

**Comparison of the Newer Preventive Therapies on  
Remineralisation of Enamel in Vitro**

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*Dedicated to my Family*

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## ABSTRACT

**Aim of the Research:** To investigate in vitro the effect of Tooth Mousse (10% w/v CPP-ACP), MI Paste Plus (10% w/v CPP-ACP, 900 ppm F) and high Fluoride concentration toothpaste (2800 ppm F) in the remineralisation of demineralised enamel subsurface lesions and to compare these to the effect of 1450 ppm Fluoride toothpaste and non-fluoride (0 ppm F) toothpaste control.

**Materials and Methods:** Enamel subsurface lesions were created in bovine enamel slabs (N=120) which were assigned randomly to five groups; (1) Fluoride-free Toothpaste (control), (2) 1450 ppm F toothpaste, (3) 2800 ppm F toothpaste, (4) 1450 ppm F toothpaste + Tooth Mousse (10% w/v CPP-ACP) and (5) 1450 ppm F toothpaste + MI Paste Plus (10% w/v CPP-ACP, 900 ppm F). The enamel slabs were subjected to a pH cycling regimen for 21 days, with daily exposure to the toothpastes solutions before and after the pH cycling, in 1450 ppm F toothpaste + Tooth Mousse and 1450 ppm F toothpaste + MI paste Plus groups the exposure to the Tooth Mousse or MI paste plus were performed after the last toothpaste exposure. QLF images were taken at the baseline and at the endpoint of the experiment. Data analysis was carried out using one-way analysis of variance (One way ANOVA). Following the completion of the study two enamel slabs were randomly selected from each group to carry out SEM analysis.

**Results:** In all five groups, both  $\Delta F$  (Average fluorescence loss) and  $\Delta Q$  (multiplication of  $\Delta F$  and area) values improved significantly within the same group after the treatment compared with the baseline values. In addition, the mean

difference in  $\Delta F$  of 0 ppm F toothpaste (control) was significantly lower than the mean difference in  $\Delta F$  of the 1450 ppm F toothpaste, 1450 ppm F toothpaste + Tooth Mousse and the 1450 ppm F toothpaste + MI paste Plus groups ( $p < 0.05$ ) but not significantly lower than the 2800 ppm F toothpaste group. Whereas the mean difference in  $\Delta Q$  of 0 ppm F toothpaste group was significantly lower when compared with all other groups ( $p < 0.05$ ). The SEM imaging revealed the presence of amorphous deposits only on the enamel surface of the 1450 ppm F toothpaste + Tooth Mousse and the 1450 ppm F toothpaste + MI paste Plus groups .

**Conclusion:** The remineralisation of enamel subsurface lesions was found in all groups with a statistically significant difference between all the test groups compared with the 0 ppm F control. The use of Tooth Mousse and MI paste Plus in conjunction with 1450 ppm F showed some increased efficacy in the remineralisation of enamel subsurface lesions, however this did not reach a significant level.

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## LIST OF ABBREVIATIONS

%	Percentage
% F	Percentage change in fluorescence
% Q	Percentage change $\Delta Q$
$\Delta F$	Average fluorescence loss
$\Delta Q$	Multiplication of $\Delta F$ and area
$\mu\text{m}$	Micrometre
ACP	Amorphous calcium phosphate
ANOVA	Analysis of Variance
BSPD	British Society of Paediatric Dentistry
Ca	Calcium
CPP	Casein phosphopeptide
CPP-ACFP	Casein phosphopeptide -amorphous calcium fluoride phosphate
CPP-ACP	Casein phosphopeptide-amorphous calcium phosphate
DEJ	Dentine- enamel junction
dmfs	Decayed, Missing and Filled Surfaces (for primary teeth)
e.g.	Example
EAPD	European Academy of Paediatric Dentistry
ESL	Enamel subsurface lesion
F	Fluoride
g	Gram
HA	Hydroxyapatite
ICADS	International Caries Detection and Assessment System
ICC	Intra-class Correlation Coefficient

Kg	Kilogram
L	Litre
mg	Milligram
min	Minutes
MIplus	MI paste plus
ml or mL	Millilitre
mm	Millimetre
Mol	Mole
N or n	Number
Na	Sodium
nm	Nanometre
°C	Degree of Celsius
OR	Odd Ratio
p	p-value
pH	Acidity
px	Pixels
PO <sub>4</sub>	Phosphate
ppm	Part per million
QLF	Quantitative Light-Induced Fluorescence
RCT	Randomised control trials
s	Second
SD	Standard deviation
SE	Standard error
SEM	Scanning Electron Microscopy
Ser(P)	Phosphoserine residue
SFGD	Slow-release fluoride glass devices
Sig	Statistical level
SMH	Surface Microhardness

SPSS	Statistics Package for the Social Sciences
TM	Tooth Mousse
TMR	Transverse Microradiography
vs	Versus
w/v	Weight per volume
w/w	Weight per weight
WSL	White spot lesion
wt	Weight



## **1.0 INTRODUCTION**

A more efficient way of oral health management is now achievable due to better understanding of the pathogenesis of dental caries and its prevention (Cummins, 2006). It has been reported that the prevalence of dental caries has remarkably declined in the last decades (Petersson and Bratthall, 1996). This was attributable primarily to the introduction and the widespread use of fluoride in developed countries, improved oral hygiene, and a greater emphasis by the dental profession on disease prevention and control. Nevertheless, dental caries for most communities remains the most prevalent oral disease and a major public health problem (Reynolds, 2008).

Dental caries can be defined as localised destruction of tooth tissue by micro-organisms. Dental plaque cariogenic bacteria produce organic acids through the fermentation of carbohydrate, and these acids are capable of causing demineralisation of the dental hard tissues. Demineralisation can be reversed in its early stages through uptake of calcium, phosphate, and fluoride through a remineralisation process. The teeth in the oral environment are in balance between continuous processes of demineralisation and remineralisation (Featherstone, 2004); once this balance is disrupted the demineralisation will be predominant, which can cause destruction and cavitation in the tooth structure (Featherstone, 2000). However, if the caries lesion is detected at an early stage it can be reversed using appropriate measures.

Remineralisation of early enamel carious lesions has now become the main aim of preventive dentistry. A shift in emphasis from a restorative approach for

treating dental caries to a therapeutic approach focused on the remineralisation of early caries lesions has taken place during the past decade (Ellwood et al., 2012), and a range of new agents have been developed to provide a non-invasive management of the early caries lesions.

The anti-cariogenic properties of milk and milk products were proven in human and animal models (Reynolds and Johnson, 1981, Rosen et al., 1984) and the chemical effect of phosphoprotein casein and calcium phosphate components were proposed as the main mechanism for this action (Harper et al., 1986, Krobicka et al., 1987). Casein phosphopeptide (CPP) has the ability to stabilise calcium and phosphate in high concentrations at the tooth surface thereby inhibiting demineralisation and enhancing the remineralisation.

Casein phosphopeptide -amorphous calcium phosphate (CPP-ACP) is a new calcium phosphate remineralisation technology that has been shown to have anti-cariogenic activity in laboratory, animal, and human in situ and clinical experiments. The ability of CPP-ACP to enhance the remineralisation of enamel subsurface lesions has been revealed in several studies (Shen et al., 2001, Reynolds et al., 2003, Cai et al., 2007, Manton et al., 2008, Morgan et al., 2008).

The synergistic effect of CPP-ACP and fluoride in caries prevention has been reported in a number of studies (Reynolds et al., 1995, Reynolds, 2008). This effect has been attributed to the formation of CPP-stabilised amorphous calcium fluoride phosphate (CPP-ACFP) (Cross et al., 2004), which results in an increase in the concentrations of fluoride ions together with bioavailable calcium and phosphate ions and their localisation at the tooth surface by the CPP.

Most of the studies that investigated the effect of CPP-ACP tested its solitary effect on caries prevention and early lesion remineralisation. On the other hand, only a few studies investigated its effect when added to the regular oral hygiene practice that involves brushing the teeth with fluoridated toothpaste twice daily.

Therefore the aim of the current study was to investigate the effect of the commercially available topical crèmes containing CPP-ACP and CPP-ACFP on the remineralisation of enamel subsurface lesions when used supplementary to fluoridated toothpaste, and to compare their effect on remineralisation to the results from the use of different concentration fluoridated toothpastes.

## 2.0 LITERATURE REVIEW

In the following section, the research literature concerning the dental caries process, along with the process of demineralisation and remineralisation will be reviewed. In addition, the role of therapeutic agents that are currently being used in the prevention and remineralisation of dental caries will also be discussed; with the main focus on fluoride and CPP-ACP preparations.

### 2.1 Dental Caries

Dental caries is defined as a localised destruction of the susceptible dental hard tissue that is caused by the bacterial acidic by-products resulting from the fermentation of dietary carbohydrate (Fejerskov et al., 2003).

Dental caries is an infectious disease initiated by the bacterial biofilm formed on the tooth surface. Bacterial biofilm is composed mainly of *Mutans Streptococci* and *Lactobacillus* which have the ability to metabolise fermentable carbohydrates to produce acidic by-products that result in a drop of the pH in the biofilm. These changes in the pH can cause a disturbance in the equilibrium between tooth substance and the surrounding plaque fluid, leading to chemical dissolution and loss of minerals from tooth surface (Featherstone, 2000).

Dental caries is a dynamic process where multiple factors play a part in the eventual outcome of the disease. This includes the pathological factors that lead to demineralisation, such as the high numbers of cariogenic bacteria, insufficient

fluoride exposure, poor oral hygiene, frequent consumption of refined carbohydrates and the inhibition of salivary function.

On the other hand, the protective factors that lead to remineralisation include good salivary flow, different salivary components such as calcium, phosphate, fluoride and protective proteins, antibacterial substances present in saliva, fluoride from extrinsic sources, and selected dietary components (Featherstone, 2004).

Dental caries is one of the most common of all human diseases. A significant shift in the epidemiology of dental caries has been noticed in many countries; this includes the distribution of dental caries, which has become profoundly skewed to a small percentage of the population (Tickle, 2002).

Before the introduction of fluoride to the market, carious lesions used to progress very fast due to the rapid progression of demineralisation (White et al., 1988, Chan, 1993). This led to straightforward detection of the large carious lesions and the tendency among dentists to restore rather than monitor suspicious or questionable lesions. However, the trend was reversed after the availability of fluoride, where caries progression slowed down, and smaller lesions became arrested and sometimes even reversed (White et al., 1978, Dodds, 1993, Stookey, 2005).

### **2.1.1 Enamel caries**

Dental enamel is a highly mineralised tissue; more than 95% of the mature dental enamel is composed of apatitic crystals. This high mineral content along with the organised structure of enamel makes it the hardest substance in the human body (Margolis et al., 2006).

Enamel is composed primarily of uniformly wide, well-oriented crystals of hydroxyapatite, packed into an organic matrix. The changes in the crystals orientation has a repetitive pattern that results in the formation of the prisms (rods) and interprismatic regions (Boyde, 1967). The interprismatic spaces (pores) are filled with water and organic material and form a fine network system of diffusion in enamel (Fejerskov et al., 2003).

The process of dental caries starts when the acidogenic bacteria in the biofilm ferment the dietary carbohydrates producing organic acids such as lactic, formic and acetic acids as a by-product. These acids have the ability to diffuse into the enamel pores between the rods where they dissociate and cause pH values of the fluid surrounding the crystals to fall below a critical value for enamel dissolution (pH=5.5) (Aoba, 2004). This drop in the pH causes dissolution of the mineral crystals especially of the carbonated hydroxyapatite (LeGeros, 1991). As a result, minerals including calcium and phosphate diffuse out of the enamel causing a net loss of enamel minerals. If this process continues cavitation will eventually take place.

### **2.1.2 Demineralisation and Remineralisation**

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).

Remineralisation on the other hand 'is the net gain of calcified material within the tooth structure, replacing that which was previously lost through demineralisation' (Fontana et al., 2010).

As discussed before, the demineralisation process resulted from the acid by-products of the acidogenic bacteria in the biofilm. As these acids diffuse into the tooth structure among the crystals, they dissolve the calcium and phosphate of the susceptible sites on the crystals into the surrounding fluid between the crystals (Featherstone, 2008).

Remineralisation is the body's natural repair mechanism for the subsurface non-cavitated carious lesions. During the remineralisation process, the calcium and phosphate, primarily from saliva, as well as from other topical sources, diffuse back into the porous subsurface region of the caries lesion (Featherstone, 2000).

The remineralisation process involves building a new surface onto the existing crystal remnants, rather than the formation of new crystal. If this happens with the presence of fluoride, the new crystal will be composed of a mineral similar to fluorapatite, resulting in a crystal surface that is much less soluble than the original carbonated hydroxyapatite mineral, and more difficult to dissolve by acid challenge from the plaque (Featherstone, 2008).

All through the day, and as part of a natural physiological process, the tooth surface is in a continuous state of demineralisation and remineralisation. Whether the caries process starts, progresses, stops, or reverses is determined by multiple factors. Caries will progress if the demineralisation predominates over remineralisation, such as in the case of acidic challenge. On the other hand, when

remineralisation takes over demineralisation, the lesion can be arrested or even reversed (Featherstone, 2004, Ellwood et al., 2012).

### **2.1.3 The Role of Saliva**

The protective role of saliva in the reversal or arrestment of the caries process is well established (Mandel and Zengo, 1973, Shannon and Edmonds, 1972).

Four main mechanisms of saliva have been described for its role in caries prevention; this includes providing a mechanical cleansing that results in less plaque accumulation on the tooth surface, the reduction of enamel solubility through the supersaturating state with regards to calcium, phosphate and fluoride, and the salivary buffering capacity that neutralises the acids produced by cariogenic bacteria or those introduced directly through diet, as well as its antibacterial activities (Mandel, 1974).

The salivary calcium and phosphate is maintained in a supersaturated state by the action of salivary proteins, in particular statherin, which binds the calcium ions and holds it readily available in solution (Lamkin and Oppenheim, 1993). Conditions that result in the impairment of the salivary flow or function can develop into rampant caries due to the insufficient calcium and phosphate that is available for remineralisation (Featherstone, 2008).

### **2.1.4 Enamel Lesion (White Spot Lesion; WSL)**

As discussed before, dental caries is now perceived as a dynamic process which is affected by several modifiers tending to push the mineral equilibrium in



one direction or another (Holt, 2001). If caries balance is tilted continually toward demineralisation, the enamel will lose enough mineral and a white spot lesion (WSL) will develop.

Histologically, four zones have been distinguished under polarised light in enamel lesions. These zones represent different stages of enamel demineralisation and remineralisation: (Darling, 1958)

- 1- The translucent zone: this represents the 'leading edge' of the enamel lesion with mineral loss of approximately 1.2% (Hallsworth et al., 1972). Its width may vary from 5-100  $\mu\text{m}$  and it is located at the deepest part of the lesion. Gaps found between rod and inter-rod enamel provide the initial source of mineral dissolution resulting in translucent appearance of this zone and in enamel structures being less evident. Analysis of material lost from this zone shows a more soluble mineral phase and the absence of calcium hydroxyapatite.
- 2- The dark zone: this represents the zone of 5–10% mineral loss (Hallsworth et al., 1972). It occurs in 90-95% of the lesions and is located between the translucent zone and the body of the lesion. Material removed from the dark zone was found to be closer to apatite than that in the translucent zone and contained carbonate and magnesium (Robinson et al., 1983).
- 3- The body of the lesion: representing an area with 20–30% of mineral loss (Hallsworth et al., 1972) and increased in the pore volume depth, it is the

area in the developing enamel lesion that ultimately loses structural integrity and precipitates surface breakdown.

The analysis of the minerals lost from this zone revealed less soluble materials that were closely related to hydroxyapatite (Robinson et al., 1983).

- 4- The surface zone: this area has a minimal loss of 1–10% in minerals (Hallsworth et al., 1972). In addition, increased pore volume is also observed in the surface zone. A great variation was found in the mineral distribution within the enamel lesion with a very thick surface zones are frequently found. The high mineral density of this zone may have resulted from the lattice ions precipitation from the oral environment.

The intact surface zone of the caries lesion has been described by Ingram and Edgar, (1994). They stated that the existing evidence indicates that during the caries process, the surface zone reforms, exhibiting larger crystallites compared with sound enamel due to the incorporation of fluoride. This demonstrates the beneficial involvement of fluoride in the process of lesion remineralisation, even at low levels.

The ‘healed’ surface zone may prevent the diffusion of the minerals into the deeper demineralised enamel crystals therefore preventing the remineralisation. Consequently, in order to be able to remineralise these deeper lesions and promote subsurface healing, alternatives to direct fluoride treatment may be required as a remineralising protocols (Meharry et al., 2012).

Aiming to bypass this ‘healed’ layer in an attempt to remineralise the subsurface zone of the lesion, two different approaches can be implemented: the

application of low levels of fluoride and calcium ions, which can penetrate deep into the subsurface lesion (Bishara and Ostby, 2008), or applying mechanical and chemical abrasion in order to reactivate the superficial enamel substrate (Ardus et al., 2007).

## **2.2 Enamel Remineralisation Therapies**

Remineralisation is an important natural process in the dynamics of dental caries. The possibility of the remineralisation of natural enamel lesions (white spot lesions), was first described by Backer Dirks in 1966. Subsequently, a great number of in situ and in vitro investigations were conducted in the following decades and showed that remineralisation occurs in newly formed artificial enamel lesions (Mellberg et al., 1986, Dijkman et al., 1990, Manning and Edgar, 1992), as well as in lesions formed under plaque in situ (Dijkman et al., 1986) and in vivo (Øgaard et al., 1988). With regard to the baseline mineral loss, it has been shown that the remineralisation of newly formed white spot lesions and in vitro formed lesions take place in the same way (Iijima et al., 1999).

With the better understanding of the caries disease, and the ability to detect enamel demineralisation in its early stage, comes the opportunity to promote ‘preventative’ therapies that can be used to facilitate the early caries lesions remineralisation. Subsequently, these therapies can reverse the early lesions leading to the preservation of tooth structure, function and aesthetics (Pretty, 2006).

Currently a range of therapeutic procedures and a variety of new agents have been developed to conservatively manage early caries lesions. These comprise of the

intensive use of topical fluoride treatments (dentifrices, varnishes, or gels), several preparations of calcium and phosphate, improved oral hygiene, and reducing the number of daily exposures to fermentable carbohydrates (Stokey, 2005).

### **2.2.1 Fluoride**

Fluoride is widely accepted as the most effective tool for caries prevention. The introduction of fluoridated toothpastes has a major role in the dental caries decline seen in developed countries over the last decades (Twetman, 2009). Current best practice and the evidence-based toolkit for prevention recommends twice-daily toothbrushing with fluoride toothpaste for dentate children (Zero et al., 2012, Department of health prevention toolkit, 2014).

Many studies have demonstrated the profound effect of fluoride on enamel demineralisation and remineralisation that resulted from regular use of toothpaste, even with low levels of fluoride (Bowen, 1995, Lynch et al., 2004).

#### **2.2.1.1 Fluoride Mechanism of Action**

Fluoride was proved to have both systemic and topical effects. The systemic effect of fluoride takes place during tooth development and involves the replacement of hydroxyapatite crystals with fluoroapatite crystals, which are characterised by being more stable and less likely to dissolve with acids. It is widely believed that the topical effect of fluoride rather than systemic delivery is more effective in caries reduction (Fejerskov et al., 1996).

The main mechanisms of fluoride action are the inhibition of demineralisation and promotion of remineralisation of tooth enamel, as the presence of fluoride ions during the demineralisation and remineralisation cycle leads to its incorporation into the crystalline structure of the carbonated hydroxyapatite (HA). However, the action of fluoride in enamel remineralisation is limited by the bio-availability of calcium and phosphate ions in saliva (ten Cate, 1999, Aoba, 2004, Edgar et al., 2004, Featherstone, 2006, Reynolds, 2008).

Fluoride ions in plaque immediately promote the formation of fluorapatite in the presence of the calcium and phosphate ions produced during demineralisation of tooth enamel by plaque bacterial organic acids. This is now believed to be the major mechanism of fluoride action in preventing enamel demineralisation (ten Cate, 1999, Reynolds, 2008).

Fluoride ions can also promote the remineralisation of previously demineralised enamel if salivary or plaque calcium and phosphate ions are available in adequate amounts when the fluoride is applied (ten Cate, 1999), for every two fluoride ions, ten calcium ions and six phosphate ions are required to form one unit cell of fluorapatite  $[\text{Ca}_{10}(\text{PO}_4)_6\text{F}_2]$  (Reynolds, 2009).

Other effects of fluoride include the inhibition of glycolysis, and reduce the production of extra-cellular polysaccharides in plaque bacteria. In addition, fluoride is a bactericidal at high concentrations, and has an effect on the cariogenic potential of the *Streptococcus mutans*.

### **2.2.1.2 Fluoride in Saliva**

The presence of the fluoride in the liquid phase at the enamel-plaque interface and its activity in the oral fluid are the most important factors in reducing enamel solubility. Several studies revealed that fluoride levels as low as 0.01-0.10 ppm were effective in the prevention of the enamel dissolution (Manly and Harrington, 1959, Larsen, 1990).

The fluoride levels in saliva and caries status has been investigated in many studies. A number of these studies showed a clear link between elevated salivary fluoride levels and low caries status (Bruun and Thylstrup, 1984, Leverett et al., 1987). In one study to evaluate the salivary fluoride level in children living in fluoridated and non-fluoridated communities, Shields et al. (1987), reported that the salivary fluoride levels in low caries children were ( $>0.04$  ppm), whereas the salivary fluoride levels in children with high caries-risk were ( $<0.02$  ppm).

### **2.2.1.3 Fluoride Delivery Systems**

Water fluoridation was the first breakthrough in preventive dentistry, which was then followed by different forms of topical fluoride applications (toothpaste, mouth rinse and professionally applied fluoride) (Marthaler and Petersen, 2005).

The effect of water fluoridation in dental caries was evaluated in several studies, and was shown to reduce the dental caries experience both in primary and permanent dentition by about 50% (Murray et al., 1991, Booth et al., 1992).

Moreover, fluoride-containing dentifrices and mouth rinses have proven to decrease caries experience significantly in several randomised, controlled clinical

trials (Biesbrock et al., 2001, Marinho et al., 2003). The dose response effect of different fluoride levels in toothpastes was also established in different clinical trials (Chesters et al., 1992, Biesbrock et al., 2003), and in a recent Cochrane review published by Walsh et al. (2010), who indicated that the caries preventive effect of fluoride toothpaste significantly increases with higher fluoride concentrations. And that the 1000 ppm F is the minimal fluoride concentration with a cariostatic activity.

Literature indicated that 1450 ppm F toothpastes display anti-caries efficacy (Walsh et al., 2010, Biesbrock et al., 2003, Chesters et al., 1992, Marinho et al., 2003). This concentration is being used widely in adult toothpastes in Europe. The current EAPD as well as the BSPD guidelines recommended the use of 1450 ppm F toothpaste, and the evidence-based toolkit for prevention also recommended the use fluoridated toothpaste (1,350-1,500 ppm fluoride) for individuals over 6 years of age (Department of health prevention toolkit, 2014).

Other preparations with higher fluoride concentrations of 2800 ppm F and 5000 ppm F are also available. It has been shown that 2800 ppm F toothpaste provides clinically-proven anti-caries benefits (Biesbrock et al., 2001). These preparations are usually given on prescription and target the groups with a high risk of caries.

The anti-caries effect of fluoride toothpaste is affected by two factors; the concentration of fluoride in the toothpaste, and the post-brushing water-rinsing behaviour.

A correlation has been found between the fluoride concentration of dentifrices ranging between 0 and 5000 ppm F and caries prevention (Tavss et al., 2003). A randomised clinical trial aimed to compare two toothpastes with different fluoride

concentrations, mainly 5000 and 1450 ppm F reported a reversal in non-cavitated fissure caries lesions with the high fluoride concentration toothpaste. In addition to that, the group using 5000 ppm F showed a significantly higher decrease in laser fluorescence of enamel than the 1450 ppm F group (Schirrmeyer et al., 2007a).

A correlation has also been found between the rinsing method after tooth brushing with caries experience and caries increment (Pitts et al., 2012). Salivary fluoride concentration measured after dentifrice application decreases significantly with increasing water volume, rinse duration and frequency of rinsing (Duckworth et al., 1991, Attin and Hellwig, 1995). Eating immediately after brushing was also found to result in a reduction in the salivary fluoride level about 12–15 fold (Sjögren and Birkhed, 1994). An average of 26% reduction in the approximal caries in preschool children was seen when a slurry rinse with the toothpaste was carried out after brushing (Sjögren et al., 1995). The fluoride concentration in proximal saliva and plaque was increased after using a dentifrice with 5000 ppm F without post-brushing water rinsing compared to a with-rinsing regimen (Nordström and Birkhed, 2009).

#### **2.2.1.4 Fluoride Limitation**

As mentioned earlier, fluoride has a strong effect on caries prevalence worldwide. However, fluoride does not provide a complete cure.

Fluoride action is limited by the bio-availability of calcium and phosphate ions. The salivary reservoirs of calcium and phosphate are rapidly depleted under acid challenges leading to a net loss of enamel minerals. Therefore, the use of a delivery system for bio-available calcium and phosphate ions is an important adjunct



to fluoride treatment for the non-invasive management of early caries lesions (Reynolds, 2009, Yamazaki et al., 2007).

#### **2.2.1.5 Fluoride Toxicity and Dental Fluorosis**

Fluoride can be toxic if used in high doses; the fluoride toxicity can be acute or chronic depending on the dose.

Acute fluoride toxicity can result if the toxic dose was ingested, however, the exact toxic dose is not known. Lidbeck et al. (1943), stated that the toxic dose was over 100 mg F/kg, while Dreisbach, (1980) estimated that it was 6-9 mg F/kg.

Signs and symptoms of acute fluoride toxicity include nausea, vomiting, hyper-salivation, abdominal pain and diarrhoea in case of ingestion of low dosage of fluoride, while convulsions, cardiac arrhythmias and comatose can result from high doses.

Chronic fluoride toxicity can result from frequent exposure to low levels of fluoride over a prolonged period of time. Depending on the ingested dose it can be expressed as dental fluorosis, skeletal fluorosis and kidney damage.

Overexposure to fluoride, especially in young children can cause fluorosis. Dental fluorosis is an alteration in the tooth structure which results from excessive fluoride ingestion during the pre-eruptive development of teeth. It has shown to have a dose response effect and it results in increased surface and subsurface porosity.

The severity of dental fluorosis was found to be associated with a number of factors namely; the dose of fluoride, timing of fluoride exposure and duration of

exposure (Dean et al., 1950). Teeth are more susceptible to fluorosis during their transitional or early maturation stage of enamel development.

Several mechanisms have been suggested in the literature to explain the development of dental fluorosis among teeth, yet the exact mechanism is still unknown. The available evidence indicates that fluoride has an effect on cell function; either directly through interactions with the developing ameloblasts or more indirectly by interacting with the extracellular matrix therefore preventing the full maturation of the tissue (DenBesten and Crenshaw, 1984, Smith et al., 1993).

Wong et al. (2011), in their Cochrane review concluded that “there should be a balanced consideration between the benefits of topical fluorides in caries prevention and the risk of the development of fluorosis”.

### **2.2.2 Milk Products and Enamel Remineralisation**

The beneficial dental effects of milk have been known for many years. This effect was attributed to the presence of calcium and phosphate in high concentrations in milk that help to prevent dilution of enamel (Birkhed et al., 1993, Bowen and Pearson, 1993) along with casein, a multi-phosphorylated protein, which stabilises milk calcium and phosphate ions in a colloidal state in micelles (Moynihan, 2000, Reynolds, 1987). As the majority of the casein and calcium and phosphate ions are bound in micelles, the calcium and phosphate may not be readily available to the tooth surface upon milk consumption (Reynolds, 1987, Holt et al., 1996). In addition to that, the diffusion of the calcium in casein micelles into the subsurface lesion is usually unlikely to occur due to the presence of a relatively intact enamel surface layer, therefore limiting the ability to remineralise enamel lesion in situ (Ferrazzano et al., 2011). In spite of this limitation and the presence of the fermentable disaccharide lactose, the anti-cariogenic activity of milk is still demonstrated when tested in a rat model, even in de-salivated rats (Reynolds and Johnson, 1981, Bowen et al., 1991), and it remineralised enamel subsurface lesions in a human in situ model (Walker et al., 2006). Other milk products like cheeses did not cause pH drops below the critical pH for enamel dissolution when tested using an in situ model (Drummond et al., 2002).

### **2.2.3 Casein Phosphopeptide - Amorphous Calcium Phosphate (CPP-ACP)**

The low solubility of calcium and phosphate ions, especially in the presence of fluoride ions, made it unsuccessful for use in remineralisation in the past. It was found to be difficult to apply and was not effectively localised at the tooth surface. An acid is required to produce the ions which are then able to diffuse into the enamel subsurface lesion (Reynolds, 2009). In addition, only very low concentrations of soluble calcium and phosphate can be used due to the intrinsic insolubility of calcium phosphates, particularly calcium fluorophosphates. Moreover, these soluble calcium and phosphate ions neither incorporate into dental plaque, nor localise at the tooth surface in order to produce effective concentration gradients to drive diffusion into the subsurface enamel (Reynolds, 2009).

Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) is a new calcium phosphate remineralisation technology which has been developed, and postulated that CPP stabilises calcium and phosphate in high concentrations in addition to fluoride ions, by binding to the pellicle and plaque at the tooth surface. This enables the calcium and phosphate ions to be freely bio-available to diffuse into the enamel subsurface lesion by the concentration gradients, and effectively promoting remineralisation in vivo (Reynolds, 2009).

Casein is the major milk protein, and accounts for almost 80% of its total protein, it presents primarily as calcium phosphate stabilised micellular complexes, and is responsible for the anti-cariogenic properties of milk (Aimutis, 2004, Azarpazhooh and Limeback, 2008, Bánóczy et al., 2009). Casein phosphopeptides (CPP) contain the cluster sequence -Ser(P)-Ser(P)-Ser(P)-Glu-Glu- that have a

remarkable ability to stabilise amorphous calcium phosphate (ACP) in a metastable solution. Through the multiple phosphoserine residues, CPP binds to form nanoclusters of ACP, preventing their growth to the critical size required for nucleation and phase transformation (Reynolds, 1998, Shen et al., 2001).

