information about mineral loss or gain and can be used where the destruction of the specimens is not an option. They also stated that QLF was easier to analyse and had less variability compared the digital photography (Cochrane et al., 2012).

Furthermore, repeatability and reproducibility of the QLF were assessed in an *in vivo* study. Fifteen Images were captured and analysed by three different specialists. The results of the study showed high inter-examiner reliability for three analysts and excellent reproducibility (Tranæus et al., 2002). These results were in line with another *in vitro* study (Pretty et al.

2002) which assessed the inter and intra-examiner reliability of QLF.

The major advantage of this technique is that it is a non-destructive method that can be used in longitudinal studies. In addition, it offers an advantage of reducing time and cost for clinical studies (Higham et al., 2005).

However, the main limitation that associated with this system is that the QLF readings can be misinterpreted due to the presence of stains, plaque and dehydration (Al-Khateeb et al., 2002).

2.4.3 Transverse Microradiography (TMR)

Transverse Microradiography (TMR) is the most common type of microradiography. It is now considered as the gold standard for mineral assessment as it has a high accuracy of evaluation of minerals' loss or gain. In addition, (TMR) gives valuable information about mineral distribution.

In this technique, thin enamel slabs are cut (µm90) and oriented perpendicularly to the tooth surface. The samples alongside a calibration aluminium wedge are placed in a film and subjected to irradiation with monochromatic x-rays. Two main parameters can be calculated, mineral loss (vol%.µm) and lesion depth (µm), by analysing the absorption of the x-rays (Arends and Ten Bosch, 1992).

The main limitation of this approach is that it is a destructive method. In addition, misinterpretation of data can result from the presence of ions that has a high absorption factor (Arends and Ten Bosch, 1992).

2.5 Research aims and hypotheses

2.5.1 Aim

The aim of this study was to investigate the efficacy of two different caries preventive regimen and therapies: MI Paste Plus combined with fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride); f-TCF Clinpro tooth crème 950 ppm F (0.21% w/w sodium fluoride) and fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride), on de/remineralisation of dental enamel under cariogenic challenge in an *in situ* randomised, cross-over study design.

2.5.2 Objectives

1- To assess and compare the effect of a different caries preventive regimen and therapies: MI Paste Plus combined with fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride); Clinpro tooth crème 950 ppm F (0.21% w/w sodium fluoride) and fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride), on progression/regression of artificial subsurface carious lesions *in situ*, using quantitative light fluorescence (QLF) technology.

2- To assess and compare the effect of a different caries preventive regimen and therapies: MI Paste Plus combined with fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride); Clinpro tooth crème 950 ppm F (0.21% w/w sodium fluoride) and fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride), on demineralisation of dental enamel *in situ*, using Surface Microhardness (SMH).

2.5.3 Null hypotheses

1. There is no difference between 0 ppm F toothpaste; MI Paste Plus combined with fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride); Clinpro tooth crème 950 ppm F (0.21% w/w sodium fluoride) and fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride), on progression/regression of artificial subsurface carious lesions *in situ* as measured by QLF.

2. There is no difference in the effect of different caries preventive regimen and therapies: MI Paste Plus combined with fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride); Clinpro tooth crème 950 ppm F (0.21% w/w sodium fluoride) and fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride), on progression/regression of artificial subsurface carious lesions *in situ* as measured by QLF.

3. There is no difference between 0 ppm F toothpaste; MI Paste Plus combined with fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride); Clinpro tooth crème 950 ppm F (0.21% w/w sodium fluoride) and fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride), on the inhibition of the demineralisation process in dental enamel under cariogenic challenge *in situ* as measured by SMH.

4. There is no difference in the effect of a different caries preventive regimen and therapies: MI Paste Plus combined with fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride); Clinpro tooth crème 950 ppm F (0.21% w/w sodium fluoride) and fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride) on the inhibition of the demineralisation process in dental enamel under cariogenic challenge *in situ* as measured by SMH

3.0 MATERIALS AND METHODS

3.1 Study Design

This is a randomised, crossover design study *in situ*. The study comprise of four study arms and making a total of 4 months for the entire study. Each study arm comprise a washout period of one week before a 21 day treatment period. Participants were wearing an intra-oral lower removable appliance continuously (except at meal times, when brushing their teeth and whilst drinking) for 3 weeks during each period. The participants then started a wash out period for a minimum of one week in order to start next arm.

During the washout period, the participants used fluoride-free toothpaste for tooth brushing. During the treatment period, the cariogenic challenge comprised of five dipping per day in a 12% sucrose solution and volunteers dipped the appliance in 50 ml of the sucrose solution for two minutes on each occasion. Volunteers also dipped their appliances twice daily for 2 minutes, in one of the test toothpaste slurry, which was prepared by mixing 1 gram of the toothpaste with 5 ml of distilled water.

For the regimen group, volunteers dipped their appliances twice daily for two minutes in 1450 ppm sodium fluoride toothpaste slurry. Also, once daily at night time, the dipping of the appliances in 1450 ppm sodium fluoride toothpaste slurry will be followed with a dipping in a Tooth Mousse Plus (10% w/v CPP-

ACP, 900ppm sodium fluoride) slurry for three minutes. These procedures were repeated for 21 days for each test period. The enamel subsurface lesions images and remineralisation were assessed using a Quantitative Light Induced Fluorescence (QLF) system under controlled conditions at baseline and after treatment. Intact enamel slabs and demineralisation were assessed using Surface Microhardness under controlled conditions at baseline and after treatment.

3.2 Ethical and regulatory aspects

Ethical approval for this study was sought from the Dental Research Ethics Committee of the University of Leeds at (Appendix 1). Informed consent was obtained from all participants. The Principal Investigators (PI) ensured that this study was conducted in full conformance with the laws and regulations of the country in which the research is conducted and the Declaration of Helsinki/Venice/Tokyo/Hong Kong/South Africa (1996).

3.2.1 Training and calibration

Prior to the commencement of the study, the Principal investigator (PI) obtained Good Clinical Practice (GCP) and laboratory training. In addition, he was calibrated to use of Diamond Wire cutting machine, QLF and Microhardness machines.

3.2.2 Sample size and power calculation

Based on data from previous *in vivo* study by Manal Allammari et al. (2010) Statistical advice was sought and the sample size was calculated. A sample size of 12 was required to achieve 91% power to detect a difference of 5% in ΔF between four materials with an estimated standard deviation of 4.5% and with a significance level (alpha) of 0.05 using one-way ANOVA-test. However, with around 15% dropout rate, 14 subjects were recruited to achieve the same power.

3.2.3 Recruitment and selection of participants

Recruitment was through using poster advertisement at the University of Leeds (Appendix 2). Fourteen subjects were recruited and completed the study. The age range of the volunteers was 22-60 years old. Volunteers were mainly from the staff at Leeds Dental Institute or students at Leeds University.

3.2.4 Inclusion Criteria

- Minimum of 18 natural teeth,
- No sign of active periodontal disease,
- Medically fit and well,
- Provide written informed consent, authorisation for the release of health information for research and medical history information before their participation,
- Between 18 to 65 years old in good health with no evidence of infectious diseases,
- Minimum unstimulated whole saliva flow rate $\geq$ 0.2 ml/min and a stimulated whole saliva flow rate $\geq$ 0.8 ml/min,
- Be able to wear the appliances 24 as required by the protocol,
- Be able to comply with the experimental procedures.

3.2.5 Exclusion criteria

- Signed informed consent not obtained by the volunteers,
- Adults taking drugs that affect the saliva rate,
- Volunteers with a complex medical history (e.g. epileptic subjects, subjects at risk of infective endocarditis, or pregnant/nursing subjects),
- Volunteers who had a course of antibiotics in the last four weeks,
- Volunteers who had antimicrobial treatment in the last two weeks,
- Volunteers with complex dental history such as periodontitis, rampant caries or salivary dysfunction,
- Volunteers with an allergy to any of the materials used in the study,
- Have any medical condition that could be expected to interfere with the subject's safety during the study period,

- Be taking any medication that could potentially react with the testing products,
- Demonstrate an inability to comply with study procedures,
- Volunteers showing signs of moderate or severe tooth wear.

3.2.6 Subject withdrawal criteria

Subjects had the right to withdraw from the study at any time for any reason. The investigator also had the right to withdraw subjects from the study if the subject failed to comply with the study protocol, experienced concurrent illness or adverse events.

3.2.7 Subject replacement

If a subject discontinues before completing all study assessments, a replacement subject would have entered into the study following an agreement between the investigator and the ethics committee.

3.2.8 Investigational products

1- Non-Fluoride toothpaste (the Boots Company PLC, Nottingham, England).

2- Fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride) (Colgate cool stripe. Colgate–Palmolive (UK) Ltd, Guildford, England).

3- MI Paste Plus 10% w/v CPP-ACP, 900 ppm F (0.2% w/w sodium fluoride) (GC MI Paste Plus™, GC Corp, Tokyo, Japan) + Fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride) (Colgate cool stripe. Colgate–Palmolive (UK) Ltd, Guildford, England) as a regimen.

4- 3M ESPE Clinpro Tooth crème - (Functionalized Tricalcium Phosphate + 950 ppm sodium fluoride).

3.3 Test methods used in de/remineralisation evaluation:

Enamel remineralisation was assessed in this study by using Quantitative Light-Induced Fluorescence (QLF) whereas enamel demineralisation was measured using Surface Microhardness (SMH).

3.3.1 Quantitative Light-Induced Fluorescence (QLF)

QLF was previously described in Section (2.4.2).

3.3.1.1 Teeth selection and cleaning

Bovine incisors were collected and stored in distilled water and 0.1% thymol (Sigma Aldrich, thymol 98%) at room temperature. Any soft tissue remnants were removed using a spoon excavator and a toothbrush with pumice powder and stone. The teeth were then visually inspected for any defect or caries. Also, all teeth were examined by trans-illumination and transmitted light using low-power microscopy (Leitz, Wetzlar®, Germany) to confirm absence of stains, caries, defects or cracks. Suitable teeth were selected.

3.3.1.2 Preparation of the enamel slabs for QLF analysis:

The selected teeth were mounted on plates using ‘green stick’ compound (Kerr, UK) on plates before teeth sectioning. The crowns were then sectioned using diamond wire saw cutting machine (Well@Walter EBNER, CH-2400 Le Loche). The slabs were obtained from the buccal flat surface. The size of each slab

was approximately 4mm x 4mm x 2mm.

Figure 3-1 Diamond wire saw cutting machine (Well® Walter EBNER, CH-2400 Le Loche).

3.3.1.3 Preparation of the Artificial Enamel Subsurface Lesion for QLF

A demineralising acid gel was used.to create enamel sub-surface like lesions

The preparation of demineralisation system: acidified hydroxyethyl cellulose gel

The demineralising gel was produced by mixing 0.1 M sodium hydroxide (BDH Analar Grade) and 0.1 M lactic acid (Sigma Aldrich D/L GPR 87% Lactic acid) to achieve a pH value of (4.5). 6% w/v hydroxyethyl cellulose (Sigma Aldrich) was then added to the solution and stirred for one hour until a consistency

similar to that of “wallpaper paste” is achieved. The mixture was left to settle for a period of 24 hours. The demineralising gel was poured into the universal tubes “Sterilin” into which the mounted teeth were submerged. The enamel slabs were varnished with two layers of an acid resistant, coloured Nail varnish (Max Factor “Glossfinity”) except for a window approximately 1mm by 2 mm. the slabs then immersed in the acid gel for a period of ten days. The slabs then removed and washed with distilled water. Methanol was used to remove the nail varnish to prepare the slabs for baseline QLF readings

3.3.1.4 Quantitative light-induced fluorescence (QLF) analysis

QLF readings were taken at baseline and at the end of each arm in a dark room using the QLF machine (QLF-D Biluminator™ 2 Inspektor Research Systems BV, Amsterdam, The Netherlands), under controlled conditions (Figure 3-2).

QLF-D Biluminator™ 2 consists of a Biluminator™ mounted on a Single Lens Reflex (SLR) camera fitted with a 60 mm macro lens. The Biluminator™ provides the light sources and filters for making white-light and QLF™-images. Fluorescence images of all enamel specimens were captured with a ‘Live View’-enabled digital full-sensor SLR camera (model 550D, Canon, Tokyo, Japan) at the following setting: shutter speed of 1/30 s, aperture value of 6.7, and ISO speed of 1600. All digital images were stored automatically on a personal computer with image-capturing software (C3 version 1.16; Inspektor Research Systems). All fluorescence images were examined with analysing software (QA2 version 1.16; Inspektor Research Systems). The analyses were performed by a single trained examiner.

The study images were captured in a standardised and controlled environment. These were achieved by using the same camera position, angels and distance. The QLF camera was fixed at a position that provided optimum illumination of the enamel block surface. To minimise the hydration effect, the slabs were dried for 15 seconds before capturing as recommended by (Al-Khateeb et al., 2002).

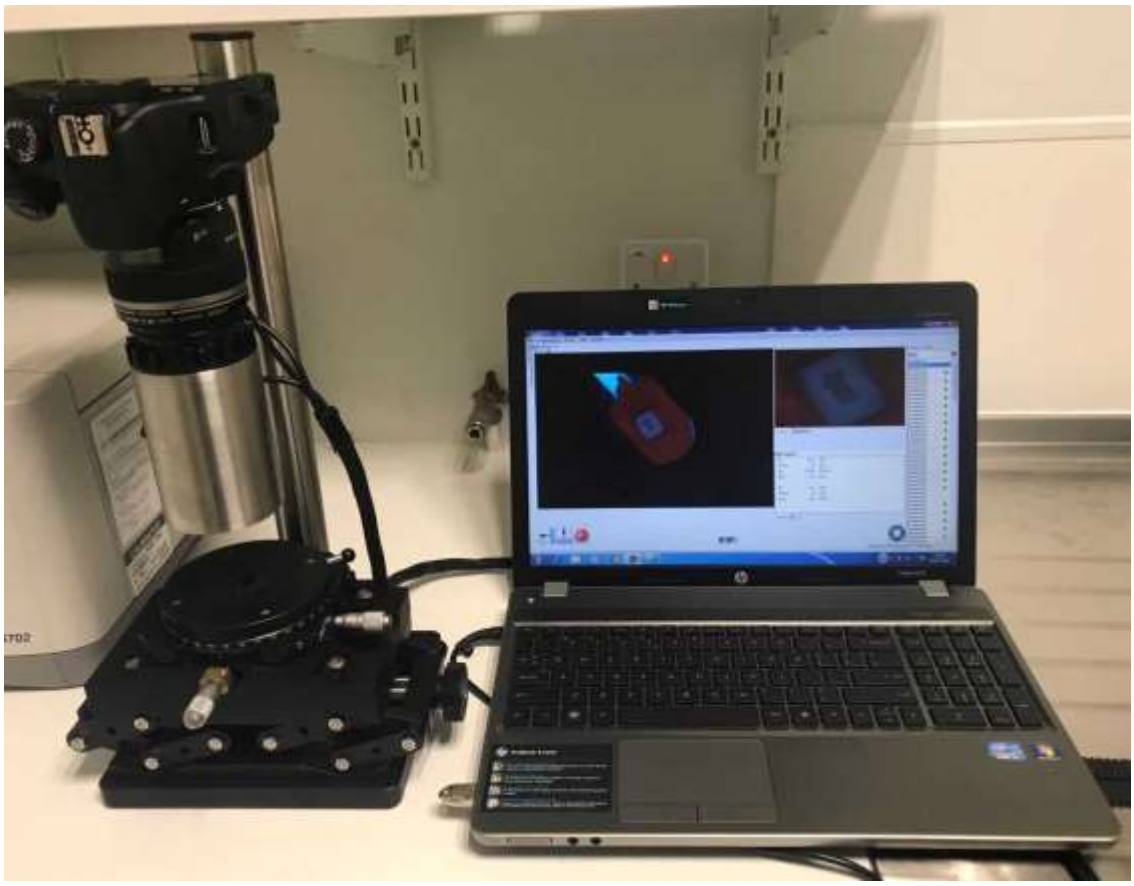

Figure 3-2 QLF machine, the SLR camera attached to the stand with standardised distance from the enamel slab.

The operator (MA) drew a patch around the white spot lesion area with its border on sound enamel (Figure 3-3). Inside this patch, the fluorescence levels of sound tissue were reconstructed by using the fluorescence radiance of the

surrounding sound enamel. The percentage difference between the reconstructed and the original fluorescence levels was calculated. The same area of interest was used for the baseline and endpoint white spot lesion image identification.

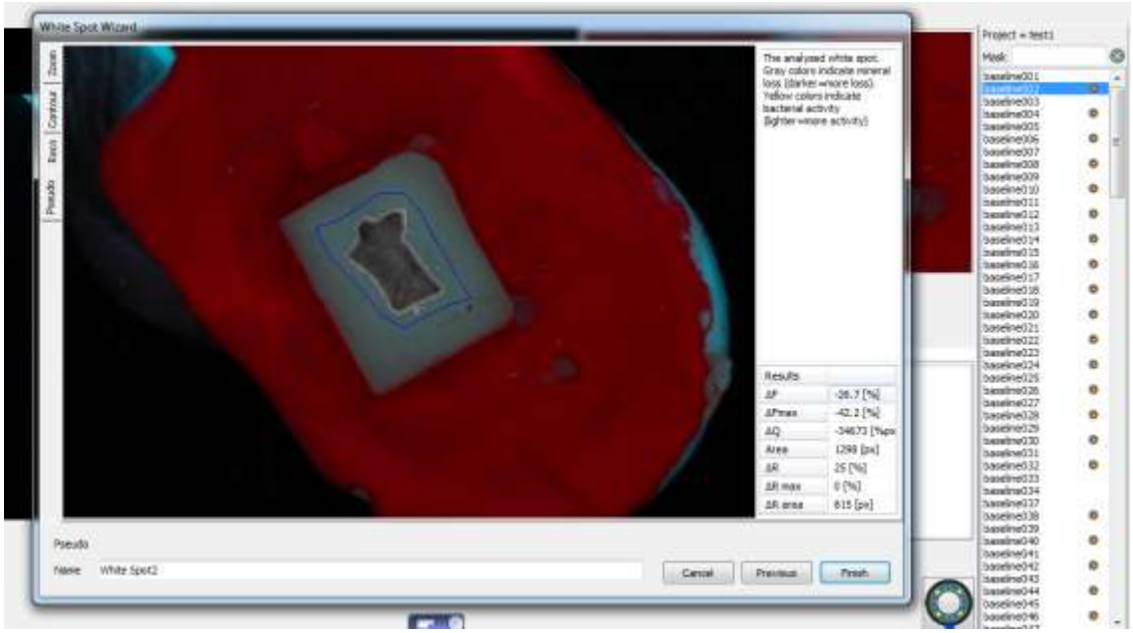

Figure 3-3: QLF image taken with the blue light shows the demineralised lesion in the centre of the enamel slab as well as a patch drawn around the lesion with the border in sound enamel.

Demineralised areas appeared as dark spots. The fluorescent radiance of a white spot lesion viewed by QLF was lower than that of sound enamel. In order to enable calculation of loss of fluorescence in the white spot lesion, the fluorescent radiance of sound tissue at the lesion site was reconstructed by interpolation from the radiance of the sound tissue surrounding the lesion. Fluorescence radiance levels less than 95% of reconstructed sound fluorescence radiance levels were considered to be artificial early caries lesions and were displayed as shades of grey where darker grey corresponds to higher

fluorescence loss. The difference between the measured values and the reconstructed values gave the resulting fluorescence loss in the lesion.

Three metrics were assessed for each enamel lesion:

1. ΔF: Percentage fluorescence loss with respect to the fluorescence of sound tooth tissue. This is a measure of lesion depth (%).
2. ΔQ: ΔF times the Area. Percentage fluorescence loss with respect to the fluorescence of sound tissue times the area. This is a measure of lesion volume (%px2).
3. Area: The surface area of the lesion expressed in pixels2 (px2).

3.3.2 Knoop Microhardness (KMH)

Knoop Microhardness (KMH) was previously described in Section (2.4.1).