The role of CPP-ACP involves the localisation of ACP on the tooth surface; this in turn leads to buffering of the free calcium and phosphate ions activities, which help to maintain a state of super-saturation with respect to enamel by suppressing demineralisation and enhancing remineralisation. The presence of CPP-ACP might permit a rapid return to resting calcium concentration and allow earlier remineralisation of enamel substrate (Reynolds, 1998).

Other roles of CPPs include binding to the *S. mutans* surface, directly and strongly, which provides a source of calcium and phosphate in the plaque fluid and reduce the loss of plaque calcium by inhibiting its diffusion. In addition to that, CPP also reduced the adherence of *S. mutans* and *S. sobrinus* within dental plaque and onto hydroxyapatite, causing a significant reduction in the proportion of the *mutans streptococci* in rat and in vitro studies (Neeser et al., 1994, Schüpbach et al., 1996, Guggenheim et al., 1999, Wernersson et al., 2006).

Van der Veen et al. (2009), reported a more than 60% reduction in the plaque *S. mutans* proportion after 12 weeks exposure to a commercially available crème containing CPP-ACP and fluoride compared to a similar crème without CPP-ACP.

The anticariogenic mechanism for CPP-ACP is believed to be multifactorial through the following mode of action (Beerens et al., 2010):

- (i) Promotion of enamel lesion remineralisation by maintaining a supersaturated state of the enamel minerals calcium and phosphate in plaque.
- (ii) Delay the formation of biofilm and inhibit bacterial adhesion to the tooth surface.
- (iii) Act as a buffering agent, which may prevent the reduction of pH in the oral micro-environment.

A commercially available topical crème; Tooth mousse™ (TM; GC Crop, Tokyo, Japan) is designed to deliver CPP-ACP via a stable and persistent carrier agent; it is provided by the manufacturer as shown in Table 2-1. MI Paste Plus™ (GC MI Paste Plus; GC Crop, Tokyo, Japan) is a commercially available product that in addition to CPP-ACP contains 900 ppm fluoride.

**Table 2-1: Tooth Mousse constitution.**

water	silica dioxide	propyl p-hydroxybenzoate
glycerol	titanium dioxide	magnesium oxide
butyl b-hydroxybenzoate	zinc oxide	guar gum
CPP-ACP (10% w/v)	xylitol	Sodium saccharin, ethyl-p-hydroxybenzoate
D-sorbitol	phosphoric acid	
sodium carboxymethylcellulose	flavouring	

Azarpazhooh and Limeback, (2008) conducted a systematic review on the efficacy of casein derivatives in clinical dentistry. The outcomes of interest of the review were caries prevention (10 studies), relief from dry mouth symptoms (one study) and treating dentin hypersensitivity (one study).

Regarding the outcome 'caries prevention' the review identified 10 studies of caries prevention via treatment with casein derivatives. Eight of these studies were randomised clinical trials with crossover designs that determined the remineralising effects of CPP-ACP by using in situ caries models. The other two studies were in vivo studies. The analysis of the studies showed that seven trials favoured CPP-ACP in comparison to control, two studies found no additional benefit and one study had contradictory findings. The authors of the review concluded that 'there is insufficient clinical trial evidence (in quantity, quality or both) to make a recommendation regarding the long-term effectiveness of casein derivatives, specifically CPP-ACP, in preventing caries in vivo'.

A more recent meta-analysis study carried out by Yengopal and Mickenautsch, (2009) to investigate whether CPP-ACP has any superior caries-preventive benefit over any other intervention or placebo.

Five in situ randomised control trials (RCT) have been pooled for the meta-analyses. These included in situ trials where participants wore appliances containing enamel slabs for short-term (7-21 days) and the enamel slabs were then analysed in the laboratory after exposure to CPP-ACP.

Exposure to CPP-ACP was found to improve the remineralisation of carious lesions significantly when compared to the control group.

The authors' conclusion indicates a short-term remineralisation effect of CPP-ACP supported by the results of the in situ clinical trials, and in vivo RCT results suggest a caries preventive effect for long-term clinical CPP-ACP use.

### **2.2.3.1 CPP-ACP Effect on Demineralisation**

CPP-ACP binds readily to both the tooth surface and the bacteria in the plaque surrounding it, depositing a high concentration of ACP in close proximity of the tooth surface. This buffers free calcium and phosphate ions that increase the calcium phosphate level in the plaque. A state of super-saturation can be maintained in this way, which results in the inhibition of enamel demineralisation whilst enhancing remineralisation (Reynolds et al., 1995, Reynolds et al., 1999).

The CPP-ACP efficacy in the prevention of demineralisation has been widely investigated in both in vitro and in situ studies. Rahiotis and Vougiouklakis, (2007) investigated the effect of CPP-ACP complex on the demineralisation of sound human dentine in an in vitro study. Dentine in test groups received Tooth Mousse, whereas no Tooth Mousse was provided to the control group. The dentine slabs were then immersed in a demineralisation solution. It was found that single application of Tooth Mousse reduced demineralisation of dentine significantly (% demineralisation= $19.6 \pm 7.8$ ) when results were compared to the control group (% demineralisation= $28.1 \pm 7.0$ ).

In a human in situ enamel demineralisation study, a 2.0% w/w sodium casinate solution used twice daily produced a  $51 \pm 19\%$  reduction in enamel mineral loss caused by frequent sugar-solution exposure. In addition, an increase in the levels of calcium and inorganic phosphate (144%, 160% respectively) in the inter-enamel



plaque were reported when 2.0% casine solution was used twice daily (Reynolds, 1987).

Another study used ultrasonic wave velocity to determine the effect of CPP-ACP, and showed no significant increase in sonic velocity observed in specimens treated with CPP-ACP before exposure to acid challenge. This suggests the preventive role of CPP-ACP in enamel demineralisation (Yamaguchi et al., 2007).

Ferrazzano et al. (2007), used the weight loss of the samples to determine demineralisation in their study. They showed that the addition of 1.0% w/v of CPP to a demineralising fluid resulted in more than 50% less mineral loss from the enamel that was exposed to a demineralising gel containing CPP compared to that exposed to gel without CPP.

Another study carried out by Giulio et al. (2009), was conducted to qualitatively evaluate by means of scanning electron microscopy (SEM) the effect of CPP-ACP on striped enamel morphology after exposure to an acid solution. The teeth were placed in acidic lactic solution for two hours three times a day for eight days, with two hours water preservation in between. The SEM micrographs showed that the stripped enamel samples exhibit a greater demineralisation compared to unstripped enamel, and CPP-ACP reduced enamel dissolution on both intact and abraded samples.

It has been reported that enamel subsurface lesions which were remineralised by CPP-ACP were more resistant to demineralisation. Iijima et al. (2004), conducted an in situ study to investigate the acid resistance of enamel lesions remineralised using a sugar-free chewing gum containing CPP-ACP. In this study subjects were required to chew either sugar-free gum containing 18.8 mg of CPP-ACP or sugar-free gum without CPP-ACP. After that, the enamel slabs were exposed to acid

challenge in vitro for eight or 16 hours. The results showed the CPP-ACP containing gum produced approximately twice the level of remineralisation as compared with the control sugar-free gum ( $17.88 \pm 0.74$  vs.  $9.02 \pm 0.74$  respectively). The eight and 16 hour acid challenge of the lesions remineralised with the CPP-ACP group led to 30.5% and 41.8% reductions in the deposited minerals respectively, compared to 65.4% and 88.0% reductions in the control group. This means sugar-free gum containing CPP-ACP remineralises enamel subsurface lesions with minerals that are more resistant to acid challenges. These observations are similar to those of Cai et al. (2003), who investigated the effects of sucking sugar-free lozenges containing different concentrations of CPP-ACP in a similar in situ model. A dose response in relation to the amount of CPP-ACP in the lozenges was observed, with percentage remineralisation (% R) values of  $7.03 \pm 0.65$  for the control lozenges and  $12.50 \pm 1.48$  and  $19.39 \pm 1.69$  for the lozenges containing 18.8mg and 56.4mg CPP-ACP, respectively.

The effects of a CPP-ACP and fluoride containing topical agents in reducing enamel demineralisation around orthodontic brackets, was evaluated in an in vivo and in vitro study (Uysal et al., 2010). In the in vivo study, 21 orthodontic patients scheduled to have first premolars teeth extracted for orthodontic reasons were involved and brackets were bonded on the maxillary and mandibular first premolars. The study subjects were randomly assigned into three groups: Tooth Mousse (CPP-ACP containing topical gel), Fluoridin N5 (sodium fluoride containing topical gel) and a control group. Following 60 days, the brackets were removed and teeth were extracted to evaluate the in vivo effects of the testing materials. Cross sectional microhardness was used to assess demineralisation in enamel around the brackets.

The in vitro study consisted of 60 extracted premolars which were divided into the same three groups. Whilst teeth from the control group were immersed in demineralisation and remineralisation solutions without exposure to any topical solution, teeth from the CPP-ACP and sodium fluoride topical gel groups were treated with a 0.5 mL coat of predetermined topical solution before immersion in cycling solutions. Samples were cycled for 14 days.

The results of both studies showed that the use of CPP-ACP and fluoride containing topical gels were more significantly efficient on preventing enamel demineralisation than the control group ( $p < 0.001$ ). No significant differences were detected between CPP-ACP and the fluoride groups against demineralisation.

Another in vitro study was carried out to investigate the effect of Tooth Mousse on the progression of enamel subsurface lesions around cemented orthodontic brackets. The results indicated the preventive effect of Tooth Mousse in reducing demineralisation when the brackets were cemented with a resin based cement, and especially when Tooth Mousse was applied with 9000 ppm sodium fluoride gel in a 50/50 w/w mix, as it has a synergistic effect resulting in minimal demineralisation (Sudjalim et al., 2007).

### **2.2.3.2 CPP-ACP Effect in Remineralisation**

Although the ability of CPP-ACP in promoting remineralisation in animals was demonstrated in 1995, it was first used for the treatment of white spots lesions in 2009.

Reynolds et al. (2003), showed that CPP-ACP technology was superior to other forms of calcium phosphate in remineralising enamel subsurface lesions. Other

studies also highlighted the role of CPP-ACP in stabilising calcium and phosphate at high levels, and in delivering the ions to the tooth surface. They also demonstrated that CPP-ACP was able to bind to bacterial cells. It was shown that the CPP-ACP bound to *Streptococcus mutans* and model plaque makes a reservoir of bio-available calcium ions (Rose, 2000, Reynolds et al., 2003).

#### ***2.2.3.2.1 In Vitro Remineralisation Studies***

In an in vitro study, a solution of 1.0% w/v of CPP-ACP was tested for its effect on enamel subsurface lesion remineralisation, the results showed that this solution replaced  $63.9 \pm 20.1\%$  of mineral lost from an enamel subsurface lesion after 10 days exposure. A dose response was also evident in this study as 0.5% CPP-ACP replaced  $51.6 \pm 20.8\%$  of minerals compared with  $43.6 \pm 18.9\%$  replacement by 0.1% CPP-ACP solution (Reynolds, 1997).

Another study reported a total mineral replacement (% R) of  $15.0 \pm 3.5\%$  when 2% CPP-ACP solution was used at pH of 7.0. It was also found that the value of mineral replacement increased to  $41.8 \pm 8.5\%$  when pH dropped to 5.5. On the other hand, when the pH fell below 5.5, the % R values also decreased, reaching  $10.3 \pm 3.3$  at pH 4.5. A significant increase in the % R up to  $57.7 \pm 8.4\%$  at pH 5.5 was reported when fluoride was incorporated into the CPP-ACP complex leading to the formation of CPP-ACFP complexes (Cochrane et al., 2008).

The CPP-ACP effect on dentine remineralisation was quantified by surface analysis in vitro using micro MIR-FTIR. Remineralisation of  $7.15 \pm 3.0\%$  was obtained when the dentine surface was exposed to Tooth Mousse. However, only  $4.1 \pm 1.5\%$  of remineralisation was obtained in the control group. The authors suggested

that the direct physical barrier provided by the CPP-ACP to demineralisation in conjunction with an increased concentration of calcium at the surface inhibiting mineral loss was the main mechanism for the increased remineralisation in the Tooth Mousse group (Rahiotis and Vougiouklakis, 2007).

Polarised light microscopy and pH cycling were used in an in vitro study to quantify enamel surface lesions. In this study, Tooth Mousse was used as a topical coating and as toothpaste, leading to a significantly increased level of mineralisation of the enamel surface lesions when compared with the control specimens. Further increases in the remineralisation of enamel surface lesions (approximately 30%) were also noted after combining the Tooth Mousse with fluoridated toothpaste (Kumar et al., 2008).

A study using sonic velocity to quantify bovine enamel mineralisation reported that using a 1:10 dilution of Tooth Mousse enhances the mineralisation of enamel under acidic conditions (0.1 M buffered lactic acid) for 10 minutes twice a day for 10 days (Yamaguchi et al., 2007).

In a recent study using DIAGNOdent™ (Jayarajan et al., 2011), both Tooth Mousse and Tooth Mousse Plus showed statistically significant enamel remineralisation compared with the control group (artificial saliva), the maximum remineralisation was obtained by the Tooth Mousse Plus group.

The effects of the CPP-ACP on the remineralisation of artificial early enamel lesions of the primary teeth were assessed in vitro study. Enamel specimens with artificial early lesions were randomly divided into three groups; distilled and deionised water as negative control, 500 ppm NaF solution as positive control, and the CPP-ACP crème as the test group. The specimens underwent the

remineralisation process twice a day for 30 days. The enamel surface microhardness (SMH) was measured before and after demineralisation, as well as 30 days after remineralisation. The SMH of the eroded enamel in the CPP-ACP crème group had increased significantly compared to that in the 500 ppm NaF solution group (Zhang et al., 2011).

#### ***2.2.3.2.2 In Situ Remineralisation Studies***

Reynolds et al. (2003), carried out an in situ study to test the effect of chewing gum containing CPP-ACP four times per day for 14 days on enamel remineraliation. They found that proportional remineralisation (% R) was  $19.0 \pm 2.5\%$  for the CPP-ACP chewing gum, whilst chewing gum with added  $\text{CaCO}_3$  produced  $8.9 \pm 1.4\%$  remineralisation. The % R values for chewing a gum containing CCP-ACP, for seven times per day for seven days were  $19.4 \pm 1.0\%$  compared with  $6.3 \pm 1.2\%$ , resulted from chewing gum containing  $\text{CaCO}_3$ . Indicating that the remineralisation capability of CPP-ACP was significantly higher compared with the chewing gums of un-stabilised calcium.

Increased enamel subsurface remineralisation up to 102% and 152% were reported after chewing gum containing 18.8 mg and 56.4 mg of CPP-ACP, respectively for 20 minutes four times per day for 14 days compared with control sugar-free gum (Cai et al., 2007). The microradiographic analysis showed that the remineralisation occurred throughout the body of the lesion. The study author reported that the remineralised apatite was more resistant to acid challenge than the normal calcium-deficient carbonated tooth enamel.

In an in situ study by Schirrmeyer et al. (2007b), a mandibular appliance was used with bovine enamel slabs placed in the buccal sulcus. Different chewing gums were used, one of them containing casein/hydrolysed casein and calcium phosphate (Phoscal™), incorrectly described as CPP-ACP, and subjects were asked to chew them four times per day for 14 days. In contrast with previous studies, no significant difference was found in remineralisation potential between the different gums, and no significant greater % R was reported after chewing gum compared to the control in this study.

Itthagarun et al. (2005), also used the in situ model with a mandibular appliance to investigate the effect of gums containing urea alone, urea with dicalcium phosphate, and urea with casein/hydrolysed casein and calcium phosphate (Phoscal™). The study subjects chewed the gums five times-per-day for 21 days. The appliance was continuously worn for the trial period and to encourage plaque formation, Dacron™ gauze was used. Results showed that chewing gum containing Phoscal™ had a significantly greater % R than the control gum. The difference between the Phoscal™/urea gum and the dicalcium phosphate dehydrate/urea gum was not significant, despite the gum with Phoscal™ having a 20% greater % R and 30% greater reduction in lesion depth.

In an attempt to enhance the positive dental effect of bovine milk, an in situ model in a double-blind cross-over design study was conducted, with the subjects drinking 200 mL of bovine milk with added CPP-ACP at two concentrations of 2 and 5 g/L, once a day for 15 days. The remineralisation of the enamel subsurface lesions exposed to the two test milks were more than for the control group with the increase in the % R values of 70% and 148% with a dose response relationship (Walker et al., 2006). In addition, about 81% and 164% increases in the % R when

drinking CPP-ACP containing milk at concentrations 2g/L and 3g/L were reported in another more recent study (Walker et al., 2009).

To investigate the CPP-ACP potential to slow the progression of enamel subsurface lesions, study using in situ model was carried out using CPP-ACP added to hard candy confections (Walker et al., 2010). The results showed that whilst the consumption of the control sugar confection resulted in a significant demineralisation (progression) in the enamel subsurface lesion, the consumption of the sugar confections containing CPP-ACP did not result in lesion progression; rather, it resulted in significant remineralisation (regression) of the lesion. The remineralisation by consumption of the sugar with 1.0% CPP-ACP confection was significantly greater than that obtained with the sugar free confection.

#### ***2.2.3.2.3 In Vivo Remineralisation Studies***

The effect of CPP-ACP in enamel remineralisation and caries prevention was assessed in a number of randomised control trials.

Morgan et al. (2008), conducted a two year randomised controlled clinical trial with 2,720 subjects. The aim was to compare the effect of two chewing gums; one containing 54 mg CPP-ACP per serving to an identical gum not containing CPP-ACP, on the prevalence of dental caries. Standardised bitewing radiographs were taken both at baseline and endpoint of the study to assess caries progression or regression over a two year period. The subjects followed a regimen of chewing the allocated gum for ten minutes three times per day, one chew being supervised at school. When transition scores were used to evaluate caries progression or regression, 18% fewer progressions were observed in the CPP-ACP group, and a



statistically significant lower chance of surface progression in the CPP-ACP gum group (OR = 0.82, p = 0.03) were reported. In this study, the efficacy of the gum containing CPP-ACP in reducing caries prevalence was believed to be marked, especially considering that study subjects were living in a community (Melbourne, Australia) with water fluoridation (1 ppm F), the incidence of caries was low, and both groups were supplied with preventive products and the positive control group also chewed a sugar-free gum three times per day, reducing their caries-risk and therefore reducing the possible incremental difference between the control and test groups.

Ferrazzano et al. (2011), conducted an in vivo study to test the effectiveness of CPP to promote remineralisation of early enamel caries. Patients with an age range of 10-16 years were selected and divided into two groups. Two demineralised enamel specimens were placed in the buccal surfaces of the upper first molars. In the first group, subjects were instructed to apply Tooth Mousse on the right sided specimen only and to use a placebo mousse on the left side. The application was done on a daily bases, three times per day for one month. In the second group, subjects had no treatment and enamel specimens were used as a control. The results of the SEM analysis showed a diffuse and homogeneous mineral coating that reduced the surface alterations only in the demineralised specimen treated with Tooth Mousse.

A more recent clinical trial (Sitthisettapong et al., 2012) was conducted to test the effect of daily application of 10% w/v CPP-ACP paste for one year when added to regular toothbrushing with fluoridated toothpaste to prevent dental caries in pre-school children.

The study population consisted of preschool children with high-caries-risk aged two to three years. Children were randomly assigned to receive either 10% w/v CPP-ACP paste (n = 150) or a placebo control (n = 146) in addition to fluoridated toothpaste. Every school day, trained teachers perform the toothbrushing with fluoridated toothpaste (1000 ppm F), followed by the application of the child's coded pastes. The assessment using The International Caries Detection and Assessment System (ICDAS) was recorded at baseline, six months, and one year.

A significant increase in mean numbers of enamel and dentin carious lesions, as well as dmfs, was found in both groups after one year with no significant difference between the two groups.

A number of randomised control trials were also conducted to evaluate the effect of CPP-ACP/ CPP-ACFP (Tooth Mousse, MI paste plus) on the remineralisation of white spot lesions.

An in vivo study was used to assess the effect a casein based product (Topocal™) incorrectly identified as CPP-ACP on post-orthodontic white spot lesions (Andersson et al., 2007). The study involved 26 subjects who were randomly assigned to either the test group using Topocal™ as a dentifrice for three months, followed by three months use of fluoridated toothpaste (1000-1100 ppm F) or a positive control group using fluoridated toothpaste and a 0.05% sodium fluoride mouth-rinse for six months. Laser fluorescence (DIAGNOdent™) in addition to the visual assessment was used to quantify the post-orthodontic white spot lesions. In this study, Topocal™ containing cream was found to have a greater capability of returning the white spot lesions to a normal appearance. At 12 months, the white

spot lesions that returned to normal in the Topocal™ group were three times more compared with that in the positive control group.

To test the efficacy of Tooth Mousse compared with a Tooth Mousse placebo without CPP-ACP, a similar white spot lesion model was used in de-banded orthodontic patients. Visual assessment using ICDASII determined a significant difference between the test and the placebo group after three months. In the severe lesions, more regression was reported with Tooth Mousse (31%) compared with a placebo (OR = 2.3, P = 0.04) and in the active lesions compared with the inactive lesions (OR = 5.07; P > 0.001) in both the test and the control group. This means diffusion of calcium and phosphate ions occurred to a greater extent into lesions with increased surface pore size (Bailey et al., 2009).

Beerens et al. (2010), investigated using a double-blind prospective randomised clinical trial the effect of CPP-ACFP (MI-Past Plus) on the remineralisation of white spot caries lesions and on plaque composition compared to that of a control group using fluoride-free paste with calcium.

Orthodontic patients (n = 54) with multiple white spot lesions were included in this randomised clinical trial. Subjects were randomly assigned to either the CPP-ACFP paste or control paste, and instructed to use these products supplementary to their normal oral hygiene. The subjects were followed up for three months and quantitative light-induced fluorescence (QLF) was used to assess the caries regression. QLF images were captured directly after the debonding and at six and 12 weeks thereafter. The results showed that both groups had a significant decrease in fluorescence loss compared to baseline and no difference was found between the groups. No significant changes in the size of the lesion area were noticed over time or between the groups.

Plaque composition was also investigated in this study, plaque samples were obtained just before debonding and at six and 12 weeks afterwards. The total counts and proportions of aciduric bacteria, *Streptococcus mutans*, and *Lactobacillus spp* were measured. A reduction in the percentage of aciduric bacteria from 47.4 to 38.1% and of *S. mutans* from 9.6 to 6.6% was reported. No difference was found between the groups. The authors concluded that there was no clinical advantage for use of the CPP-ACFP paste supplementary to normal oral hygiene over the time span of 12 weeks.

In another clinical trial (Bröchner et al., 2011), the study subject were divided into two groups. Subjects in the first group were instructed to apply Tooth Mousse once daily (in the evening) and to brush their teeth with standard fluoride toothpaste (1100 ppm F) in the morning. While In the control group, the subjects were instructed to brush their teeth with standard fluoride toothpaste two times a day (morning and evening). This was done for a study period of 4 weeks, during this period the use of additional preventive measures based on fluoride was prohibited. QLF was used in this trial to assess the white spot lesion regression or progression after fixed orthodontic treatment.

The result of this study showed that statistically significant reductions of the  $\Delta F$  values, approximately 30–35%, were observed in both study groups. However, there was no difference between the groups. Regarding the lesion area, a reduction by 58% was noticed in the CPP–ACP group and 26% in the fluoride group, this difference was close to reaching statistical significance ( $p = 0.06$ ).

Akin and Basciftci, (2012) carried out a randomised clinical trial, investigated different treatments for white spot lesions after fixed orthodontic therapy. The

treatment modalities that were compared included sodium fluoride mouth rinse, CPP-ACP, and the microabrasion technique.

Eighty patients (with 966 affected teeth) who had developed multiple decalcified enamel lesions after fixed orthodontic therapy were involved in the study and were divided into four groups. The control group participants were instructed to just brush their teeth. The participants in the fluoride group were instructed to use 20 ml of neutral 0.025% sodium fluoride rinse for 30 seconds twice daily. Rinsing was done immediately after brushing with a toothpaste containing fluoride. In the CPP-ACP group, participants were instructed to use Tooth Mousse twice a day in addition to fluoride toothpaste for six months. In the fourth group, the participants underwent treatment by the microabrasion technique, using mixture of 18% hydrochloric acid.

Using a digital camera, standardised intraoral images were taken for the affected teeth surfaces after debonding, and after six months of treatment. Image-processing software was used for the analysis of the white spot lesions.

In all groups, the area of the white spot lesions decreased significantly. However, the success rates were significantly different between the groups. The highest success rate was observed for the microabrasion group (97%). The success rate of CPP-ACP group (58%) was significantly higher than that of the fluoride group (48%) and the control group (45%).

The authors concluded that the use of CPP-ACP can be more beneficial than a fluoride rinse for post orthodontic remineralisation. And an effective treatment for

cosmetic improvement of longstanding white spot lesions can be achieved by microabrasion technique.

A new technique has been described for the management of the white spot lesions (Ardu et al., 2007). This technique combines microabrasion with the application of CPP-ACP cream. Microabrasion was suggested to remove the hypermineralised superficial layer of enamel, followed by daily home application of CPP-ACP, this treatment has the ability to eliminate WSLs without involving invasive restorative procedures.

This technique has been tested *in vitro*, and quantitative light-induced fluorescence were used to assess the effects of application of CPP-ACP paste and microabrasion treatment on the regression of white spot lesions (Pliska et al., 2012). In this *in vitro* study, artificially-induced white spot lesions in bovine enamel were randomly assigned to one of four treatment groups including CPP-ACP paste only, microabrasion only, microabrasion and CPP-ACP, and a control group. Samples in the control group were rubbed with a cotton swab and deionised water twice a day. In the CPP-ACP paste group, samples were rubbed with 1:1 diluted MI paste and deionised water for 20 seconds twice daily. The samples were not rinsed before returning to the remineralisation solution. The samples in the microabrasion group were treated with 35% phosphoric acid at the start of the two-week treatment period and then rubbed for 20 seconds with deionised water twice a day for the whole of the two-week treatment period. Finally, the same microabrasion application was applied in the microabrasion and paste group at the start of the two-week period, followed by twice-daily application of 1:1 diluted MI paste and deionised water for

20 seconds. Again, samples were not rinsed before returning to the remineralisation solution

The results showed that a statistically significant gain in fluorescence was associated with the microabrasion only, as well as the microabrasion and CPP-ACP treatments; 6.8% and 8.2% respectively. However, the CPP-ACP treatment alone does not significantly improve the fluorescence value of the white spot lesions; 3.1% compared to the control group; 2.9%.

### **2.2.3.3 CPP-ACP and Erosion**

According to ten Cate and Imfeld, (1996), dental erosion is ‘the physiological result of a pathogenic, chronic, localised loss of dental hard tissue that is chemically etched away from the tooth surface by acid and/or chelation without bacterial involvement’. Dental erosion is a multi-factorial disease; extrinsic and intrinsic acids in addition to behavioural factors play important roles in the development of dental erosion (ten Cate and Imfeld, 1996, Bartlett, 1997, Lussi et al., 2003).

It was found that the addition of CPP-ACP to a commercially available sports drink (Powerade™) resulted in a significant reduction in the erosive potential of the beverage. A 0.125% w/v of CPP-ACP decreased the depth of the enamel erosive lesions more than that obtained with de-ionised water in vitro (Ramalingam et al., 2005).

A laboratory based study using twice daily, 10 minute exposures of enamel and dentine specimens to 0.1 M calcium buffered lactic acid and subsequent storage in artificial saliva over a four-week period, investigated the effect of exposure to a 10:1 dilution of Tooth Mousse (containing 0.1% w/v CPP-ACP) for 10 minutes

prior to demineralisation on enamel and dentine mineralisation (Oshiro et al., 2007). The study reported that exposure to CPP-ACP reduced the erosive-like surface changes to both enamel and dentine to a minimal extent when compared with the control and negative control specimens.

In vitro study looked at the effect of Tooth Mousse on enamel erosion by a cola soft drink. In this study the use of a Tooth Mousse slurry after acidic challenge from the cola soft drink was shown to return the hardness of the exposed enamel toward the baseline value (Panich and Poolthong, 2009).

The protective effect of commercially novel agents against erosion was investigated in vitro (Wang et al., 2011). Both GC Tooth Mousse (10% CPP-ACP) and GC MI past plus (10% CPP-ACP, 900 ppm F) were tested in this study in which human teeth was exposed to a cycling regimen for four days. The cycle involved incubation of the specimens in human saliva for two hours to allow the formation of pellicle. Following that, the specimens were exposed to remineralisation solution for 3 minutes and demineralisation solution (orange juice, pH 3.6) for another three minutes; this was done twice daily. Two procedures were used in this experiment; in the first, the tested agent was applied prior to the erosive attack and in the second, after the erosive attack. This study failed to show a significant protective effect for both GC Tooth Mousse and GC MI past plus against erosion (by means of difference in surface nanohardness  $\Delta$ SNH) compared to the control group.

To test whether CPP-ACP could reduce enamel wear rates under severe erosive conditions, simulating heavy attrition and gastric regurgitation, Ranjitkar et al. (2009), subjected enamel specimens to 10,000 wear cycles at a load of 100N and pH 1.2 in a tooth wear machine. Two test groups (CPP-ACP paste and non CPP-



ACP paste with the same formula) and a control group were included in this study. In the test groups, the pastes were applied for 5 minutes, whereas no paste was applied in the control group. The results of this study showed that both CPP-ACP and non CPP-ACP pastes significantly reduce the enamel wear, with smoother and more polished wear facets noticed compared with the control group. The mean wear rate of the CPP-ACP group was also significantly lower than that of the non CPP-ACP group.

Vongsawan et al. (2010), tested the effectiveness of high calcium milk and CPP-ACP on enamel erosion caused by chlorinated water. In this study the enamel specimens were soaked in chlorinated water (pH 5.0, resembling gas-chlorinated swimming pool water) for 72 hours at room temperature, followed by emersion in artificial saliva for 30 minutes. Before this procedure, the experiment agents were applied to the enamel specimens of the test groups; a 0.5 mm layer of CPP-ACP for five minutes, and immersion in bovine milk for five minutes, whilst the control group received no treatment. The results found a significantly greater decrease of the surface microhardness value in the control group compared to the test groups.

#### **2.2.3.4 CPP-ACP and Fluoride**

The synergistic effect of CPP-ACP and fluoride in caries reduction is well established (Reynolds et al., 1995, Reynolds et al., 2008). This effect may be attributable to the formation of CPP-stabilised amorphous calcium fluoride phosphate (Cross et al., 2004), resulting in the increased incorporation of fluoride

ions into plaque, together with increased concentrations of bio-available calcium and phosphate ions.

In their study on rats, Reynolds et al. (1995), reported a significant reduction in the caries scores in the group received 0.5% CPP-ACP plus 500 ppm F compared with those in the group received either 0.5% CPP-ACP or 500 ppm F alone. This may be attributed to the formation of CPP-stabilised amorphous calcium fluoride phosphate, which increases the concentration of bio-available calcium and phosphate ions in addition to the increased incorporation of fluoride ions into plaque (Cross et al., 2004).

In an in situ study investigating the ability of CPP-ACP to increase the incorporation of fluoride into plaque and in promoting enamel remineralisation, CPP-ACP and fluoride containing dentifrices (1100 ppm NaF, 2800 ppm NaF, 2% CPP-ACP, 2% CPP-ACP plus 1100 ppm NaF, placebo) and mouth-rinses (2% CPP-ACP plus 450 ppm F, de-ionised water) were tested (Reynolds et al., 2008). The mouth-rinse was used three times daily for five days and the dentifrices were rinsed with water slurries four times a day for 14 days. A significant increase in the fluoride incorporation into plaque was found after the addition of 2% CPP-ACP to the 450 ppm F mouth-rinse ( $33.0 \pm 17.6$  nmol/mg dry wt) and this was more than a 100% increase compared with the fluoride mouthwash alone. The study also showed that remineralisation of 2% CPP-ACP containing dentifrice was comparable with that achieved with 2800 ppm F dentifrice, whilst the dentifrice containing 2% CPP-ACP plus 1100 ppm F was superior to all other formulations.

Kariya et al. (2004), in an in vitro study demonstrated that the addition of fluoride to the CPP-ACP improved its acid-resisting effect. The same findings were also reported by Lennon et al. (2006), as they showed that the enamel lost when

using paste containing casein calcium phosphate followed by a toothpaste containing 250 ppm F in an erosive cycle, was less than that using 250 ppm F and casein calcium phosphate alone. This was attributed to the capacity of the fluoride to improve the crystalline tooth structure, generation of fluorapatite, and accelerated remineralisation.

In another in vitro study Kumar et al. (2008), reported that the use of CPP-ACP in combination with fluoridated toothpaste showed a higher remineralisation potential and a 13.1% reduction in the lesion depth in comparison to 7% in the fluoridated toothpaste group and 10.1% in the other CPP-ACP groups. The negative control group showed an increase in lesion depth by 23%. The study authors recommended the use of CPP-ACP as a self-applied topical coating after brushing the teeth with fluoridated toothpaste in children with high caries-risk.

The effect of CPP-ACP in enamel remineralisation along with the effect of slow-release fluoride glass devices (SFGD) were investigated in vitro under pH cycling condition (Al-Mullahi and Toumba, 2010). In this study the effect of CPP-ACP and SFGD were tested separately and together. Enamel surface microhardness SMH was measured at baseline and after 10 days of pH cycling regime. The results of this study showed the enamel SMH values in all the test groups (SFGD, CPP-ACP and SFGD + CPP-ACP) were significantly higher than that in the control group. No significant difference was noticed in enamel SMH between the SFGD and CPP-ACP groups. Although SFGD + CPP-ACP group produced greater improvement in enamel SMH in comparison to SFGD or CPP-ACP alone this difference was not statistically significant.

## **2.3 Model Systems used to Study the Caries Process in Enamel**

Different approaches have been used for studying the caries process. The gold standard for these types of studies is a well-conducted and controlled randomised clinical trial. However, conducting a clinical trial is both time consuming and expensive. Therefore, a variety of models have been used as alternatives. These include:

### **2.3.1 In vitro Model**

In this model, tooth specimens were used and the application of the test material is done in a laboratory setting. An improvement of this approach has resulted from the development of the analytical technology of determining mineral gain or loss, to or from the tissue or its supernatant (Higham et al., 2005). The advantages of this approach are that it can be inexpensive and performed over a short period of time. However, limitations have been recognised for the in vitro model as it cannot replicate the oral environment, therefore, the information they provide on enamel demineralisation and remineralisation can be different from what actually occur in the oral cavity (Higham et al., 2005).

#### **2.3.1.1 Artificial Caries Lesions**

Using artificial enamel caries lesions in an in vitro model has been widely used in studying the process of demineralisation and remineralisation. Early caries lesion is characterised by having an intact surface layer with underlying subsurface demineralisation (Silverstone, 1973). Different methods has been used to create an

artificial carious lesions, this includes acidified gels (Silverstone, 1973, Arends and Davidson, 1975), and lactate buffers (Featherstone et al., 1978), With the later been shown to produce artificial lesions with characteristic features similar to that of natural caries i.e. apparently intact surface layer, a lesional centre and an inner zone (Silverstone, 1973).

### **2.3.1.2 pH Cycling Model**

The pH cycling model is an in vitro model that involves a process of alternating demineralisation and remineralisation, hence providing a better simulation of the caries process.

This model has become the method of choice for many caries researchers, and has been used widely to investigate caries-preventive agents on the dynamics of enamel demineralisation and remineralisation (Featherstone, 1996, ten Cate et al., 2006, White et al., 1994).

### **2.3.2 Animal Caries Models**

Animal caries models were used since the 1940s. Through this model, different variables involved in the caries process can be controlled such as the oral microflora where super-infection with cariogenic bacteria can be done, as well as controlling the delivery of the diet. Therefore, this model can provide information that is more related to the actual lesions that results from in vivo conditions. Nevertheless, objections of this model arises as the process of demineralisation and

remineralisation in animals are not related to the real process in human teeth in the human oral environment (Higham et al., 2005).

### **2.3.3 In situ Model**

This model involves the use of an intra-oral appliance or device that contains specimens of dental tissue. Many studies used the in situ models to study the dental caries and its prevention, this involves the study of fluoride mechanism of action, fluoride delivery systems, as well as other remineralising agents (Zero, 1995).

In contrast to in vitro or animal experimentation, in situ studies are performed in the human mouth, which make it more related to the actual demineralisation and remineralisation process in the oral environment. At the same time this model allows flexibility of experimental design and facilitates the control of different experimental variables in ways not achievable with clinical trials (Zero, 1995).

## **2.4 Methods used in Demineralisation and Remineralisation**

### **Evaluation**

Different laboratory methods are currently used in the measurement of enamel mineralisation. These methods allow detection of the mineral loss and gain in enamel even with small changes, this includes:

#### **2.4.1 Microradiography**

This technique is based on comparing the amount of x-ray absorbed from the tooth sample with that of a simultaneous exposed standard.

Transverse Microradiography (TMR) is considered to be the gold standard for the measurement of smooth surface enamel mineralisation. The accurate and reliable measurements it produces make it the most practical and widely accepted analytical technique (Arends and Ten Bosch, 1992, Damen et al., 1997).

TMR became a suitable tool for detecting small changes in mineral density profiles, especially after the development of the computer-aided video- image analysis (Damen et al., 1997). It involves the investigation of 80  $\mu\text{m}$  and 150  $\mu\text{m}$  planoparallel sections of enamel and dentine, which are cut from the sample.

The lesion mineral content is represented by  $\Delta Z$  which calculated from the lesion depth and the mineral distribution profile.

Disadvantages of the TMR technique includes the fact that destruction of the tooth sample is needed in preparation, therefore it is only useful for in vitro and in situ studies. In addition, the preparation of the slices is a very technique sensitive procedure as testing slices must have an identified and homogeneous thickness (Ten

Bosch and Angmar-Månsson, 1991). Add to that the expensive radiographic consumables that are also required.

The technique advantages include reasonable accuracy of quantitative mineral loss or gain, and determining mineral distribution (Arends and Ten Bosch, 1992). The other two types of microradiography are the longitudinal microradiography and wavelength independent microradiography which allow repeated measurements of the mineral content by using non-destructive techniques.

#### **2.4.2 Quantitative Light-Induced Fluorescence (QLF)**

Fluorescence is a phenomenon by which an object is excited by a particular wavelength of light and the fluorescent (reflected) light is of a larger wavelength. When the excitation light is in the visible spectrum, the fluorescence will be of a different colour (Pretty, 2006).

Benedict, (1928) was the first to describe the enamel auto-fluorescence, and suggested its use in the detection of dental caries (Benedict, 1929).

The demineralisation of enamel was found to result in a reduction of its auto-fluorescence. de Josselin de Jong et al. (1990), suggested that the observed differences in fluorescence between sound and carious enamel could be explained by altered amounts of light scattering and absorption. When dental enamel is demineralised, it becomes porous and the saliva liquids fill the pores causing a decrease in the light path in the enamel (Tranæus et al., 2001). In a caries lesion, the light scattering is much stronger than that in sound enamel (Ten Bosch, 1996) and the absorption of light per unit of volume is smaller, as a result, the fluorescence is less strong in the caries lesion.



Quantitative Light-Induced Fluorescence (QLF) is a novel device for the detection of early demineralisation of enamel. It is reported to be a valuable and non-destructive tool for early detection, quantification, and longitudinal monitoring of early (non-cavitated) carious lesions (Kühnisch and Heinrich-Weltzien, 2004).

QLF is a two-stage process for the detection and assessment of demineralisation and remineralisation. It involves the acquisition of images using a camera-based system (Yin et al., 2007) by subjecting the enamel to a beam of monochromatic blue light at a wavelength of 370 nm. The resultant auto-fluorescence with a yellow-green colour of human enamel is then detected and measured after filtration with a high band pass filter at 520 nm (Adeyemi et al., 2006).

Following that, the analysis of the images is carried out using proprietary software which involves using a patch to define areas of sound enamel around the lesion of interest. Subsequently, the software employs the pixel values of the sound enamel to reconstruct the surface of the affected area and thus allow an estimation of the degree of fluorescence lost for each pixel, considering the lesion is at a given threshold, typically 5.0% (De Josselin de Jong et al., 1995). This means that all pixels with a loss of fluorescence greater than 5.0% of the average sound value will be considered to be part of the lesion. Once the pixels have been assigned 'sound' or 'lesion' the software then calculates the average fluorescence loss in the lesion, known as %  $\Delta F$ , and then the total area of the lesion in  $\text{mm}^2$ . A multiplication of these two variables results in a third metric output,  $\Delta Q$  (Pretty, 2006).

The enamel dentinal junction (DEJ) is believed to be the source of the auto-fluorescence. The presence of the DEJ or dentine underneath the enamel is

necessary to provide sufficient fluorescence contrast between the lesion and the sound enamel. Studies have shown that when underlying dentine is removed, the enamel fluorescence is lost (Van Der Veen and De Josselin de Jong, 2000).

Studies have revealed that QLF could detect demineralisation that resulted from an eight hour exposure to the decalcification solution (lactic acid-Carbopol solution) and was able to quantify changes in lesion severity associated with longer demineralisation (Ando et al., 1997). It can also follow the progression of the lesion longitudinally in the same tooth.

The QLF device has been validated by comparison with transverse microradiography TMR. A good correlation was found between QLF and TMR (Al-Khateeb et al., 1997) and thus indicates the validity of the QLF as a measure of mineral content (Higham et al., 2005). It has shown to be reliable and reproducible as well.

Different types of carious lesions have been assessed by the QLF. The QLF sensitivity has been reported at 0.68 and specificity at 0.70 for the occlusal caries which is comparable with other systems. The correlations of QLF metrics and lesion depth have also been reported to be up to 0.82 (Pretty, 2006).

The presence of confounding factors that adversely affect the reliability of measurements is associated with all techniques; therefore, optimal conditions should be determined and implemented to ensure valid results.

In vitro and in vivo studies using the QLF technique have shown larger standard deviations or errors for the QLF technique compared to other techniques, such as microradiography (four to five times greater) and confocal laser scanning light microscopy. This was mainly noticed in the in vitro studies. In this case, the

thickness of enamel and dentin is the only confounding factor, taking into consideration the work under the standardised conditions (Ando et al., 2003).

This was confirmed in an in vitro study (Ando et al., 2003). The results of this study imply that fluorescence radiance measurements in either sound or demineralised enamel will depend on enamel thickness for lesions of the same size, which means that two specimens with same severity of demineralisation but with different enamel thickness would have different values for fluorescence radiance due to the differences in enamel thickness. These findings draw attention to the importance of standardising the thickness of enamel within the same group when conducting in vitro fluorescence studies assessing enamel lesion severity.

Another factor that could potentially influence the QLF image and consequently the analysis of the demineralisation loss is the extent of hydration of the lesion (Angmar-Mansson and Bosch, 2001). This is particularly important when monitoring the lesions longitudinally. In order to achieve reliable results, the application of compressed air for 15 seconds prior to the QLF imaging is suggested (Pretty et al., 2004).

The ambient light may also influence the results of QLF test, however, in areas where QLF is to be used, a light level of 88 lux can be employed without significantly affecting the reported values (Pretty et al., 2002a).

### **2.4.3 Microhardness Test**

The microhardness technique is very sensitive to changes in mineral density and can provide indirect evidence of mineral loss or gain (Featherstone and Zero,

1992). For that reason, it has been used to determine demineralisation and remineralisation effects on dental enamel (Koulourides and Volker, 1964).

This method measures the resistance of enamel surface to indenter penetration, which represents the degree of the superficial enamel layer porosity that in turn indicates mineral loss or gain in subsurface lesions (Koulourides, 1971). The measurements involve micro- and nano- indentation of a Knoop or Vickers diamond tip of defined geometrical dimensions, and its load and duration on the tooth surface (Lussi et al., 2006).

Microhardness testing is a rapid and inexpensive technique and it has been in use in the dentistry field for a long time (Lussi et al., 2006). A flat and polished tooth specimen surface is needed to get effective microhardness measurements. In addition to that, the measurements obtained do not necessarily represent the surface of the whole specimen since different specimen sites have varying microhardness values. Which is considered the main disadvantage of the technique (Caldwell et al., 1957).

#### **2.4.4 Scanning Electron Microscopy (SEM)**

The principle of the SEM procedure involves scanning a fine beam of electrons across the surface of a specimen in synchronism with the spot of the display cathode ray tube (CRT). This produces a highly magnified image of three dimensional appearances, which is derived from the action of the electron beam scanning across the surface of the specimen.

SEM scans qualitative alterations of the surface, the size and the shape of features as well as observing the roughness on the surface of objects. Moreover, SEM can reveal the minerals precipitate in pellicle from mineral dissolution. The advantages of this technique includes that no sample preparation is required. The micro-structural images produced by the SEM are readily interpreted. However, SEM only provides subjective and qualitative assessment without comprehensive information about surface alterations of specimens.

## **2.5 Methods Used for Early Caries Detection**

An increase in the research activity surrounding caries diagnostic methods has been noticed in recent years. The assessment of early caries lesions has received a particular concern (Pretty, 2006), since the early detection of the caries process is crucial to prevent the progression of the caries lesion.

Several methods are currently available for the early detection of dental caries:

### **2.5.1 International Caries Detection and Assessment System (ICDAS)**

The ICDAS system has been developed in order to standardise caries detection and diagnosis among practitioners. The system employs an evidence-based approach for detecting early and later stage caries, and classifies lesions based on their clinical visual appearance. Depending on the severity of the lesion the system has a coded classification from 0-6 (Pitts and Ekstrand, 2013).

### **2.5.2 QLF**

Discussed before in 2.4.2

### **2.5.3 Fibre Optic Transillumination**

Fibre optic transillumination uses the optical properties of enamel. Through a device composed of small aperture in the form of a dental hand-piece it uses a high intensity white light to enhance the optical properties of enamel, thereby providing the ability to distinguish between early enamel and early dentine lesions. However, the system has a disadvantage of being subjective rather than objective. This property makes the longitudinal monitoring of the lesion a complex process (Pretty, 2006).

### **2.5.4 Laser Fluorescence**

Laser Fluorescence (DIAGNODent) system measures enamel fluorescence quantitatively using 655 nm red light. It supports the detection of non-cavitated, occlusal pit-and-fissure caries, as well as smooth surface caries at their early stage. Clinical investigations have demonstrated high sensitivity ( $\geq 0.92$ ) in detection of occlusal caries and reproducibility tests resulted in Cohen's Kappa value of 0.93 in vivo (Hibst et al., 2001).

No image of the tooth is produced by the system; alternatively it displays a numerical value on two LED displays. The first numerical value displays the current reading, whilst the second displays the peak reading for that examination (Pretty, 2006).

### **2.5.5 Digital Radiographic Techniques**

Digital radiography has contributed to an increase in the diagnostic yield of dental radiographs. It offers the potential of image enhancement by applying a range of algorithms making their diagnostic performance at least as good as the conventional radiographs (Pretty, 2006). Several studies revealed a significantly lower sensitivity and specificity of digital radiographs on assessing small proximal caries (Verdonschot et al., 1992). However, these radiographs have the advantage of using a lower radiation dose for the patient in order to produce the tooth image (Pretty, 2006).

### **2.5.6 Electronic Caries Monitor (ECM)**

The Electronic caries monitor (ECM) is a device which employs a single, fixed-frequency alternating current in an attempt to measure the ‘bulk resistance’ of tooth tissue (Longbottom and Huysmans, 2004). A number of physical factors were found to affect ECM results, including the temperature of the tooth (Huysmans et al., 2000), the thickness of the tissue (Wang et al., 2000), and the hydration of the material and the surface area (Longbottom and Huysmans, 2004).

### **2.5.7 Ultrasound Techniques**

The principle behind the Ultrasound technique is that sound waves can pass through gases and liquids as well as solids and the boundaries between them (Hall and Girkin, 2004). By utilising a sonar device in which a beam of ultrasound wave is

directed against the tooth surface, images of tissues can be acquired by collecting the reflected sound waves.



## **2.6 Research Aims, Objectives, And Hypothesis**

### **Aim:**

To investigate in vitro the effect of Tooth Mousse (10% w/v CPP-ACP), MI Paste Plus (10% w/v CPP-ACP, 900 ppm F) and high fluoride concentration toothpaste (2800 ppm F) in the remineralisation of demineralised enamel subsurface lesion and to compare it to the effect of 1450 ppm F toothpaste and 0 ppm F toothpaste control.

### **Objectives:**

- To assess using QLF the effect of Tooth Mousse (10% w/v CPP-ACP) and MI Paste Plus (10% w/v CPP-ACP, 900 ppm F) when used supplementary to fluoride toothpaste (1450 ppm F) in the remineralisation of demineralised enamel subsurface lesions in vitro under pH cycling condition.
- To compare the additional effect of Tooth Mousse and MI Paste Plus with that of fluoride toothpaste (1450 ppm F) alone, high fluoride toothpaste (2800 ppm F) and non-fluoride toothpaste.

### **The null hypothesis:**

- There is no difference in the enamel remineralisation that results from Tooth Mousse, MI Paste Plus, 1450 ppm F toothpaste, 2800 ppm F toothpaste and non-fluoridated toothpaste.

## **3.0 MATERIALS AND METHODS**

This was an in vitro study designed to investigate the remineralisation of the enamel subsurface lesions under pH cycling condition using different remineralising agents. The methodology adopted in the present study including preparation of tissue samples and the pH cycling protocol as well as the materials and equipment used will be described in this section.

### **3.1 Power Calculation**

Statistical advice was sought and the sample size was calculated by using data from a previous PhD thesis ‘Investigations into the effect of casein phosphopeptide-amorphous calcium phosphate on enamel demineralisation and remineralisation’ (Manton, 2009). A total of 22 enamel slabs per group were needed. This calculation was based on the assumption that the standard deviation of the response variable is 2.0, power 90%, 0.05 significance level and a true difference between treatments would be adjusted to 3 units. This was based on the calculations by MGH Biostatistics Centre software (Schoenfeld, 2010).

### **3.2 Experiment Materials**

- Non-Fluoride toothpaste (the Boots Company PLC, Nottingham, England).
- Fluoride toothpaste 1450 ppm F (0.32% w/w sodium fluoride) (Colgate cool stripe. Colgate–Palmolive (UK) Ltd, Guildford, England).
- High fluoride toothpaste 2800 ppm F (0.619 w/w sodium fluoride) (Duraphate®. Colgate–Palmolive (UK) Ltd, Guildford, England).
- Tooth Mousse 10% w/v CPP-ACP (GC Tooth Mousse™, GC Corp, Tokyo, Japan).
- 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).

### **3.3 Study Groups**

- Group 1: Fluoride-free Toothpaste (0 ppm F) (negative control).
- Group 2: Fluoride toothpaste (1450 ppm F) (positive control).
- Group 3: High Fluoride toothpaste (2800 ppm F).
- Group 4: Tooth Mousse (10% w/v CPP-ACP) + Fluoride toothpaste (1450 ppm F).
- Group 5: MI paste plus (10% w/v CPP-ACP + 900 ppm F) + Fluoride toothpaste (1450 ppm F).

### 3.4 Enamel Slab Preparation

All enamel slabs used in the present study were obtained from bovine incisors. Approval for collection of bovine teeth was sought from the Food Standards Agency (Appendix 1) The teeth were obtained from an abattoir and stored immediately in distilled water and 0.1% thymol (Sigma Aldrich) at room temperature. Before sectioning, the teeth were cleaned using a spoon excavator and a toothbrush to remove any soft tissue remnants. To detect any defects, caries or cracks, all teeth were screened by trans-illumination and transmitted light using a low-power microscopy (Leitz, Wetzlar®, Germany).

Each tooth was mounted using ‘green stick’ impression compound (Kerr, UK) on plates. The crowns were sectioned using water cooled, diamond wire saw, cutting machine (Well@Walter EBNER, CH-2400 Le Loche) (Figure 3-1). The buccal and palatal surfaces of each crown were separated, and each buccal section was cut into two slabs that were approximately 6 x 5 x 3 mm in size.

**Figure 3-1: Diamond wire saw apparatus used for the teeth sectioning (Well® Walter EBNER, CH-2400 Le Loche).**



Each enamel slab was mounted on a plastic rod using “sticky wax” to hold the slab in the demineralising gel. The rod was secured to the lid of a “Sterilin” type universal tube so that when the top was screwed onto the tube, the tooth was suspended in the centre of the tube free space (Figure 3-2). Two coats of an acid resistant, coloured nail varnish (Max Factor “Glossfinity”) were then applied on the enamel slabs, except for a small window of approximately 2 x 3 mm on the centre of each slab that was left exposed (Figure 3-3). An interval of 24 hours was left between the two applications to allow the nail varnish to dry completely.

Once the enamel slabs were prepared, they were kept moist in plastic containers at room temperature to prevent dehydration.

**Figure 3-2: Enamel slab suspended in the “Sterilin” type universal tube.**



**Figure 3-3: Enamel slabs covered with nail varnish except for a small window.**



### **3.5 Preparation of the Enamel Sub-Surface Lesion**

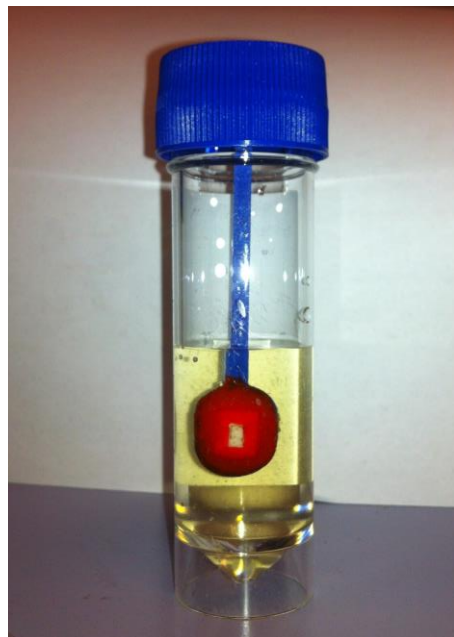
In order to obtain a sub-surface caries-like lesion an acid demineralising gel was prepared.

#### **3.5.1 The preparation of demineralisation system: acidified hydroxyethyl cellulose gel**

The gel was prepared by adding 0.1 M sodium hydroxide (BDH Analar Grade) to 0.1 M lactic acid (Sigma Aldrich D/L GPR 87% Lactic acid) to give a pH value of 4.5 and then 6% w/v hydroxyethyl cellulose (Sigma Aldrich) was added to the solution and stirred for one hour until a consistency similar to that of “wallpaper paste” was achieved. The mixture was left to settle for 24 hours. Once the demineralising gel was ready for use, it was poured into the universal tubes

“Sterilin” into which the mounted teeth were then submerged (Figure 3-4). The enamel slabs were immersed in acid gel for 10 days to produce an artificial enamel subsurface lesion. The enamel slabs were removed from the acid gel and washed with distilled water, the nail varnish was then removed using methanol to prepare the enamel slabs for the baseline QLF measurements.

**Figure 3-4: Enamel slabs suspended in the acid gel.**



### **3.6 Quantitative Light-Induced Fluorescence (QLF) Measurements**

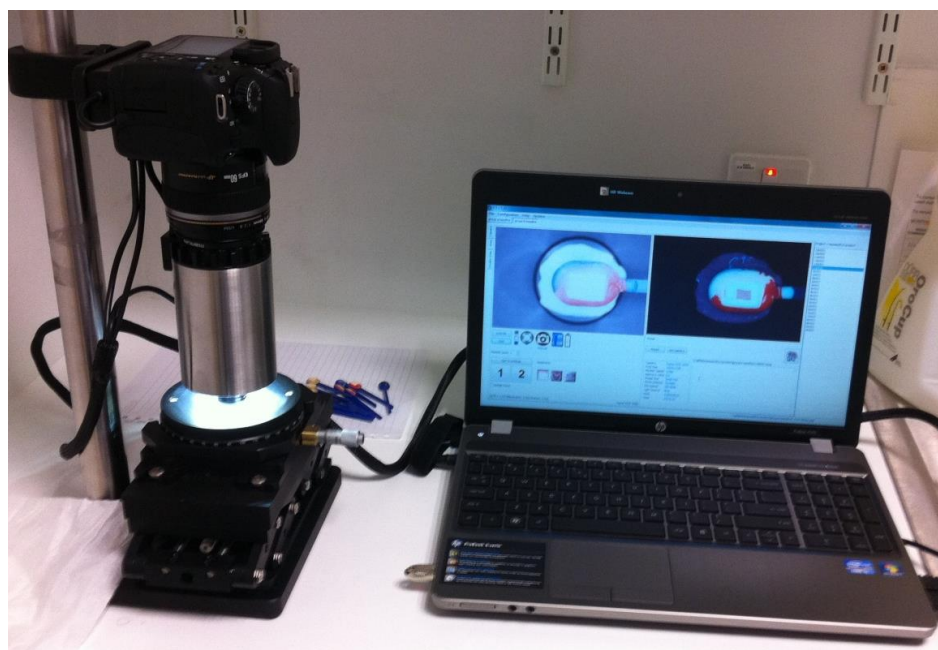
For each enamel slab, QLF measurements were taken after the creation of the enamel subsurface lesion and at the end of the 21 days experiment period using the QLF machine (QLF-D Biluminator™ 2) (Inspektor Research Systems BV, Amsterdam, The Netherlands) as shown in Figure 3-5, Under controlled conditions. All the slabs were dried for 15 seconds with compressed air prior to imaging, and were then examined in a dark room.

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.

To ensure that images of the enamel slab were always captured in the same camera positions and from the same angles, the camera was attached to a stand in the same position for all the images. The QLF camera was fixed at a position that provided optimum illumination of the enamel block surface. The camera specimen distance was standardised using the jig thereby controlling specimen stability light intensity and magnification.



**Figure 3-5: QLF machine, the SLR camera attached to the stand with standardised distance from the enamel slab.**

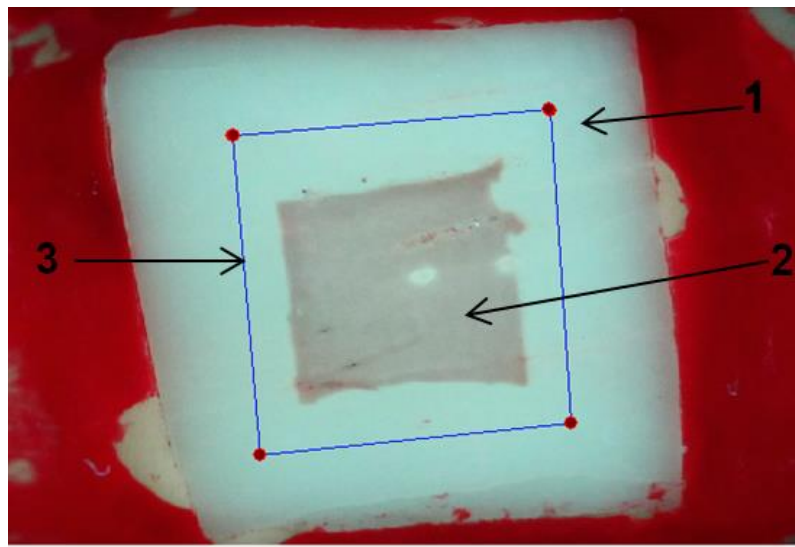


A patch was drawn around the white spot lesion site by the study examiner with its borders on sound enamel as can be seen in Figure 3-6. Inside this patch, the fluorescence levels of sound tissue were reconstructed by using the fluorescence radiance of the surrounding sound enamel. The percentage difference 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.

Demineralised areas appeared as dark spots. The fluorescent radiance of a white spot lesion viewed by QLF was lower than that of sound enamel. As already described in page 48, 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.