3.3.2.1 Teeth selection and cleaning

Teeth selection and cleaning were previously stated in Section (3.3.1.1)

3.3.2.2 Preparation of the Enamel Slabs for Microhardness testing

The selected teeth were mounted on plates using ‘green stick’ compound (Kerr, UK) on plates before teeth sectioning. The crowns were then sectioned using diamond wire saw cutting machine (Well@Walter EBNER, CH-2400 Le Loche). The slabs were obtained from the buccal flat surface. The size of each slab was approximately 2mm width, 4mm length and 2 mm in depth. To ensure accuracy of the microhardness reading, the slabs were polished while wet using fine grit abrasive paper (P1000 Wet & Dry paper, 3M) in combination with 5 µm and 1µm alumina paste used to remove the outermost enamel layer (100-200 µm) and achieve a flat surface.

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3.3.2.3 Baseline Microhardness Assessment:

Each enamel slab was subjected to baseline microhardness testing before being placed in the *in situ* intra-oral appliances for the study. After sterilisation, an assessment of the enamel microhardness was made using a Knoop indenter with a load of 100 g applied for 15 seconds. Five indentations were applied and measured across the enamel specimen. A clean, sterile indenter was used for reading the enamel slabs.

Figure 3-4: The computer-aided Duramin Indenter Machine (Struers A/S, DK 26-10, Denmark).

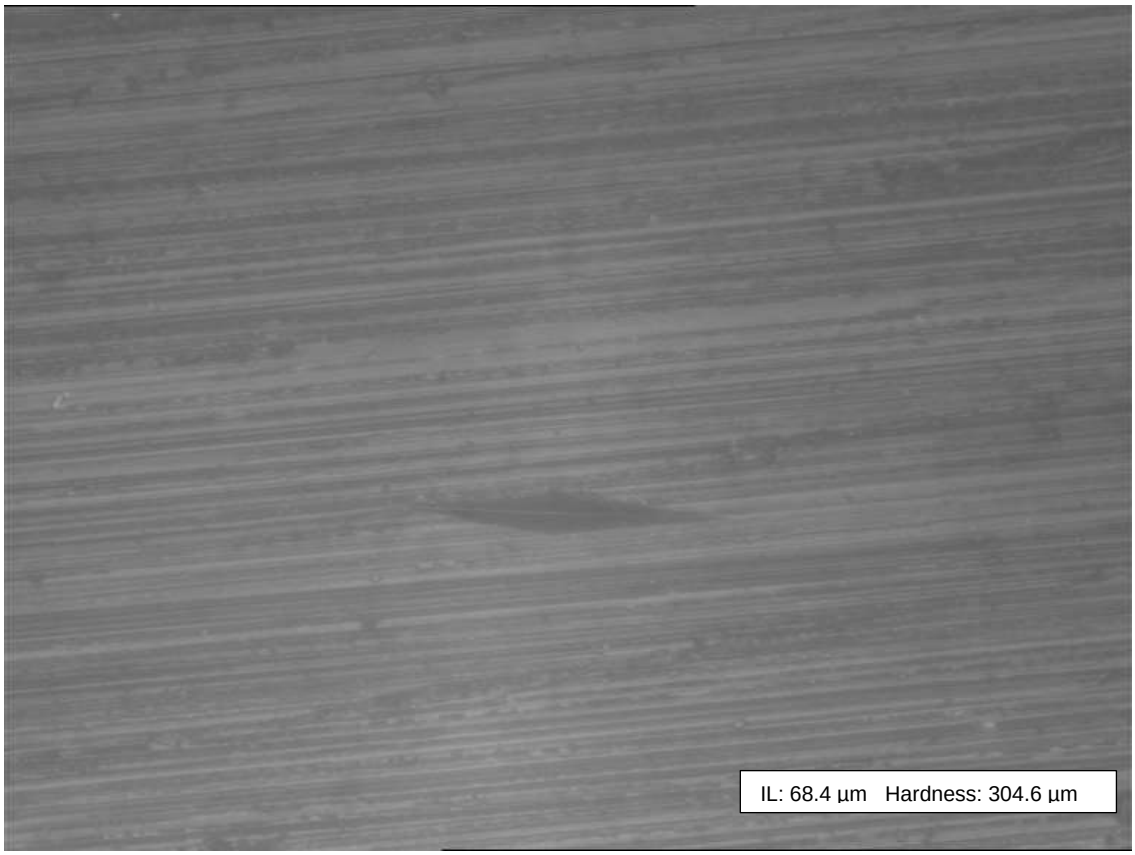


Figure 3-5: Microscopic image of diamond shape indentation on the enamel surface of the enamel at baseline.

3.3.2.4 Orientation of Microhardness Testing on Enamel Slabs:

Indentation occurred on the buccal surface of a tooth only. The surface was made flat and shiny during preparation processes to ensure an optimal surface for microhardness testing. The indents on the slab were applied at five points across the enamel slab as shown below:

2

4 1 5

3

3.3.2.5 Indenter Sterilisation:

Indenter sterilisation was conducted after microhardness testing of any potentially contaminated slabs using Virkon Surface disinfectant.

3.3.2.6 Sterilisation and storage of enamel slabs

Once the slabs were prepared, they were kept moist in de-ionised distilled water at room temperature (20^{o}C). The slabs were immersed in 12% sodium hypochlorite for 24 hours to eliminate prions. The slabs were then stored damp in sealed containers and sent to the Department of Immunology of the University of Liverpool, where they were exposed to gamma radiation (4080 Gy). The slabs were handled at all time using disposable medical gloves.

3.3.3 Experimental appliance

The appliance used in the study was a mandibular removable appliance, which was introduced by Koulourides et al. (1974). It consisted of a labial arch with two acrylic buccal posterior flanges and metal clasps to aid retention (Figure 3-6). Two enamel slabs were secured in the buccal flange of the appliance for each phase. The enamel slabs were assigned to the left side of the appliance and secured in the buccal flange with sticky wax, care was taken to ensure that the wax did not cover the exposed surface of the enamel. The slabs were then covered by 0.15mm Dacron gauze and secured with sticky wax. The gauze was used to act as a plaque retention factor.

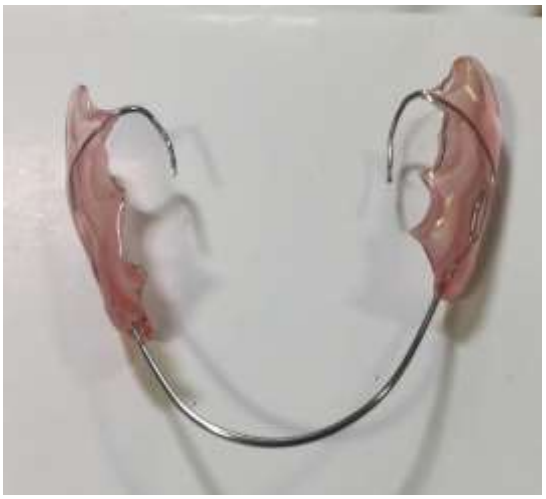


Figure 3-6: Lower removable appliance with two buccal acrylic flanges.

3.4 Randomisation and blindness

The randomisation of the enamel slabs for both QLF and Microhardness tests was applied in order to allocate them to the subjects. In addition, subjects were randomised in order to organise the order of the toothpastes they used. Randomisation was performed by using a randomisation website http://www.randomization.com (Appendix 3). When the slabs were analysed with QLF or Microhardness, the investigator (MA) did not know which group the slab belong to, making the analysis blinded.

3.5 Study procedures

3.5.1 Informed consent

Advertisement posters were placed at public areas at the University of Leeds. Participant information sheet was given to potential participants (Appendix 4). A minimum period of seven days was given to participants before they were invited for the first screening visit. After adequate explanation of study aims, methods and potential risks and benefits, an informed verbal and written consent was obtained by the chief investigator (MA) from participants prior to participation (Appendix 5). The date and time of the consent was then recorded in the participant's records.

3.5.2 Screening visit

A subject screening record and a Case Record Form (CRF) was used to document the screening evaluation along with any reason for failure (Appendix 6). At the screening visit, a CRF was completed for all attended subjects. Eligible subjects were randomised to receive study products and a CRF was completed for all randomised subjects. The following were performed during assessed during the screening visit:

3.5.2.1 Demography

Participants' date of birth and gender were recorded in the CRF.

3.5.2.2 Medical History

The investigator took the medical history of each subject including details of any relevant medical or surgical history, allergies or drug sensitivity. The Investigator also reported into the CRF details of any concomitant medications.

3.5.2.3 Oral examination

At the start of the study, the subject was given a dental examination to determine their decayed, missing and filled teeth (DMFT Index). The findings were recorded in the CRF.

3.5.2.4 Salivary flow rate

A referenced salivary flow rate (an unstimulated whole saliva flow rate $\geq$ 0.2 ml/min and a stimulated whole saliva flow rate $\geq$ 0.8 ml/min) was measured to ensure that a standard remineralisation effect of the saliva of all volunteers was achieved.

3.5.2.5 Measurements made for *in situ* oral appliances

The subjects were seated in a comfortable position in a dental chair. In order to protect clothing, the subject wore a bib. The subjects used a dental mouth rinse to keep the oral cavity clean during the impression procedure.

A colour coded transparent impression tray (Polytray Dentsply) was chosen to fit each subject. The chosen tray was filled with an alginate impression material (Xantalgin®, Kulzer) and placed in the subject's mouth to give an impression of the lower jaw/arch. A wax bite was taken. The impressions and wax bite was disinfected in Perform-ID (Schulke & Mayr) before transporting to the dental laboratory for *in situ* intra-oral appliance construction.

3.6 Treatment Phases

3.6.1 Wash-in period

In order to eliminate the effect of any carryover dental products, there was a wash-in phase for seven days prior to the start of the first arm of the study. Subject used fluoride free toothpaste during this period. Each participant had a wash-out period with non-fluoridated toothpaste between each study arm.

3.6.2 Acclimatisation period

The subjects wore the *in situ* oral appliances for 2-7 days before commencing arm one (i.e. concurrent with the wash-in phase of study arm 1). During this period subjects wore the appliances at all times (except when eating, drinking, or brushing their teeth).

If the subject experienced discomfort, they returned to the study site for the appliance to be adjusted. They then continued with the acclimatisation period.

3.6.3 Plaque build-up period

At the start of each study arm, the *in situ* intra-oral appliances were disinfected, and two enamel slabs were inserted into each applaince. On the same or the following working day (Day-2) subjects were then fitted with the *in situ* intra-oral appliances containing the two enamel slabs. The subjects were given the appropriate investigational product, instructions, and a diary card (Appendix 6 and 7).

The subject wore the *in situ* intra-oral appliances at all times (except when eating, drinking, brushing their teeth and exposure to the investigational products) for a minimum of 48 hours immediately prior to dosing.

3.6.4 Investigational product use period

Each subject wore their *in situ* intra-oral appliance (with the two enamel slabs) in their mouth for the entire duration of each study arm, i.e. from the start of the plaque build-up phase through to the removal of the two enamel slabs after 21 days. Each subject was instructed to remove the *in situ* intra-oral appliances from their mouth and immerse it in the allocated investigational product as instructed for 21 days.

3.6.5 Day 21

On Day 21, the subjects returned to the study site. Both enamel slabs were removed from the *in situ* intra-oral appliances. The subjects returned their diary cards and used investigational product containers to site staff. Subjects were asked if they had continued to comply with the study restrictions. Any adverse events or concomitant medications were recorded in the CRF.

At the end of each of the three study arms, the subjects left the *in situ* intra-oral appliances at the study site for preparation of the subsequent study arm.

3.6.6 Washout

In order to eliminate any effect of the previous product, the washout phase between study arms was at least seven days.

3.6.7 Follow up visit

The participants attended a follow- up visit after the final visit. In this visit, medical history was checked, and optional application of fluoride varnish was delivered. Subjects also were advised to recommence use of fluoride-containing toothpaste when brushing their teeth.

3.7 Study regime

The subjects were randomised to start with one of the test regimes using specially designed appliances with fixed enamel slabs. The study regime is shown in Table 3-1.

Table 3-1: Study regimes of the four arms

1st Arm	7 days	Wash-in before starting the 1st arm
	48 hours(plaque build-up phase)	The subject wore the in situ intra-oral appliances at all times in their mouth (except when eating, drinking or brushing their teeth) for a minimum of 48 hours immediately before dispensing the study products.
	21 days	During day, the participants dipped the appliance 5 times daily for two minutes into 12%- 50 ml sucrose solution. Volunteers dipped their appliances twice daily for 2 minutes, in slurry of the experimental materials which was prepared by mixing 1 g of the toothpaste with 5 ml of distilled water. For the regimen group, volunteers dipped their appliances twice daily for two minutes in a toothpaste slurry of 1450 ppm sodium fluoride followed with a dipping in a slurry of MI Paste Plus (10% w/v CPP-ACP, 900ppm sodium fluoride) for three minutes once daily at night.

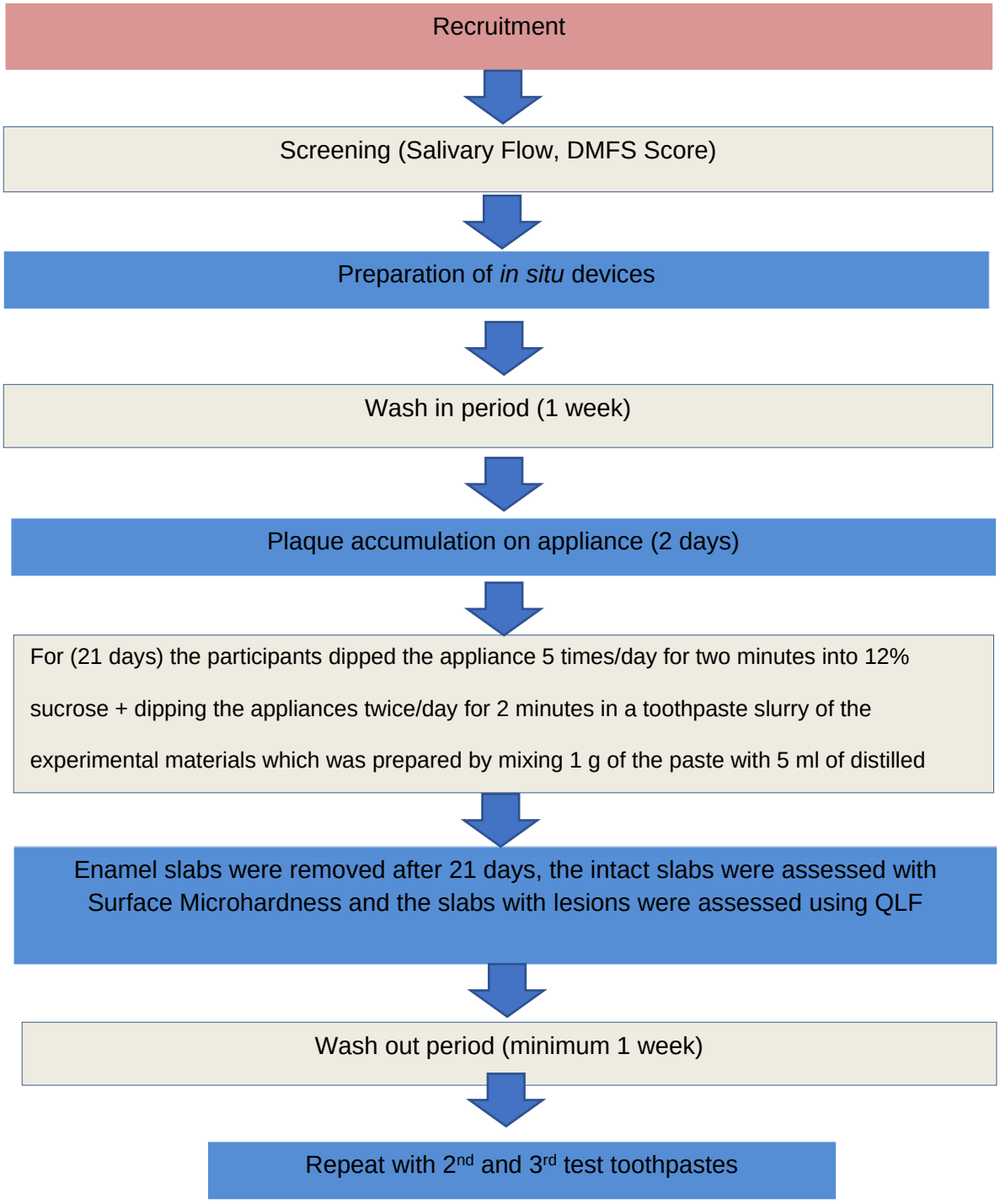
2nd Arm	7days	Wash-out before starting the 2nd arm
	48 hours(plaque build-up phase)	The subject wore the in situ intra-oral appliances at all times in their mouth (except when eating, drinking or brushing their teeth) for a minimum of 48 hours immediately before dispensing the study products.
	21 days	During day, the participants dipped the appliance 5 times daily for two minutes into 12%- 50 ml sucrose solution. Volunteers dipped their appliances twice daily for 2 minutes, in slurry of the experimental materials which was prepared by mixing 1 g of the toothpaste with 5 ml of distilled water. For the regimen group, volunteers dipped their appliances twice daily for two minutes in a toothpaste slurry of 1450 ppm sodium fluoride followed with a dipping in a slurry of MI Paste Plus (10% w/v CPP-ACP, 900ppm sodium fluoride) for three minutes once daily at night.

3rd Arm	7 days	Wash-out before starting the 3rd arm
	48 hours(plaque build-up phase)	The subject wore the in situ intra-oral appliances at all times in their mouth (except when eating, drinking or brushing their teeth) for a minimum of 48 hours immediately prior to dispensing the study products.
	21 days	During day, the participants dipped the appliance 5 times daily for two minutes into 12%- 50 ml sucrose solution. Volunteers dipped their appliances twice daily for 2 minutes, in slurry of the experimental materials which was prepared by mixing 1 g of the toothpaste with 5 ml of distilled water. For the regimen group, volunteers dipped their appliances twice daily for two minutes in a toothpaste slurry of 1450 ppm sodium fluoride followed with a dipping in a slurry of MI Paste Plus (10% w/v CPP-ACP, 900ppm sodium fluoride) for three minutes once daily at night.

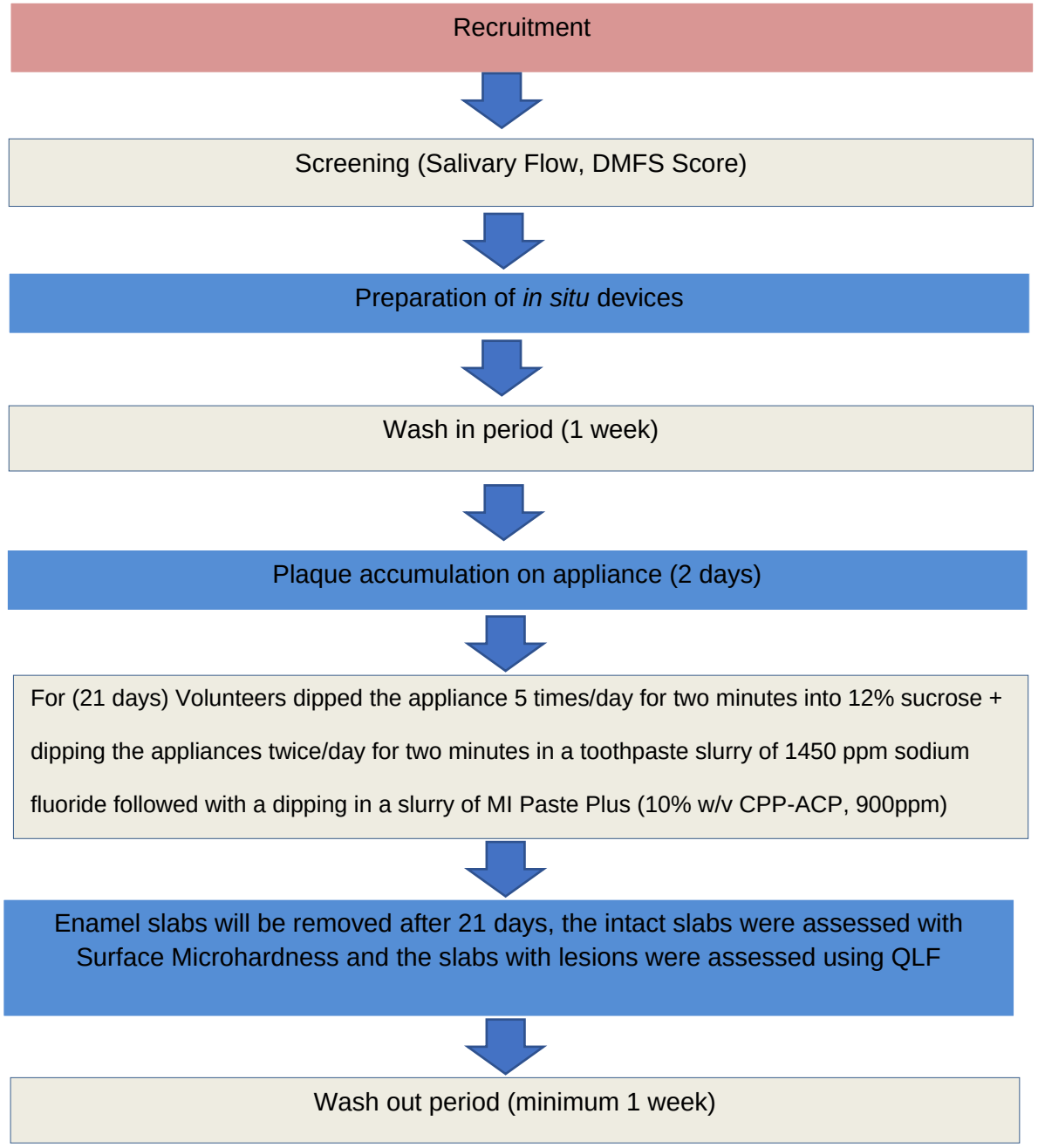
4th Arm	7days	Wash-out before starting the 4th arm
	48 hours (plaque build-up phase)	The subject wore the in situ intra-oral appliances at all times in their mouth (except when eating, drinking or brushing their teeth) for a minimum of 48 hours immediately before dispensing the study products.
	21 days	During day, the participants dipped the appliance 5 times daily for two minutes into 12%- 50 ml sucrose solution. Volunteers dipped their appliances twice daily for 2 minutes, in slurry of the experimental materials which was prepared by mixing 1 g of the toothpaste with 5 ml of distilled water. For the regimen group, volunteers dipped their appliances twice daily for two minutes in a slurry of toothpaste 1450 ppm sodium fluoride followed with a dipping in a slurry of MI Paste (10% w/v CPP-ACP, 900ppm sodium fluoride) for three minutes once daily at night.