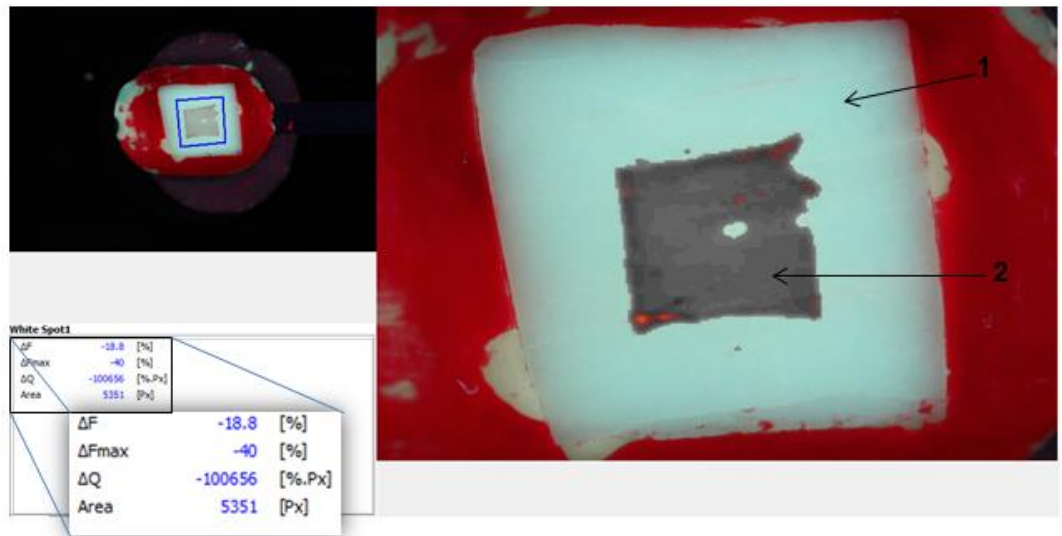
**Figure 3-6: QLF image taken with the blue light shows the demineralised lesion in the centre of the enamel slab as well as a patch drawn around the lesion with the border in sound enamel. (Arrow 1: sound enamel, arrow 2: demineralised enamel, arrow 3: patch drawn around the lesion).**



For each enamel lesion the following three metrics were obtained: (see Figure 3-7)

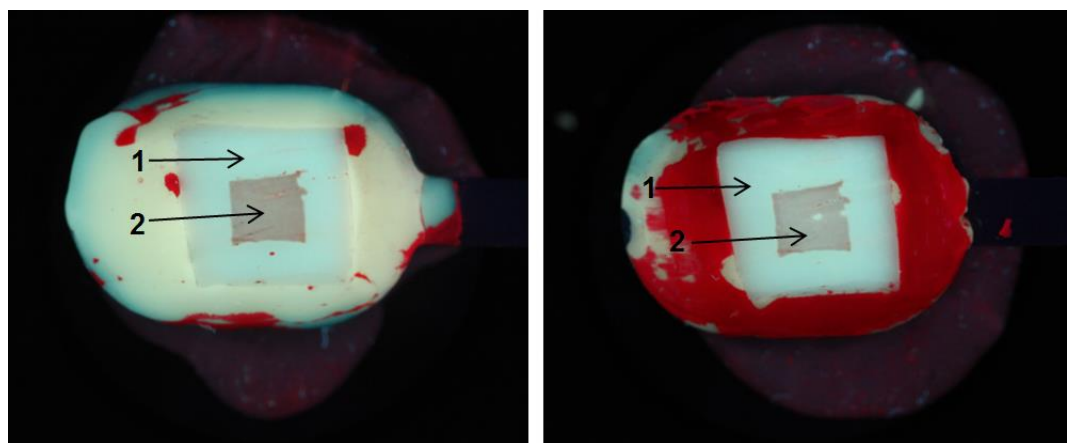
1.  $\Delta F$ : Percentage fluorescence loss with respect to the fluorescence of sound tooth tissue; related to lesion depth (%),
2.  $\Delta Q$ : The  $\Delta F$  times the Area. Percentage fluorescence loss with respect to the fluorescence of sound tissue times the area. Related to lesion volume ( $\% \text{ px}^2$ ),
3. Area: The surface area of the lesion expressed in pixels<sup>2</sup> ( $\text{px}^2$ ).

**Figure 3-7: Example of the blue light image analysis results including  $\Delta F$ ,  $\Delta Q$  and the lesion area values. (Arrow 1: sound enamel, arrow 2: demineralised enamel).**



**Figure 3-8: Examples of the blue light image of demineralised enamel lesions before and after pH cycling for each group. (Arrow 1: sound enamel, arrow 2: demineralised enamel).**

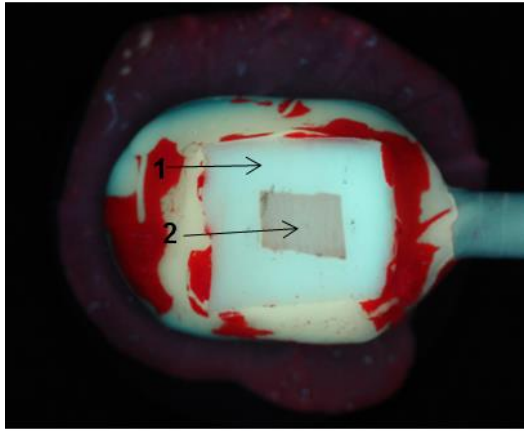
**Group 1: 0 ppm F**



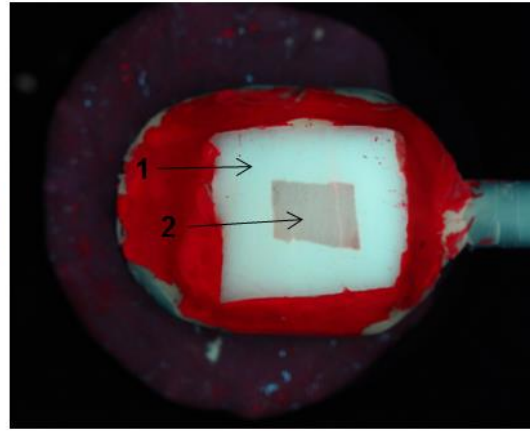
**Lesion before pH cycling**

**Lesion after pH cycling**

**Group 2: 1450 ppm F**

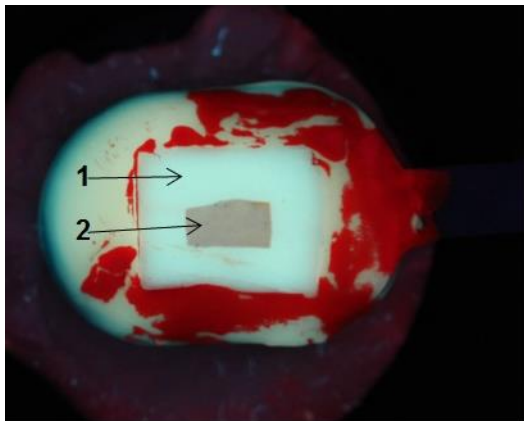


**Lesion before pH cycling**

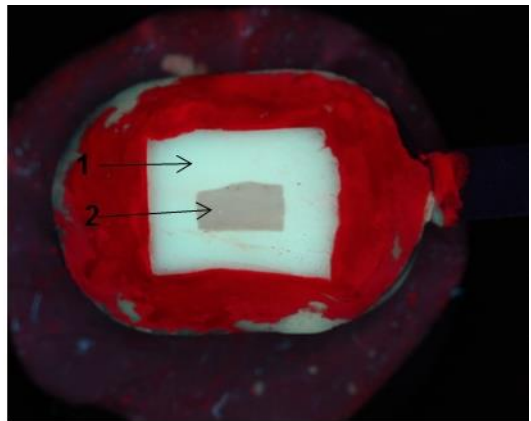


**Lesion after pH cycling**

**Group 3: 2800 ppm F**

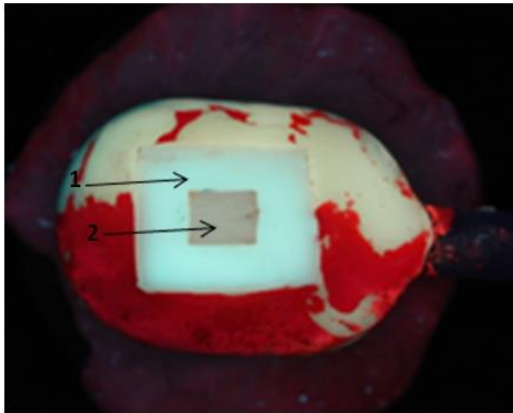


**Lesion before pH cycling**

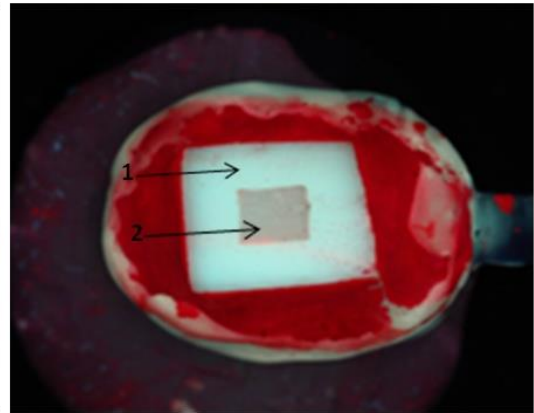


**Lesion after pH cycling**

**Group 4: 1450 ppm F + TM**

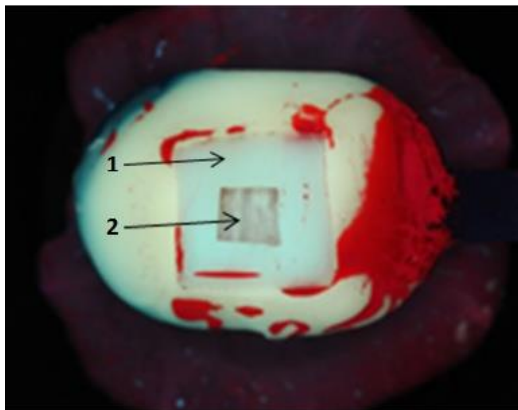


**Lesion before pH cycling**

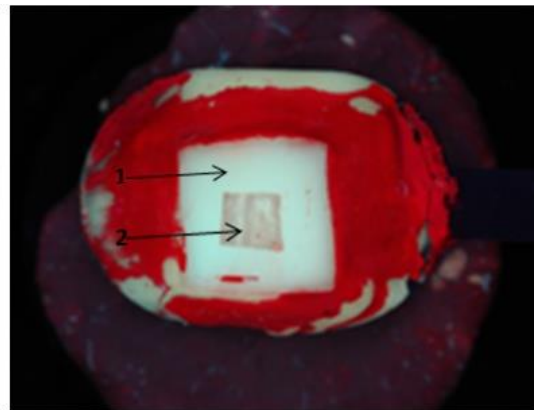


**Lesion after pH cycling**

**Group 5: 1450 ppm F + MIplu**



**Lesion before pH cycling**



**Lesion after pH cycling**

### **3.7 The $\Delta F$ Range of the Artificial Lesions**

After performing the QLF baseline analysis for all enamel slabs, the range of  $\Delta F$  values were found to vary between -7.66 and -31.98. The enamel slabs with the  $\Delta F$  range (-14.12 to -26.65 with an average of -20.75) were selected to be involved in the experiment in order to pick up the differences in  $\Delta F$  after treatment.

### **3.8 Randomisation and Blindness**

All enamel slabs were randomly assigned to five groups using a table of random numbers. When the slabs were analysed with QLF, the investigator did not know to which group the enamel slab belongs, making the analysis completely blinded.

### **3.9 The pH Cycling Regime**

Each enamel slab was attached to plastic rod (holder). The enamel slabs were rinsed with distilled water for 1 minute then dipped in toothpaste slurry for 30 minutes. After that the enamel slabs were rinsed with distilled water for 1 minute and placed in day time artificial saliva for 60 minutes. The enamel slabs were then exposed to first demineralisation challenge by dipping in acetic acid solution (pH 4.8) for 5 minutes, then rinsed with distilled water for 1 minute and placed in day time artificial saliva. This process was repeated until the enamel slabs were subjected to 5 demineralisation challenges. After the last cycle the enamel slabs were dipped in toothpaste slurry for 30 minutes.

In 0 ppm F, 1450 ppm F and 2800 ppm F groups, enamel slabs were then placed in the night time artificial saliva. While in 1450 ppm F + TM and 1450 ppm F + MIplus groups, and following the toothpaste dipping, the enamel slabs were rinsed gently with distilled water and then dipped in the Tooth Mousse or MI paste plus slurries for 30 minutes and finally were placed in the night time artificial saliva. The acetic acid was changed after each exposure. The day time saliva and the night time saliva were changed every day. The enamel slabs were kept in incubator at 37°C at all times except during the dipping in the toothpaste slurry or the demineralisation solution.

### **3.10 Study Protocol**

#### **Group 1: Non-Fluoride Toothpaste (0 ppm F)**

Non-Fluoride toothpaste (0 ppm F), which served as a negative control, was used twice daily. One application was done before the pH cycling regimen and one at the end of the pH cycling before placing the enamel slabs in the night time saliva. The enamel slabs were placed in the toothpaste slurry for 30 minutes each time.

#### **Group 2: Fluoride Toothpaste (1450 ppm F)**

Fluoride toothpaste (1450 ppm F), which served as a positive control, was applied twice daily. One application was done before the pH cycling regimen and one at the end of the pH cycling before placing the enamel slabs in the night time saliva. The enamel slabs were placed in the toothpaste slurry for 30 minutes each time.

### **Group 3: High Fluoride Toothpaste (2800 ppm F)**

High Fluoride toothpaste (2800 ppm F) was applied twice daily. One application was done before the pH cycling regimen and one at the end of the pH cycling before placing the enamel slabs in the night time saliva. The enamel slabs were placed in the toothpaste slurry for 30 minutes each time.

### **Group 4: Tooth Mousse (10% w/v CPP-ACP)**

Fluoride toothpaste (1450 ppm F) was applied twice daily and Tooth Mousse (10% w/v CPP-ACP) was applied once a day.

The first toothpaste application was done before the pH cycling regimen and the second application at the end of the pH cycling. The enamel slabs were placed in the toothpaste slurry for 30 minutes each time. Following the last application, the enamel slabs were gently washed with distilled water and placed in the Tooth Mousse slurry for 30 minutes and then placed in night time saliva.

### **Group 5: MI paste plus (10% w/v CPP-ACP + 900 ppm F)**

Fluoride toothpaste (1450 ppm F) was applied twice daily, and MI paste plus (10% w/v CPP-ACP + 900 ppm F) applied once a day.

The first toothpaste application was done before the pH cycling regimen and the second application at the end of the pH cycling. The enamel slabs were placed in the toothpaste slurry for 30 minutes each time. Following the last application, the enamel slabs were gently washed with distilled water and placed in the MI paste plus slurry for 30 minutes and then placed in night time saliva.



### **3.10.1 Experiment material slurries**

#### **1. Toothpaste slurry**

Toothpaste slurries were prepared by mixing the toothpaste with artificial day saliva in a volume ratio 1:4 (toothpaste: saliva) by weight, using a WhirliMixer® (Fisons) for 1 minutes.

The toothpastes used were: non-fluoride toothpaste, 1450 ppm fluoride toothpaste and 2800 ppm fluoride toothpaste.

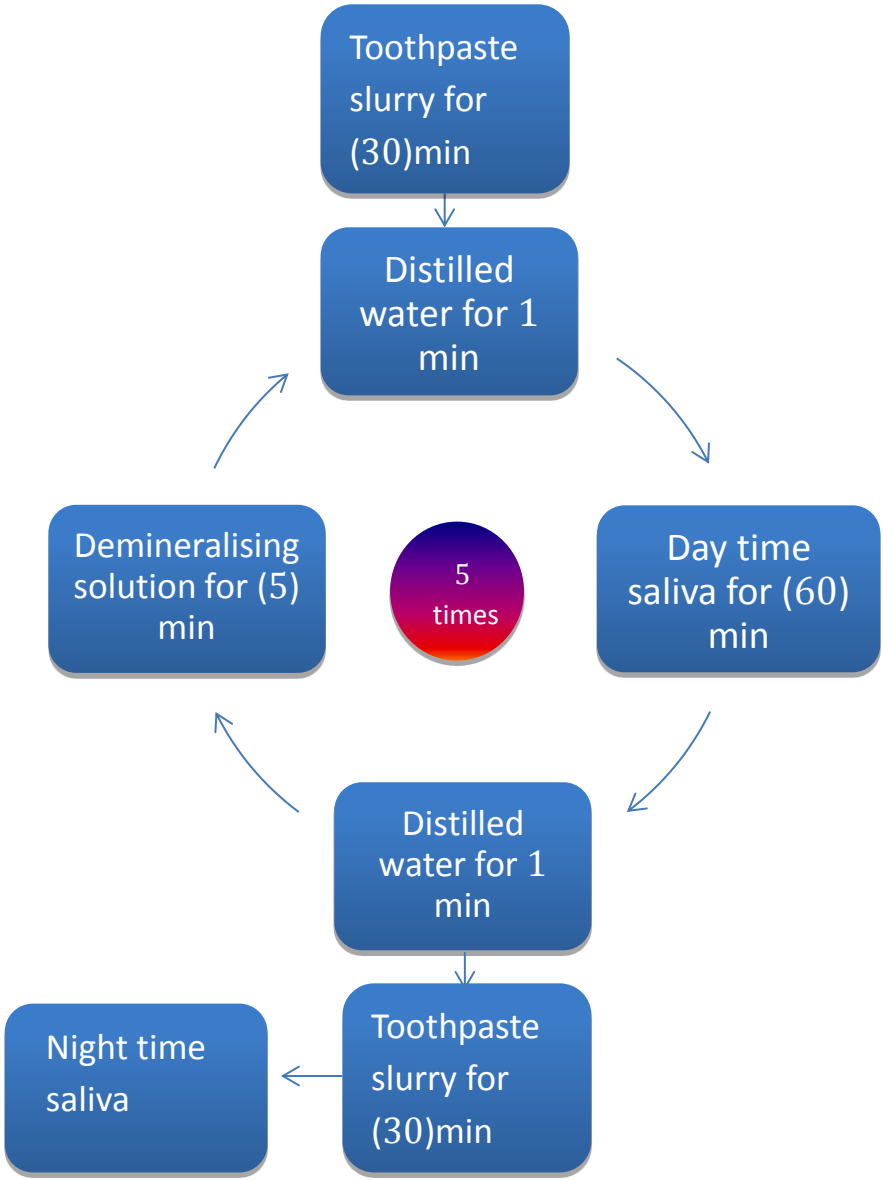
#### **2. Tooth Mousse and MI paste plus slurries**

The slurries of these two products were prepared by mixing 1 g of Tooth Mousse (10% w/v CPP-ACP) or MI paste plus (10% w/v CPP-ACP + 900 ppm F) with 4 ml of distilled water (Reynolds., et al 2008) using a WhirliMixer® (Fisons) for 1 minute.

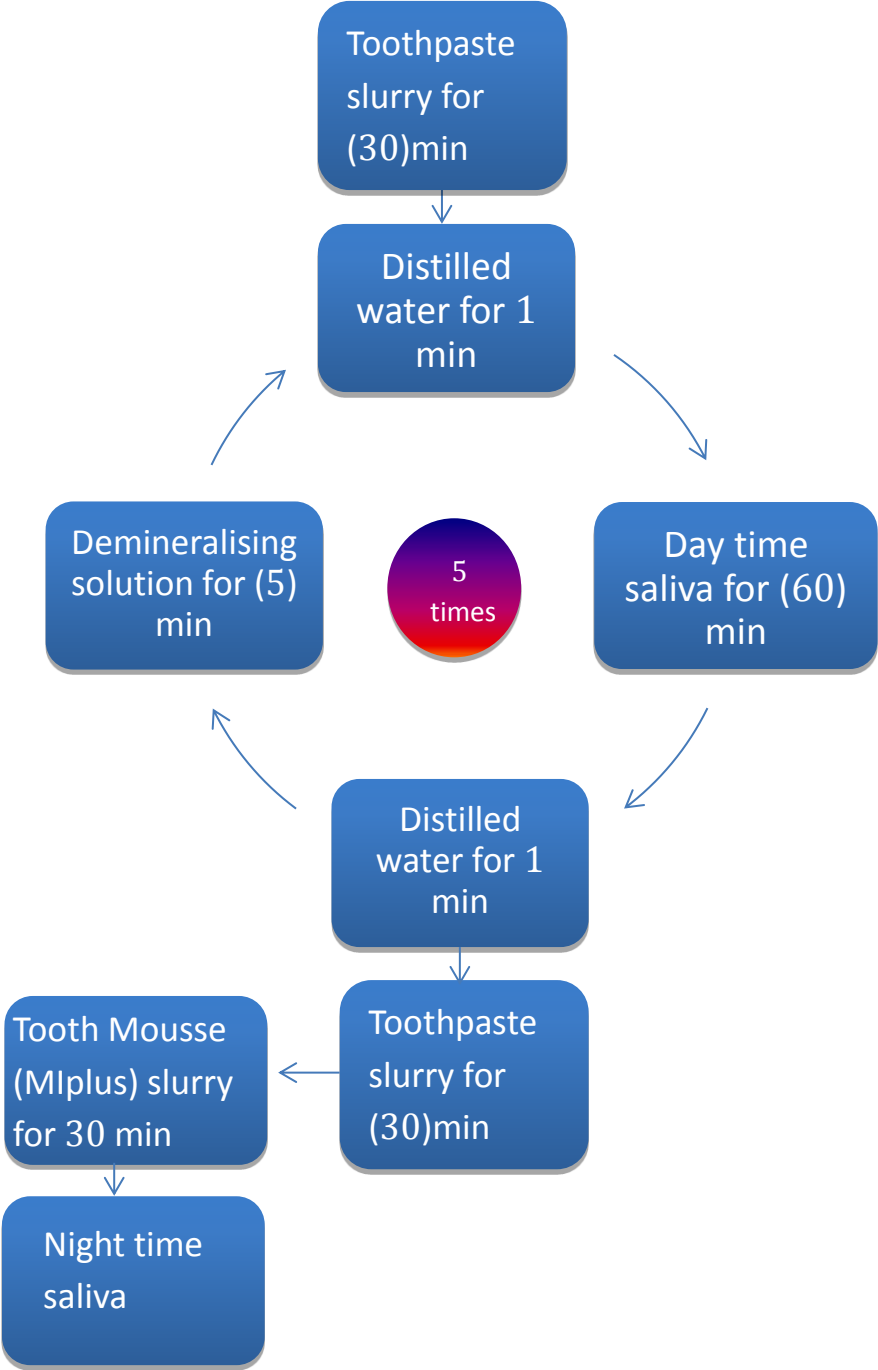
The pH values for the Tooth Mousse and the MI paste plus slurries were measured using pH meter (ORION- model 920A) and were found to have pH value of 7.0.

The study design and experimental protocol are shown in the Figure 3-9, 3-10, 3-11 and 3-12.

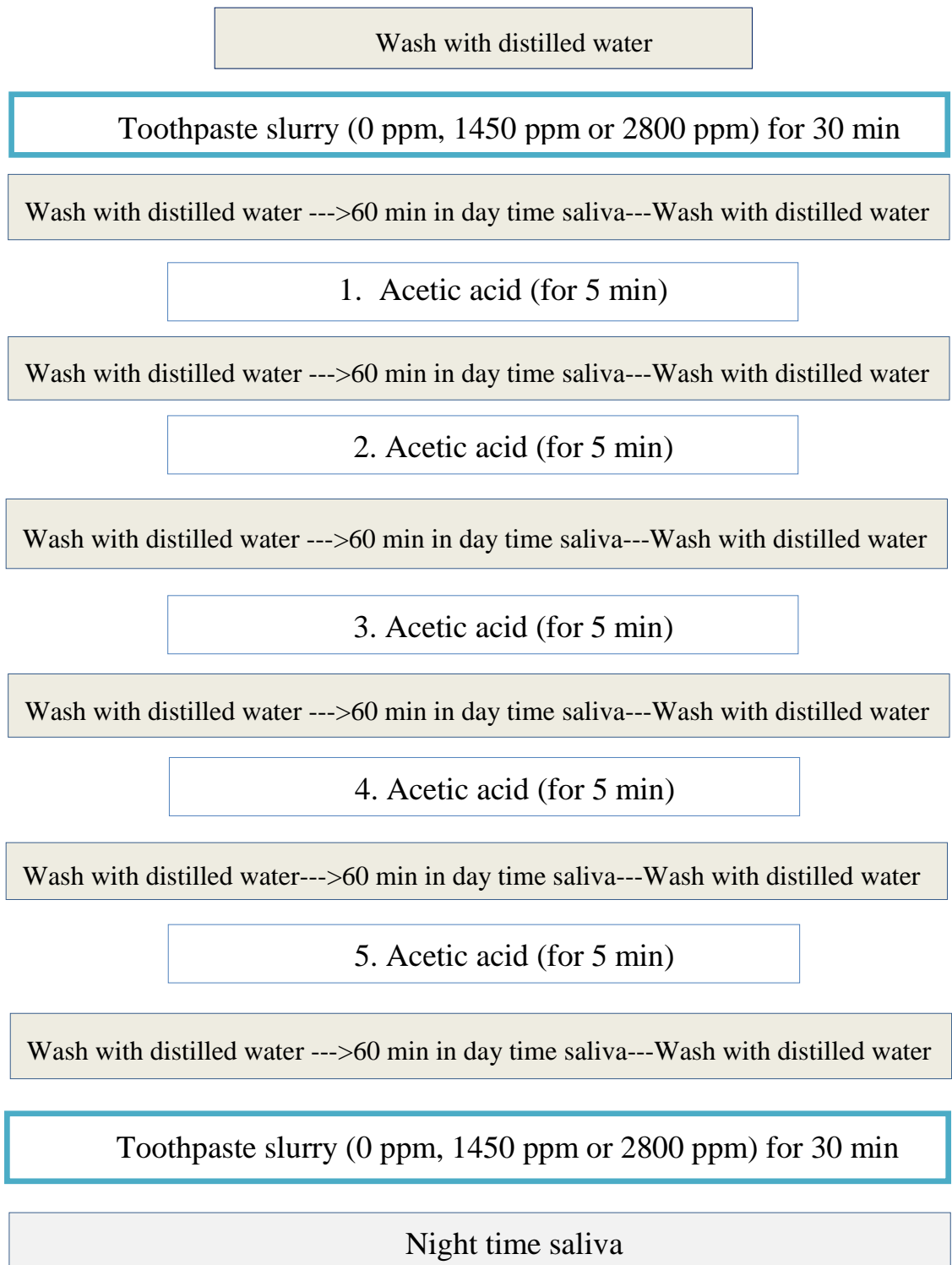
**Figure 3-9: Flow chart of the pH cycling protocol followed for 0 ppm F, 1450 ppm F and 2800 ppm F toothpastes groups.**



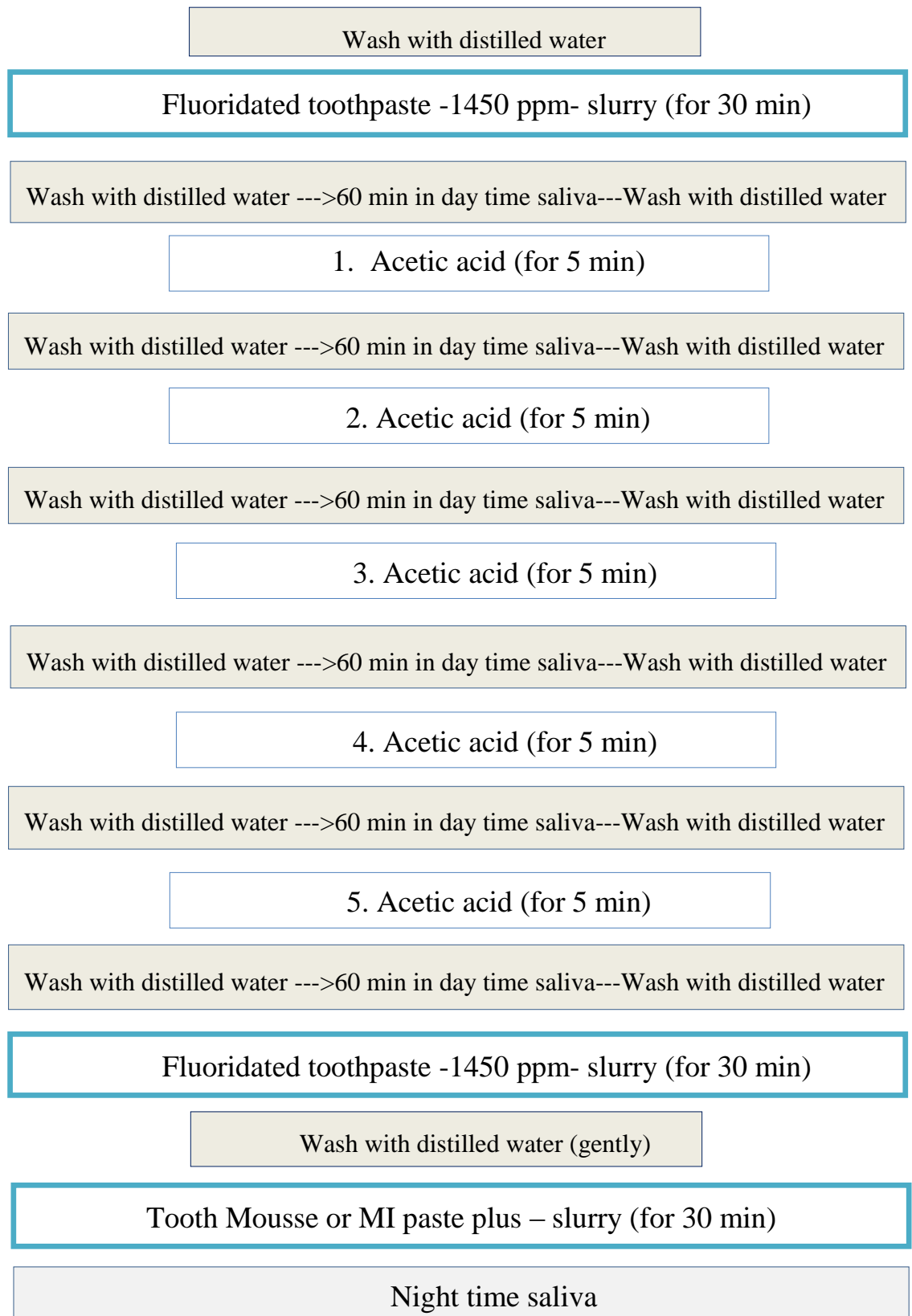
**Figure 3-10: Flow chart of the pH cycling protocol followed for 1450 ppm F +TM and 1450 ppm F + MIplus groups.**



**Figure 3-11: Flow chart for 0 ppm F, 1450 ppm F and 2800 ppm F toothpaste groups.**



**Figure 3-12: Flow chart for 1450 ppm F+ TM and 1450 ppm F + MIplus groups.**



### 3.11 Preparation of Solutions Used in the Study

#### 3.11.1 Artificial saliva

Two artificial saliva solutions were used in this study. The first solution was used for day time during the pH cycling, between the acid exposures. The second solution was used to store the slabs during the night. The day saliva was supersaturated solution that allowed remineralisation of enamel slabs, the night saliva was a saturated solution that maintained the enamel condition and did not provide any minerals exchange.

The artificial saliva composition was based on the electrolyte composition of natural saliva and it was advised to be used in order to eliminate any precipitation on the enamel surface (as provided by Dr RP Shellis, Department of Oral and Dental Science, University of Bristol, Bristol, UK).

##### 3.11.1.1 The preparation of day time artificial saliva

The formulation of the day time saliva is shown in Table 3-1.

**Table 3-1: Composition of the day time saliva solution.**

<b>Contents</b>	<b>Concentration g/L</b>
<b>Calcium carbonate</b>	0.07
<b>Magnesium carbonate (hydrated basic)</b>	0.019
<b>Potassium di-hydrogen phosphate</b>	0.554
<b>HEPES buffer (acid form)</b>	4.77
<b>Potassium chloride</b>	2.24

Using 900 ml distilled water 1.8 ml 1 mol/L HCl and the above components were stirred using a magnetic stirrer until they all dissolved. The solution pH was then adjusted to 6.8 by adding KOH solution and was made up to 1L with de-ionised water.

### 3.11.1.2 The preparation of night time artificial saliva

The formulation of the night time saliva is shown in Table 3-2.

**Table 3-2: Composition of the night time saliva solution.**

<b>Contents</b>	<b>Concentration g/L</b>
<b>Calcium carbonate</b>	0.05
<b>Magnesium carbonate (hydrated basic)</b>	0.019
<b>Potassium di-hydrogen phosphate</b>	0.068
<b>HEPES buffer (acid form)</b>	4.77
<b>Potassium chloride</b>	2.24

Using 900 ml distilled water, 1.8 ml 1 mol/L HCl and the above components were stirred using magnetic stirrer until they all dissolved. The solution pH was then adjusted to 6.8 by adding KOH solution and was made up to 1L with de-ionised water.

### 3.11.2 Acetic acid buffer

The preparation of acetic acid solution was done according to ten Cate et al. (2006), the constitution of acetic acid is shown in Table 3-3.

**Table 3-3: Constituents of Acetic acid.**

Contents	Concentration g/L
Calcium chloride	1.66
Potassium di-hydrogen phosphate	1.22
Acetic acid	30.02

The above contents and 1 L distilled water were stirred using centrifuge until fully dissolved. The pH was adjusted using pH meter (ORION- model 920A) by adding KOH solution to reach pH 4.8.

### 3.12 Training and Calibration

The study investigator had received training to use the QLF machine (QLF-D Biluminator™ 2) and was familiar with the QLF software before the study.

The training was provided by the manufacturer (Inspektor Research Systems BV, Amsterdam, The Netherlands), and included the image capturing as well as image analysis. The investigator assessment of sound, demineralised enamel and the border of the lesion during image analysis were calibrated.



### **3.13 Intra-Examiner Reproducibility**

The study investigator randomly retested 15% of the enamel slabs with the QLF at the end of the experiment. Intra-examiner reproducibility was tested using the Bland-Altman plot as well as using Intra-class Correlation Coefficient (ICC).

### **3.14 Statistical Analysis**

The data were analysed using SPSS statistical software package for windows version 20.0. Descriptive statistics were used to calculate the mean, median, range, and standard deviation.

The normality of the data distribution was assessed using Shapiro-Wilk test and Kolmogorov-Smirnov test.

Paired sampled t-tests were performed to compare the changes in remineralisation at baseline and after treatment within the same group.

One way ANOVA was used to compare between the five groups when the data were normally distributed, and Kruskal-Wallis Test was used when the data were not normally distributed. Furthermore, Bonferroni test or Mann-Whitney U test was used to assess if there was any significant difference between each of the groups. The test calculated the 95% confidence interval as well. The significance level was set at a level of  $p < 0.05$ .

### 3.15 Scanning Electron Microscopy SEM

Two enamel slabs from each group were selected randomly for the SEM analysis. Samples were mounted on stubs using carbon discs (12mm Agar) for gold sputtering. Specimens were then sputter coated with 300Å layer of gold using Polaron E5000 sputter coater (Figure 3-13). Microstructural analysis was undertaken using a Scanning Electron Microscope (HITACHI S-3400N) (Figure 3-14) operated at a 20 kV accelerating voltage. Standardised scanning angles were used and the entire surface of each enamel disc was scanned and imaged using high power magnification.

**Figure 3-13: enamel slabs coated with gold for the SEM analysis.**



**Figure 3-14: Scanning Electron Microscopy (HITACHI S-3400N).**



## 4.0 RESULTS

### 4.1 Quantitative Light-induced Fluorescence (QLF) Results

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

$\Delta F$ : Percentage fluorescence loss with respect to the fluorescence of sound tooth tissue. Related to lesion depth (%).

$\Delta Q$ :  $\Delta F$  times the Area. Percentage fluorescence loss with respect to the fluorescence of sound tissue times the area. This is related to lesion volume (%px<sup>2</sup>).

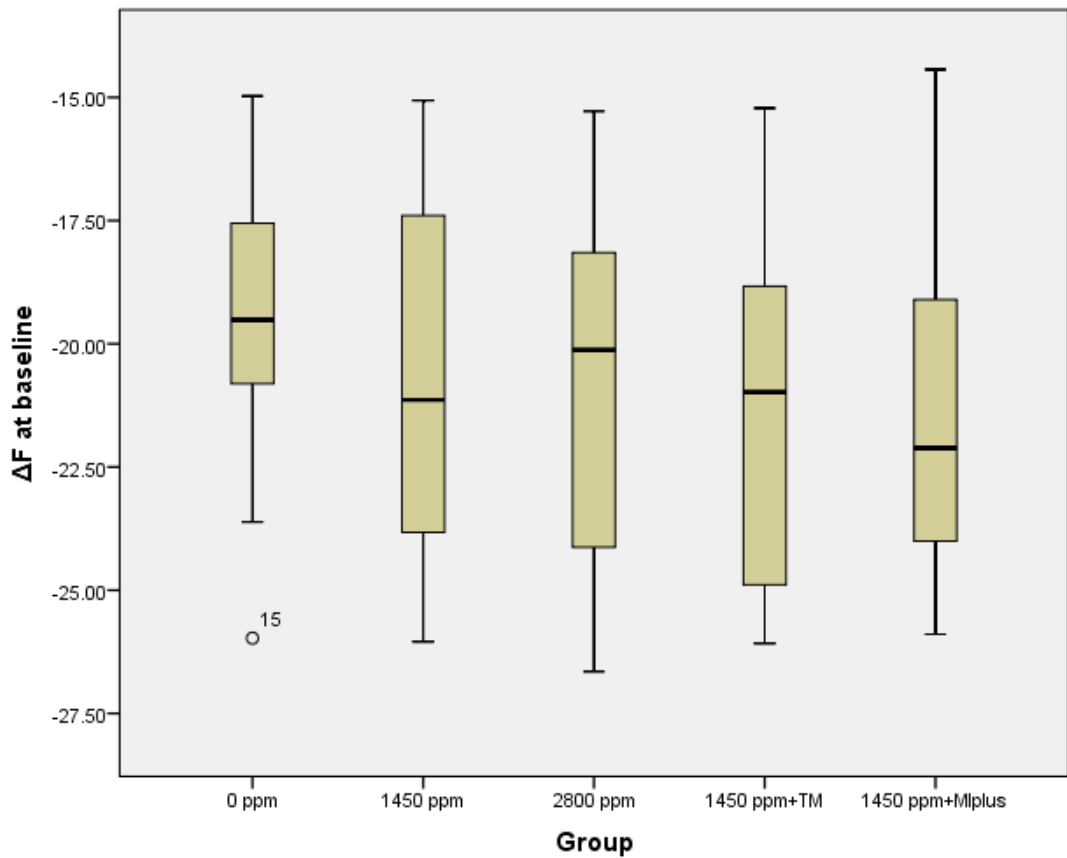
Area: The surface area of the lesion expressed in pixels<sup>2</sup> (px<sup>2</sup>).

#### 4.1.1 The mean fluorescence loss $\Delta F$

The values of  $\Delta F$  at baseline for all groups were checked to see if there was a difference between the groups. The normality tests (Shapiro-Wilk test and Kolmogorov-Smirnov test) were carried out to check the normality of the data. The data were considered normally distributed if the p values from these tests were not statistically significant ( $p > 0.05$ ). p values for all groups except for group 3 were not statistically significant therefore data was considered to be normally distributed. (Appendix 2).

The boxplot (Figure 4-1) for the distribution of the  $\Delta F$  at the baseline showed that all groups had almost the same range, and group 1 (non-fluoride toothpaste) had the narrowest variation with one outlier in the  $\Delta F$  compared to the other groups.

**Figure 4-1: Boxplot for the distribution of the  $\Delta F$  values at baseline for all groups. Error bars represent SD, the line in the box of Box-and-whisker plot is the median value of the data.**



One way ANOVA test (Table 4-1) was performed to assess if there was any statistically significant difference in  $\Delta F$  values at the baseline between the lesions assigned to the five groups. No statistically significant difference was found.

**Table 4-1: One way ANOVA between groups for  $\Delta F$  values at baseline**

	<b>Sum of Squares</b>	<b>df</b>	<b>Mean Square</b>	<b>F</b>	<b>Sig.</b>
<b>Between Groups</b>	49.062	4	12.265	1.108	.356
<b>Within Groups</b>	1273.357	115	11.073		
<b>Total</b>	1322.419	119			

#### 4.1.1.1 Difference in $\Delta F$ within each group

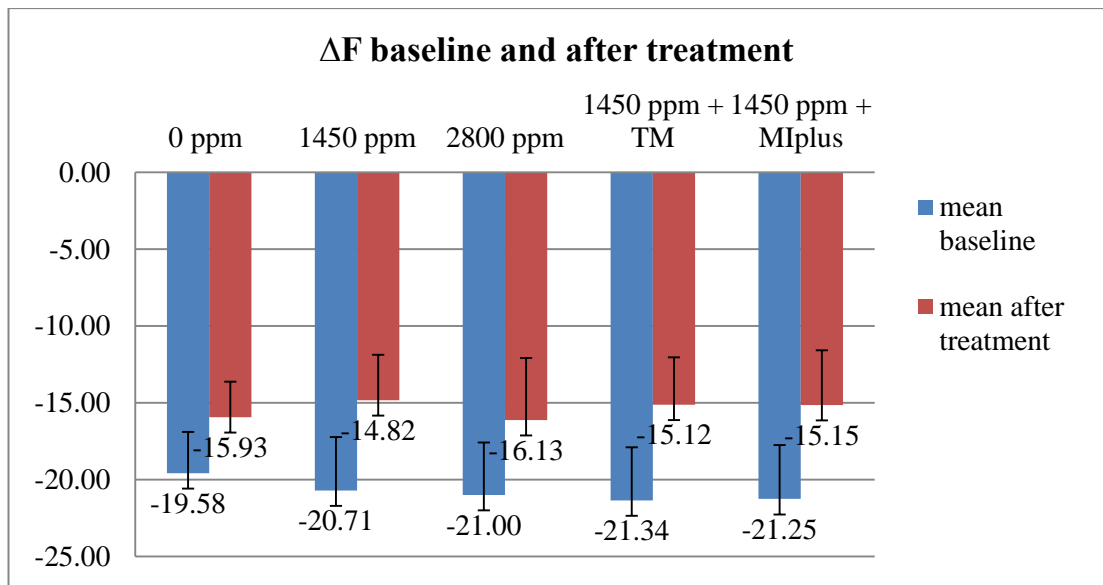
The  $\Delta F$  mean values both at baseline and after treatment are shown in Table 4-2. It can be seen that there was an improvement in  $\Delta F$  values for all the groups in the study.

**Table 4-2: The mean values of  $\Delta F$  at baseline and after treatment for all groups.**

<b>Group</b>	<b>Mean <math>\Delta F</math> at baseline <math>\pm</math> SD</b>	<b>Mean <math>\Delta F</math> after treatment <math>\pm</math> SD</b>	<b>Mean Difference in <math>\Delta F</math> at baseline and after treatment <math>\pm</math> SD</b>
<b>0 ppm</b>	-19.58 $\pm$ 2.69	-15.93 $\pm$ 2.32	3.647 $\pm$ 1.89
<b>1450 ppm</b>	-20.71 $\pm$ 3.50	-14.82 $\pm$ 2.96	5.887 $\pm$ 2.03
<b>2800 ppm</b>	-21.00 $\pm$ 3.41	-16.13 $\pm$ 4.04	4.869 $\pm$ 2.66
<b>1450 ppm + TM</b>	-21.34 $\pm$ 3.45	-15.12 $\pm$ 3.08	6.219 $\pm$ 2.57
<b>1450 ppm + MIplus</b>	-21.25 $\pm$ 3.51	-15.15 $\pm$ 3.57	6.107 $\pm$ 2.53

Figure 4-2 shows the change in the mean of  $\Delta F$  at baseline and after treatment with the standard deviation for all groups.

**Figure 4-2:  $\Delta F$  mean values at baseline and after treatment for all groups.**



To assess whether the change in  $\Delta F$  at baseline and after treatment was significantly different within the same group, paired T-Test was used.

The results of the paired T-Test are shown in Table 4-3. It can be seen that there was a statistically significant improvement in the  $\Delta F$  values after treatment compared with that at baseline in all groups ( $p < 0.001$ ).

**Table 4-3: Paired sampled T-Test results for  $\Delta F$  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	
<b>0 ppm</b>	$\Delta F$ at baseline – $\Delta F$ after treatment	-3.696	1.847	.377	-4.476	-2.916	<b>.000*</b>
<b>1450 ppm</b>		-5.887	2.035	.415	-6.746	-5.028	<b>.000*</b>
<b>2800 ppm</b>		-4.869	2.663	.544	-5.994	-3.745	<b>.000*</b>
<b>1450 ppm + TM</b>		-6.219	2.567	.524	-7.303	-5.136	<b>.000*</b>
<b>1450 ppm + MIplus</b>		-6.107	2.529	.516	-7.175	-5.039	<b>.000*</b>

\* Statistically significant



#### 4.1.1.2 Difference in $\Delta F$ between all groups

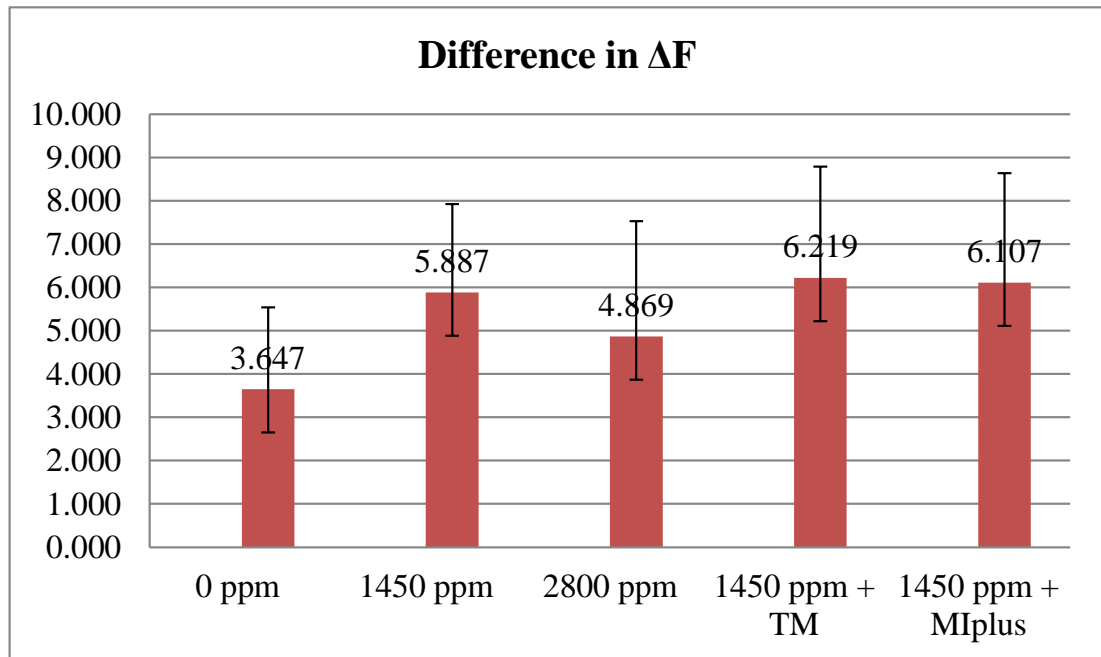
The  $\Delta F$  difference (change) was measured using the following formula:

$$\text{Difference in } \Delta F = \Delta F \text{ after treatment} - \Delta F \text{ at baseline}$$

##### Descriptive statistics

Figure 4-3 shows the difference in  $\Delta F$  in the five tested groups. In all groups the difference in  $\Delta F$  was positive, meaning that there was decrease in  $\Delta F$  (mean fluorescence loss) after treatment compared to that at baseline. The highest reduction in  $\Delta F$  was seen in the 1450 ppm fluoride toothpaste with Tooth Mousse (1450 ppm F + TM) group with mean difference of  $(6.22 \pm 2.57)$ , followed by the 1450 ppm fluoride toothpaste with MI paste plus (1450 ppm F + MIplus) with mean difference of  $(6.11 \pm 2.53)$ . The least reduction was noticed in the non-fluoride toothpaste (0 ppm F) group with mean difference of  $(3.65 \pm 1.89)$ . The 1450 ppm fluoride toothpaste (1450 ppm F) group and the 2800 ppm fluoride toothpaste (2800 ppm F) group also show reduction in the  $\Delta F$  value with mean difference of  $(5.89 \pm 2.03)$ ,  $(4.87 \pm 2.66)$  respectively (Table 4-4).

**Figure 4-3: Means of the difference in  $\Delta F$  at baseline and after treatment of all groups.**



**Table 4-4: Descriptive statistics for the difference in  $\Delta F$  at baseline and after treatment for all groups.**

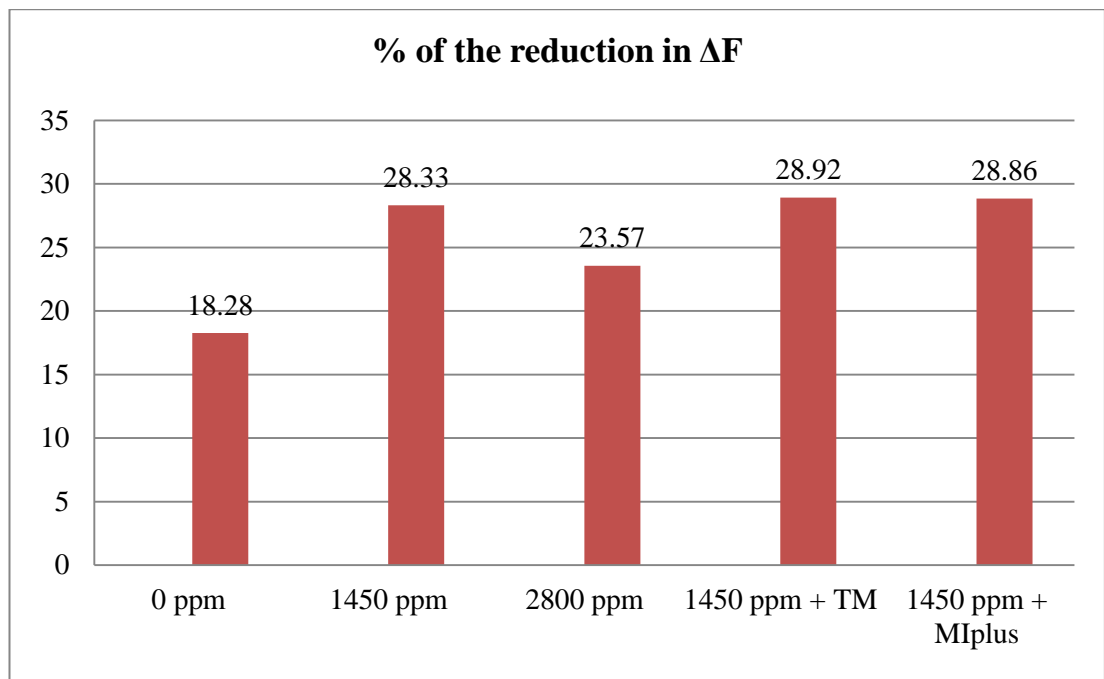
group	N	Minimum	Maximum	Mean	Median	Std. Deviation
<b>0 ppm</b>	24	-.16	7.56	3.6474	3.8830	1.89233
<b>1450 ppm</b>	24	1.83	9.62	5.8874	5.9815	2.03452
<b>2800 ppm</b>	24	1.23	11.69	4.8692	4.8155	2.66296
<b>1450 ppm + TM</b>	24	3.09	12.04	6.2194	5.7170	2.56674
<b>1450 ppm + MIplus</b>	24	1.63	10.13	6.1070	6.3555	2.52932

The percentage change in  $\Delta F$  at baseline and after treatment (% F) was calculated using the following formula:

$$\text{(Difference in } \Delta F \text{ at baseline and after treatment / } \Delta F \text{ at baseline)} \times 100$$

Figure 4-4 shows the % F values for all groups, % F values were similar for 1450 ppm F, 1450 ppm F + TM and 1450 ppm F + MIplus groups. Lower values were observed for 0 ppm F and 2800 ppm F (18.28 and 23.57% respectively).

**Figure 4-4: The % F values for all groups.**

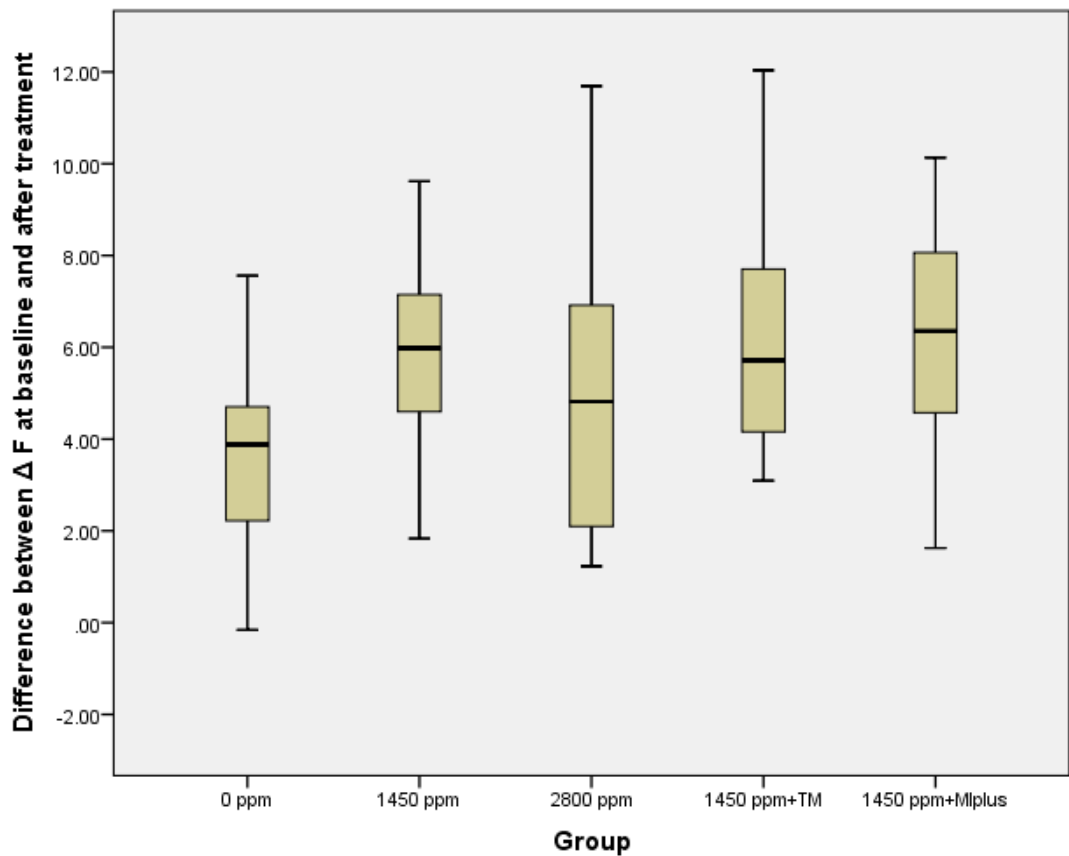


#### Determination of the normality of the data

In order to check if the  $\Delta F$  differences between the baseline and after treatment were normally distributed, data normality test was carried out. This included Shapiro-Wilk test and Kolmogorov-Smirnov test (Appendix 3). All the p values were not statistically significant therefore data was considered to be normally distributed ( $p > 0.05$ ).

The boxplot (Figure 4-5) of difference in  $\Delta F$  at baseline and after treatment showed that the median values were close in the 1450 ppm F, 1450 ppm F + TM and 1450 ppm F + MIplus groups (5.98, 5.72 and 6.36 respectively), whereas the median value was lowest for 0 ppm F group. The boxplot also showed that 2800 ppm F had the widest variation in the  $\Delta F$  differences compared to the other groups, from 1.23 to 11.69.

**Figure 4-5: Boxplot for the difference in  $\Delta F$  at baseline and after treatment for all groups.**



One way ANOVA test (Table 4-5) was performed to assess if the difference in  $\Delta F$  was statistically significant between the five groups. It showed that the mean difference in  $\Delta F$  was statistically significant between the groups ( $p < 0.001$ ).

**Table 4-5: One way ANOVA between groups for the difference in  $\Delta F$  at baseline and after treatment.**

	<b>Sum of Squares</b>	<b>df</b>	<b>Mean Square</b>	<b>F</b>	<b>Sig.</b>
<b>Between Groups</b>	113.946	4	28.486	5.124	<b>.001*</b>
<b>Within Groups</b>	639.334	115	5.559		
<b>Total</b>	753.280	119			

\* Statistically significant

In order to determine which groups were statistically significant different, pairwise comparisons were conducted using a Bonferroni test. The Bonferroni tests corrects for multiple testing. The results of the Bonferroni tests are shown in Table 4-6.

It can be seen that the mean difference in  $\Delta F$  of the 0 ppm F control group was significantly lower compared to the 1450 ppm F, 1450 ppm F + TM and 1450 ppm F + MIplus groups, ( $p < 0.05$ ). However, no significant difference was observed between the 0 ppm F and 2800 ppm F groups.

**Table 4-6: Multiple comparisons of the difference in  $\Delta F$  at baseline and after treatment between all test groups and control.**

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
<b>0 ppm</b>	1450 ppm	-2.24004*	.68065	.013	-4.1882	-.2919
	2800 ppm	-1.22179	.68065	.753	-3.1700	.7264
	1450 ppm+TM	-2.57204*	.68065	.003	-4.5202	-.6239
	1450 ppm+MIplus	-2.45958*	.68065	.004	-4.4077	-.5114
<b>1450 ppm</b>	0 ppm	2.24004*	.68065	.013	.2919	4.1882
	2800 ppm	1.01825	.68065	1.000	-.9299	2.9664
	1450 ppm+TM	-.33200	.68065	1.000	-2.2802	1.6162
	1450ppm+MIplus	-.21954	.68065	1.000	-2.1677	1.7286
<b>2800 ppm</b>	0 ppm	1.22179	.68065	.753	-.7264	3.1700
	1450 ppm	-1.01825	.68065	1.000	-2.9664	.9299
	1450 ppm+TM	-1.35025	.68065	.497	-3.2984	.5979
	1450 ppm+MIplus	-1.23779	.68065	.716	-3.1860	.7104
<b>1450 ppm+TM</b>	0 ppm	2.57204*	.68065	.003	.6239	4.5202
	1450 ppm	.33200	.68065	1.000	-1.6162	2.2802
	2800 ppm	1.35025	.68065	.497	-.5979	3.2984
	1450 ppm+MIplus	.11246	.68065	1.000	-1.8357	2.0606
<b>1450 ppm+MIplus</b>	0 ppm	2.45958*	.68065	.004	.5114	4.4077
	1450 ppm	.21954	.68065	1.000	-1.7286	2.1677
	2800 ppm	1.23779	.68065	.716	-.7104	3.1860
	1450 ppm+TM	-.11246	.68065	1.000	-2.0606	1.8357

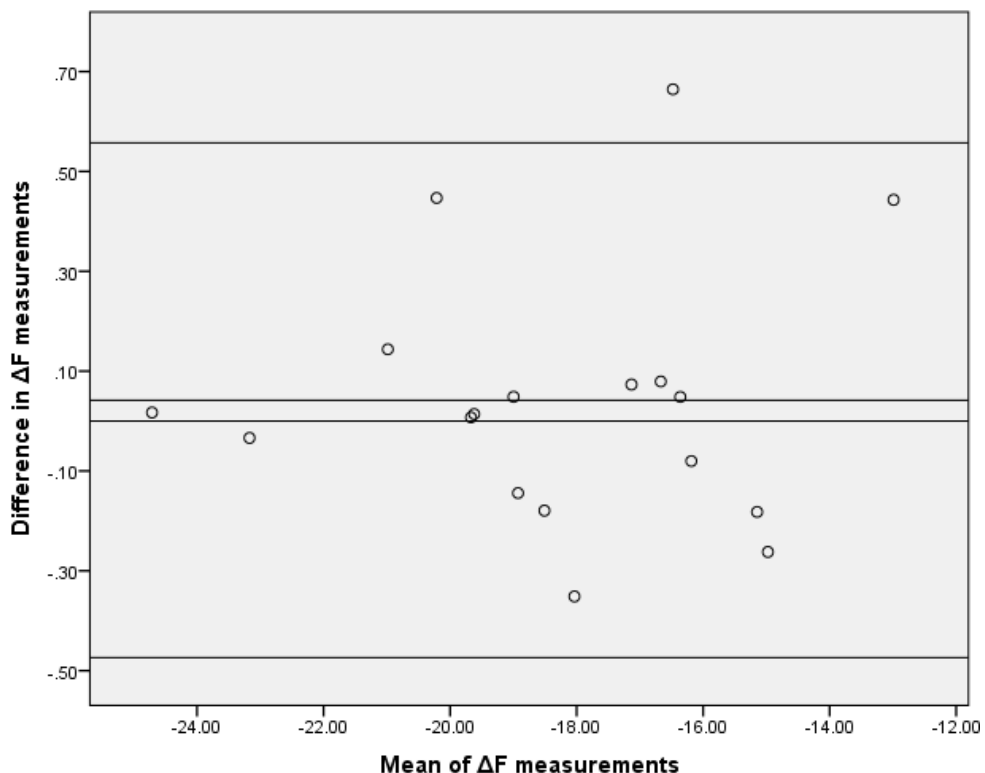
\*. The mean difference is significant at the 0.05 level.