3.8 Flow Charts

3.8.1 Flow chart regime of the study arms using fluoride 1450 ppm F, non-fluoride and Clinpro toothpastes.



3.8.2 Flow chart regime of the study arm using MI Paste Plus(10% w/v CPP-ACP, 900 ppm sodium fluoride) + fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride) as a regimen.



3.9 Compliance

Volunteers' compliance was checked using the following methods:

- Each volunteer had a case record form to monitor and record each step during the study (Appendix 5).
- By checking the dipping diary during volunteers' visits. The subject recorded the date, start time and duration of each immersion in the diary card provided.
- By collecting the used sucrose solution bottles and measuring the remnants of each bottle and record it in disposed form.
- The used toothpastes were checked after each study arm.

4.0 RESULTS

4.1 Quantitative Light-induced Fluorescence (QLF) Results

Three main parameters for QLF were statistically analysed, these were:

1. ΔF: Percentage fluorescence loss with respect to the fluorescence of sound tooth tissue. This is a measure of lesion depth (%).
2. ΔQ: ΔF times the Area. Percentage fluorescence loss with respect to the fluorescence of sound tissue times the area. This is a measure of lesion volume (%px_2).
3. Area: The surface area of the lesion expressed in $pixels_2$ (px_2).

4.1.1 The mean fluorescence loss ΔF

4.1.1.1 Normality of ΔF data

Shapiro-Wilk and Kolmogorov-Smirnov tests were used to assess the data normality of ΔF. The tests gave a P value of >0.05 which indicated that the data was normally distributed.

The box- and- whisker plots show (Figure 4-1) the median, maximum value and minimum value, first and third quartiles within the data set. It can be seen that the ΔF readings at the baseline for all groups had almost the same range of distribution.

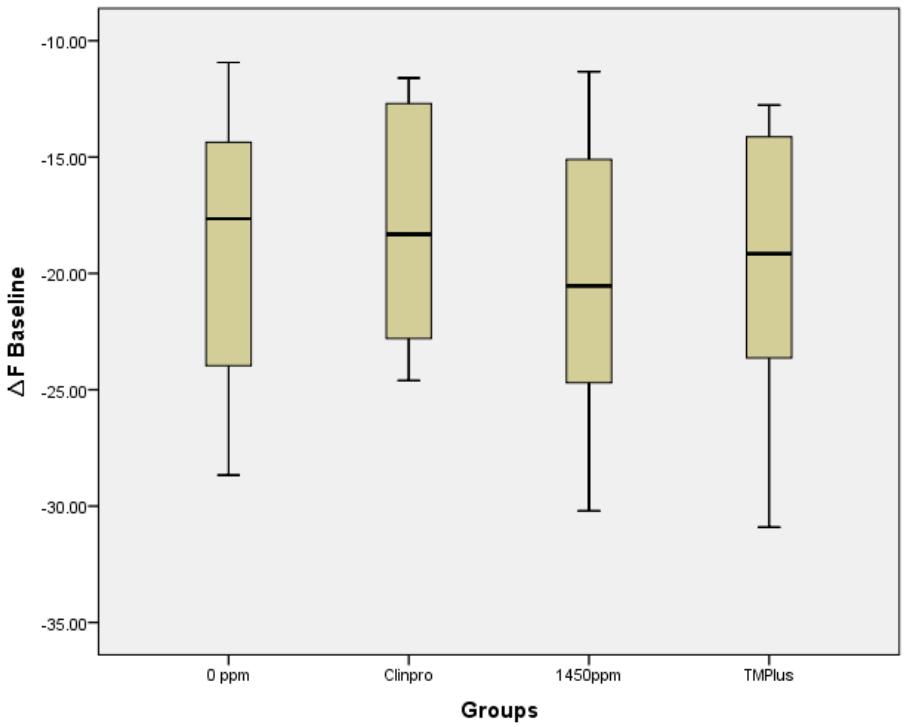


Figure 4-1: Boxplot for the distribution of the ΔF values at baseline, demonstrating the upper and lower values, Standard deviation (SD) and the median value of the data.

One-way ANOVA test was performed to assess if there was any statistical significant difference at baseline readings of ΔF between the lesions assigned to the four groups (Table 4-1). The results revealed that no statistical significant difference was detected ($p > 0.05$).

Table 4-1: One-way ANOVA between groups for ΔF values at baseline.

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	44.16	3	14.72	.44	.72
Within Groups	1715.39	52	32.98		
Total	1759.56	55			

4.1.1.2 Difference in ΔF within each treatment group

Table 4-2 shows the mean ΔF readings at start and end points. An improvement in ΔF values after treatment was seen across all groups except the negative control group (0 ppm F).

Table 4-2: Mean values of ΔF at baseline and after treatment for all groups.

Group	Mean ΔF at baseline ± SD	Mean ΔF after treatment ± SD	Mean Difference in ΔF at baseline and after treatment ± SD
0 ppm	-17.82 ± 1.36	-20.04 ± 1.08	-2.214 ± 3.76
1450 ppm	-20.00 ± 1.56	-14.73 ± 0.89	5.275 ± 3.78
Clinpro	-18.49 ± 1.47	-16.06 ± 1.01	2.428 ± 4.16
MI Paste Plus	-19.71 ± 1.71	-14.88 ± 1.20	4.826 ± 5.14

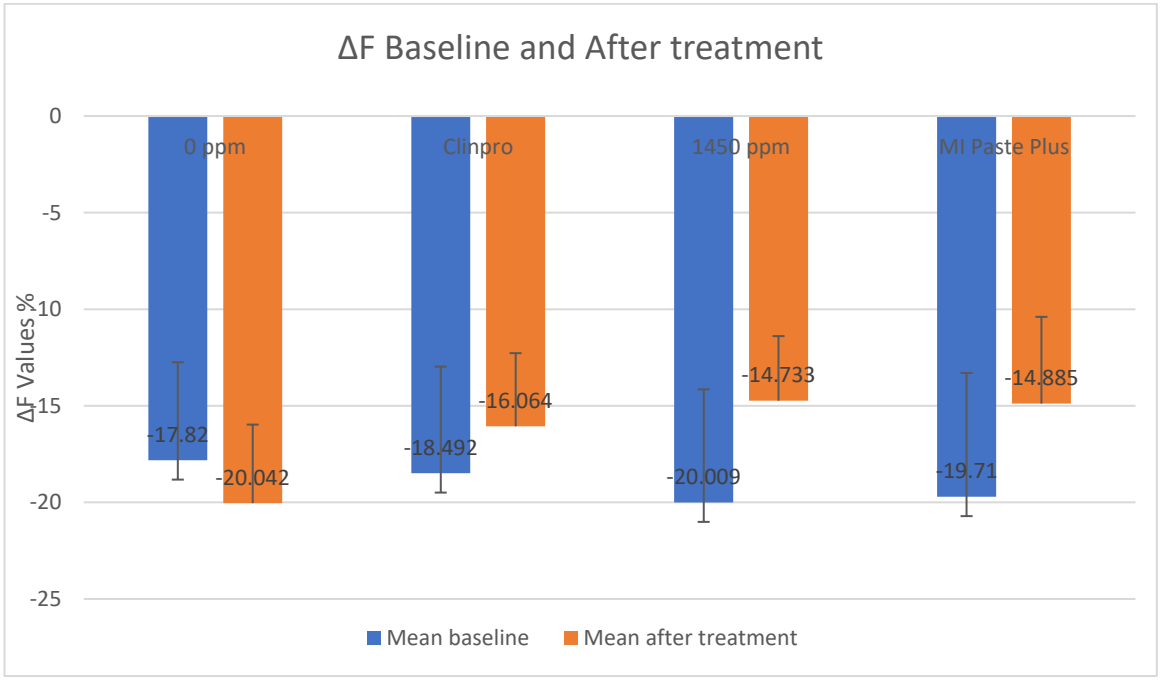


Figure 4-2: ΔF mean values at baseline and after treatment for all groups.

Paired T-Test was used to compare the mean ΔF at baseline and after treatment. The results show that in all groups except the negative control group, there was a statistical significant decrease in ΔF endpoint values compared with that at baseline ($p < 0.05$) (Table 4-3).

Table 4-3: Paired sampled T-Test results for ΔF values at baseline and after treatment.

		Paired Differences					Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference		
					Lower	Upper	
0 ppm	**ΔF Baseline - ΔF After Treatment**	2.21	3.76	1.00	0.04	4.38	**.046***
Clinpro		-2.42	3.78	1.01	-4.61	-0.24	**.032***
1450 ppm		-5.27	4.16	1.11	-7.68	-2.87	**.000***
MI Paste Plus		-4.82	5.14	1.37	-7.79	-1.85	**.004***

*** Statistically significant**

4.1.1.3 Difference in ΔF between all groups

The following formula was applied to measure the difference in ΔF

Difference in ΔF = mean ΔF after treatment – mean ΔF at baseline

ΔF differences for all groups are shown in (Table 4.4) (Figure 4-3). Positive values were seen in all treatment groups except the negative control group (0 ppm F). This indicated that there was an improvement in (ΔF) after treatment in all groups except the negative control group (0 ppm F) with a mean difference

of -2.21±3.76. The greatest improvement was shown in the 1450 ppm F group with a mean difference of 5.275± 4.165, followed by MI Paste Plus group with a mean of 4.83±5.14. A reduction in ΔF difference was also noticed in the Clinpro group with a mean of 2.43±3.78.

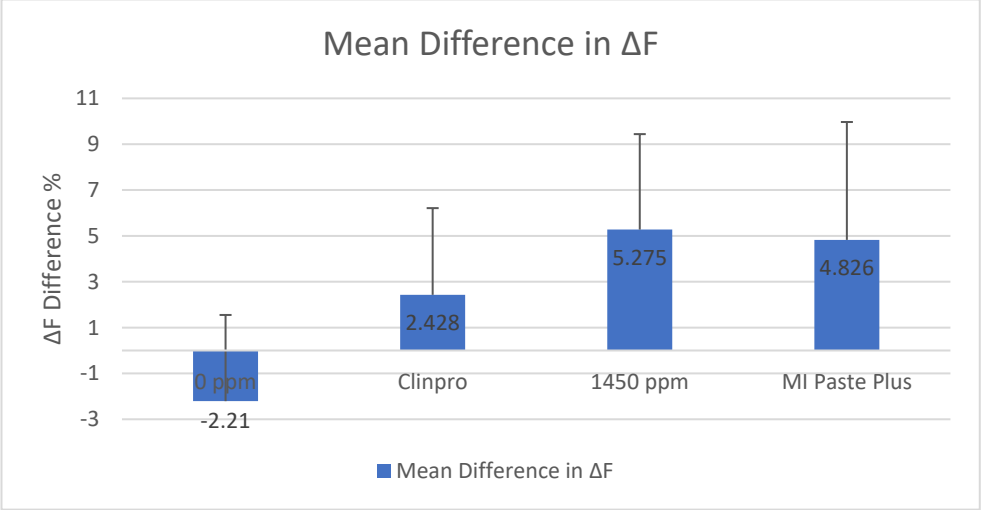


Figure 4-3: The mean difference in ΔF in the four groups.

Table 4-4: Descriptive statistics for the difference in ΔF at baseline and after treatment for all groups.

	N	Mean	Std. Deviation	95% Confidence Interval for Mean		Minimum	Maximum
				Lower Bound	Upper Bound		
0 ppm	14	2.21	3.76	0.04	4.38	-2.23	11.60
Clinpro	14	-2.42	3.78	-4.61	-.024	-7.17	3.87
1450 ppm	14	-5.27	4.16	-7.68	-2.87	-13.13	-0.20
MI Paste	14	-4.82	5.14	-7.79	-1.85	-13.93	6.50

The following formula: was used to determine the percentage change in ΔF (% F):

(Difference in ΔF at baseline and after treatment / ΔF at baseline) × 100

It can be seen that both 1450 ppm F and MI Paste Plus groups had comparable values (26.33% and 24.48%) respectively. Lowest value (-12.41%) was detected in the negative control group (0 ppm F) as seen figure 4.4.

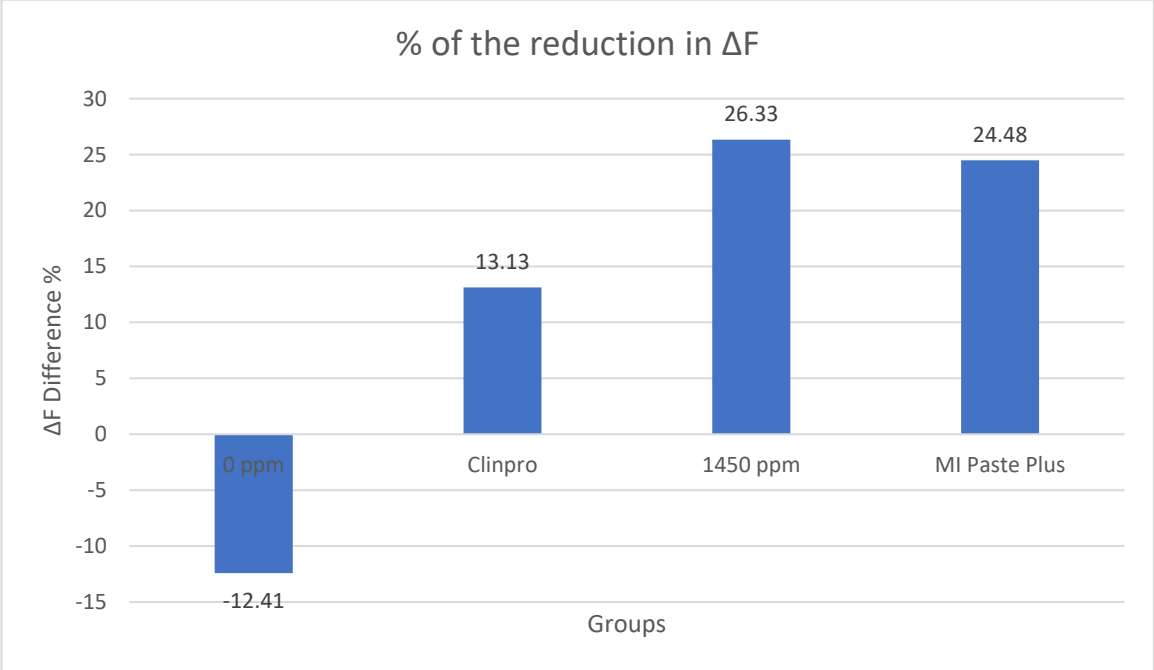


Figure 4-4: The % change in ΔF values from baseline for all groups.

As the ΔF difference data were normally distributed, a parametric one-way ANOVA test was carried out to check whether the difference in ΔF was statistically significant between the four groups (Table 4-5). It showed that there was a statistical significant difference between the four groups ($p < 0.001$).

Table 4-5: One-way ANOVA between groups for the difference in ΔF from baseline.

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	494.50	3	164.83	9.12	**.000***
Within Groups	939.88	52	18.07		
Total	1434.39	55			

*** Statistically significant**

Pairwise comparisons were applied using a Bonferroni test to identify which groups differed significantly. The results show that significant lower values were observed in the negative control group (0 ppm F) compared to the other groups ($p < 0.05$). However, the tests did not find any other statistical significant differences between the other groups (Table 4-6).

Table 4-6: Multiple comparisons of the difference in ΔF at baseline and after treatment between all test groups and control.

Pairwise Comparisons						
(I) Groups	(J) Groups	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
Clinpro	Free	-4.64*	1.60	**.034**	-9.05	-0.23
	NaF	2.84	1.60	0.49	-1.56	7.25
	MI Paste	2.39	1.60	0.85	-2.01	6.80
0 ppm	Clinpro	4.64*	1.60	**0.03**	0.23	9.05
	NaF	7.49*	1.60	**0.00**	3.08	11.89
	MI Paste	7.04*	1.60	**0.00**	2.63	11.44
1450 ppm	Clinpro	-2.84	1.60	0.49	-7.25	1.56
	Free	-7.49*	1.60	**0.00**	-11.89	-3.08
	MI Paste	-.45	1.60	1.00	-4.85	3.95
MI Paste Plus	Clinpro	-2.39	1.60	0.85	-6.80	2.01
	Free	-7.04*	1.60	**0.00**	-11.44	-2.63
	NaF	0.45	1.60	1.00	-3.95	4.85

***. The mean difference is significant at the .05 level.**

4.1.1.4 Intra-examiner reproducibility for ΔF

Nine slabs were randomly chosen for re-analysis. These represented 15% of the total slab number. The intra-examiner reproducibility was found to be 0.93 which indicates an excellent reproducibility.

4.1.2 ΔQ: Lesion volume

4.1.2.1 Normality of ΔQ data

Shapiro-Wilk and Kolmogorov-Smirnov tests were used to assess the data normality of ΔQ. The tests gave a P value of >0.05 which indicated that the data was normally distributed.

One-way ANOVA test was performed to assess if there was any statistical significant difference at baseline readings of ΔQ between the lesions assigned to the four groups. The results revealed that no statistical significant difference was detected ($p > 0.05$).

4.1.2.2 Difference in ΔQ within each group

Table 4-7. Shows the mean ΔQ readings at the start and after treatment. An improvement in ΔQ values after treatment was seen in all groups except the negative control group (0 ppm F).

Table 4-7: The mean values of ΔQ at baseline and after treatment for all groups.

Group	Mean ΔQ at baseline ± SD	Mean ΔQ after treatment ± SD	Mean Difference in ΔQ at baseline and after treatment ± SD
0 ppm	-16534.30± 9675.85	-19257.35± 10619.05	-2723.04± 10187.03
Clinpro	-20672.69± 9589.30	-8761.64± 6003.50	11911.04 ± 8011.96
1450 ppm	-20640.28 ± 10230.24	-6862.19± 4029.04	13778.09± 10047.09
MI Paste Plus	-22751.23± 10414.47	-7524.83± 6667.17	15305.69± 8792.48

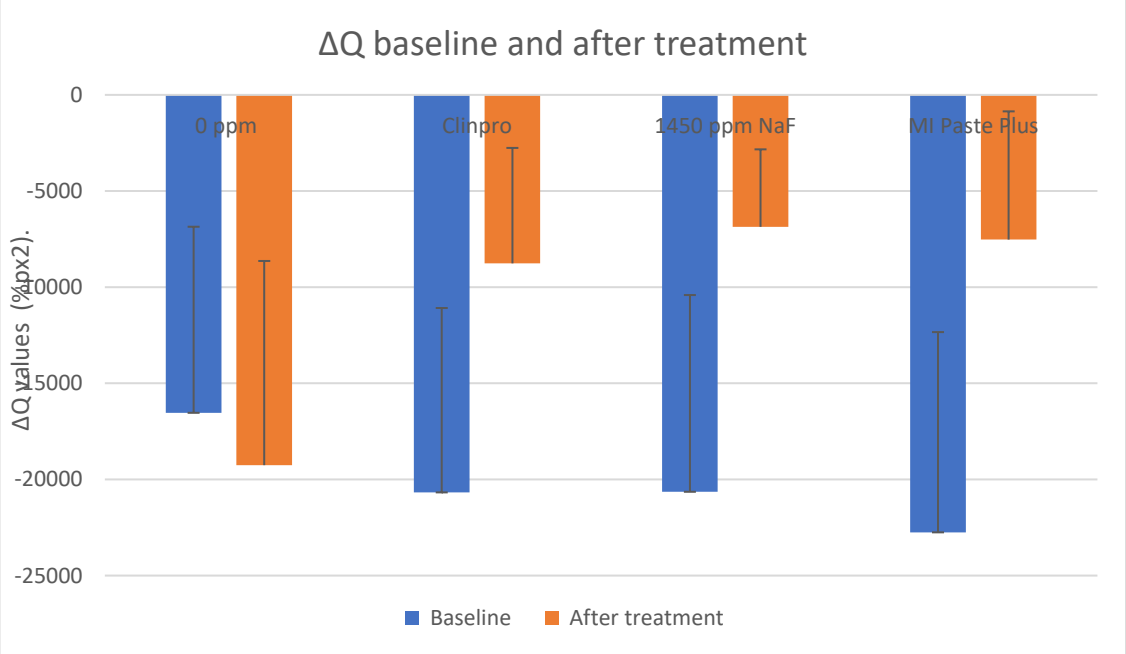


Figure 4-5: ΔQ mean values at baseline and endpoint for all groups.

Paired T-Test was used to compare the mean ΔQ at baseline and after treatment. The results show that in all groups except the negative control group (0 ppm F), there was a statistical significant decrease in ΔQ values compared with that at baseline ($p < 0.05$) (Table 4-8).

Table 4-8: Paired sampled T-Test results for ΔQ values at baseline and after treatment.

		Paired Differences					Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference		
					Lower	Upper	
0 ppm	ΔQ at baseline – ΔQ after treatment	-2723.04	10187.03	2722.59	-3158.7	-8604.86	**0.33**
Clinpro		11911.	8011.96	2141.28	-16537	-7285.07	**0.00***
1450ppm		13778.	10047.09	2685.2	-19579.1	-7977.07	**0.00***
MI Paste Plus		15305.6	8792.48	2349.23	-20382.3	-10229.06	**0.00***

*** Statistically significant**

4.1.2.3 Difference in ΔQ between all groups

The following formula was applied to measure the difference in ΔQ

Difference in ΔQ = mean ΔQ after treatment – mean ΔQ at baseline

ΔQ differences for all groups are shown in (Table 4.9) (Figure 4-6). Positive values were seen in all treatment groups except the negative control group (0 ppm F). This indicated that there was an improvement in (ΔQ) after treatment in all groups except the negative control group with a mean difference of -2723.04±10187.03. The greatest improvement was shown in the MI Paste Plus group with a mean difference of 15305.69±8792.48 followed by 1450 ppm F

group with a mean of 13778.09±10047.09. A reduction in ΔQ difference was also noticed in the Clinpro group with a mean of 11911.04± 8011.96.

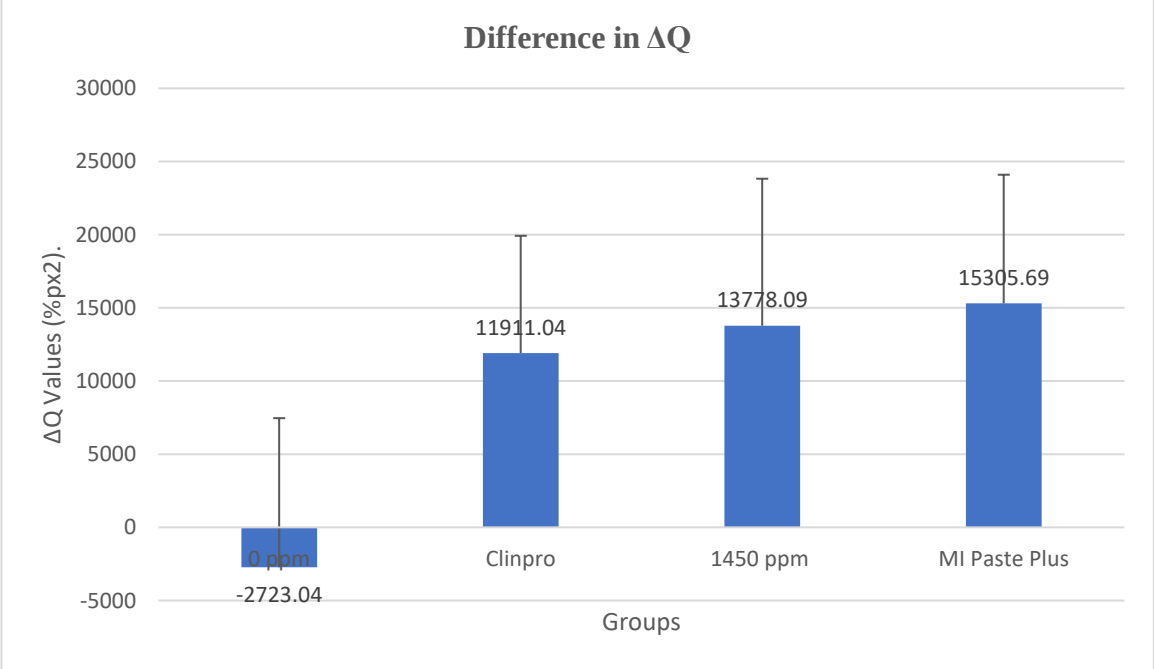


Figure 4-6: Means of the difference in ΔQ at baseline and after treatment of all groups.

Table 4-9: Descriptive statistics for the difference in ΔQ from baseline for all groups.

	N	Mean	Std. Deviation	95% Confidence Interval for Mean		Minimum	Maximum
				Lower Bound	Upper Bound		
0 ppm	14	-2723.04	10187.03	-3158.77	-8604.86	-21570.66	20269
Clinpro	14	11911.04	8011.96	-16537.01	-7285.07	-30713	3578.33
1450ppm	14	13778.09	10047.09	-19579.11	-7977.07	-29672.66	949
MI Paste Plus	14	15305.69	8792.48	-20382.31	-10229.06	-31171.33	1244.67

The following formula: was used to determine the percentage change in ΔQ (% Q)

(Difference in ΔQ at baseline and after treatment / ΔQ at baseline) × 100

Figure 4-7 shows the percentage for Q values. It can be seen that both 1450 ppm F and MI Paste Plus groups had comparable percentages (66.75% and 67.27%) respectively. Lowest percentage (-16.46%) was detected in the negative control group (0 ppm F).

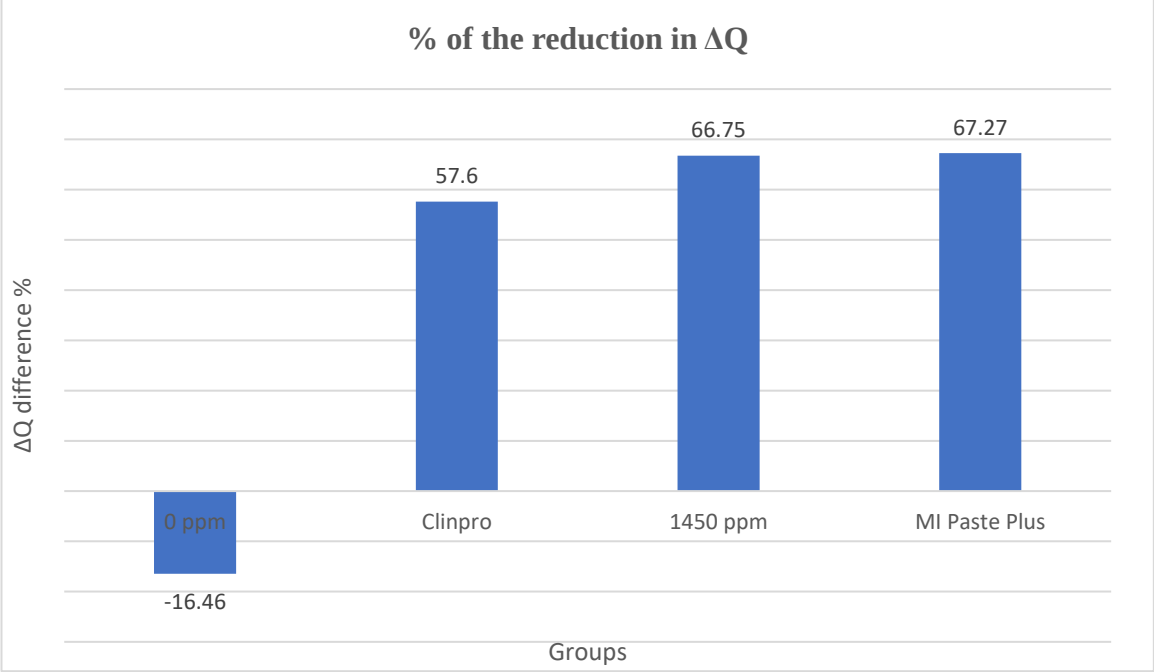


Figure 4-7: The % Q values for all groups.

As the ΔQ difference data were normally distributed, a parametric one-way ANOVA test was carried out to check whether the difference in ΔQ was statistically significant between the four groups (Table 4-11). It showed that there was a statistical significant difference between the groups ($p < 0.001$).

Table 4-10: One-way ANOVA between groups for the difference in ΔQ at baseline and after treatment.

ANOVA					
	Sum of Squares	**Df**	**Mean Square**	**F**	**Sig.**
Between Groups	2900880047	3	966960015.6	11.172	**0.00**
Within Groups	4500849650	52	86554800.96		
Total	7401729697	55			

Pairwise comparisons were applied using a Bonferroni test to identify which groups were differ significantly (Table 4-11). The results show that significantly lower values were observed in the negative control group (0 ppm F) compared to the other treatment groups ($p < 0.05$). However, the tests did not find any other statistical significant differences between the other treatment groups.

Table 4-11: Multiple comparisons of the difference in ΔQ at baseline and after treatment between all test groups and control.

Pairwise Comparisons						
(I) Groups	(J) Groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Clinpro	Free	-14634.09*	3516.38	**0.001**	-24279.37	-4988.81
	NaF	1867.04	3516.38	1.00	-7778.23	11512.32
	MI Paste	3394.64	3516.38	1.00	-6250.63	13039.92
0 ppm	Clinpro	14634.09*	3516.38	**0.001**	4988.81	24279.37
	NaF	16501.14*	3516.38	**0.00**	6855.86	26146.42
	MI Paste	18028.73*	3516.38	**0.00**	8383.45	27674.01
1450 ppm	Clinpro	-1867.04	3516.38	1.00	-11512.326	7778.23
	Free	-16501.14*	3516.38	**0.00**	-26146.42	-6855.86
	MI Paste	1527.59	3516.38	1.000	-8117.68	11172.87
MI Paste Plus	Clinpro	-3394.64	3516.38	1.000	-13039.92	6250.63
	Free	-18028.73*	3516.38	**.000**	-27674.01	-8383.45
	NaF	-1527.59	3516.38	1.000	-11172.87	8117.68

***. The mean difference is significant at the .05 level.**

4.1.2.4 Intra-examiner reproducibility for ΔQ

Nine slabs were randomly chosen for re-analysis. These represent 15% of the total slab number. The intra-examiner reproducibility found to be 0.96 which indicates an excellent reproducibility.

4.1.3 Area of the White spot lesion

4.1.3.1 Normality of lesion area values

Shapiro-Wilk and Kolmogorov-Smirnov tests were used to assess the data normality of lesion area. The tests gave a P value of <0.05 which indicated that the data was not normally distributed.

Non-parametric Kruskal-Wallis test was performed to assess if there was any statistical significant difference at baseline readings of lesion area between the lesions assigned to the four groups. No statistical significant difference was detected between the four groups (p >0.05).

4.1.3.2 Difference in lesion area within each group

Table 4-12 shows the mean lesion area readings at the start and after treatment. A decrease in lesion area values after treatment was seen in all groups except the negative control group (0 ppm F).

Table 4-12: The mean values of lesion area at baseline and after treatment for all groups.

Group	Mean ΔF at baseline ± SD	Mean ΔF after treatment ± SD	Mean Difference in ΔF at baseline and after treatment ± SD
0 ppm	822.94±357	1011.09± 368.19	188.15 ± 394.60
1450 ppm	1092.21± 323.95	441.23 ± 223.54	-636.69 ± 383.79
Clinpro	1106.23± 469.30	520.78± 310.88	-585.45 ± 482.43
MI Paste Plus	1249.23± 481.36	487.80 ± 434.89	-761.42 ± 285.58

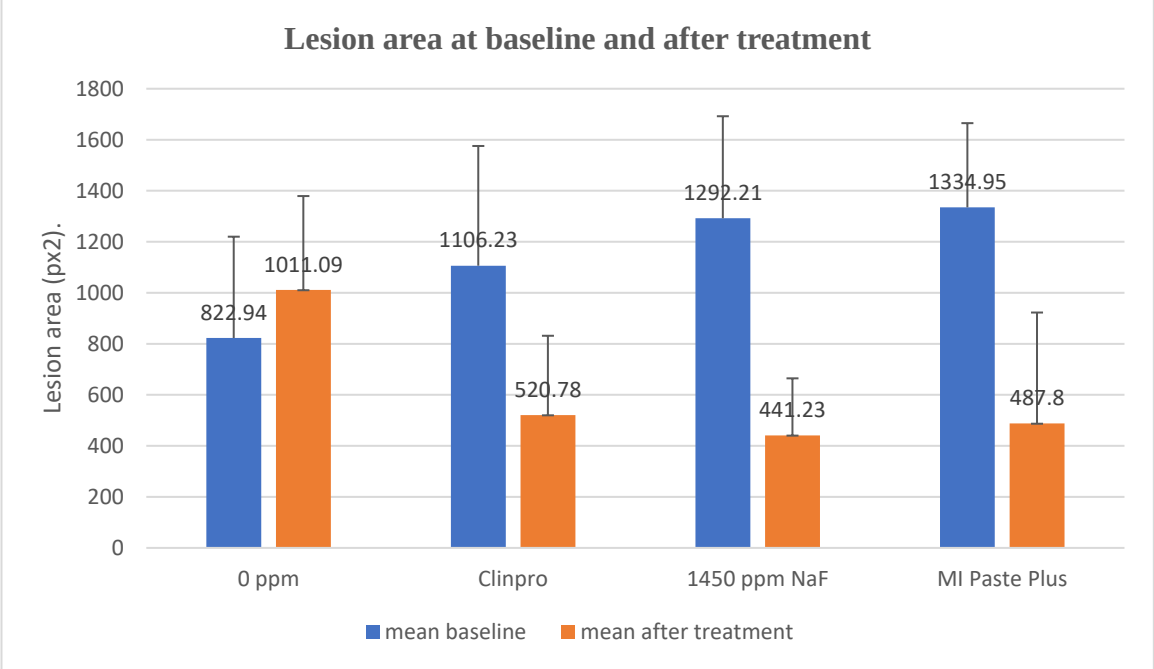


Figure 4-8: Lesion area at baseline and after treatment for all groups.

A non-parametric test Wilcoxon's matched pairs test was used to compare the mean lesion area at baseline and after treatment. The results show that in all groups except the negative control group (0 ppm F), there was a statistical significant decrease in lesion area values compared with that at baseline ($p < 0.05$) (Table 4-13).

Table 4-13: Paired sampled T-test results for the lesion area values at baseline and after treatment for all groups.

		Paired Differences					Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference		
					Lower	Upper	
0 ppm	**ΔF Baseline – ΔF After Treatment**	-188.7	396.57	105.98	-417.71	40.23	0.09
Clinpro		-585.4	482.43	128.93	306.90	864	**.001***
1450 ppm		-636.6	383.79	102.57	905.16	1279.9	**.000***
MI Paste Plus		-761.4	285.58	76.32	971.30	1527.1	**.000***

*** Statistically significant**

4.1.3.3 Difference in lesion area between all groups

The following formula was applied to measure the difference in lesion area

Difference in lesion area = mean lesion area after treatment – mean lesion area at baseline

Lesion area differences for all groups are shown in table 4-14 and figure 4.9. Negative values were seen in all treatment groups except the control group (0 ppm F). This indicated that there was a reduction in lesion area after treatment in all treatment group except the negative control group with a mean difference of -188.73± 396.57. The greatest reduction in lesion area was shown in the MI Paste Plus group with a mean difference of -761.4± 285.58 followed by 1450 ppm F group with a mean of -636.6± 383.79. A reduction in lesion area was also noticed in the Clinpro group with a mean of 585.45± 482.43.

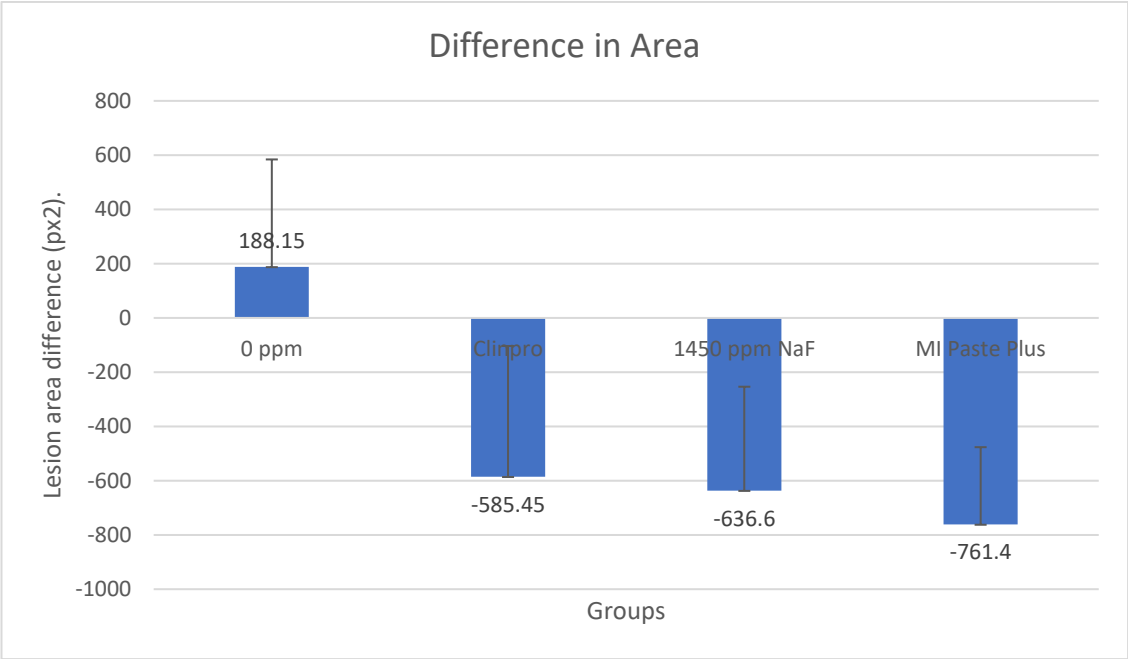


Figure 4-9: Means of the difference in the lesion area.

Table 4-14: Descriptive statistics for the difference in the lesion area at baseline and after treatment for all groups.