#### 4.1.1.3 Intra-examiner reproducibility for $\Delta F$

The intra-examiner reproducibility was tested using the Bland-Altman plot (Figure 4-6). 18 enamel slabs (15%) were randomly selected and re-analysed. The mean of the differences or the bias was 0.04% which is very close to 0 indicating that there was a good level of agreement. The 95% limits of agreement was 0.56, -0.47, this variation was not considered clinically important and therefore the intra-examiner reproducibility was considered acceptable.

The Intra-class Correlation Coefficient (Table 4-7) was found to be (0.99) which also represent a very good reproducibility.

**Figure 4-6: Bland-Altman plot for reproducibility of  $\Delta F$  measurements at the end of the experiment.**



**Table 4-7: Intra-class Correlation Coefficient for  $\Delta F$  measurements.**

	Intra-class Correlation	95% Confidence Interval		F Test with True Value 0			
		Lower Bound	Upper Bound	Value	df1	df2	Sig
Single Measures	.995	.986	.998	415.576	15	15	.000
Average Measures	.998	.993	.999	415.576	15	15	.000

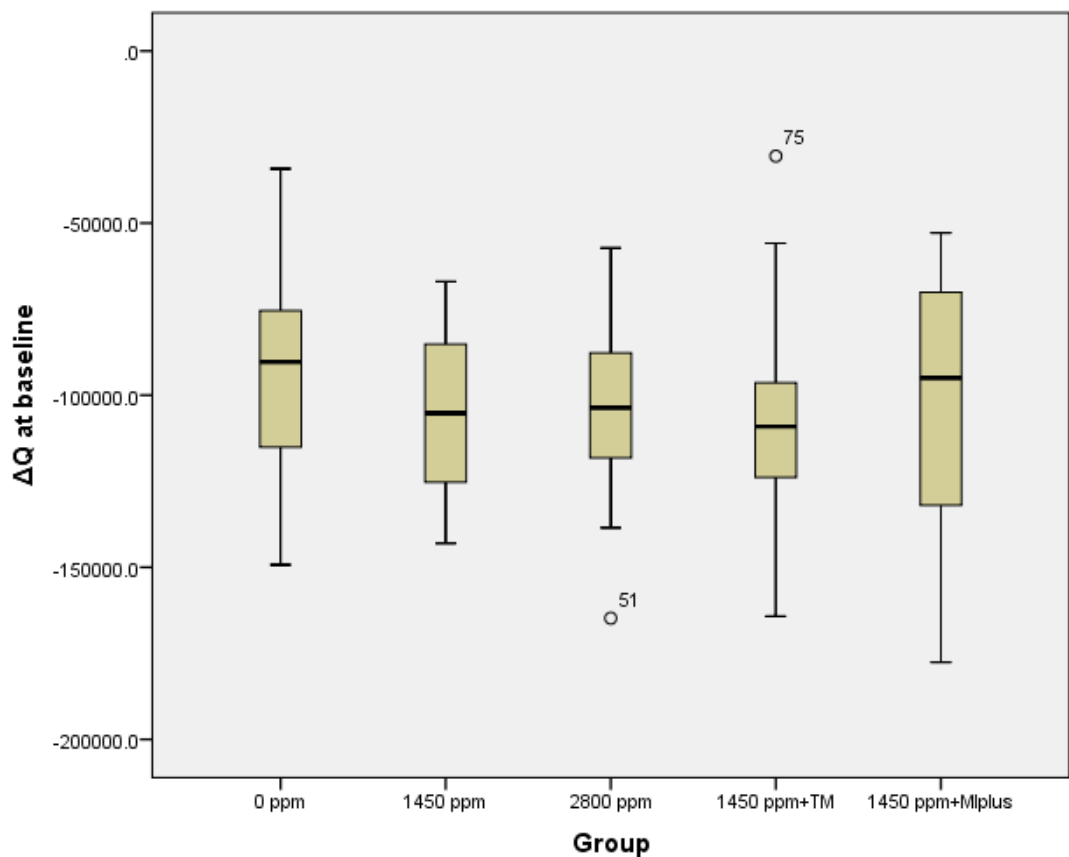


#### 4.1.2 $\Delta Q$ : $\Delta F$ times the Area. Percentage fluorescence loss with respect to the fluorescence of sound tissue times the area. Lesion volume

Normality tests (Shapiro-Wilk test and Kolmogorov-Smirnov test) were done for the  $\Delta Q$  values at baseline and showed that the data were normally distributed for all groups (Appendix 4).

The boxplot (Figure 4-7) for the distribution of the  $\Delta Q$  at the baseline showed that the median for all groups were close, and two outliers can be seen in 2800 ppm F and 1450 ppm F + TM groups.

**Figure 4-7: Boxplot for the distribution of the  $\Delta Q$  values at baseline for all groups.**



One way ANOVA test (Table 4-8) was performed to assess if there was any statistically significant difference in  $\Delta Q$  at the baseline between the lesions assigned to the five groups. No statistically significant difference was found.

**Table 4-8: One way ANOVA results for  $\Delta Q$  values at baseline.**

	Sum of Squares	df	Mean Square	F	Sig.
<b>Between Groups</b>	2324304435.923	4	581076108.981	.671	.613
<b>Within Groups</b>	99600988807.981	115	866095554.852		
<b>Total</b>	101925293243.905	119			

#### 4.1.2.1 Difference in $\Delta Q$ within each group

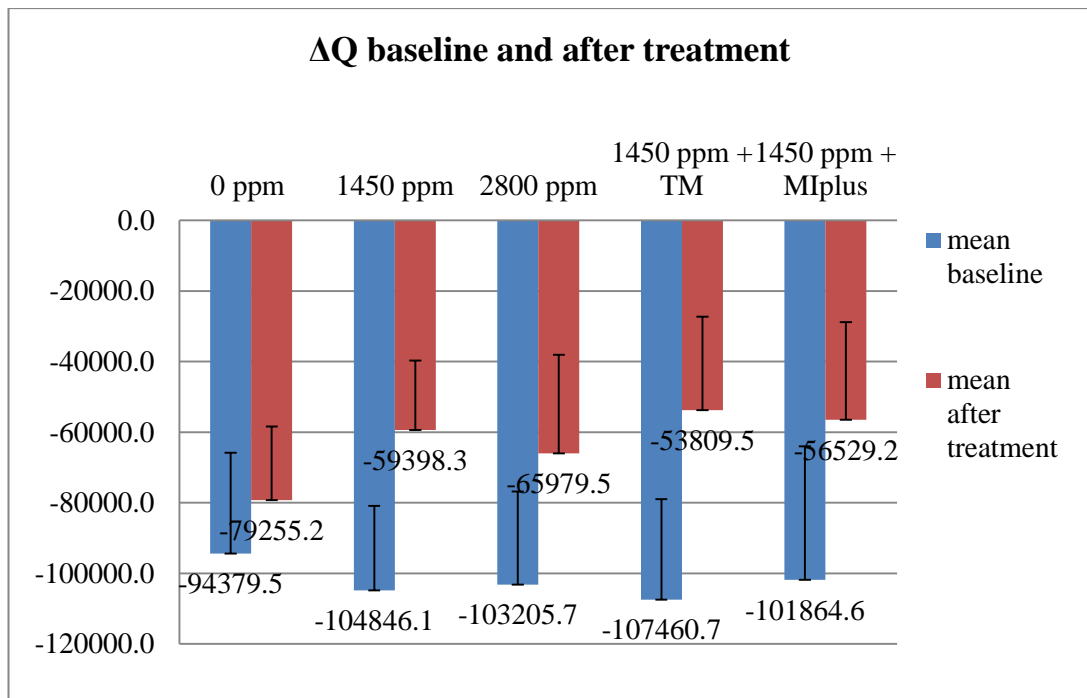
The  $\Delta Q$  mean values both at baseline and after treatment are shown in Table 4-9. It can be seen that there was an improvement in  $\Delta Q$  values for all the groups in the study.

**Table 4-9: The mean values of  $\Delta Q$  at baseline and after treatment for all groups.**

Group	Mean $\Delta Q$ at baseline $\pm$ SD	Mean $\Delta Q$ after treatment $\pm$ SD	Mean Difference in $\Delta Q$ at baseline and after treatment $\pm$ SD
<b>0 ppm</b>	-94379.5 $\pm$ 28516.7	-79255.2 $\pm$ 20899.1	15124.3 $\pm$ 25091.00
<b>1450 ppm</b>	-104846.1 $\pm$ 23932.3	-59398.3 $\pm$ 19694.7	45447.8 $\pm$ 17787.43
<b>2800 ppm</b>	-103205.7 $\pm$ 26417.8	-65979.5 $\pm$ 27936.8	37226.3 $\pm$ 22747.10
<b>1450 ppm + TM</b>	-107460.7 $\pm$ 28483.4	-53809.5 $\pm$ 26529.1	53651.2 $\pm$ 25712.28
<b>1450 ppm + MIplus</b>	-101864.6 $\pm$ 37885.5	-56529.2 $\pm$ 27745.8	45335.4 $\pm$ 22596.46

Figure 4-8 shows the change in the mean of  $\Delta Q$  at baseline and after treatment with the standard deviation for all groups.

**Figure 4-8:  $\Delta Q$  mean values at baseline and after treatment.**



To assess whether the change in  $\Delta Q$  at baseline and after treatment was significantly different within the same group, paired T-Test was carried out and the results (Table 4-10) showed that there was a statistically significant improvement in the  $\Delta Q$  values after treatment compared with that at baseline in all groups ( $p < 0.01$ ).

**Table 4-10: Paired sampled T-Test results for  $\Delta Q$  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	
<b>0 ppm</b>	$\Delta Q$ at baseline – $\Delta Q$ after treatment	-15124.3	25091.0	5121.7	-25719.3	-4529.3	<b>.007*</b>
<b>1450 ppm</b>		-45447.8	17787.4	3630.8	-52958.8	-37936.9	<b>.000*</b>
<b>2800 ppm</b>		-37226.3	22747.1	4643.2	-46831.5	-27621.0	<b>.000*</b>
<b>1450 ppm+TM</b>		-53651.2	25712.3	5248.5	-64508.5	-42793.8	<b>.000*</b>
<b>1450 ppm + MIplus</b>		-45335.4	22596.5	4612.5	-54877.0	-35793.7	<b>.000*</b>

\* Statistically significant

#### 4.1.2.2 Difference in $\Delta Q$ between groups

The  $\Delta Q$  difference (change) was measured using the following formula:

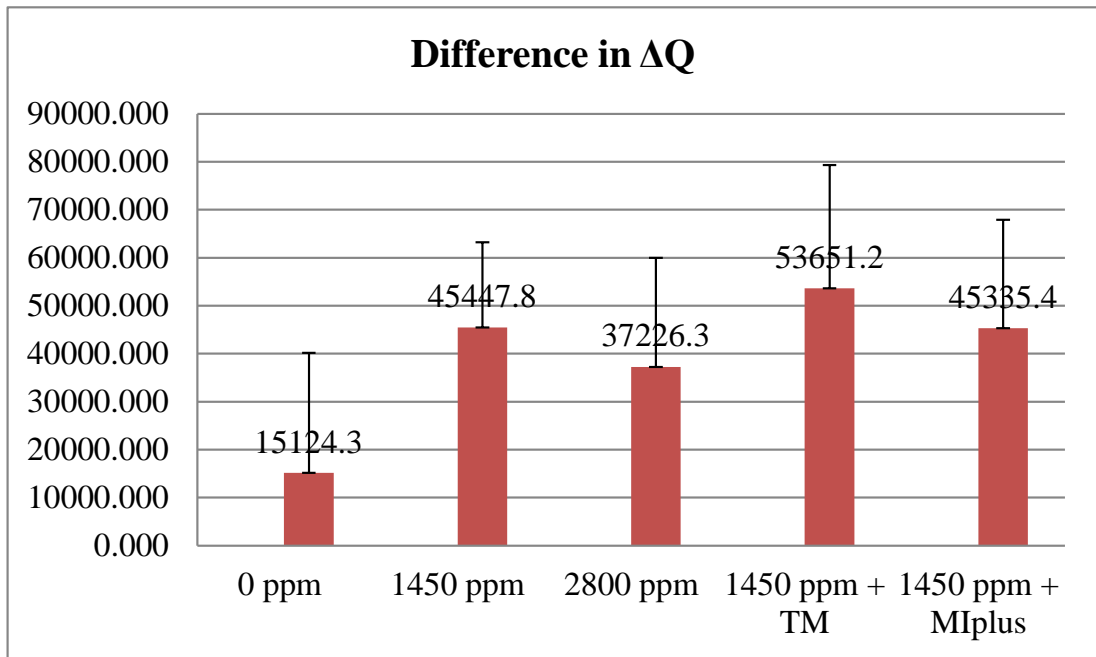
$$\text{Difference in } \Delta Q = \Delta Q \text{ after treatment} - \Delta Q \text{ at baseline}$$

##### Descriptive statistics

Figure 4-9 shows the difference in  $\Delta Q$  in the five tested groups. In all groups the difference in  $\Delta Q$  was positive, meaning that there was decrease in  $\Delta Q$  after treatment compared to that at baseline.

The highest reduction in  $\Delta Q$  was seen in the 1450 ppm F + TM group with mean difference of  $53651.2 \pm 25712.28$ , followed by the 1450 ppm F with mean difference of  $45447.8 \pm 17787.43$ . The least reduction was noticed in the 0 ppm F group with mean difference of  $15124.3 \pm 25091.00$ . The 1450 ppm F + MIplus and the 2800 ppm F groups also show reduction in the  $\Delta Q$  values with mean difference of  $45335.4 \pm 22596.46$  and  $37226.3 \pm 22747.10$  respectively (Table 4-11).

**Figure 4-9: Means of the difference in  $\Delta Q$  at baseline and after treatment of all groups.**



**Table 4-11: Descriptive statistics for the difference in  $\Delta Q$  at baseline and after treatment for all groups.**

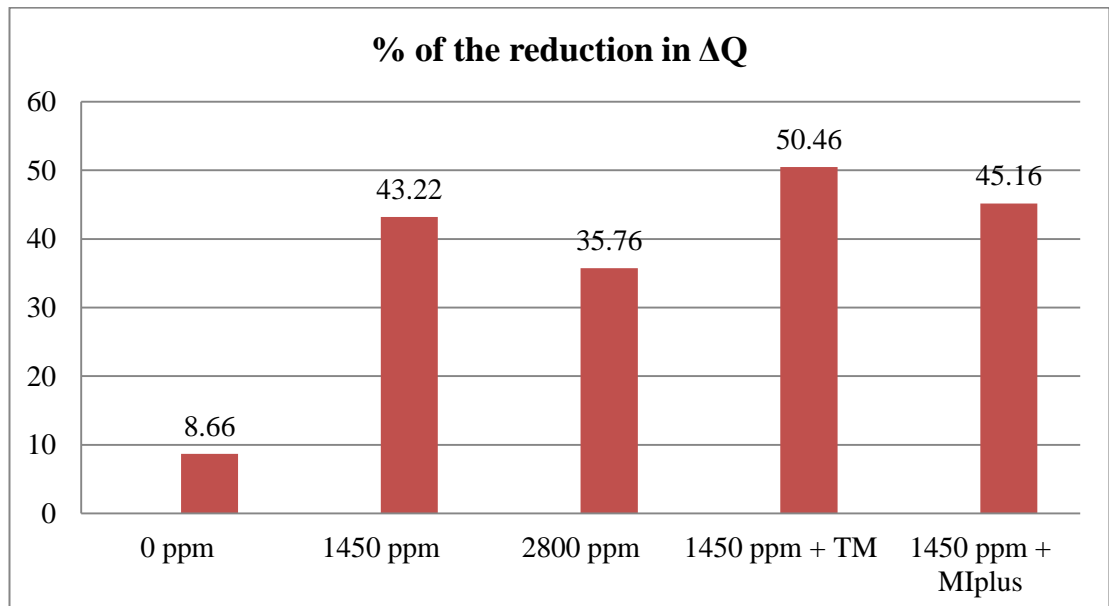
group	N	Minimum	Maximum	Mean	Median	Std. Deviation
<b>0ppm</b>	24	-45843.9	65353.6	15124.3	17391.6	25091.0
<b>1450ppm</b>	24	20777.1	77293.1	45447.8	40542.1	17787.4
<b>2800ppm</b>	24	-1802.8	88930.1	37226.3	35997.3	22747.1
<b>1450ppm+TM</b>	24	13234.7	107616.9	53651.2	48669.3	25712.3
<b>1450ppm+TM<sup>+</sup></b>	24	13391.3	94102.8	45335.4	43069.4	22596.5

The percentage of the changes in  $\Delta Q$  at baseline and after treatment (% Q) was calculated using the following formula:

$$\text{(Difference in } \Delta Q \text{ at baseline and after treatment / } \Delta Q \text{ at baseline)} \times 100$$

Figure 4-10 shows the % Q values for all groups. % Q value was the highest for the 1450 ppm F + TM with % Q of 50.46%, followed by that for 1450 ppm F + MIplus and 1450 ppm F (45.16 and 43.22% respectively). The 2800 ppm F had % Q value of 35.76% and the least was observed for 0 ppm F with 8.66%.

**Figure 4-10: The % Q values for all groups.**

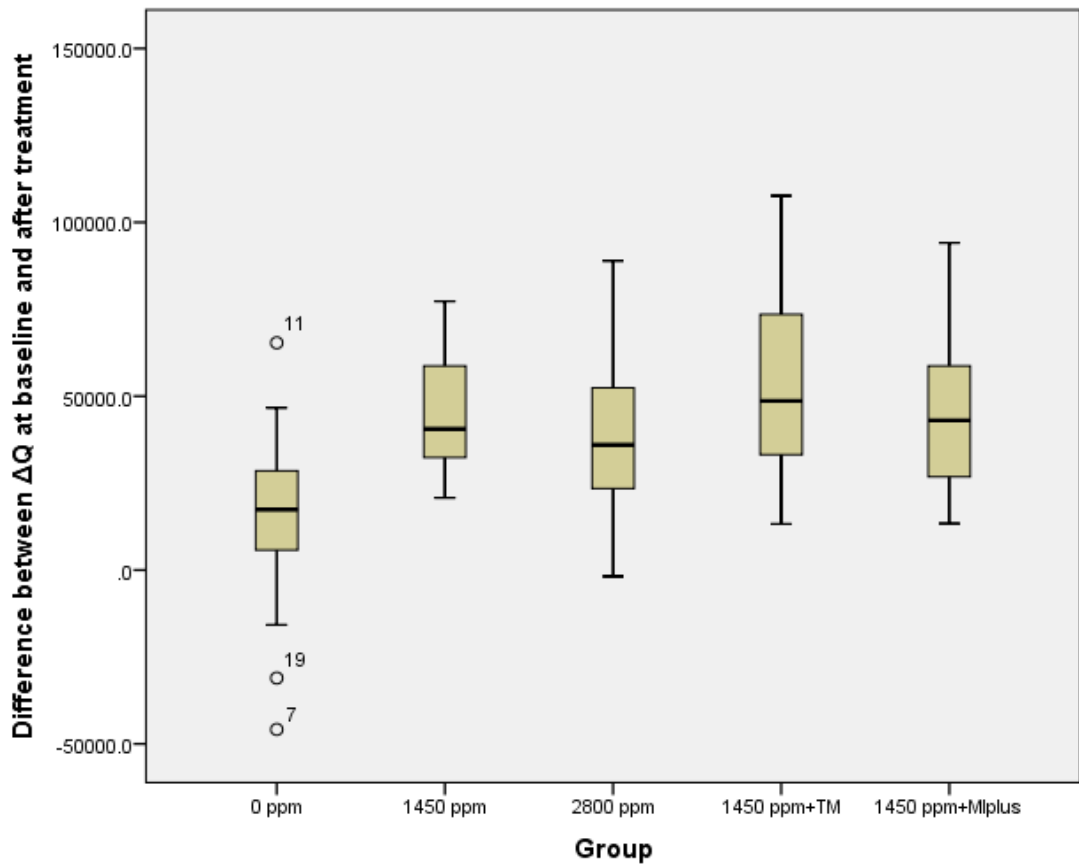


#### Determination of the normality of the data

Shapiro-Wilk test and Kolmogorov-Smirnov test (Appendix 5) was carried out to check if the difference in  $\Delta Q$  at baseline and after treatment was normally distributed. The data were considered normally distributed, as they were not statistically significant.

The boxplot (Figure 4-11) of difference in  $\Delta Q$  at baseline and after treatment shows that the medians are close in the 1450 ppm F, 2800 ppm F, 1450 ppm F + TM and 1450 ppm F + MIplus groups, whereas the median value was lowest in the 0 ppm F group, which also had three outliers.

**Figure 4-11: Boxplot for the difference in  $\Delta Q$  at baseline and after treatment for all groups.**





One way ANOVA test (Table 4-12) was performed to assess if the difference in  $\Delta Q$  was statistically significant between the five groups. It showed that the mean difference in  $\Delta Q$  was statistically significant between the groups ( $p < 0.001$ ).

**Table 4-12: One way ANOVA between groups for the difference in  $\Delta Q$  at baseline and after treatment.**

	Sum of Squares	df	Mean Square	F	Sig.
<b>Between Groups</b>	20854236820.3	4	5213559205.1	9.893	<b>.000</b>
<b>Within Groups</b>	60607363029.0	115	527020548.1		
<b>Total</b>	81461599849.4	119			

In order to determine which groups were statistically significant different, pairwise comparisons were conducted using Bonferroni test. The Bonferroni tests corrects for multiple testing. The results of the Bonferroni tests are shown in Table 4-13. It can be seen that the mean difference in  $\Delta Q$  of the 0 ppm F was statistically significant lower compared to all groups ( $p < 0.05$ ).

**Table 4-13: Multiple comparisons of the difference in  $\Delta Q$  at baseline and after treatment between all test groups and control.**

(I) Group No	(J) Group No	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
<b>0 ppm</b>	1450ppm	-30323.5	6627.1	.000*	-49291.6	-11355.4
	2800ppm	-22102.0	6627.1	.011*	-41070.0	-3133.9
	1450ppm+TM	-38526.9	6627.1	.000*	-57495.0	-19558.8
	1450ppm+MIplus	-30211.1	6627.1	.000*	-49179.2	-11243.0
<b>1450 ppm</b>	0ppm	30323.5	6627.1	.000*	11355.4	49291.6
	2800ppm	8221.6	6627.1	1.000	-10746.5	27189.7
	1450ppm+TM	-8203.3	6627.1	1.000	-27171.4	10764.8
	1450ppm+MIplus	112.5	6627.1	1.000	-18855.6	19080.5
<b>2800 ppm</b>	0ppm	22102.0	6627.1	.011*	3133.9	41070.0
	1450ppm	-8221.6	6627.1	1.000	-27189.7	10746.5
	1450ppm+TM	-16424.9	6627.1	.146	-35393.0	2543.2
	1450ppm+MIplus	-8109.1	6627.1	1.000	-27077.2	10859.0
<b>1450 ppm + TM</b>	0ppm	38526.9	6627.1	.000*	19558.8	57495.0
	1450ppm	8203.3	6627.1	1.000	-10764.8	27171.4
	2800ppm	16424.9	6627.1	.146	-2543.2	35393.0
	1450ppm+MIplus	8315.8	6627.1	1.000	-10652.3	27283.9
<b>1450 ppm + MIplus</b>	0ppm	30211.1	6627.1	.000*	11243.0	49179.1
	1450ppm	-112.5	6627.1	1.000	-19080.5	18855.6
	2800ppm	8109.1	6627.1	1.000	-10859.0	27077.2
	1450ppm+TM	-8315.8	6627.1	1.000	-27283.9	10652.3

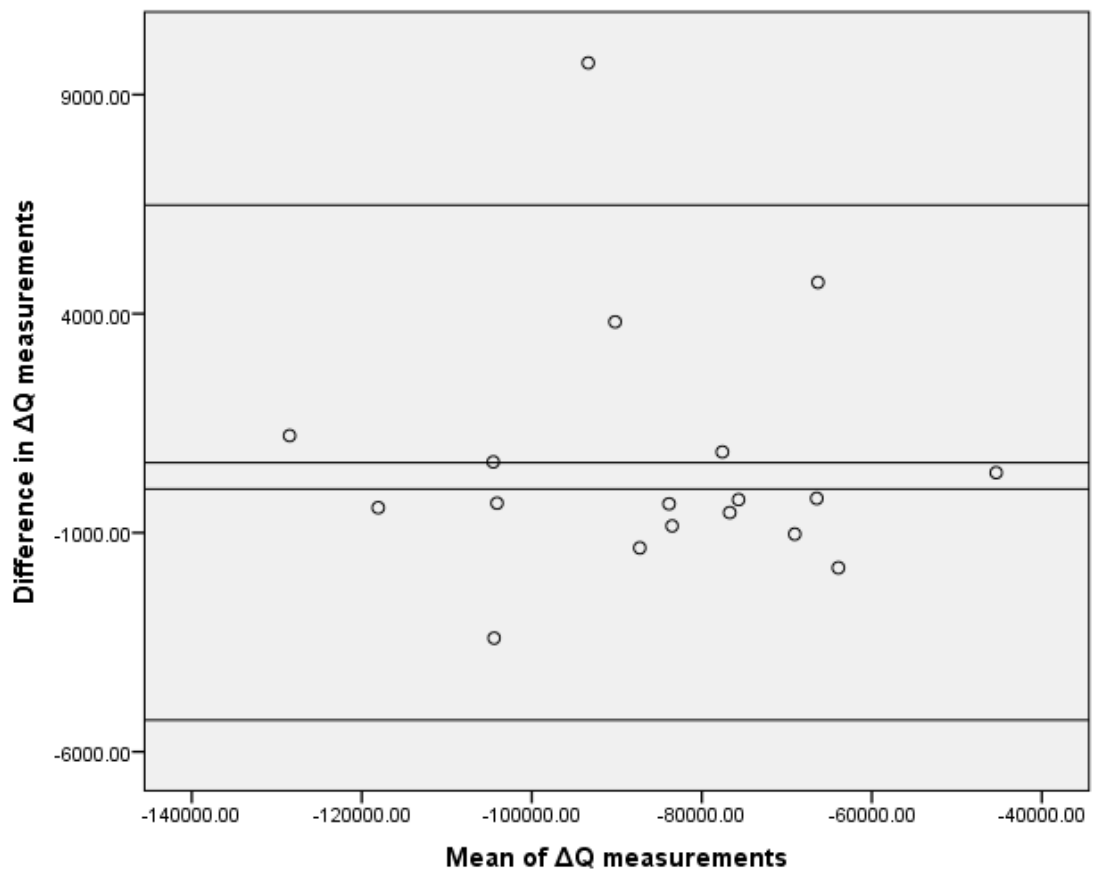
\*. The mean difference is significant at the 0.05 level.

### 4.1.2.3 Intra-examiner reproducibility for $\Delta Q$

The intra-examiner reproducibility was tested using the Bland-Altman plot (Figure 4-12). 18 enamel slabs (15%) were randomly selected and re-analysed. The mean of the differences or the bias was 599.1 $\mu\text{m}^2$ . The 95% limits of agreement were 6477.3, -5279.0 this variation was not considered clinically important and therefore the intra-examiner reproducibility was considered acceptable.

The Intra-class Correlation Coefficient (Table 4-14) was found to be (0.99) which also represent a very good reproducibility.