	N	Mean	Std. Deviation	95% Confidence Interval for Mean		Minimum	Maximum
				Lower Bound	Upper Bound		
0 ppm	14	-188.73	396.57	-417.71	40.23	-760	744.33
Clinpro	14	-585.45	482.43	306.90	864	-252	1844
1450ppm	14	-636.6	383.79	905.16	1279.94	-136.	1129
MI Paste Plus	14	-761.4	285.58	971.30	1527.17	264	1305.33

The following formula: was used to determine the percentage change in lesion area (% Area)

(Difference in lesion area at baseline and after treatment/lesion area at baseline) × 100

Figure 4-10 shows the % change in the lesion area. It can be seen that both 1450 ppm F and MI Paste Plus groups had comparable percentages (58.3.75% and 60.96%) respectively. Lowest percentage (-22.86%) was detected in the negative control group (0 ppm F).

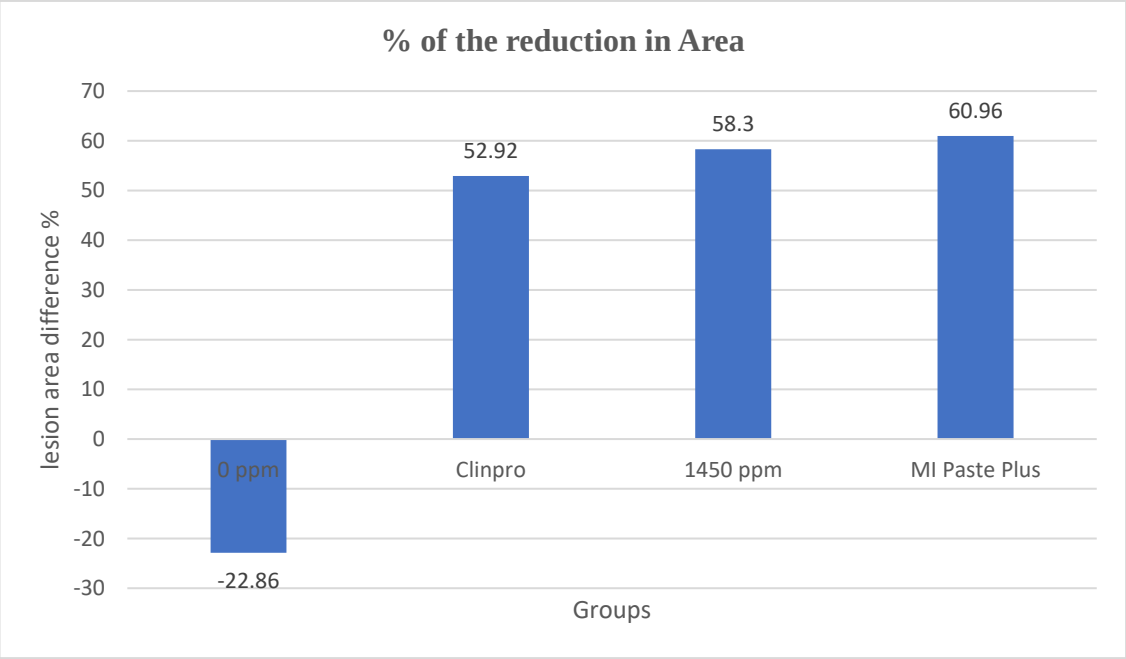


Figure 4-10: The % area difference values for all groups.

As the lesion area difference data were normally distributed, a parametric one-way ANOVA test was carried out to check whether the difference in lesion area was statistically significant between the four groups (Table 4-15). it showed that there was a statistical significant difference between the four groups ($p < 0.001$).

Table 4-15: One-way ANOVA between groups for lesion area values difference

ANOVA					
	Sum of Squares	**df**	**Mean Square**	**F**	**Sig.**
Between Groups	7980995.69	3	2660331.69	16.99	**.000**
Within Groups	8138582.65	52	156511..205		
Total	16119577.73	55			

Pairwise comparisons were applied using a Bonferroni test to identify which groups differed significantly (Table 4-16). The results showed that significant higher values were observed in the negative control group (0 ppm F) compared to the other groups ($p < 0.05$). However, the tests did not find any other statistical significant differences between the other groups

Table 4-16: Multiple comparisons of the difference in area at baseline and after treatment between all test groups and control.

Pairwise Comparisons						
(I) Groups	(J) Groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Clinpro	Free	783.47*	149.52	**0.001***	373.32	1193.62
	NaF	-51.23	149.52	1.00	-461.38	358.91
	MI Paste	175.97	149.52	1.00	-586.12	234.17
0 ppm	Clinpro	-783.4762	149.52	**0.00***	-1193.62	-373.32
	NaF	-834.71	149.52	**0.00***	-1244.86	-424.56
	MI Paste	-959.45	149.52	**0.00***	-1369.60	-549.30
1450 ppm	Clinpro	51.23	149.52	1.00	-358.91	461.38
	Free	834.71	149.52	**0.00***	424.56	1244.86
	MI Paste	-124.73	149.52	1.00	-534.88	285.41
MI Paste Plus	Clinpro	175.97	149.52	1.00	-234.17	586.12
	Free	959.45	149.52	**0.00***	549.30	1369.60
	NaF	124.73	149.52	1.00	-285.41	534.88

***. The mean difference is significant at the .05 level.**

4.1.3.4 Intra-examiner reproducibility for lesion area

Nine slabs were randomly chosen for re-analysis. These represent 15% of the total slab number. The intra-examiner reproducibility was found to be 0.97 which indicates an excellent reproducibility.

4.1.4 Summary of the results for all QLF three parameters (Remineralisation assessment)

1- In all QLF parameters (ΔF, ΔQ, lesion area), there was an evidence of significant remineralisation in MI Paste Plus combined with fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride) as a regimen, Clinpro tooth crème and fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride) groups when compared with that at baseline as well as the control negative control group.

2- There was no statistically significant difference in remineralisation between MI Paste Plus combined with fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride) as a regimen,Clinpro tooth crème and fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride).

4.2 Microhardness results

In this study, enamel microhardness was assessed at baseline and after 21 days by measuring the resistance to the penetration of the Knoop microhardness indenter. Five readings for each slab were carried out, and the mean readings for each slab were used.

4.2.1 Test of normality of Indentation length

In order to check the normality of indentation length data, the Shapiro-Wilk test was used. It can be seen from Table 4-17 that some of the values were normally distributed as they had P-value <0 .05. Therefore, a non-parametric test was used to analyse the data.

Table 4-17: Tests of Normality (indent length - KMH).

Groups	Shapiro-Wilk Sig.
Baseline 0ppm F	0.08
Baseline Clinpro	0.78
Baseline 1450 NaF ppm	0.21
Baseline MI Paste Plus	0.54
Treatment 0ppm F	**0.01***
Treatment Clinpro	0.79
Treatment 1450 NaF ppm	0.41
Treatment MI Paste Plus	**0.03***
Mean Difference 0ppm F	0.20
Mean Difference Clinpro	**0.03***
Mean Difference 1450 ppm NaF)	**0.008***
Mean Difference MI Paste Plus	0.51

*** Significant.**

4.2.2 Difference in indentation length within the same group

As the indentation length data were not normally distributed, non-parametric test Wilcoxon's Matched pairs test was implemented to assess the differences within the same group at baseline and after treatment. The results of the test gave ($p < 0.001$) values in all groups, which means that there was a statistical significant change after treatment when compared with baseline readings in all groups. Table 4.18 and figure 4-11 demonstrated the mean differences in indentation length at baseline and after treatment. The highest after treatment reading (indentation length) was seen in the negative control group (0 ppm) with 79.50 µm. However, all other groups had comparable post-treatment readings.

Table 4-18: Change from baseline (indent length - KMH).

Treatment	Baseline (µm)	SD	Test (µm)	SD	Diff (µm)	SD Mean Diff	Sign.
0 ppm F	68.36	3.87	79.50	11.35	11.3	9.39	**0.001***
Clinpro	68.65	3.60	72.50	5.22	3.85	3.82	**0.001***
1450 ppm NaF	68.05	3.38	71.6	2.84	3.01	2.21	**0.005***
MI Paste Plus	68.32	2.49	71.85	2.67	3.53	2.35	**0.001***

*** Significant Wilcoxon's Matched pairs test, p<0.01.**

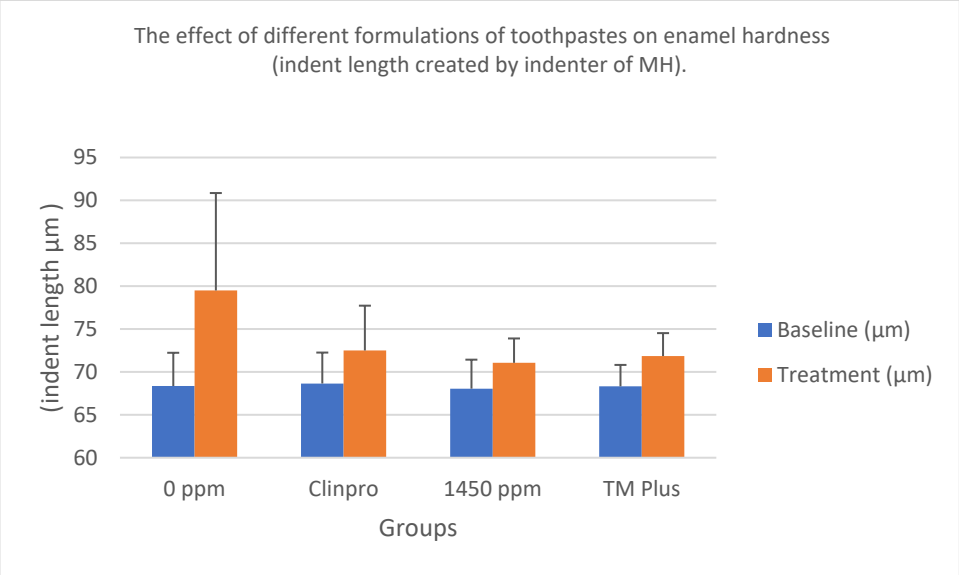


Figure 4-11: indent length(µm) baseline and after (indent length created by Knoop microhardness indenter (KMH).

4.2.3 Difference in Indent length between all groups

The following formula was applied to measure the difference in indent length:

Difference in indent length = indent length after treatment - indent length at baseline

Descriptive statistics:

Figure 4-12 illustrates the difference in Indent Length in the four tested groups. A positive difference in indentation length was found in all groups. This indicated that there was more enamel hardness loss after treatment in all groups compared to that at baseline.

The highest increase in Indent Length was seen in the 0 ppm F with a mean difference of 11.3 ± 9.30. The other three groups had comparable difference

values with the least increase was noticed in the 1450 ppm NaF group 3.01 ± 2.21(Table 4-18).

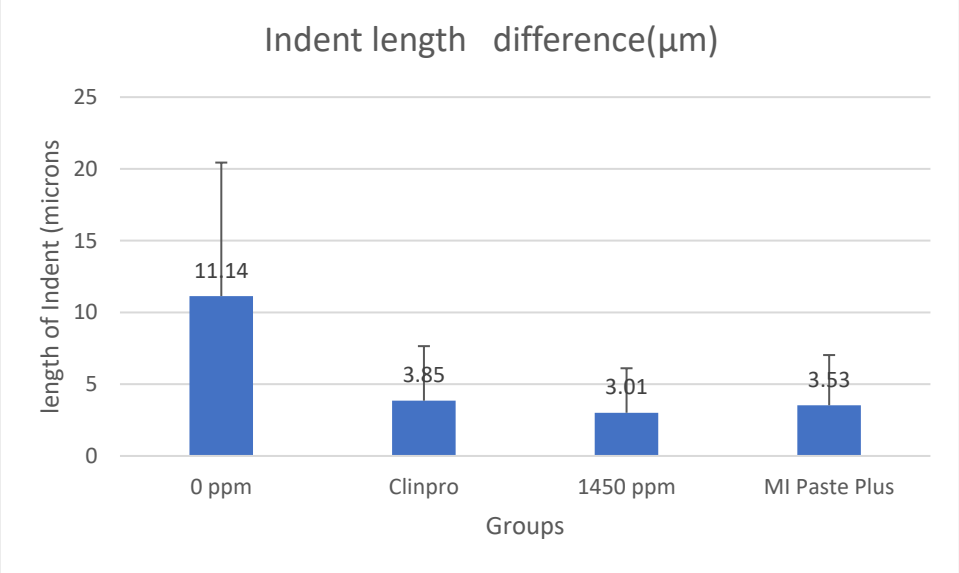


Figure 4-12: The effect of different formulations of toothpastes on enamel hardness (indent length created by indenter of MH) after cariogenic challenge.

The following formula was applied to calculate the percentage of the changes in Indent Length (% IL)

(Difference in IL at baseline and after treatment / IL at baseline) × 100

Figure 4-13 demonstrates the % IL values for all groups. It can be seen that the greatest % IL difference value was found in the negative control group (0 ppm) with % IL of 16.53%, followed by that for Clinpro+ MI Paste Plus (5.6 and 5.1% respectively). The least difference was seen in 1450 ppm NaF group at 4.42%.

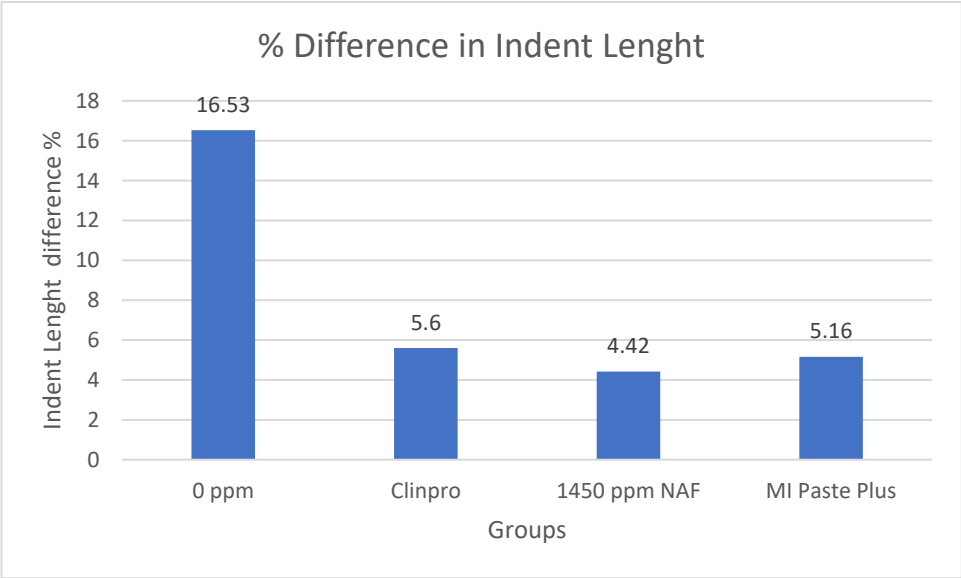


Figure 4-13: The % IL values for all groups.

As the data were not normally distributed, a nonparametric test (Kruskal-Wallis Test) was carried out to assess if the difference in the enamel hardness (indent length - KMH) was statistically significant between the four groups. It indicated that the mean difference in enamel hardness was statistically significant between the groups ($p < 0.05$).

To identify which groups were statistically significant different, Kruskal-Wallis test with pairswise comparisons, $p<0.05$ was performed (Table 4-19). The results revealed that the mean change in (indent length) was statistically significantly different in the negative control group (0 ppm F) when compared to other groups. However, the tests did not find any other statistical significant differences between the other test groups ($p > 0.05$).

Table 4-19: Comparison between different formulations of toothpastes on the hardness of enamel using the length of the indent (Mean Difference) created by KMH.

Groups	Mean Diff (µm)	SE Difference	Sig. (2-tailed)
0 ppm F vs MI Paste Plus	7.77	6.16	**0.019***
0 ppm F vs 1450 ppm NaF	8.29	6.16	**0.015***
0 ppm F vs Clinpro	7.45	6.16	**0.011***
MI Paste Plus vs 1450 NaF ppm F	0.52	6.16	.935
MI Paste Plus vs Clinpro	-0.32	6.16	0.853
1450 NaF ppm vs Clinpro	-0.84	6.16	0.917

***Significant. Kruskal-Walis test with pairs wise comparisons, p<0.05.**

4.2.4 Intra-examiner reproducibility for indentation length

Nine slabs were randomly chosen for re-analysis. These represent 15% of the total slab number. The intra-examiner reproducibility was found to be 0.98 which indicates an excellent reproducibility.

4.2.5 Summary of the Microhardness results (Demineralisation assessment)

1- In all groups, there was an evidence of enamel hardness loss compared to that at baseline.

2- There was evidence of significant enamel hardness loss in the negative control group (0 ppm F) after treatment when compared with the other groups.

3- There was no significant statistical difference in the efficacy to inhibit enamel demineralisation between MI Paste Plus combined with fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride) as a regimen, Clinpro tooth crème and fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride).

5.0 DISCUSSION

The effects of several preventive agents like CPP-ACP and f-TCP have been investigated but there is still considerable controversy surrounding the use of these products over the use of fluoride products alone. These newer agents are more expensive than conventional F products and it is important that they are shown to be efficacious, provide an additional benefit over F toothpaste alone, and are cost-effective to use. Few *in situ* studies have investigated the ability of these products to inhibit enamel demineralisation and enhance enamel remineralisation. Moreover, to the best our knowledge QLF has never been reported to have been used to assess enamel de/remineralisation *in situ.* Also, previous studies mainly investigate the effect of the agents on their own, despite the fact that some are used in addition to daily use of fluoride toothpaste. It is important to study the efficacy of these agents that are prescribed for daily use in the way that they are used routinely.

Therefore, the aim of this work was to compare the efficacy of MI Paste Plus combined with fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride) as a regimen, with f-TCF Clinpro tooth crème 950 ppm F (0.32% w/w sodium fluoride) and fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride), on remineralisation of the enamel subsurface caries-like lesion in an *in situ* randomised, cross-over design study.

5.1 *In situ* model

The ability of the test products to inhibit enamel demineralisation and enhance remineralisation was investigated using an *in situ* model in the present study. The *in situ* model used in this study has been widely used in dental research as it has several advantages over the other models. The main advantage is that it has the ability to allow the investigation to take place in the oral environment. Another advantage is that it is less time consuming and provides more control over the experimental variables compared to clinical trials (Zero, 1995).

In situ models also have several limitations. The main disadvantage is that the sample size is usually small due to the nature of and complexity of the model and care should be exercised while extrapolating the results to the wider population. Also, the *in situ* model is highly reliant on participants' compliance, requiring the participation of highly motivated participants (Zero, 1995).

In the present *in situ* study, written and verbal instructions were given to the participants at every visit. A dipping diary was also used to help volunteers comply with the study protocol. Also, the toothbrushes and toothpastes were checked after each study arm to assess the compliance with the study protocol.

5.2 Study design

Two main designs are used for *in situ* studies, cross-over and parallel designs. The present *in situ* model used the cross-over design. The cross-over approach offers several benefits. The foremost advantage is that it requires less sample size compared with the parallel design. This is achieved by using the same

participant as their own control. However, this also means that care has to be exercised to reduce the risk of having a residual effect which may affect the results. To reduce the carryover effect, a wash period between the arms is required (Zero,1995; Stephen et al. 1992). In this present study design, a cross-over method was used with a minimum of seven days wash-out period between study arms. In addition, all participants had a wash-in period of one week to minimise any residual effect of the previous toothpastes they used to have before starting the study. Seven days wash-out period has been used in several *in situ* studies (Zero,1995; Stephen et al. 1992).

5.3 Sample size

The sample size was calculated following statistical advice. The sample size was carried out using data from previous *in vivo* study by Manal Allammari et al. (2010) to detect a difference of 5% in ΔF between four materials with an estimated standard deviation of 4.5% and with a significance level (alpha) of 0.05. Based on these values, a sample size of 12 participants was required. However, 14 participants were recruited to ensure the completeness of the study and to overcome potential dropout.

5.4 Standardisation

Standardisation is an essential factor when designing *in situ* studies and indeed any clinical study as this would minimise variations between subjects (Zero, 1995).

In the present study, standardisation was achieved by several means. During subject recruitment, specified inclusion and exclusion criteria were applied according to the recommendation by Curzon and Hefferren, (2001).

Standardisation was also achieved in the present model by asking participants to use only the assigned toothpaste for each arm. No other fluoride-containing food or products were used during the study. Other factors like saliva and medical history were carefully considered in our inclusion and exclusion criteria.

5.5 Hard tissue substrates

Several types of hard tissue substrates can be used in caries research. Human enamel is considered the substrates of choice (Curzon and Hefferren, 2001). However, several disadvantages limit its use. Human enamel is difficult to obtain in a large quantity and good quality. In addition, human teeth associated with great variations in their compositions and response as they are usually obtained from different sources. Moreover, a large flat enamel surface is rarely found as most of the human teeth used are posterior teeth (Zero, 1995; Mellberg, 1992).