**Figure 4-12: Bland-Altman plot for reproducibility of  $\Delta Q$  measurements.**



**Table 4-14: Intra-class Correlation Coefficient for  $\Delta Q$  measurements.**

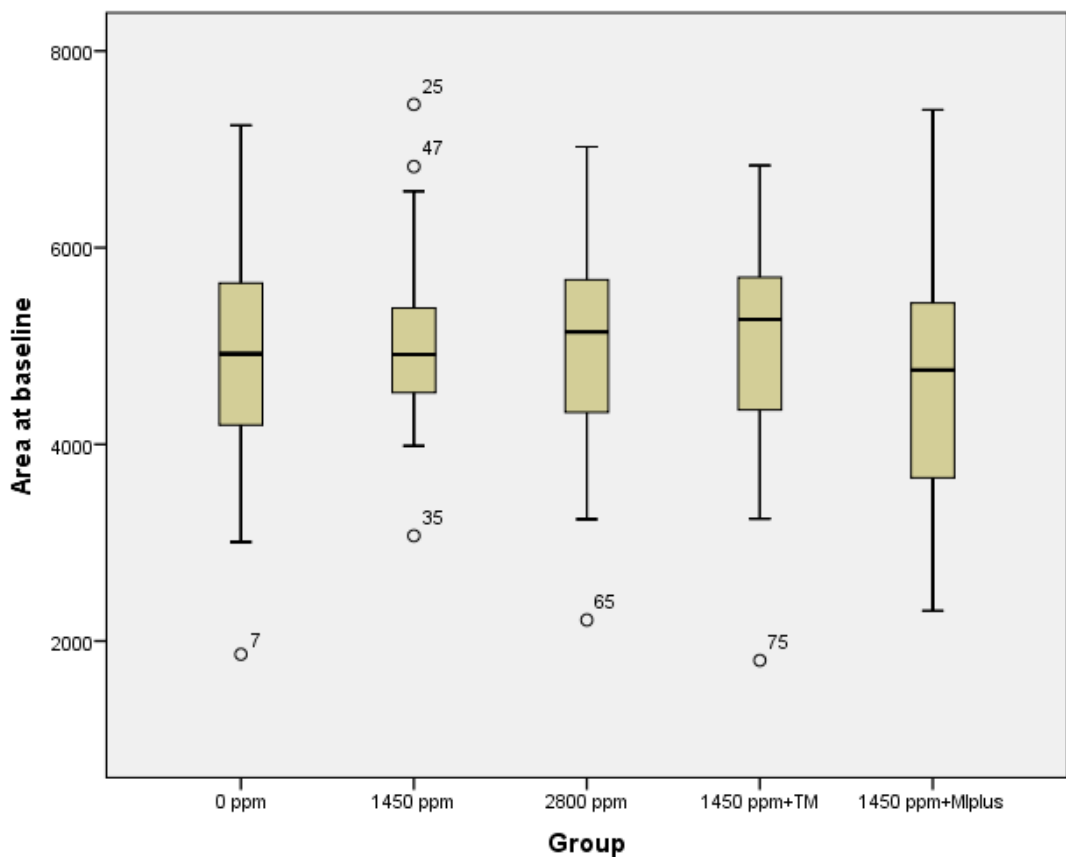
	Intra-class Correlation	95% Confidence Interval		F Test with True Value 0			
		Lower Bound	Upper Bound	Value	df1	df2	Sig
Single Measures	.985	.957	.995	129.212	15	15	.000
Average Measures	.992	.978	.997	129.212	15	15	.000

### 4.1.3 Area of the White spot lesion.

The values of the white spot lesion area at baseline for all groups were checked to see if there was a difference between the groups. The normality tests (Shapiro-Wilk test and Kolmogorov-Smirnov test) showed that the data were normally distributed (Appendix 6).

The boxplot (Figure 4-13) for the distribution of the area at the baseline showed that the 0 ppm F, 2800 ppm F and 1450 ppm F + TM groups had a comparable range with one outlier each. 1450 ppm F has the narrowest variation in the area with three outliers, and the 1450 ppm F + MIplus group had the broadest variation compared to the other groups.

**Figure 4-13: Boxplot for the distribution of the Area values at baseline for all groups.**



One way ANOVA test (Table 4-15) was performed to assess if there was any statistically significant difference in the area values at the baseline between the lesions assigned to the five groups. No statistically significant difference was found.

**Table 4-15: One way ANOVA results for area values at baseline.**

	<b>Sum of Squares</b>	<b>df</b>	<b>Mean Square</b>	<b>F</b>	<b>Sig.</b>
<b>Between Groups</b>	1979387.8	4	494846.9	.373	<b>.828</b>
<b>Within Groups</b>	152584256.0	115	1326819.6		
<b>Total</b>	154563643.8	119			

#### **4.1.3.1 Difference in lesion area within each group**

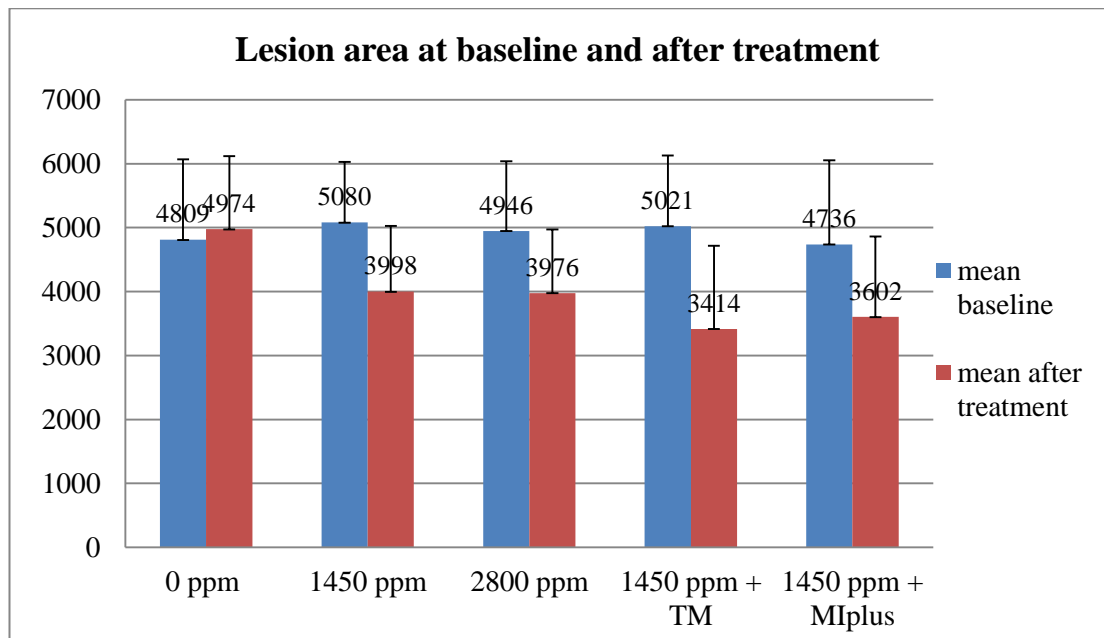
The lesion area mean values both at baseline and after treatment are shown in Table 4-16. It can be seen that there was a decrease in the lesion area for all test groups in the study, however the lesion area increased in the negative control group (0 ppm F).

**Table 4-16: The mean values of lesion area at baseline and after treatment for all groups.**

Group	Mean area at baseline $\pm$ SD	Mean area after treatment $\pm$ SD	Mean Difference in area at baseline and after treatment $\pm$ SD
<b>0 ppm</b>	4809 $\pm$ 1257.29	4974 $\pm$ 1145.66	165 $\pm$ 1129.03
<b>1450 ppm</b>	5080 $\pm$ 949.18	3998 $\pm$ 1027.02	-1082 $\pm$ 746.53
<b>2800 ppm</b>	4946 $\pm$ 1089.81	3976 $\pm$ 994.36	-971 $\pm$ 1018.20
<b>1450 ppm + TM</b>	5021 $\pm$ 1109.12	3414 $\pm$ 1304.13	-1607 $\pm$ 1292.69
<b>1450 ppm + MIplus</b>	4736 $\pm$ 1317.02	3602 $\pm$ 1259.90	-1134 $\pm$ 630.32

Figure 4-14 shows the change in the mean of area at baseline and after treatment with the standard deviation for all groups.

**Figure 4-14: Lesion area at baseline and after treatment for all groups.**



To assess whether the change in the area at baseline and after treatment was significantly different within the same group, paired T-Test was used.

The paired T-Test results shown in Table 4-17 showed that there was a statistically significant improvement in the lesion area values after treatment compared with that at baseline in all groups ( $p < 0.001$ ), except in the 0 ppm F group.

**Table 4-17: 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	
<b>0 ppm</b>	Area at baseline – Area after treatment	-165.2	1129.0	230.5	-641.9	311.6	<b>.481</b>
<b>1450 ppm</b>		1081.7	746.5	152.3	766.5	1396.9	<b>.000*</b>
<b>2800 ppm</b>		970.8	1018.2	207.8	540.8	1400.7	<b>.000*</b>
<b>1450 ppm + TM</b>		1607.3	1292.7	263.7	1061.4	2153.1	<b>.000*</b>
<b>1450 ppm + MIplus</b>		1133.9	630.3	128.7	867.8	1400.1	<b>.000*</b>

\*Statistically significant



#### 4.1.3.2 Difference in lesion area between groups

The lesion area difference (change) was measured using the following formula:

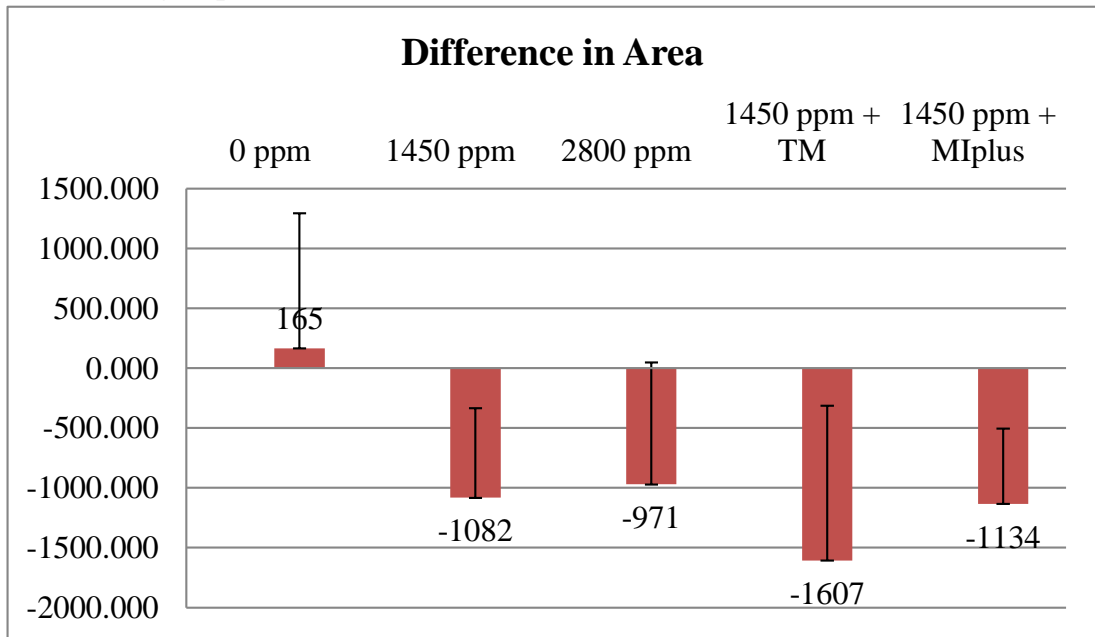
$$\text{Difference in lesion area} = \text{lesion area after treatment} - \text{lesion area at baseline}$$

##### Descriptive statistics

Figure 4-15 shows the difference in the lesion area for the five tested groups. In the 1450 ppm F, 2800 ppm F, 1450 ppm F +TM and the 1450 ppm F + MIplus groups, the difference in the lesion area was negative, meaning that there was a decrease in the lesion area after treatment compared to that at baseline. While in the 0 ppm F group, the value was positive meaning that there was increase in the area of the lesion after treatment.

The highest reduction in area was seen in the 1450 ppm F + TM group with mean difference of  $1607.00 \pm 1292.69$ , followed by the 1450 ppm F + MIplus group with mean difference of  $1134.00 \pm 630.32$ . The 1450 ppm F and the 2800 ppm F groups also showed a reduction in the area values with mean difference of  $1082.00 \pm 746.53$  and  $971.00 \pm 1018.20$  respectively. An increase in the area was noticed in the 0 ppm F group with a mean difference of  $165.00 \pm 1129.03$  (Table 4-18).

**Figure 4-15: Means of the difference in the lesion area at baseline and after treatment of all tested groups.**



**Table 4-18: Descriptive statistics for the difference in the lesion area at baseline and after treatment for all groups.**

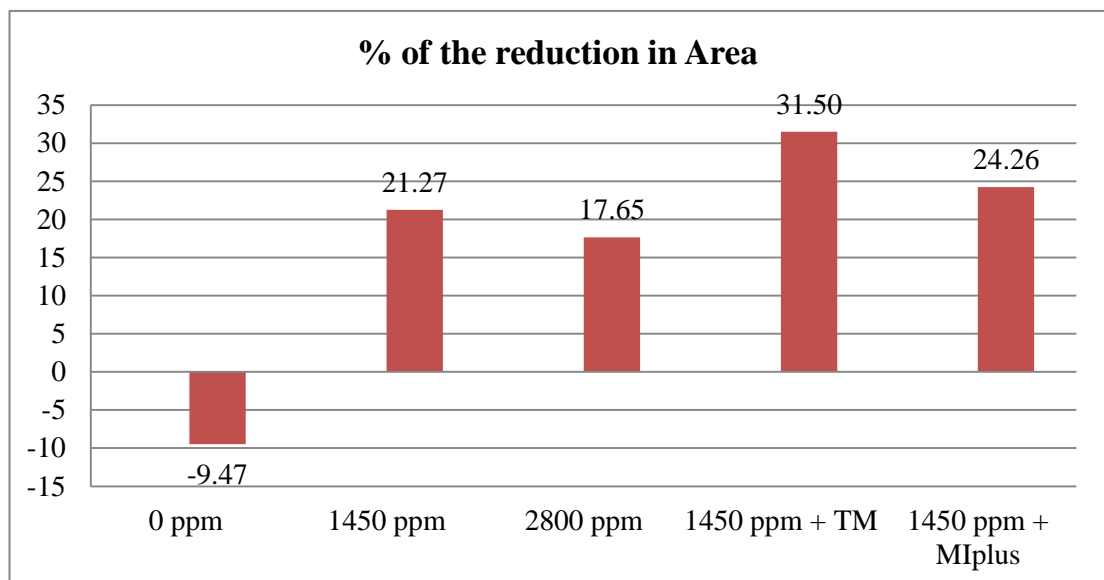
group	N	Minimum	Maximum	Mean	Median	Std. Deviation
<b>0ppm</b>	24	-3090	2465	165.17	-29.50	1129.0
<b>1450ppm</b>	24	-3466	-202	-1081.7	-1044.5	746.5
<b>2800ppm</b>	24	-3710	573	-970.8	-491.5	1018.2
<b>1450ppm+TM</b>	24	-5272	44	-1607.3	-1190.5	1292.7
<b>1450ppm+TM<sup>+</sup></b>	24	-2810	-10	-1133.9	-1149.0	630.3

The percentage change in lesion area at baseline and after treatment (% Area) was calculated using the following formula:

$$\text{(Difference in area at baseline and after treatment / area at baseline)} \times 100$$

Figure 4-16 shows the % Area values for all groups, % Area value was the highest for the 1450 ppm F + TM at 31.50%, followed by that for 1450 ppm F + MIplus, 24.26%, and 1450 ppm F, 21.27%. The 2800 ppm F had % Area value of 17.65 % while 0 ppm F produced a negative % Area value of -9.47 %.

**Figure 4-16: The % F values for all groups.**

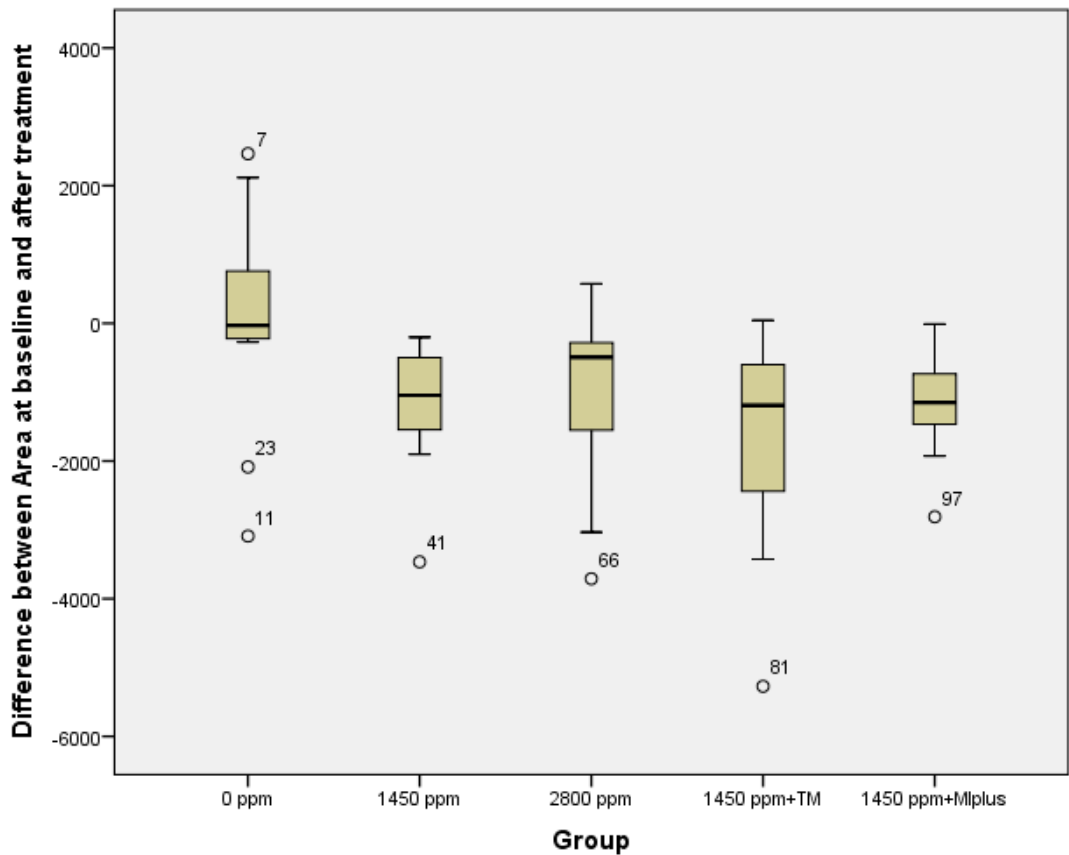


#### Determination of the normality of the data

In order to check if the differences in the lesion area at baseline and after treatment are normally distributed, data normality test was carried out. This included Shapiro-Wilk test and Kolmogorov-Smirnov test. The data were considered not normally distributed, as they were some data that are statistically significant (Appendix 7).

The boxplot (Figure 4-17) of difference in the lesion area at baseline and after treatment shows skewness of the data in the 0 ppm F group. The medians are close in the 1450 ppm F, 1450 ppm F + TM and 1450 ppm F + MIplus groups, whereas the median value was highest in the 0 ppm F group which also had three outliers whereas the other groups have one outlier each.

**Figure 4-17: Boxplot for the difference in the lesion area at baseline and after treatment for all groups.**



Nonparametric test (Kruskal-Wallis Test) was performed to assess if the difference in the lesion area is statistically significant between the five groups (Table 4-19). It showed that the mean difference in area was statistically significant between the groups ( $p < 0.001$ ).

**Table 4-19: Kruskal-Wallis Test results for the difference in the lesion area at baseline and after treatment.**

	<b>Difference between Area at baseline and after treatment</b>
<b>Chi-Square</b>	37.938
<b>df</b>	4
<b>Asymp. Sig.</b>	<b>.000</b>
a. Kruskal Wallis Test	
b. Grouping Variable: Group No	

In order to determine which groups were statistically significant different, two-independent sample test using Mann-Whitney U test was performed. The test showed that the mean difference in the lesion area of the 0 ppm F group was statistically significant lower compared to all groups ( $p < 0.001$ ).

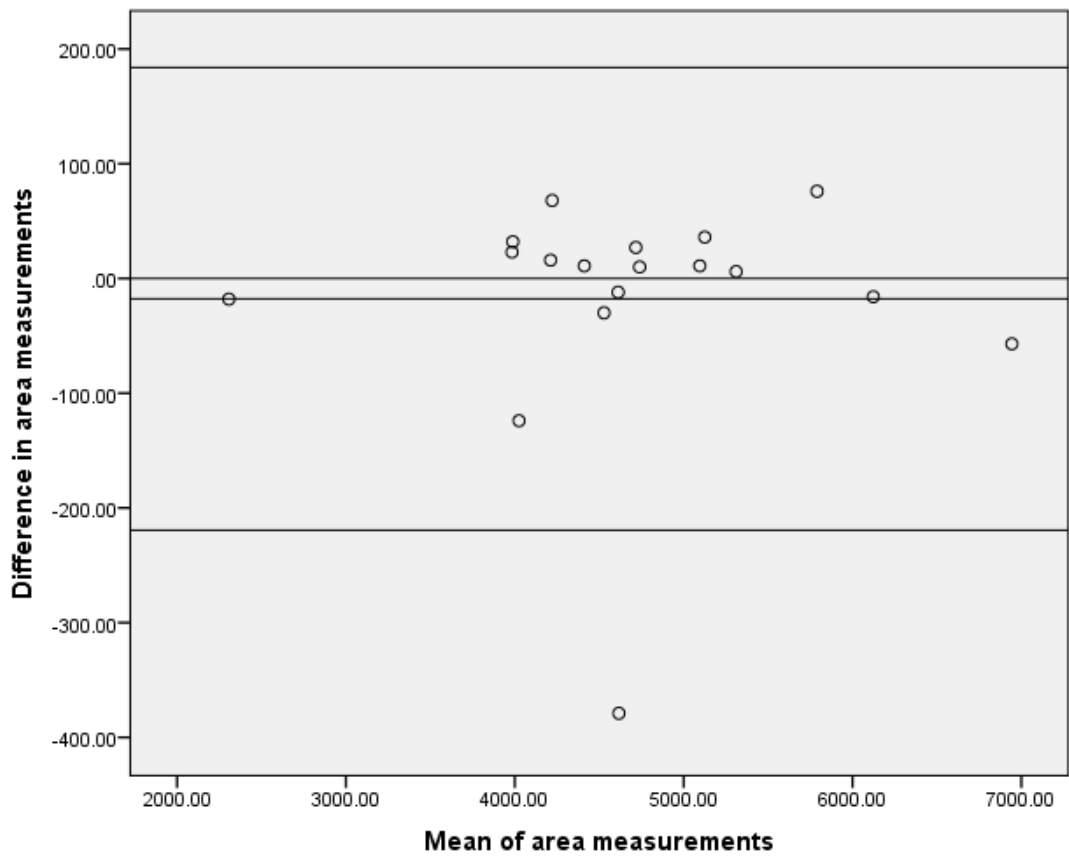
No other statistically significant differences were found between the other groups ( $p > 0.05$ ).

#### **4.1.3.3 Intra-examiner reproducibility for lesion area**

The intra-examiner reproducibility was tested using the Bland-Altman plot (Figure 4-18). 18 enamel slabs (15%) were randomly selected and re-analysed. The mean of the differences or the bias was  $-17.78 \text{ px}^2$ . The 95% limits of agreement were 183.7, -219.4 this variation was not considered clinically important indicating good agreement between the readings.

The Intra-class Correlation Coefficient (Table 4-20) was found to be (0.99) which also represent a very good reproducibility.

**Figure 4-18: Bland-Altman plot for reproducibility of lesion area measurements.**



**Table 4-20: Intra-class Correlation Coefficient for lesion area measurements.**

	Intraclass Correlation	95% Confidence Interval		F Test with True Value 0			
		Lower Bound	Upper Bound	Value	df1	df2	Sig
Single Measures	.992	.977	.997	246.267	15	15	.000
Average Measures	.996	.988	.999	246.267	15	15	.000

#### **4.1.4 Summary of the results for all three parameters.**

**The  $\Delta F$  results:** the results showed that there was statistically significant improvement in the  $\Delta F$  values between the baseline and after treatment for all groups. The greatest improvement was seen in 1450 ppm F + TM group followed by that for the 1450 ppm F + MIplus, 1450 ppm F and 2800 ppm F groups. The least improvement in the  $\Delta F$  values was produced by the 0 ppm F group.

The difference in  $\Delta F$  at baseline and after treatment in 1450 ppm F + TM, 1450 ppm F + MIplus and 1450 ppm F groups was statistically significant higher than that for the 0 ppm F group. No statistical significant difference was found between the 2800 ppm F and the 0 ppm F groups.

**The  $\Delta Q$  results:** A statistically significant improvement in the  $\Delta Q$  values between the baseline and after treatment was found in all groups. The highest improvement was achieved by the 1450 ppm F + TM group followed by that for 1450 ppm F, 1450 ppm F + MIplus and 2800 ppm F groups. The least improvement was produced by the 0 ppm F group.

The difference in  $\Delta Q$  at baseline and after treatment in all test groups was statistically significant higher than that for the 0 ppm F group.

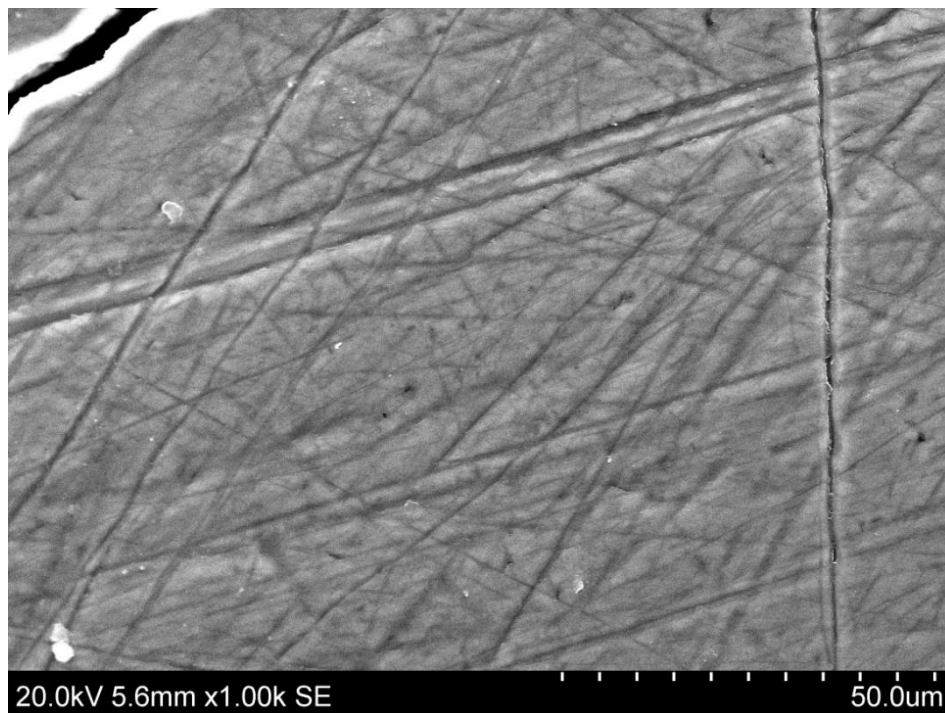
**For the lesion area results:** A reduction in the area of the white spot lesion was seen in the 1450 ppm F + TM, 1450 ppm F + MIplus, 1450 ppm F and 2800 ppm F groups, while an increase in the lesion area was the case in the 0 ppm F group.

The difference in the lesion area at baseline and after treatment was statistically significant for the test groups compared with the 0 ppm F group. No significant difference was found between the test groups.

## 4.2 Scanning Electron Microscopy (SEM) results

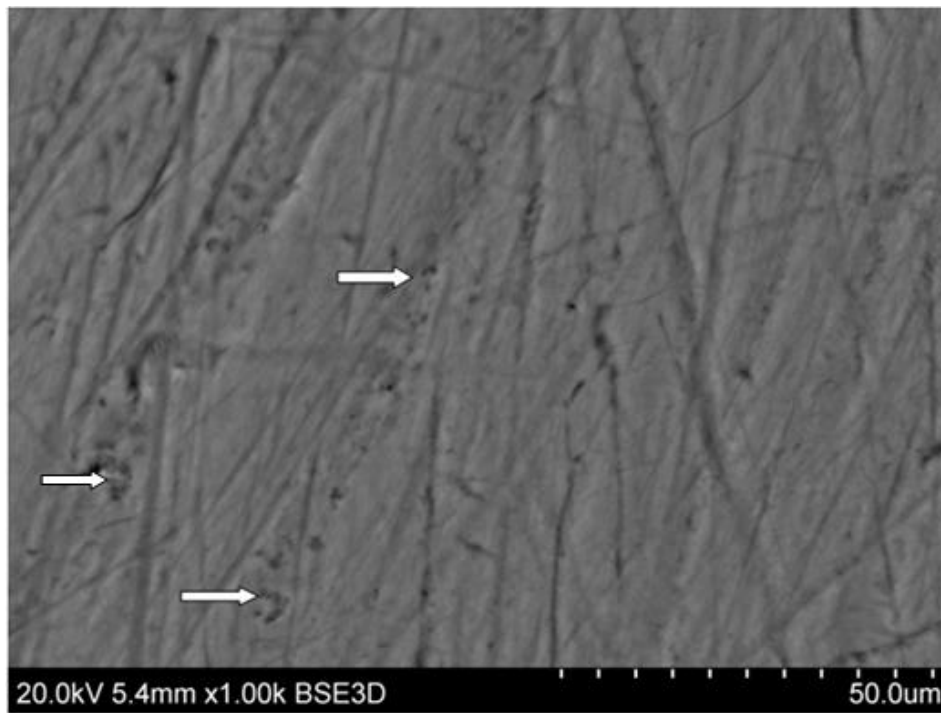
In order to further investigate the results seen in the previous section, especially in relation to the groups containing TM and MI paste plus, SEM images are presented here for these groups after 21 days of pH cycling.