Enamel slabs that used in the present study were obtained from bovine incisors as it was difficult to obtain human enamel in a large number. Bovine enamel has been commonly used as an alternative to human teeth over the past years (Hollanders et al., 2018; Yassen et al., 2011). The key advantage of using bovine teeth is that it is relatively easy to obtain a large number of teeth in sound quality. Furthermore, unlike human teeth, bovine teeth have less variability in their composition and response. Also, a large and flat intact enamel can be simply obtained (Sønju Clasen and ØGaard, 1999; Zero, 1995; Mellberg, 1992).

On the other hand, the caries progression in bovine enamel is faster than human teeth as it has more porous enamel. Also, the chemical and mechanical structures of bovine teeth are not the same as the human teeth (Sønju Clasen and ØGaard, 1999; Zero, 1995; Mellberg, 1992). Despite these differences, the

use of bovine enamel as an alternative to human enamel for caries research is acceptable (Zero, 1995; Mellberg, 1992).

Prior to the use of bovine teeth, the possible occurrence of an infectious disease called bovine spongiform encephalopathy (BSE), or mad cow disease was considered. This disease attacks bovines and produces a progressive neurodegenerative lesion, by means of an infectious prion (Prusiner, 1982). In order to eliminate prions, the slabs were immersed in 12% sodium hypochlorite for 24 hours. It has been shown that sodium hypochlorite did not have an effect on the mineral content of dentine or its crystal structure (Driscoll et al., 2002).

5.6 Artificial caries lesion

In caries research, enamel subsurface caries-like lesion is commonly used to mimic the white spot lesions in the natural environment. Two main methods are mainly used to create artificial lesions; chemical and oral microbiota models. Both models produce a relatively similar amount of enamel demineralisation. (Abufarwa et al., 2018).

Two methods can be used to create enamel subsurface like lesion using the chemical model; buffered solutions or acidified gels. In the present model, artificial lesions were created using an acidified gel chemical model as described earlier in section 2.4.3. This model has been used in several studies and produced consistent enamel lesions. In addition, the acidified gel is easy to prepare (Bataineh et al., 2017).

5.7 Intra-oral appliance design

In the present study, a lower removable appliance with two buccal acrylic flanges was used. This design was introduced by (Koulourides et al., 1974). Two enamel slabs were inserted into the acrylic flanges and secured with wax. This was then covered with mesh to accumulate dental plaque. The slabs were placed buccally to protect the slabs and biofilm from the tongue movement and minimise disturbance. To ensure standardisation, slabs were placed in the left side for all participants' appliances.

This design has a number of advantages. Several *in situ* studies have used this design without causing damage to the oral structure or causing discomfort. It also allows for normal daily oral hygiene as it is easy to remove during brushing as well as during eating and drinking (Malinowski et al., 2018). However, the removable appliances have some disadvantages, such as the issue of having bulky acrylic appliances that might cause discomfort for the participants. In addition, the removable appliances might cause mucosal damage or dental caries secondary to plaque accumulation. To overcome these limitations, we were regularly monitoring the participants' oral health during the study. The participants were clinically examined during each visit. In addition, the participants were instructed to return to the study site if they experienced any discomfort for the appliance to be adjusted.

Our patient information sheet lacked some information about the potential disadvantage of wearing the removable appliances. Future work should

consider reviewing the patient information sheet and explaining all the potential disadvantages of wearing the appliances.

5.8 Use of Quantitative Light-induced Fluorescence (QLF)

QLF is a quantitative system to detect early lesion as well as measuring enamel remineralisation based on the concept that the change in mineral content of tissue affects its autofluorescence. The key benefit of QLF system is that it is a non-destructive method to assess the enamel de/remineralisation. This advantage allows for easy conduction of longitudinal evaluation of the lesions (Karlsson, 2010). Also, QLF has been validated against the gold standard TMR with a statistically positive correlation (r = 0.63) (Cochrane et al., 2012). Moreover, excellent intra-examiner and inter-examiner reproducibly were found in several *in vitro* and *in vivo* studies (Bataineh et al., 2017; Al-Khateeb et al., 2002; Pretty et al., 2002; Tranæus et al., 2002). In the current study the intra-class correlation for the intra-examiner coefficient was found to be (0.96) which represented an excellent reproducibly.

However, QLF's readings can be affected by several confounders (Al-Khateeb et al., 2002). Therefore, the study images were captured in a standardised and controlled environment. These were achieved by using the same camera position, angels and distance. In addition, the slabs were dried for 15 seconds to minimise the hydration effect as recommended by (Al-Khateeb et al., 2002). To reduce the light effect, pictures were taken and analysed in a dark room. The images were taken and analysed by a single operator (MA) to ensure

consistency. The operator (MA) had received training by the manufacturer before the use of QLF.

In this *in situ* study, three parameters were assessed; ΔF which represents the percentage fluorescence loss and is related to lesion depth, the lesion surface area and lesion volume (ΔQ). However, (ΔQ) was considered as the main parameter in the present study. Ando et al. (2004) concluded in their study that ΔF and lesion area may not correlate. This means that the lesion area may not change while the ΔF changes or instead the lesion area may sustain while there are changes in ΔF. Therefore, it was recommended to use (ΔQ) as the primary parameter when assessing lesion progression or regression.

5.9 Use of Microhardness

One of the methods that were used to assess demineralisation and remineralisation is microhardness. This system measures the resistance of the enamel structure to the force applied by the indenter. As discussed earlier in section 1.4.1, the microhardness testing is classified into surface microhardness (SMH) and cross-sectional microhardness (CSMH) (White et al., 1992; Arends and Ten Bosch, 1992). In the present study, Knoop SMH was used to assess the ability of test products to inhibit enamel demineralisation. The use of SMH has several benefits. It is non-destructive and easy to use. Also, it is sensitive to mineral change (Arends and Ten Bosch, 1992; Zero et al., 1990). Moreover, microhardness was validated by comparison with the gold

standard TMR, and a significant positive correlation was found (Lippert and Lynch, 2014).

However, the main drawback is that SMH is less sensitive to assess deep lesions. Another disadvantage is that it does not give much details about mineral distribution and lesion shape (Arends and Ten Bosch, 1992; Zero et al., 1990).

Several loads have been used ranging from 50g up to 500g for caries research. A 100g load using the Knoop diamond was applied in this study. This load was shown to be needed to facilitate optical perceptibility (Davidson et al., 1974).

5.10 Remineralisation potential of the test products on artificial subsurface like lesions using QLF

The first part of this *in situ* study was conducted to assess and compare the remineralisation ability of three different products; 1450 ppm NaF, Clinpro tooth crème and a combination of MI Paste Plus with 1450 ppm NaF as a regimen using QLF. The 0 ppm F toothpaste was used as a negative control group. Artificial lesions were created and assessed at baseline and after treatment under controlled conditions using QLF.

The results showed that there was a statistically significant improvement in remineralisation in all groups except the negative control group compared with that at baseline in all QLF parameters. Whereas, the negative control group showed significant lesion progression in all QLF parameters. This was expected due to the lack of fluoride in the negative control group and the use of intense cariogenic challenge (five dipping a day in a sugary solution for 2 minutes during the whole arm).

The highest (ΔQ) improvement was seen in the regimen group (MI Paste Plus+1450 ppm NaF. However, multiple comparison tests with Bonferroni correction did not find any statistically significant differences between MI Paste Plus with 1450 ppm NaF as a regimen compared with1450 ppm NaF and Clinpro tooth crème.

5.10.1 Effect of CPP-ACPF + 1450 ppm NaF as a regimen

The ability of CPP-ACP products to enhance enamel remineralisation has been shown in several *in vitro* and clinical studies (Reynolds, 2009). A recent systematic review concluded that CPP-ACP had a significant long-term remineralisation ability when compared with placebo (Li et al., 2014). Moreover, the synergic effect of CPP-ACP and fluoride has been demonstrated in multiple studies (Mendes et al., 2018; Tao et al., 2018; Altenburger et al., 2010; Reynolds et al., 2008). The synergic effect is thought to be due to the formation of CPP-stabilised amorphous calcium fluoride phosphate. (CPP-ACFP).

The present findings agreed with the previous studies as the regimen group (CPP-ACPF + 1450 ppm NaF) showed significant remineralisation improvement compared with that at the baseline as well as the negative control group.

5.10.2 The benefits of using CPP-ACFP as a regimen with fluoride over the use of fluoride products alone

The benefits of using CPP-ACFP as a regimen with fluoride over the use of fluoride products have been investigated in the literature. However, there is still much controversy surrounding the benefits of this approach over conventional use of fluoride products alone.

Although the results demonstrated a trend towards more enamel remineralisation in the regimen group (CPP-ACPF + 1450 ppm NaF), our model did not find any significant difference in term of using CPP-ACPF as a

regimen with 1450 ppm NaF over the use of 1450 ppm F alone. These findings were in agreement with several *in vitro* and *in situ* studies (Bataineh et al., 2017; Vyavhare et al., 2015; Huang et al., 2013; Sitthisettapong et al., 2012; Bröchner et al., 2011; Beerens et al., 2010). However, the current study conflicts the findings published by Mendes et al.(2018) who compared the remineralisation ability of four different products (Placebo, 1.23% acidulated phosphate fluoride, MI Paste™ and MI Paste Plus Plus™) *in vivo*. The study participants were using routine dental care (1450 ppm F) in all groups. The study found a significant lesion regression with MI Paste™ and MI Paste Plus Plus™ at 90 days compared to other groups. However, the enamel regression was only significant after 30 days. Furthermore, our findings are not in agreement with the results demonstrated by Reynolds et al. (2008) who conducted a randomised, double-blind cross-over *in situ* study to assess the enamel remineralisation ability of different toothpaste slurries; (i) placebo, (ii) 1100 ppm NaF, (iii) 2800 ppm NaF, (iv) 2% CPP-ACP and (v) 2% CPP-ACP Plus 1100 ppm NaF. The study concluded that CPP-ACP Plus 1100 ppm NaF as a regimen showed significant more remineralisation compared with the other groups, over two weeks. In this study the application of the experimental pastes was not followed according to the manufacturer recommendations.

In the present study, the MI Paste+1450 ppm F regimen could be expected to have more significant remineralisation efficacy compared with other experimental pastes due to the presence of higher concentration of fluoride. However, this was not the case. There are several possible explanations for these findings. One explanation could be the duration of exposure; our study

duration was 21 days per study arm. Several studies have reported significant remineralisation effect with a longer period of exposure (Mendes et al., 2018; Hegde and Moany, 2012). Another possible reason could be the application sequence of the pastes. In the present *in situ* study , the MI Paste Plus was applied immediately after the use of fluoridated toothpaste (1450 ppm NaF). Therefore, the formation of fluorapatite ions might block the enamel pores preventing the diffusion of calcium and phosphate ions. A recent *in vitro* study tested the different application sequence of CPP-ACFP and fluoride and concluded that the application of CPP-ACFP before the fluoride therapy provides the optimum remineralisation results (Al-Batayneh et al., 2017). However, the evidence related to this aspect is very limited.

5.10.3 Comparison of remineralisation ability of CPP-ACFP and Clinpro Tooth Crème

Few clinical studies have directly compared the remineralisation potential of CPP-ACFP and f-TCP Clinpro tooth crème. In the present *in situ* study, the participants were instructed to use these products as per manufacturer directions.

In both groups, the present study found significant remineralisation in all QLF parameters compared with that at the baseline as well as the negative control group. However, the study failed to find any significant difference between them when compared to each other.

The findings of the current study are consistent with a randomised controlled *in situ* study that was published by (Vanichvatana and Auychai, 2013). In their study, the remineralisation ability of CPP-ACFP and Clinpro tooth crème were compared with fluoridated toothpaste (1450 ppm F). The study found that all the test products produced significant remineralisation. However, no significant difference was detected between the groups. The previous findings were also in agreement with other *in vitro* studies (Jo et al., 2014; Joshi et al., 2013).

In contrast with previous findings, Shen et al. (2011) conducted a randomised controlled trial and reported higher significant remineralisation in CPP-ACP and CPP-ACFP groups when compared with Clinpro tooth crème and different concentrations of fluoridated toothpaste. However, a shortcoming of the previously mentioned study was it lacked any sample size calculation. Moreover, the duration of the study arm was only for ten days, and the

application CPP-ACP and CPP-ACFP products were not following the manufacturers' recommendations.

5.11 The ability of the test products to inhibit enamel demineralisation using microhardness

In this part of the study, the inhibitory effect on demineralisation of three different products; 1450 ppm NaF, Clinpro tooth crème and a combination of MI Paste Plus with 1450 ppm NaF as a regimen was assessed using microhardness test *in situ.* The non-fluoridated toothpaste was used as a negative control group. Intact bovine enamel slabs were assessed at baseline and after 21 days of intensive cariogenic challenge (five dipping a day using 12% sucrose, two minutes for each dipping). The use of sound enamel slabs is the preferable approach to assess enamel demineralisation and lesion formation (Mellberg, 1992).

It was evident that there was an enamel hardness loss in all groups compared to the values at baseline. The highest enamel hardness loss was seen in the negative control group (0 ppm F). This finding was expected due to the intensive cariogenic challenge and the lack of fluoride in the negative control group.

The statistical tests revealed that there was a statistically significant difference in term of enamel hardness loss between the negative control group and the other test groups. However, no significant differences were detected between the other groups. This means that significant enamel demineralisation was observed in the negative control group compared with that at other groups.

The effect of the CPP-ACP and CPP-ACFP agents in inhibiting enamel demineralisation has been well reported in the literature. This is believed to be due to the ability to stabilise and localise ACP on the tooth surface as well as on the plaque (Reynolds 2009; Reynolds, 2008). Our findings correlate favourably with several studies that showed a significant effect of reducing enamel demineralisation when using CPP-ACP and CPP-ACFP agents (Bröchner et al., 2011; Robertson et al., 2011; Uysal et al., 2010).

The ability of f-TCP Clinpro tooth crème to inhibit enamel demineralisation was also investigated in this part of the study. The results revealed a significant impact on inhibiting enamel demineralisation when compared with the negative control group, this finding is in agreement with studies published previously (Jo et al., 2014; Vanichvatana and Auychai, 2013).

When comparing the test product with each other, the statistical analysis did not find any significant difference in their efficacy. It would have been reasonable to have expected the regimen group to have a higher efficacy due to the presence of a high concentration of fluoride. A positive correlation has been reported between the fluoride dose and effect on preventing enamel demineralisation and enhancing remineralisation (Marinho et al., 2016; Walsh et al., 2010). The reason for this contradictory result is not entirely clear, but there are several possible explanations. It could be the application order as described earlier. CPP-ACP and CPP-ACFP behave better when applied before the application of the fluoridated toothpaste (Al-Batayneh et al., 2017). In this study, CPP-ACFP was applied immediately after the application of the fluoridated toothpaste. Another possible explanation is the length of the study.

In our protocol, the period of each arm was 21 days. CPP-ACP and CPP-ACFP have been reported to produce a significant inhibitory effect with more extended periods (Mendes et al., 2018; Hegde and Moany, 2012).

Another possible speculation which is supported by limited evidence is the time of CPP-ACFP application in relation to the cariogenic challenge. CPP-ACFP and CPP-ACP have been shown to work better when applied 10 minutes before the cariogenic challenge (Yamaguchi et al., 2007; Yamaguchi et al., 2006). In our model, the CPP-ACFP was applied after the cariogenic challenge.

5.12 Remineralising potential of f-TCP technologies

Interestingly, the results of our study found that the ability of f-TCP Clinpro crème to enhance enamel remineralisation and inhibit enamel demineralisation did not differ statistically than 1450 ppm F and CPP-ACFP regimen. It should be emphasised that f-TCP Clinpro crème has less fluoride concentration that 1450 ppm and CPP-ACFP regimen groups. Therefore, f-TCP Clinpro crème may be of benefit for patients at high caries and at risk of developing fluorosis such as children under age of three years old.

The results indicate that f-TCP can be a promising technology to enhance enamel remineralisation and inhibit demineralisation of the enamel. However, more studies are needed to confirm these findings.

5.13 Suggestion for future research

The results of current *in situ* study, have been promising in term of the ability of f-TCP Clinpro tooth crème (950 ppm F) to enhance remineralisation and inhibiting demineralisation of enamel. The current literature does not provide sufficient evidence to support this. Future well-conducted randomised clinical studies on this topic are therefore required to confirm these findings.

The results of this study did not find any significant difference of using MI Paste Plus combined with fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride) as a regimen over the use of routine fluoridated toothpaste (1450 ppm F). We suggest further research to investigate the effect of using different application sequence than the one we used. In addition, further well-conducted randomised studies with more extended period are needed.

5.14 Null hypotheses outcome

1. The nul hypothesis “ There is no difference between 0 ppm F toothpaste; MI Paste Plus combined with fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride); Clinpro tooth crème 950 ppm F (0.21% w/w sodium fluoride) and fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride), on progression/regression of artificial subsurface carious lesions *in situ* as measured by QLF” can be rejected as significant differences were detected in the enamel remineralisation between 0 ppm toothpaste and all other tested products.

2. The null hypothesis “There is no difference in the effect of different caries preventive regimen and therapies: MI Paste Plus combined with fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride); Clinpro tooth crème 950 ppm F (0.21% w/w sodium fluoride) and fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride), on progression/regression of artificial subsurface carious lesions *in situ* as measured by QLF” can be accepted as no significant differences were detected in the enamel remineralisation between the tested products.

3. The null hypothesis “There is no difference between 0 ppm F toothpaste; MI Paste Plus combined with fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride); Clinpro tooth crème 950 ppm F (0.21% w/w sodium fluoride) and fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride), on the inhibition of the demineralisation process in dental enamel under cariogenic challenge *in situ* as measured by SMH” can be rejected as significant differences were detected in the enamel demineralisation between 0 ppm group and all other tested products.

4. The null hypothesis “There is no difference in the effect of a different caries preventive regimen and therapies: MI Paste Plus combined with fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride); Clinpro tooth crème 950 ppm F (0.21% w/w sodium fluoride) and fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride) on the inhibition of the demineralisation process in dental enamel under cariogenic challenge *in situ* as measured by SMH*”* can be accepted as no significant differences were detected in the enamel demineralisation between the tested products.

6.0 CONCLUSION

From the results of this study it can be concluded that:

1- 1450 ppm NaF toothpaste was as effective as calcium phosphate delivery technologies in remineralising enamel subsurface lesions and inhibiting enamel demineralisation. Within the limitations of this study, it seems appropriate that dentists should advise patients to use conventional fluoride 1450 ppm especially in term of cost effectiveness.

2- QLF was shown to be able to assess enamel de/remineralisation in our *in situ* model. QLF offers significant advantages include being non-destructive and time saving. However, standardised and controlled environment are required to minimise the effect of the confounders such as the ambient light and the hydration effects.

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APPENDICES

Appendix 1: Ethical approval from the Dental Research Ethics Committee of University of Leeds.

Inbox

Dear Mannaa

DREC reference: 140616/MA/205
Study title: The use of caries preventive agents alone and in regimens for prevention of enamel demineralisation under cariogenic challenge in situ

Thank you for submitting the above Ethics application to the Dental Research Ethics Committee. Your application has been reviewed and I am pleased to inform you that your application has been accepted.

Documents reviewed

Document name	Version number and date
Protocol	Version 5 11/07/2016
Participant information sheet	Version 4 05/09/2016
Consent form	Version 3 05/09/2016
Poster	Version 3 05/09/2016

With best wishes for the success of your project.

Please note: You are expected to keep a record of all your approved documentation, as well as documents such as sample consent forms, signed consent forms, participant information sheets and all other documents relating to the study. This should be kept in your study file, and may be subject to an audit inspection. If your project is to be audited, you will be given at least 2 weeks' notice.

It is our policy to remind everyone that it is your responsibility to comply with Health and Safety, Data Protection and any other legal and/or professional guidelines there may be.

Kind regards,

For and on behalf of
Dr Julia Csikar
DREC Chair

Appendix 2: Advertisement poster of the study

Version 3 05th September 2016

UNIVERSITY OF LEEDS

Are you aged 18-65?

Are you fit and healthy?

Would you be interested in participating in a research study?

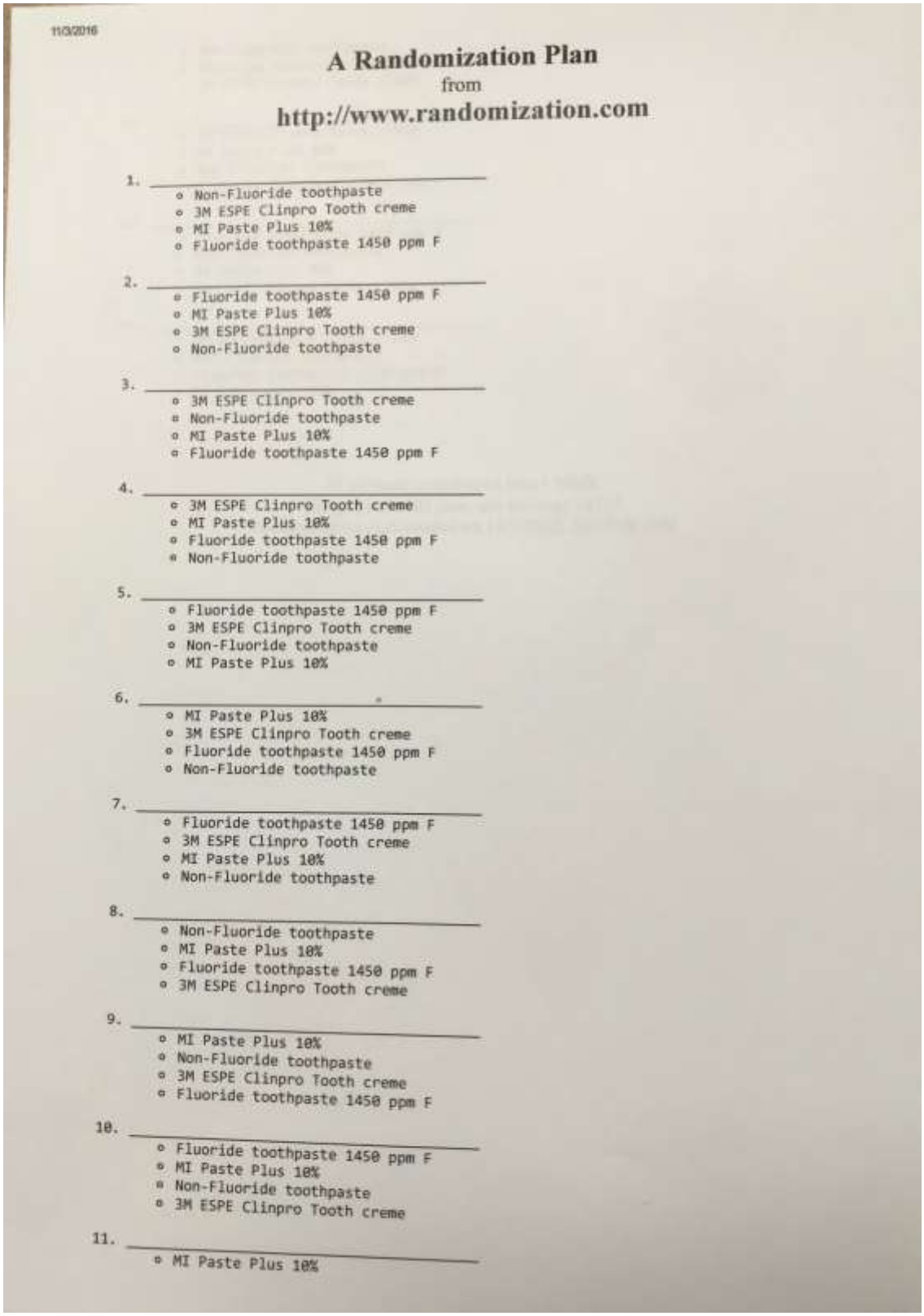
We are studying the effect of using a different preventive toothpastes for prevention of enamel demineralization (weakening).

What we will need from you is to wear an oral appliance, and to keep the appliance in a sugar solution and in slurries of toothpastes (which we will provide) on certain times during the day

You will receive £100 for each completed phase for taking part in the study as a compensation for your time and inconvenience

For further information, please contact Mannaa Aldowsari,
School of Dentistry - University of Leeds
Tel: 07843925822 or
Email: dnmka@leeds.ac.uk

Appendix 3: Randomisation



11/3/2016

A Randomization Plan
from
http://www.randomization.com

1. _____
   - Non-Fluoride toothpaste
   - 3M ESPE Clinpro Tooth creme
   - MI Paste Plus 10%
   - Fluoride toothpaste 1450 ppm F

2. _____
   - Fluoride toothpaste 1450 ppm F
   - MI Paste Plus 10%
   - 3M ESPE Clinpro Tooth creme
   - Non-Fluoride toothpaste

3. _____
   - 3M ESPE Clinpro Tooth creme
   - Non-Fluoride toothpaste
   - MI Paste Plus 10%
   - Fluoride toothpaste 1450 ppm F

4. _____
   - 3M ESPE Clinpro Tooth creme
   - MI Paste Plus 10%
   - Fluoride toothpaste 1450 ppm F
   - Non-Fluoride toothpaste

5. _____
   - Fluoride toothpaste 1450 ppm F
   - 3M ESPE Clinpro Tooth creme
   - Non-Fluoride toothpaste
   - MI Paste Plus 10%

6. _____
   - MI Paste Plus 10%
   - 3M ESPE Clinpro Tooth creme
   - Fluoride toothpaste 1450 ppm F
   - Non-Fluoride toothpaste

7. _____
   - Fluoride toothpaste 1450 ppm F
   - 3M ESPE Clinpro Tooth creme
   - MI Paste Plus 10%
   - Non-Fluoride toothpaste

8. _____
   - Non-Fluoride toothpaste
   - MI Paste Plus 10%
   - Fluoride toothpaste 1450 ppm F
   - 3M ESPE Clinpro Tooth creme

9. _____
   - MI Paste Plus 10%
   - Non-Fluoride toothpaste
   - 3M ESPE Clinpro Tooth creme
   - Fluoride toothpaste 1450 ppm F

10. _____
    - Fluoride toothpaste 1450 ppm F
    - MI Paste Plus 10%
    - Non-Fluoride toothpaste
    - 3M ESPE Clinpro Tooth creme

11. _____
    - MI Paste Plus 10%

- Non-Fluoride toothpaste
- Fluoride toothpaste 1450 ppm F
- 3M ESPE Clinpro Tooth creme

12. _____
- 3M ESPE Clinpro Tooth creme
- MI Paste Plus 10%
- Non-Fluoride toothpaste
- Fluoride toothpaste 1450 ppm F

13. _____
- Fluoride toothpaste 1450 ppm F
- Non-Fluoride toothpaste
- MI Paste Plus 10%
- 3M ESPE Clinpro Tooth creme

14. _____
- 3M ESPE Clinpro Tooth creme
- Non-Fluoride toothpaste
- Fluoride toothpaste 1450 ppm F
- MI Paste Plus 10%

14 subjects randomized into 1 block
To reproduce this plan, use the seed 16173
Randomization plan created on 11/3/2016, 10:16:46 AM

11/3/2016

A Randomization Plan

from

http://www.randomization.com

1. MH subject ID 2
2. MH subject ID 3
3. MH subject ID 1
4. MH subject ID 1
5. MH subject ID 7
6. MH subject ID 6
7. MH subject ID 10
8. MH subject ID 14
9. MH subject ID 5
10. MH subject ID 5
11. MH subject ID 4
12. MH subject ID 9
13. MH subject ID 11
14. MH subject ID 8
15. MH subject ID 3
16. MH subject ID 11
17. MH subject ID 12
18. MH subject ID 10
19. MH subject ID 4
20. MH subject ID 14
21. MH subject ID 11
22. MH subject ID 6
23. MH subject ID 7
24. MH subject ID 4
25. MH subject ID 13
26. MH subject ID 9
27. MH subject ID 13
28. MH subject ID 13
29. MH subject ID 12
30. MH subject ID 2
31. MH subject ID 2
32. MH subject ID 11
33. MH subject ID 1
34. MH subject ID 14
35. MH subject ID 1
36. MH subject ID 10
37. MH subject ID 13
38. MH subject ID 3
39. MH subject ID 5
40. MH subject ID 9
41. MH subject ID 5
42. MH subject ID 10
43. MH subject ID 14
44. MH subject ID 12
45. MH subject ID 6
46. MH subject ID 2
47. MH subject ID 7
48. MH subject ID 9
49. MH subject ID 4
50. MH subject ID 12
51. MH subject ID 3
52. MH subject ID 8
53. MH subject ID 8
54. MH subject ID 6
55. MH subject ID 8
56. MH subject ID 7

56 subjects randomized into 1 block
To reproduce this plan, use the seed 16111

11/3/2016

A Randomization Plan

from

http://www.randomization.com

1. QLF subject ID 2
2. QLF subject ID 4
3. QLF subject ID 12
4. QLF subject ID 5
5. QLF subject ID 12
6. QLF subject ID 10
7. QLF subject ID 12
8. QLF subject ID 4
9. QLF subject ID 2
10. QLF subject ID 9
11. QLF subject ID 10
12. QLF subject ID 12
13. QLF subject ID 5
14. QLF subject ID 8
15. QLF subject ID 2
16. QLF subject ID 5
17. QLF subject ID 9
18. QLF subject ID 9
19. QLF subject ID 14
20. QLF subject ID 6
21. QLF subject ID 6
22. QLF subject ID 1
23. QLF subject ID 7
24. QLF subject ID 6
25. QLF subject ID 11
26. QLF subject ID 11
27. QLF subject ID 13
28. QLF subject ID 7
29. QLF subject ID 3
30. QLF subject ID 3
31. QLF subject ID 8
32. QLF subject ID 14
33. QLF subject ID 5
34. QLF subject ID 13
35. QLF subject ID 11
36. QLF subject ID 1
37. QLF subject ID 4
38. QLF subject ID 4
39. QLF subject ID 9
40. QLF subject ID 11
41. QLF subject ID 6
42. QLF subject ID 14
43. QLF subject ID 7
44. QLF subject ID 7
45. QLF subject ID 10
46. QLF subject ID 14
47. QLF subject ID 10
48. QLF subject ID 1
49. QLF subject ID 2
50. QLF subject ID 1
51. QLF subject ID 3
52. QLF subject ID 13
53. QLF subject ID 13
54. QLF subject ID 8
55. QLF subject ID 8
56. QLF subject ID 3

56 subjects randomized into 1 block
To reproduce this plan, use the seed 19073

Appendix 4: Patient information sheet

Volunteer Information Sheet

UNIVERSITY OF LEEDS

Thank you for expressing an interest in our forthcoming study. Please find below some more information about the study, which we hope will answer some of the questions you may have.

Version 4, Dated 05/09/2016

Study Title: "The Use of Caries Preventive Agents Alone and In Regimen for Prevention of Enamel Demineralisation Under Cariogenic Challenge *In situ*"

"The Effect of Using A Different Preventive Toothpastes For Prevention of Enamel Demineralization (Weakening) Under Sugary Challenge."

Ethics Committee Ref No: 140616/MA/205

You are being invited to take part in the above research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully, and discuss it with others if you wish. Ask us if there is anything that is not clear, or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study?
This study is part of my Professional Doctorate in Paediatric Dentistry Degree. The aim of this project is to study the effect of using additional preventive toothpastes, over and above the daily use of the conventional fluoride toothpaste for prevention of enamel demineralization (weakening).

Why have I been chosen?
We hope to recruit volunteers for this study. All we ask of volunteers is that they are willing to take part in the study; that they are at least 18 to 65 years old; that they are in general good health; that they are not pregnant or planning to become pregnant during the course of the study as pregnancy hormones can effect the

stability of the oral environment (even though pregnant women are excluded, should you become pregnant whilst in the study there is no significant risk to the baby, but please let us know immediately); and that they have at least 18 natural teeth. Before you are enrolled on the study, you will need to be 'screened'. This will involve a short dental examination, to enable us to establish whether you meet all our criteria and a professional scaling and polishing if there is any tartar. We will also ask you a few simple questions about your general health, this information is kept confidential. You will also have the opportunity to ask us any questions you may have about the study.

Do I have to take part?
Participation in this study is entirely voluntary. If you decide you would like to take part, you will be given a copy of this information sheet to keep, and we will ask you to complete and sign a form which gives your written consent to take part. If after reading this and thinking about the information given, you decide you would not like to take part that is fine. Even if you decide you would like to take part in the study, but you later decide you no longer wish to continue, you can withdraw at any time, and you do not have to give us a reason unless you want to. If you do decide to withdraw from the study at any point, please let us reassure you that your future dental care at the Dental Hospital will in no way be compromised. If you are a member of staff of the Trust or University, you are under no obligation whatsoever to take part in this study, but if you decide you would like to take part, you can still withdraw at any point without having to give a reason.

What will happen to me if I take part?
If you decide you would like to take part and our screening procedures identify you as a suitable volunteer, then we have to have your written consent in order to recruit you. You do not have to give consent on the day of the dental examination, but you will have one week to decide.

At the first appointment we will collect a sample of saliva by asking you to spit in a tube for 2 minutes. You will also be asked to use fluoride-free toothpaste for 1 week before each sampling appointment. A lower dental impression using alginate impression will be taken to construct a removable appliance for your lower teeth . You will wear this removable appliance which contains two sterilised bovine enamel chips. These sections are the parts that we will do our test on. It will be given to you before each part of the study and should be worn at all times, except at mealtimes, drinking, toothbrushing, and dipping time. You will also be asked to keep the appliance in solutions of sucrose and in slurries of toothpastes on certain times during the day which we will provide. You will be provided with fluoride-free toothpaste in each part of the study, which you will use twice daily to brush your teeth normally for 7 days between the study phases and on one of the study phases for 21 days.

We will show you how to remove and re-insert the appliance, and you will have opportunity to ask any questions, and to make sure you are happy and confident in using the appliance.

The study consists of 4 periods. The duration of each experimental period will be one month making a total of four months for the whole study.

You will need to visit us two times in each period. These visits are to provide you with the appliance and collect it from you at the end of each study period. The appliance will be collected by the researcher (Mr.Mannaa Aldowsari), and the enamel pieces will be removed in the laboratory.

Once you decide to take part in the study, you will be given an appointment card showing the days and times we would like you to attend our test centre at the Leeds Dental Institute. You will have opportunity to ask any questions, and to make sure you are happy and confident before starting the study.

What do I have to do?
You will need to agree to have the appliances in your mouth, and to agree to remove the device when eating and/or drinking and at toothbrushing time. You will also need to come into the test centre at the dates and times agreed. We realise that this may cause you some inconvenience. In recognition of any inconvenience and out of pocket expenses you will incur, you will be paid a fee of **£100 per each period** for taking part in the study which will be paid at the end of the last period.

What are the side effects of taking part?
We hope there will be no side effects for volunteers. You as volunteer may have a small amount of discomfort from the appliance which usually occurs during the first couple of days. However in previous studies where the same appliance has been used, volunteers became used to it after a couple of days. If there is any pain, discomfort, or any other problems with the appliance at home (e.g. accidentally broken) please contact us on the telephone number **07843925822 (Mr. Mannaa Aldowsari -Researcher)** and we will arrange an appointment to adjust the appliance if needed

The other slight risk to you as a volunteer would be a very small chance of enamel demineralisation, because you will be using fluoride-free toothpaste during the study. However, previous tests have shown that teeth regain lost minerals naturally by saliva. As an extra precaution, you will have a topical fluoride gel professionally applied at the end of the study.

We are confident that no damage will be caused to you or your teeth if you take part in this study.

What are the possible disadvantages or risks of taking part?
The only disadvantage to you as a volunteer will be that you will be asked to wear and remove the device at the times specified. For the first few days of the study, you may find this to be a slight inconvenience. However, once you get into

routine of working with the appliance, we are sure you will find it easy to remember what to do and when.

What are the possible benefits of taking part?
There is no direct immediate benefit to the research participants. However, part icipants will help us to study the effect of additional preventive therapeutic aids, over and above the daily use of a fluoride toothpaste for prevention of enamel demineralisation.

What if new information becomes available?
If any new information becomes available during the study, we will of course let you know as soon as possible, and explain the changes to you. You will have the chance to ask us any questions before deciding whether you would like to continue with the study. If the changes are significant, it may be that we ask for your written consent to continue with the study. Please let us stress that, should this occur, you will be under no obligation whatsoever to continue with the study, and you may still withdraw at any point without giving a reason.

What happens when the research study stops?
At the end of the study, you will need to come back to the test centre. A dentist will remove the device; give you a final check up and a professional tooth cleaning. We will then collect all our data, and examine it in our laboratory. Once this is done, we will write a report on our findings. We will not identify any of our volunteers by name in our report. Any information we collect about you during our study will be kept strictly confidential.

Is this research part of your university studies?

Yes, I will submit the results of this study as my research thesis to fulfil the research component of my Professional Doctorate in Paediatric Dentistry degree. I would like to thank you for helping me complete this research.

What if something goes wrong?
If you have any concerns about your treatment during the study, please do not hesitate to contact any one of us, and we will do our best to help.
If you feel that the matter is serious, it may be that you will be entitled to appropriate compensation, from the sponsor of the study (University of Leeds). The sponsor, without armal commitment should compensate you without having to prove that it is at fault. This applies in cases where it is likely that such injury has resulted from any new drugs or procedures carried out in accordance with the protocol of the study. The sponsor “will not compensate you where such injury results from any procedure which is not in accordance with the protocol for the study”. Your right to claim compensation for the injury where you can prove negligence is not affected.
In the very unlikely event of you being harmed as a result of taking part in the study no special indemnity arrangements are in place. Your right at law to claim compensation for injury where you can prove negligence is not affected.

Will my taking part in this study be kept confidential?
If you consent to take part in this study, any information we gather will be kept confidential. You will not be identified by name in any reports or publications.

What will happen to the results of the research study?
We will report on the results of this study as a research thesis for a higher degree. We hope to publish the results in an international journal and present our findings at both national and international meetings. You will not be identified by name in any reports we write.

Who is organizing and funding the research?
The research will be carry out by the principal investigator, postgraduate student Mannaa Aldowsari, under the supervision of Prof. M.S. Duggal, Dr Jinous F Tahmassebi and Dr Marina Malinowski. The study is being sponsored by University of Leeds

Contact for further information: If you would like any further information at all, either before or during the study, please do not hesitate to contact any one of us:

Name	Telephone	e-mail
Mannaa Aldowsari	07843925822	dnmka@leeds.ac.uk
Professor Monty Duggal	(0113)3436177	M.S.Duggal@leeds.ac.uk
Dr Jinous F Tahmassebi	(0113)3433955	J.Tahmassebi@leeds.ac.uk
Dr Marina Malinowski	(0113) 343 6150	denmma@leeds.ac.uk

Appendix 5: Consent form

UNIVERSITY OF LEEDS

DREC No:
Patient Identification Number:

CONSENT FORM

Title of Project: ***The use of caries preventive agents alone and in regimen for prevention of enamel demineralisation under cariogenic challenge in situ.***

Name of Researcher: Mannaa Aldowsari

Please initial box

1. I confirm that I have read and understand the information sheet dated **5th September 2016 (version 4)** for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of any of my medical notes and data collected during the study may be looked at by clinicians involved in the study, from regulatory authorities or from the NHS Trust where it is relevant to my taking part in research. ☐

4. I agree to take part in the above study. ☐

_____	_____	_____
Name of Volunteer	Date	Signature
_____	_____	_____
Name of Person taking consent	Date	Signature

When completed, 1 for volunteer; 1 for researcher site file;
1 (original) to be kept in medical notes

Appendix 6: Case record form (CRF).

School of Dentistry

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Leeds LS2 9LU

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F +44 (0) 113 343 6165
E dentistry@leeds.ac.uk

UNIVERSITY OF LEEDS

Participant Initials: __ __ __ **Participant DOB:** __ __ / __ __/ __ __ __ __

Screening No.: __ __ __

Participant ID No.: __ __ __

Randomisation No.: __ __ __

Case Record Form

THE USE OF CARIES PREVENTIVE AGENTS ALONE AND IN REGIMENS FOR PREVENTION OF ENAMEL DEMINERLISATION UNDER CARIOGENIC CHALLENGE *IN SITU*.

Ethics Committee Ref No: 140616/MA/205

Academic Supervisors
Dr Jinous F Tahmassebi

(Paediatric Dentistry department)

Professor Monty Duggal

Research Investigator
Mannaa Aldowsari

(Paediatric Dentistry department)

Dr Marina Malinowski

(Paediatric Dentistry department)

Participant Information Sheet Version No. __ Dated: __ __/__ __/__ __ __ __	Yes ☐ No ☐ Date given __ __/__ __/__ __ __ __
Participant Consent Form Version No. __ Dated: __ __/__ __/__ __ __ __	Yes ☐ No ☐ Date given __ __/__ __/__ __ __ __ Time of consent: __ __: __ __ (hh:mm)

Screening Visit

- ### Medical History

Any medical conditions to report? ☐ Yes ☐ No

List any relevant previous and current medical conditions (including allergies) and surgeries that have been experienced by the participant in the table below*

Medical Condition	Start Date	On-going	Stop date (If applicable)
	…/……/……..	YES/ NO	…/……/……..
	…/……/……..	YES/ NO	…/……/……..
	…/……/……..	YES/ NO	…/……/……..
	…/……/……..	YES/ NO	…/……/……..
	…/……/……..	YES/ NO	…/……/……..

***Note that if any treatment(s) is/are currently being taken for any of the above conditions this (these) must be recorded in the following table (Current/Concomitant medication)**

- ### Current/ Concomitant medication

Drug's name	Reason for medication	Dosage	Date started	Date stopped (If applicable)

Researcher's Signature:…….. **Date** __ __/__ __/__ __ __ __

SCREENING VISIT

Inclusion Criteria Checklist*

	Yes	No
1. Age ≥ 18 years < 65	☐	☐
2. General Health		
• No relevant medical history and under no medication that is known to affect the oral cavity, the oral flora status and salivary flow rate	☐	☐
• Non-pregnant female volunteers	☐	☐
3. Dental examination		
i. Presence of at least 18 natural teeth	☐	☐
ii. Free from visual signs of untreated caries or periodontal disease or any other adverse dental oral health conditions that could be exacerbated by the study interventions	☐	☐
iii. Unstimulated salivary flow rate ≥ 0.2 ml/min	☐	☐
iv. Stimulated salivary flow rate ≥ 0.8 ml/min	☐	☐
4. Compliance		
• Fully understands and is willing, able and likely to comply with the study procedures and restrictions	☐	☐
5. Consent		
• Demonstrates understanding of the study and willingness to participate as evidenced by voluntary written informed consent	☐	☐

***Note: If any of the above questions are answered "No", the participant should be discontinued from the study as a "Screen failure" on the study conclusion page.**

Researcher's Signature:…….. **Date** __ __ / __ __/ __ __ __ __

SCREENING VISIT

Exclusion Criteria Checklist*

	Yes	No
1. Age: < 18 years > 65	☐	☐
2. General Health:		
• Current or recurrent disease that could affect the oral cavity or interfere with the dental examination and/or wearing of oral appliance	☐	☐
• Severe psychiatric, physical and medical disorders requiring treatment or making the participant unlikely to give informed consent or to cope with the procedures required by the study protocol	☐	☐
• Pregnant/ intending to become pregnant/ lactating female participant	☐	☐
3. Medication		
• Medication that is known to affect the oral cavity, oral flora status and salivary flow rate	☐	☐
• Antimicrobial therapy within 14 days prior to screening or during the study	☐	☐
• Antibiotic treatment within 28 days prior to screening or during the study	☐	☐
4.Dental details		
• Dental disease that require immediate treatment	☐	☐
• Oral surgery or extraction 6 weeks prior to screening or during the study	☐	☐
• Wearing of prostheses or orthodontic appliances that could affect the study procedures	☐	☐
• Unstimulated salivary flow rate < 0.2ml/min	☐	☐
• Stimulated salivary flow rate < 0.8 ml/min	☐	☐
5.Clinical trials		
• Participation in another clinical study or receipt of an investigational drug within 30 days of the screening and during the study	☐	☐

6.Other • Known or suspected intolerance/ hypersensitivity to study materials or ingredients that will be used in the study	☐ ☐ ☐ ☐

***Note: If any of the above questions are answered "Yes", the participant should be discontinued from the study as a "Screen failure" on the study conclusion page.**

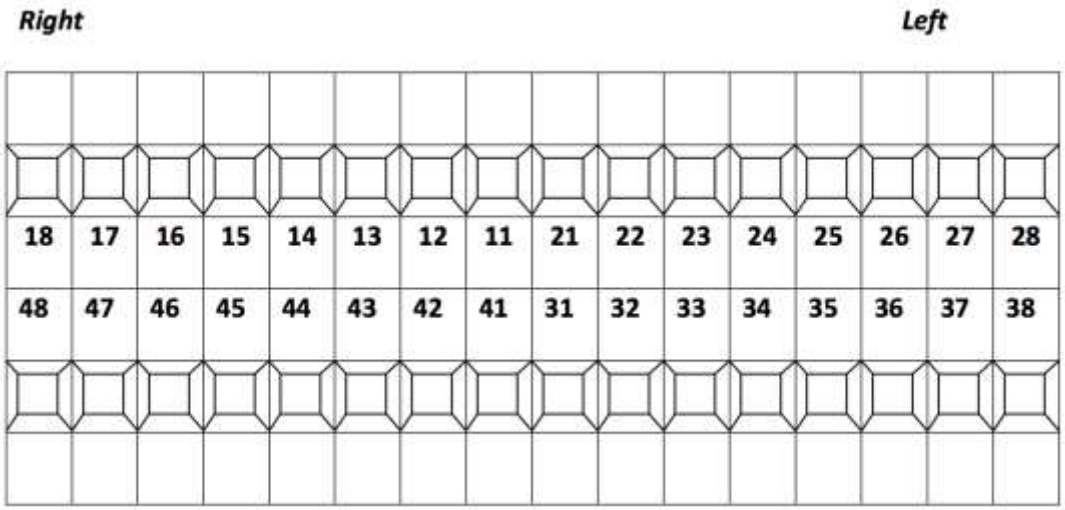
Researcher's Signature:…….. **Date** __ __ / __ __/ __ __ __ __

SCREENING VISIT **Time of Dental Examination.:** __ __: __ __

- **Oral Cavity Examination**

Soft tissue	
☐ Normal ☐ Abnormal	Describe abnormality:…… …… ……..

- **Dental Examination**



Total DMFT:	No. of teeth:

Note: Participant must have at least 18 natural teeth to be eligible for inclusion

- **Salivary Flow Rate**

Unstimulated: _. _ _ ml/min (Must be ≥ 0.2 ml/min) Stimulated _. _ _ ml/min (Must be ≥ 0.8 ml/min)

Researcher's Signature:…….. **Date** __ __/__ __/__ __ __ __

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SCREENING VISIT

Fitness and Eligibility to Participate in the study

In the investigator's opinion, on the basis of the screening assessments and inclusion and exclusion criteria, is the participant eligible to participate in the next part of the study? Yes ☐ No ☐

Researcher's Signature:…….. **Date** __ __ / __ __/ __ __ __ __

Screening Visit Checklist

Personal Data sheet completed	Yes ☐	No ☐
Medical History checked	Yes ☐	No ☐
Dental Examination completed	Yes ☐	No ☐
Inclusion Criteria Sheet completed	Yes ☐	No ☐
Exclusion Criteria Sheet completed	Yes ☐	No ☐
Eligibility Sheet completed	Yes ☐	No ☐

Researcher's Signature:…….. **Date** __ __/__ __/__ __ __ __

<table>
<tr><td rowspan="2">First Visit

Date__ __ / __ __/ __ __ __ __</td><td>• Lower alginate impression</td><td>☐</td></tr>
<tr><td colspan="2">• Any problems? YES ☐ NO ☐
If Yes, give details……..
…….
……</td></tr>
<tr><td colspan="2">Researcher's Signature</td><td>Date</td></tr>
<tr><td colspan="2">……..</td><td>__ __/__ __/__ __ __ __</td></tr>
<tr><td rowspan="5">Second Visit

Date__ __ / __ __/ __ __ __ __</td><td>• Fitting of appliance</td><td>☐</td></tr>
<tr><td colspan="2">• Any problems? YES ☐ NO ☐
If Yes, give details……..
…….
……..</td></tr>
<tr><td>Have there been any changes to the participant's medication record since the last visit?
(*If yes please complete the Concomitant Medication page)

Have there been any changes to the inclusion/exclusion criteria since the last visit?

Is the participant still eligible to continue in the study?</td><td>YES ☐ NO ☐

YES ☐ NO ☐

YES ☐ NO ☐</td></tr>
<tr><td>• Instructions & restrictions explained</td><td>YES ☐ NO ☐</td></tr>
<tr><td>• Appointment arranged after 3 weeks?</td><td>YES ☐ NO ☐
__ __/__ __/__ __ __ __</td></tr>
</table>

<table>
<tr><td>Researcher’s Signature</td><td colspan="2">Date</td></tr>
<tr><td>……..</td><td colspan="2">__ __/__ __/__ __ __ __</td></tr>
<tr><td rowspan="3">Third Visit

Date__ __ / __ __/ __ __ __ __</td><td>• Removal of appliance</td><td>☐</td></tr>
<tr><td colspan="2">• Any problems? YES ☐ NO ☐
If Yes, give details……
……..
……
……
……
……</td></tr>
<tr><td>Have there been any changes to the participant’s medication record since the last visit?

(*If yes please complete the Concomitant Medication page)

Have there been any changes to the inclusion/exclusion criteria since the last visit?

Is the participant still eligible to continue in the study?</td><td>YES ☐ NO ☐

YES ☐ NO ☐

YES ☐ NO ☐</td></tr>
<tr><td>Researcher’s Signature</td><td colspan="2">Date</td></tr>
<tr><td>……..</td><td colspan="2">__ __/__ __/__ __ __ __</td></tr>
</table>

<table>
<tr><td rowspan="6">Fourth Visit

Date__ __ / __ __/ __ __ __ __</td><td colspan="2">• Fitting of appliance</td><td>☐</td></tr>
<tr><td colspan="3">• Any problems? YES ☐ NO ☐

If Yes, give details…….
…….
……
……..
……</td></tr>
<tr><td>Have there been any changes to the participant's medication record since the last visit?

(*If yes please complete the Concomitant Medication page)</td><td colspan="2">YES ☐ NO ☐</td></tr>
<tr><td>Have there been any changes to the inclusion/exclusion criteria since the last visit?</td><td colspan="2">YES ☐ NO ☐</td></tr>
<tr><td>Is the participant still eligible to continue in the study?</td><td colspan="2">YES ☐ NO ☐</td></tr>
<tr><td colspan="2">• Instructions & restrictions explained</td><td>☐</td></tr>
<tr><td colspan="2">• Appointment arranged after 3 weeks?</td><td>YES ☐ NO ☐
__ __ /__ __/__ __ __ __</td></tr>
<tr><td colspan="2">Researcher's Signature</td><td colspan="2">Date</td></tr>
</table>

<table>
<tr><td colspan="2">……..</td><td>__ __/__ __/__ __ __ __</td></tr>
<tr><td rowspan="3">Fifth Visit

Date__ __ / __ __/ __ __ __ __</td><td>• Removal of appliance</td><td>☐</td></tr>
<tr><td colspan="2">• Any problems? YES ☐ NO ☐

If Yes, give details……
……
……
……
……
……</td></tr>
<tr><td>Have there been any changes to the participant’s medication record since the last visit?

(*If yes please complete the Concomitant Medication page)

Have there been any changes to the inclusion/exclusion criteria since the last visit?

Is the participant still eligible to continue in the study</td><td>YES ☐ NO ☐

YES ☐ NO ☐

YES ☐ NO ☐</td></tr>
<tr><td colspan="2">Researcher’s Signature</td><td>Date</td></tr>
<tr><td colspan="2">……..</td><td>__ __/__ __/__ __ __ __</td></tr>
</table>

sixth Visit Date__ __ / __ __/ __ __ __ __	• Fitting of appliance	☐
	• Any problems? YES ☐ NO ☐ If Yes, give details……. ……. …… …….. ……	
	Have there been any changes to the participant's medication record since the last visit? (*If yes please complete the Concomitant Medication page) Have there been any changes to the inclusion/exclusion criteria since the last visit? Is the participant still eligible to continue in the study	YES ☐ NO ☐ YES ☐ NO ☐ YES ☐ NO ☐
	• Instructions & restrictions explained	☐
	• Appointment arranged after 3 weeks?	YES ☐ NO ☐ __ __/__ __/__ __ __ __

Researcher's Signature	Date
……..	__ __/__ __/__ __ __ __

<table>
<tr><td rowspan="3">Seventh Visit

Date__ __ / __ __/ __ __ __ __</td><td>• Removal of appliance</td><td>☐</td></tr>
<tr><td colspan="2">• Any problems? YES ☐ NO ☐

If Yes, give details……
……..
……
……
……</td></tr>
<tr><td>Have there been any changes to the participant's medication record since the last visit?

(*If yes please complete the Concomitant Medication page)

Have there been any changes to the inclusion/exclusion criteria since the last visit?

Is the participant still eligible to continue in the study</td><td>YES ☐ NO ☐

YES ☐ NO ☐

YES ☐ NO ☐</td></tr>
<tr><td colspan="2">Researcher's Signature</td><td>Date</td></tr>
<tr><td colspan="2">……..</td><td>__ __/__ __/__ __ __ __</td></tr>
</table>

Eighth Visit Date__ __ / __ __/ __ __ __ __	• Fitting of appliance	☐
	• Any problems? YES ☐ NO ☐ If Yes, give details……. …… …… …….. ……	
	Have there been any changes to the participant's medication record since the last visit? (*If yes please complete the Concomitant Medication page) Have there been any changes to the inclusion/exclusion criteria since the last visit? Is the participant still eligible to continue in the study	YES ☐ NO ☐ YES ☐ NO ☐ YES ☐ NO ☐
	• Instructions & restrictions explained	☐
	• Appointment arranged after 3 weeks?	YES ☐ NO ☐ __ __/__ __/__ __ __ __

Researcher's Signature	Date
……..	__ __/__ __/__ __ __ __

<table>
<tr><td rowspan="4">Ninth Visit

Date__ __ / __ __/ __ __ __ __</td><td>• Removal of appliance</td><td>☐</td></tr>
<tr><td colspan="2">• Any problems? YES ☐ NO ☐

If Yes, give details……
……..
……
……</td></tr>
<tr><td>Have there been any changes to the participant’s medication record since the last visit?

(*If yes please complete the Concomitant Medication page)

Have there been any changes to the inclusion/exclusion criteria since the last visit?</td><td>YES ☐ NO ☐

YES ☐ NO ☐</td></tr>
<tr><td>• Application of topical fluoride</td><td>YES ☐ NO ☐</td></tr>
<tr><td colspan="2">Researcher’s Signature</td><td>Date</td></tr>
<tr><td colspan="2">……..</td><td>__ __/__ __/__ __ __ __</td></tr>
</table>

UNSCHEDULED VISIT - APPLIANCE MODIFICATION SHEET

Modifications

	Yes*	No
Has the participant required any modification to their appliance? **If yes*, please specify in the comments' box below.	☐	☐

Comments
(Record each date a modification was required and details of modification conducted)

Date	Reason	Clinician's Signature
	…… …… …… …… ……	
	…… …… …… …… ……	
	…… …… …… …… ……	
	…… …… …… …… ……	
	…… …… …… …… ……	

Study Conclusion

Did the participant complete the entire study? Yes ☐ No* ☐

If " Yes", Date completed: _ _/ _ _/ _ _ _ _

If "No" is checked, please complete the following (Please check as an appropriate):

Screen Failure ☐

Adverse Event ☐

Lost to follow-up ☐

Protocol Deviation (please specify details) ☐
……
……
……
……

Withdrawal of Volunteer (please specify details) ☐
……
……
……
……

Other (please specify details) ☐
……
……
……
……

Researcher's Signature

I confirm that I have reviewed all the data collected in this Case Report Form and take responsibility that the information is accurate and complete.

Researcher's Name……

Researcher's Signature…… Date __ __ / __ __/ __ __ __ __

Appendix 7: Dipping diary and instruction sheet for non-regimen groups.

THE USE OF CARIES PREVENTIVE AGENTS ALONE AND IN REGIMEN FOR PREVENTION OF ENAMEL DEMINERALISATION UNDER CARIOGENIC CHALLENGE IN SITU.

BRIEF INFORMATION FOR COMPLETION OF THE FOLLOWING DIARY CARD

- ✓ Brushing should occurs initially in the morning and before bed (Please remove appliance when brushing- brushing is for 2 mins.
- ✓ Guidelines for the frequency of dipping in a sugar solution and in a slurry of toothpaste**:**

  Each dipping in sugar solution = 2 minutes and the time should be recorded in the diary log.

  Dipping in sugar solution is 5 times per day. The interval between dipping in sugar solution is not less than 1 hour.

  Dipping in slurry toothpaste is 2 times per day (morning and evening) for 2 minutes.

  Example of completed diary card:

Date	Morning Brushing	Morning Dip in slurry of toothpaste (2 min)	Dipping in sugar solution for 2 min					Evening Dip in slurry of toothpaste (2 min)	Evening brushing
			1st time	2nd time	3rd time	4th time	5th time		
09/11/16	yes	8:00am	9:00am	12:00pm	3:00pm	6:00pm	8:oopm	9:00pm	**yes**

Thank you for your cooperation

Participant Initials: __ __ __ **Screening No.: __ __ __**	**Leg no: _ _** **Randomisation No: __ __ __**

Dipping Diary Sheet:

Fluoride –free toothpaste (washout period) start date: _ _/ _ _/ _ _ _ _

Dental device using start date _ _/ _ _/ _ _ _ _

Dental device dipping start date _ _/ _ _/ _ _ _ _

Date	Morning Brushing	Morning Dip in slurry of toothpaste (2 min)	Dipping in sugar solution for 2 min					Evening Dip in slurry of toothpaste (2 min)	Evening brushing
			1st time	2nd time	3rd time	4th time	5th time		

Thank you for your cooperation

Appendix 8: Dipping diary and instruction sheet for the regimen groups.

THE USE OF CARIES PREVENTIVE AGENTS ALONE AND IN REGIMEN FOR PREVENTION OF ENAMEL DEMINERALISATION UNDER CARIOGENIC CHALLENGE IN SITU.

Regimen Phase

BRIEF INFORMATION FOR COMPLETION OF THE FOLLOWING DIARY CARD

- ✓ Brushing should occurs initially in the morning and before bed (Please remove appliance when brushing- brushing is for 2 mins.

- ✓ Guidelines for the frequency of dipping in a sugar solution and in a slurry of toothpaste**:**

  Each dipping in sugar solution = 2 minutes and the time should be recorded in the diary log.

  Dipping in sugar solution is 5 times per day. The interval between dipping in sugar solution is not less than 1 hour.

  Dipping in slurry toothpaste is 2 times per day (morning and evening) for 2 minutes.

- ✓ Apply a pea-sized amount of tooth mousse plus (MI Paste) to your teeth's surface using your finger and Leave undisturbed for 3 minutes. (Please do not remove the appliance while applying the MI paste).

  Example of completed diary card:

Date	Morning Brushing	Morning Dip in slurry of toothpaste (2 min)	Dipping in sugar solution for 2 min					Evening Dip in slurry of toothpaste (2 min)	Evening brushing	T M Pl ap
			1st time	2nd time	3rd time	4th time	5th time			
09/11/16	yes	8:00am	9:00am	12:00pm	3:00pm	6:00pm	8:oopm	9:00pm	**yes**	**ye**

Thank you for your cooperation

Participant Initials: __ __ __ **Screening No.:** __ __ __	**Arm no:** _ _ **Randomisation No:** __ __ __

Dipping Diary Sheet:

Fluoride –free toothpaste (washout period) start date: _ _ / _ _ / _ _ _ _

Dental device using start date _ _ / _ _ / _ _ _ _

Dental device dipping start date _ _ / _ _ / _ _ _ _

Date	Morning Brushing	Morning Dip in slurry of toothpaste (2 min)	Dipping in sugar solution for 2 min					Evening Dip in slurry of toothpaste (2 min)	Evening brushing	MI Paste Plusapplication
			1st time	2nd time	3rd time	4th time	5th time			

Thank you for your cooperation