**Figure 4-19: SEM image for sound enamel surface shows natural surface without porosities.**

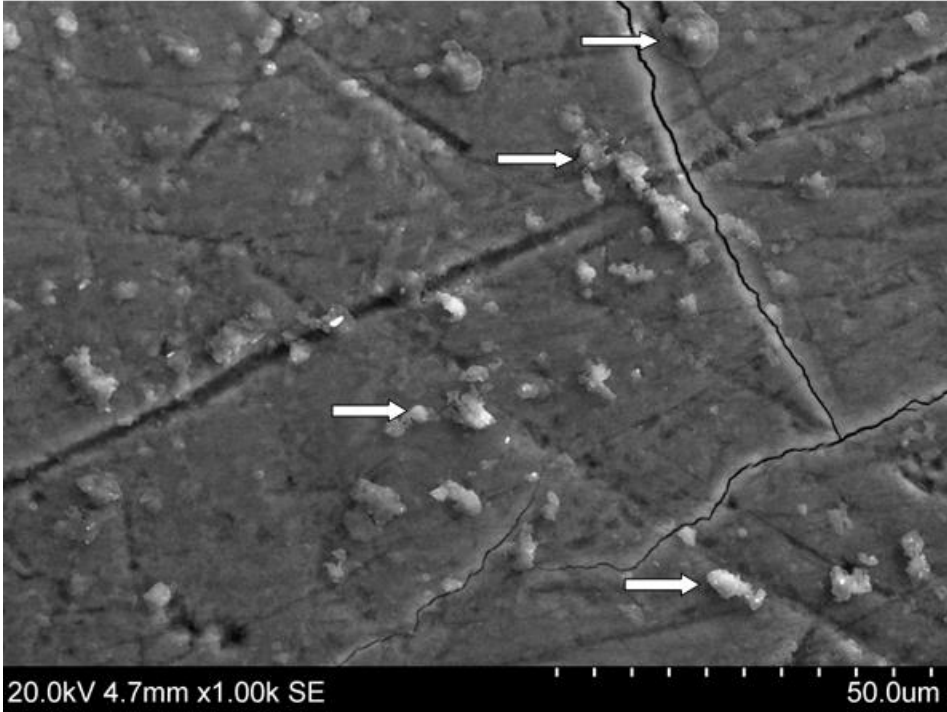




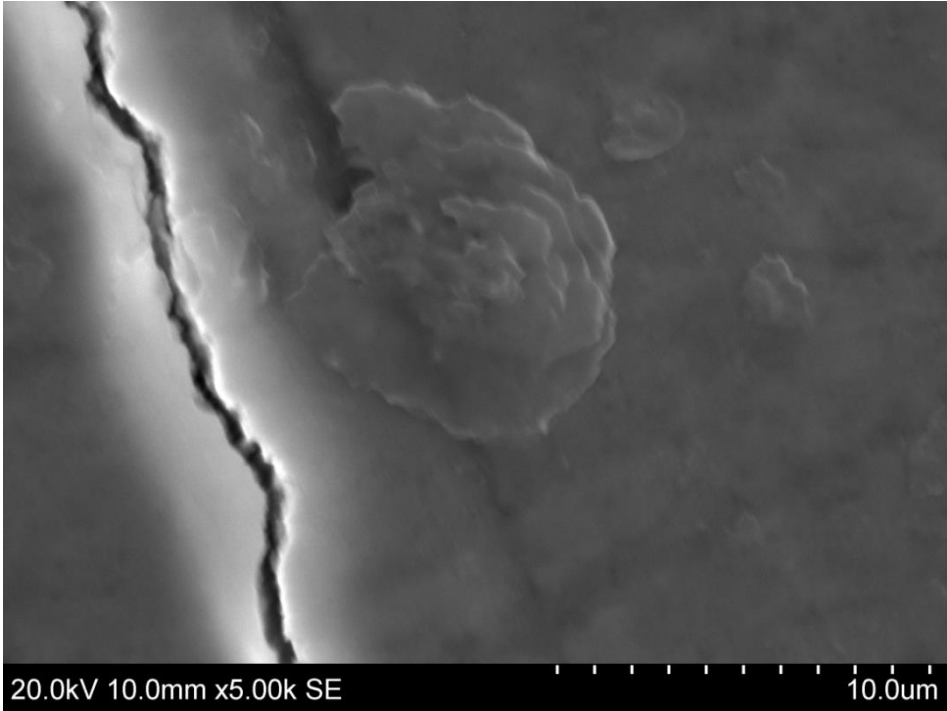
**Figure 4-20: SEM image of the demineralised enamel subsurface lesion area treated with non-fluoride toothpaste (0 ppm F) for 21 days in vitro under pH cycling. There is evidence of micro porosities possibly due to exposure to the acid gel (arrows).**



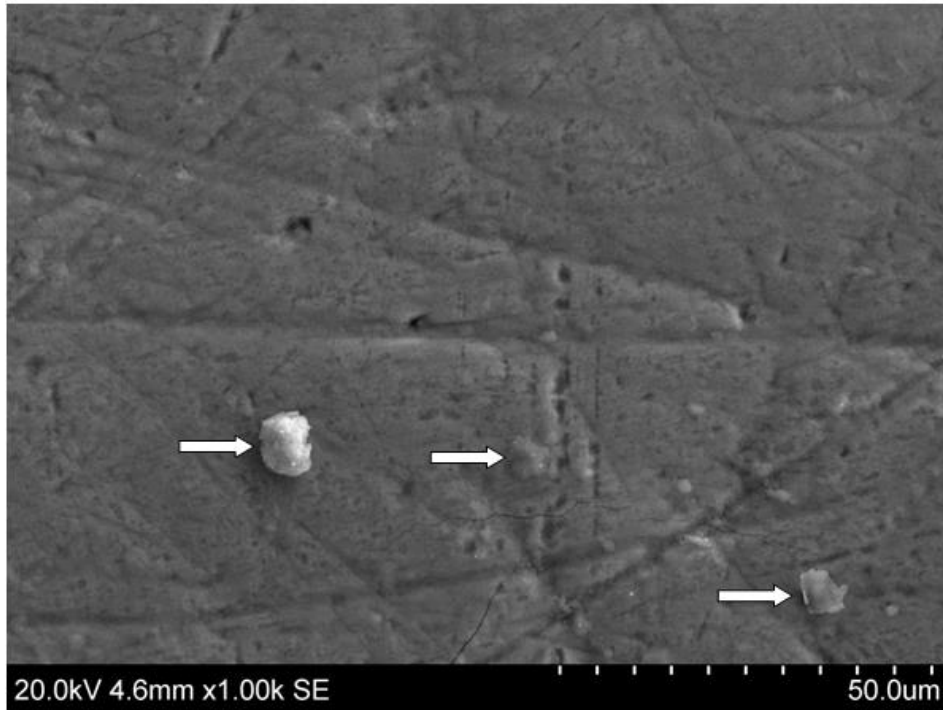
**Figure 4-21: SEM image of the demineralised enamel subsurface lesion area treated with fluoride toothpaste and Tooth Mousse (1450 ppm F + TM) for 21 days in vitro under pH cycling. Less clear evidence of micro porosities is visible. The surface is covered with amorphous deposits (arrows).**



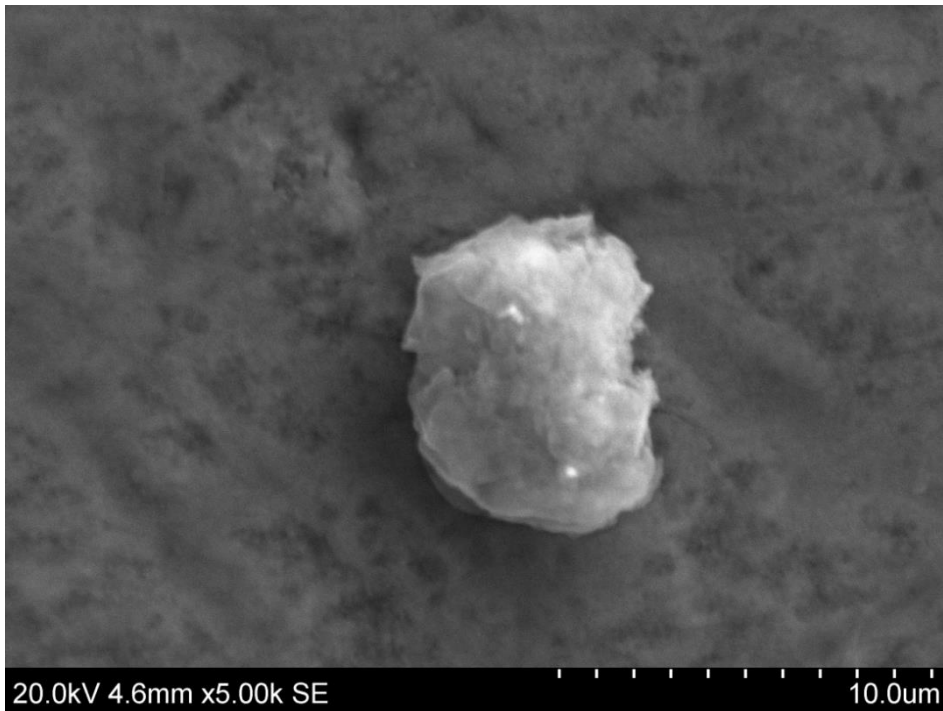
**Figure 4-22: A high magnification of one of the deposits seen in Figure 4-21 showing an amorphous structure.**



**Figure 4-23: SEM image of the demineralised enamel subsurface lesion area treated with fluoride toothpaste and MI paste plus (1450 ppm F + MIplus) for 21 days in vitro under pH cycling. Some deposits can be seen on the enamel surface (arrows).**



**Figure 4-24: A high magnification of one of the deposits seen in Figure 4-23.**



## 5.0 DISCUSSION

The potential of the remineralisation of enamel lesion has been demonstrated by Backer Dirks (1966), who noticed spontaneous disappearance of the white spot lesions in young individuals as a result of the remineralising ability of saliva. Subsequently, many studies were conducted to assess the effect of various preparations of calcium, phosphate, and fluoride on enamel remineralisation.

The search of the available literature revealed many studies including in vitro, in situ and in vivo studies that had investigated the effect of CPP-ACP, CPP-ACFP and fluoride on enamel remineralisation. In the majority of these studies the CPP-ACP and/or CPP-ACFP preparations were used alone and were compared with other remineralising agents such as fluoride containing toothpaste or mouthrinses, or they were compared with a negative control group or placebo.

There appears to be shortage of literature investigating the effect of adding the CPP-ACP and CPP-ACPF crèmes to the regular oral hygiene practice that involves toothbrushing with fluoridated toothpaste twice daily.

Therefore the current in vitro study aimed to investigate the additional effect of using CPP-ACP as well as the CPP-ACFP crème as an adjunct to the regular use of fluoridated toothpaste (1450 ppm F) on the remineralisation of enamel subsurface lesions, and to compare this effect to those results from the solitary use of fluoridated toothpastes (1450 ppm F or 2800 ppm F) or to the effect of non-fluoridated toothpaste as a control.

## **5.1 In vitro model**

The present study used an in vitro model to study enamel remineralisation. The in vitro model of studying the caries process and studying the effect of different anti-cariogenic agents on enamel demineralisation and remineralisation is widely accepted and has been adopted in numerous studies.

The key advantage of the in vitro model is providing the ability to carry out single variable experiments under highly controlled conditions (White, 1995). It also allows the use of a wide range of analytical techniques for substrate analysis that might not be accessible in the in vivo model. Other advantages include being inexpensive and a quick approach (White, 1995).

In spite of that, significant limitations are inherited in the in vitro model; the most particular is the inability to replicate the complex biological processes involved in caries (White, 1995). This model also lacks many important protective biochemical processes available in the oral environment that reverse the process of demineralisation and promote the remineralisation including the composition of saliva, the salivary flow rate, and salivary pellicle formation on the enamel (White, 1992, West et al., 1998).

## **5.2 Study design**

Five groups were used in this study; in addition to the CPP-ACP and CPP-ACPF crème groups, negative control (non-fluoride toothpaste) and positive control (fluoride toothpaste 1450 ppm F), a high fluoride toothpaste (2800 ppm F) group was also included in the present study. A randomised, single-blinded design was used to prevent the introduction of bias in the study.

### **5.2.1 Bovine teeth**

Preferably, human teeth should be used for the study of the caries process. However, due to the difficulty of obtaining human teeth in large numbers; bovine teeth were used in the current study.

Over the past years the use of bovine teeth in dental research has increased (Yassen et al., 2011).

The characteristics of bovine teeth make it a good substitute for human teeth in caries research. This includes the presence of a large flat surface that has no previous exposure to caries challenges. In addition to that, bovine teeth have less variability in composition than human teeth which results in a less variable response to the cariogenic challenge as well as to the anti-cariogenic agents (Mellberg, 1992).

The mineral distribution in carious lesions in bovine teeth is reportedly similar to that found in human teeth, and the structural changes are comparable in human and bovine teeth (Edmunds et al., 1988).

On the other hand, bovine enamel was found to be softer in comparison to human enamel, represented by the higher rate of lesion progression and lesion depth (Amaechi et al., 1999). The bovine enamel does not have the exact structure and chemistry of human enamel, which led some researchers to question their continued use in dental research (Titley et al., 1988, Arends et al., 1989, Wennber and Orstavik, 1990). However a number of in vitro studies (Brighenti et al., 2006, Lennon et al., 2006, ten Cate et al., 2006) have successfully used the bovine enamel in order to evaluate the effect of different anti-cariogenic agents on enhancing enamel remineralisation and inhibiting enamel demineralisation.

### **5.2.2 Enamel slabs preparation and storage**

The buccal section of the bovine teeth was used in the present study to allow more uniform thickness of enamel as well as more flat surfaces. The enamel slabs were stored in distilled water and 0.1% thymol (Sigma Aldrich) with the aim of inhibition of the bacterial growth and prevention of enamel slabs dehydration. The antimicrobial properties of thymol were proven through its ability to perforate cell membranes, and subsequently destroy the pathogens that may be present on the teeth (Shapiro and Guggenheim, 1995). At the same time thymol has no detrimental effect on enamel but a few studies showed that it can affect dentine permeability (Preston et al., 2007).

### **5.2.3 Artificial carious lesions**

Two methods were used in previous studies in order to create artificial caries-like lesions. They included using either buffered solutions or acidified gels. It has been shown that the lesion produced by the acidified gels were generally shallow and smaller in size compared with those produced by the buffered solutions, which are characterised by being large and deep (Gray, 1966, Featherstone and Rodgers, 1981, Issa, 2004). This was attributed to the slow diffusion rate with the acidified gels that maintain the dissolved mineral ions in close proximity to the sample allowing their re-precipitation within the enamel surface leading to the growth of residual mineral crystals and the formation of an intact surface layer (Clark, 2001).

In the current study the enamel subsurface lesion was created using acidified hydroxyethyl cellulose gel as it is easy to use and produce consistent lesions with uniformed areas of demineralisation. In addition to that, lesions produced by

hydroxyethyl cellulose gel were found to be more rigid when compared with the lesions produced by acetic acid buffer (Issa, 2004).

The enamel slabs were immersed in the acidified gel for 10 days in order to produce enamel lesion with average  $\Delta F$  of approximately 20.7, which was close to the  $\Delta F$  values in a study carried out by Manton (2009).

#### **5.2.4 pH cycling**

In order to replicate the continuous process of demineralisation and remineralisation in the oral environment, the pH cycling model has been suggested by ten Cate and Duijsters, (1982). This model involves a process of alternating demineralisation and remineralisation of artificial enamel lesions that aims to simulate the carious process in the oral environment including the various demineralisation challenges from moderate to severe (ten Cate et al., 2006). The pH cycling model has been used widely to investigate caries-preventive agents on the dynamics of enamel de- and remineralisation (Featherstone, 1996, ten Cate et al., 2006).

Most of the previous studies that have used the pH cycling model have repeated the cycle for a 14 day period (ten Cate and Duijsters, 1982, Malinowski et al., 2007), however in the current study, a 21 day period of pH cycling was implemented in order to allow sufficient time to produce changes in the pre-demineralised enamel slabs. The pH cycling regime involved exposing the enamel slabs to 5 demineralisation challenges during the day. Acetic acid buffer (ten Cate et al., 2006) at pH 4.8 was used and was applied for 5 minutes. This demineralisation challenge represents the acid in the cariogenic challenge.



Both day time and night time artificial saliva were used in this study. The day time saliva was supersaturated with calcium and phosphate in order to allow remineralisation of enamel slabs during the day and it was used in between the demineralisation challenges for 60 minutes. On the other hand, the night time saliva was a saturated solution and it was used overnight to maintain the enamel condition without providing any mineral exchanges.

The pH cycling protocol used for the current study was developed at the University of Leeds and it has been used in a previous caries study (Al-Mullahi and Toumba, 2010) at the Leeds Dental Institute-Paediatric Dentistry Department.

### **5.2.5 Experiment materials**

The toothpastes used in the current study included non-fluoride toothpaste which was used as negative control in order to assess the remineralisation results from the artificial saliva.

The other toothpastes used included the 1450 ppm F which is the most common fluoride concentration in adult toothpaste preparations in the United Kingdom, and the 2800 ppm F toothpaste which usually is prescribed for high caries-risk individuals.

The application of Tooth Mousse or MI Paste Plus was done in the form of slurries as was used in previous studies (Reynolds et al., 2008, Al-Mullahi and Toumba, 2010). The application of the slurries to the enamel slabs was carried out after the exposure to the fluoride toothpaste, with no rinsing afterwards and was done in accordance with the manufacturer's instructions.

### **5.3 Quantitative Light-Induced Fluorescence (QLF)**

In the present study, QLF was used to assess the enamel demineralisation and remineralisation at baseline and following the treatment. This technique has been used for the detection and quantitative analysis of early caries as a strong correlation was found between the mineral loss and fluorescence loss in enamel after demineralisation. The advantage of this technique is being non-destructive which allows the longitudinal monitoring of early carious lesions.

QLF was validated by comparison with transverse microradiography (Al-Khateeb et al., 1997) and a good correlation coefficient ( $r = 0.84$ ) was found between fluorescence changes and mineral loss.

The intra-examiner and inter-examiner repeatability of QLF has been assessed in different studies. Pretty et al. (2002b), reported high reproducibility of QLF for the in vitro caries lesions. The intra-class correlation coefficients (ICCs) for intra-examiner reliability was (0.93) and for the inter-examiner reliability (0.96). In this study the assessment of the intra and inter-examiner repeatability was limited to the analysis stage of the QLF technique.

Excellent repeatability and reproducibility of the QLF method was reported in vivo for both the image capture and analysis stages (Tranæus et al., 2002). The inter-examiner reliability showed ICCs between 0.95 and 0.98 for the image capturing stage. A close ICCs was found for the analytical stage with intra-examiner reliability between 0.93 and 0.99 and inter-examiner reliability between 0.95 and 0.99.

In the present study the intra-class correlation coefficients (ICCs) for inter-examiner reliability for the image analysis was found to be (0.99) which represent a very good reproducibility.

Many confounders were found to influence the QLF imaging, therefore it was important to standardise the techniques while carrying out the present study. The image capture was carried out under controlled conditions at baseline and after the end of the experiment. All images were captured in a dark room in order to control the ambient light effect. To control the extent of hydration of the lesions, all the slabs were dried for 15 seconds with compressed air prior to imaging.

The enamel and dentine thickness were found to influence the fluorescence, however; this confounding factor could not be absolutely standardised in the present study. Since only profound differences in the total thickness of the dental tissue was found to influence the caries assessment with the QLF (Ando et al., 2003), the impact of this factor is believed to be minimal on the results of the current study.

The analysis of the QLF images is highly subjective as it is based on the investigator assessment of sound or demineralised enamel. In order to minimise the potential for operator bias, the study investigator had received training from the manufacturer and was familiar with the QLF software before the study.

#### **5.4 Results of Quantitative Light-Induced Fluorescence (QLF)**

As mentioned before, QLF produces three parameters that include; the  $\Delta F$  which represents the percentage fluorescence loss and related to lesion depth, the surface area of the lesion as well as  $\Delta Q$  which is the  $\Delta F$  times the area and represent the lesion volume. All these values were calculated in this study; however, the  $\Delta Q$  value was considered as the main indicator for the mineral loss and the lesion progression or regression in the present study. Since it was indicated that the lesion area may increase or decrease while the  $\Delta F$  value maintained the same or

alternatively the lesion area may be maintained despite the increase or decrease in  $\Delta F$  value (Ando et al., 2004). For this reason the independent evaluation for these two values may not give a good evaluation of the lesions progression or regression.

#### **5.4.1 Effect of fluoridated toothpaste in remineralising enamel subsurface lesions**

The current study assessed the effect of fluoridated toothpastes on remineralising enamel subsurface lesions. Two fluoride concentrations were assessed; the 1450 ppm F (0.32% w/w sodium fluoride), and the 2800 ppm F (0.619 w/w sodium fluoride), along with non-fluoridated toothpaste as a negative control.

The study results showed that all these groups produced remineralisation of the enamel subsurface lesions. The highest remineralisation was achieved using the 1450 ppm F toothpaste followed by that produced by 2800 ppm F toothpaste and the least remineralisation was produced in the negative control group, 0 ppm F toothpaste. A statistical significance difference was found between both fluoride groups and the negative control when the  $\Delta Q$  and lesion area values were compared; however, the comparison of the  $\Delta F$  values between the groups showed a significant difference between the 1450 ppm F and the 0 ppm F groups, and no significant difference between 2800 ppm F and 0 ppm F was found. In addition, no statistical significance difference was found between the 1450 ppm F and the 2800 ppm F in any of the three parameters.

Overall, in the present study it was noted that the higher fluoride concentration of 2800 ppm toothpaste did not follow the expected dose response curve. However, when  $\Delta Q$  was used as a outcome measure, a significant difference was observed

between 0 ppm F and 2800 ppm F, but this was not evident for  $\Delta F$ . A possible explanation for this anomaly is that  $\Delta Q$  reflects the total demineralisation which also takes into account the area of the lesion and the depth.  $\Delta F$  is an average measure of fluorescence loss which is a reflection of the average demineralisation in the measured lesion. With 2800 ppm F, although the area of the lesion seems to have reduced in size (improved  $\Delta Q$ ), the average mineral loss and depth of the lesion across the remaining lesion did not significantly improve with 2800 ppm F compared to the 0 ppm F control.

This was an interesting result, given that it has been shown in a randomised, double-blind clinical trial (Biesbrock et al., 2001) that 2800 ppm F dentifrice reduced caries experience by 20.4%, which was 85% greater reduction than that produced by the 1700 ppm F, and statistically significant comparable to the 1100 ppm F. This clinical study had clearly shown a dose response. A dose response effect of different fluoride levels in toothpastes was also shown in Cochrane review by Walsh et al. (2010).

It would be interesting to speculate why in our model this dose response was not observed for remineralisation of subsurface caries-like lesions. A possible explanation could be that an exceedingly high F concentration in toothpaste slurry produced a surface mineral rich layer which in turn compromised the remineralisation of the body of the lesion. Some evidence to support this hypothesis can be found in the literature. The remineralisation of white spots lesion by fluoride was investigated in previous studies, ten Cate et al. (1981), showed that the topical applications of high-concentration fluoride solution resulted in a higher initial, but lower subsequent, rates of mineral deposition. A higher mineral deposit in the body of the lesion was also reported when no fluoride was added to the remineralising

solution compared with that of 2 ppm F (Lammers et al., 1990). Although the fluoride effect in promoting remineralisation is well established, longitudinal epidemiological studies showed that fluoride might inhibit complete lesion remineralisation (Dirks, 1966, Pot and Groeneveld, 1976). It was found that the remineralisation process of surface softened enamel was completely different from that for the remineralisation of subsurface lesions from a kinetic point of view (ten Cate and Arends, 1978). The surface softened lesion was found to remineralise faster and more completely than the subsurface lesion (Gelhard, 1982).

Ogaard et al. (1988), found that the application of fluoride on established carious lesions underneath orthodontic appliances may arrest the lesions, however the complete repair of the lesions might be inhibited due to the formation of fluorapatite in the surface area. The deposition of fluorapatite in the surface layer can result in the blockage of the surface pores and subsequently restrict the diffusion into the lesions. The resulting surface would probably be much more caries-resistant compared to the original enamel, but it still retained a demineralised appearance (ten Cate and Duijsters, 1982).

On the other hand, the 2800 ppm F toothpaste was shown to produce more remineralisation (89%) compared with that of 1100 ppm F toothpaste (Reynolds et al., 2008). The microradiography analysis revealed that the fluoride toothpaste tended to promote remineralisation predominantly in the surface layer. However, an in situ model was used in this study making the direct comparison with our study difficult.

#### **5.4.2 Effect of CPP-ACP and CPP-ACFP on remineralising enamel subsurface lesions**

In the present study the effect of both CPP-ACP (Tooth Mousse) and CPP-ACFP (MI Paste Plus) on the remineralisation of enamel subsurface lesions were assessed. Both materials were used as one application after the use of fluoridated toothpaste (1450 ppm F).

The results showed that in all QLF parameters ( $\Delta F$ ,  $\Delta Q$  and lesion area) the 1450 ppm + TM and 1450 ppm + MIplus groups produced remineralisation of the enamel subsurface lesion which was statistically significant compared with the baseline as well as in comparison to the remineralisation produced in the negative control group (0 ppm F). This remineralisation was also found to be higher in comparison with that produced by the 1450 ppm F and 2800 ppm F groups; however, this difference failed to reach a significant level. This might be attributed to the in vitro model used in the present study, which might not be sensitive enough to express these differences. As in the current model both CPP-ACP and ACP-ACFP were used directly after the use of the 1450 ppm F toothpaste, the use of F toothpaste might cause a blockage of the surface pores of the lesion by the formation of the fluorapatite in the surface layer which in turn can prevent the diffusion of the calcium and phosphate ions from the CPP-ACP into the body of the lesion to promote remineralisation as discussed earlier (section 1.3.1).

The release of calcium, phosphate and fluoride ions from the CPP complex in dental plaque intra-orally is believed to be promoted by low plaque pH (Cochrane et al., 2010). In their in vitro study Cochrane et al. (2008), reported an increase in the remineralisation by a 2% CPP-ACP solution from  $15.0 \pm 3.5\%$  to  $41.8 \pm 8.5\%$  when

the solution pH dropped from 7.0 to 5.5, and that the remineralisation was increased to  $57.7 \pm 8.4$  at pH 5.5 when fluoride was incorporated into the CPP-ACP. This might explain why in our in vitro model the remineralisation produced by the CPP-ACP and CPP-ACFP solutions increased, however it did not reach significance as the measured pH value for these solutions in our study were 7.0.

The positive effect of CPP-ACP (Tooth Mousse) and CPP-ACFP (MI Paste Plus) in the remineralisation of enamel lesions has been shown in previous in vitro studies (Kumar et al., 2008, Jayarajan et al., 2011, Zhang et al., 2011). However, different methodologies were used in these studies compared to the current study.

In Jayarajan et al. (2011), and Zhang et al. (2011), studies the CPP-ACP/CPP-ACFP crème were used alone, and showed a significant difference in enamel remineralisation when compared with fluoride or a negative control. Whereas in the present study, the CPP-ACP and CPP-ACFP crèmes were used as an adjunct to the fluoride treatment with 1450 ppm F. In their study, Kumar, et al. (2008), revealed an additive effect for the application of the CPP-ACP crème after the fluoride toothpaste treatment, which is consistent with the results of the current study, however it was not reported whether this additional effect was statistical significance or not.

The CPP-ACP was shown to promote the remineralisation of enamel subsurface lesions in situ models, This resulted from the localisation of the CPP-ACP in the plaque matrix resulting in a significant increase of the calcium and phosphate ions in supragingival plaque (Reynolds et al., 2003) as well as its bounding to the surface of bacterial cells producing a reservoir of bio-available calcium ions (Rose, 2000, Reynolds et al., 2003).



Reynolds et al. (2008), used the in situ model to assess the remineralisation effect of 2% CPP-ACP dentifrice. This study showed that the remineralisation produced by the 2% CPP-ACP dentifrice was better than a single use of 1100 ppm F containing dentifrice, as well as similar to that produced by the 2800 ppm F. The highest remineralisation among all groups was that produced by dentifrice with 2% CPP-ACP plus 1100 ppm F.

The additive effect of the CPP-ACP and fluoride was reported to be resulted from the interaction between the CPP-ACP with fluoride ions to produce ACFP (Cross et al., 2004, Reynolds et al., 2008) which in turn promote the localisation of the fluoride ion at the tooth surface. The presence of fluoride ions along with the calcium and phosphate ions in plaque increases the level of fluorapatite saturation; consequently promote the remineralisation of enamel with fluorapatite during an acid challenge.

Unexpectedly, in the present study, the CPP-ACP group produced more remineralisation than the CPP-ACFP group. However, this difference was not found to be statistically significant. The reason of such finding may be again caused by the use of higher fluoride concentration in the CPP-ACFP group which might results in the formation of the surface hypomineralised layer that inhibit the diffusion of calcium and phosphate into the body of the lesion, bearing in mind that the use of the CPP-ACFP as well as the CPP-ACP crèmes were done after the application of the 1450 ppm F twice daily which in turn can be responsible for the formation of the surface mineral rich zone.

Another explanation for this finding is the formation of calcium fluoride precipitate (crystals) when the fluoride ions are present along with calcium ions from CPP-ACP resulting in less fluoride and calcium ions available to diffuse down

the body of the lesion to promote remineralisation. This is particularly significant when using in vitro models that is lacking in natural salivary proteins especially the calcium stabilising proline-rich protein and statherin.

The additive effect of CPP-ACP to the normal oral hygiene practice using fluoride toothpaste on the remineralisation of the white spot lesions was shown in a number of randomised control trials, Bailey et al. (2009), reported (31%) more remineralisation of the white spot lesions in the Tooth Mousse group compared with the placebo cream. However the application of the cream was done morning and night after normal oral hygiene procedures using fluoridated dentifrice (1000 ppm F as NaF) for 12 weeks. Akin and Basciftci, (2012) also reported a high success rate using CPP-ACP Tooth Mousse in treating white spot lesions which was statistically significant than that produces with the control group or that for 0.025% sodium fluoride mouthrinse.

On the other hand Beerens et al. (2010), and Bröchner et al. (2011), failed to show the additive clinical benefits of CPP-ACFP and CPP-ACP in the remineralisation of white spot lesions when compared to the normal oral hygiene practice with fluoride toothpaste. However, the application of the CPP-ACP/ CPP-ACFP crèmes in these two trials were done once a day and for study period of 4 weeks in Bröchner et al. (2011) study, compared with that for Bailey et al. (2009), where the application was done twice daily for 12 weeks period.

Although comparing the result of the in vitro study with that of an in vivo study might not be valid, the same trend was noticed between the current study and that for the Beerens et al. (2010), and Bröchner et al. (2011), as the CPP-ACP/ CPP-ACFP was applied once a day, and did not result in a significant improvement in the

white spot lesions compared with that resulted from the solitary use of 1450 ppm F toothpaste.

## **5.5 Scanning Electron Microscopy (SEM)**

The scanning electron microscope produces highly magnified images through the interaction between the electron beams with the specimen surface. SEM analysis can provide useful information about structural changes of the tooth surfaces that take place during the study. SEM imaging has been used extensively to study the enamel and dentine surfaces, this includes studies of different types of tooth surface loss as well as the studies of dentine hypersensitivity and different agents used for its managements.

In the current study SEM imaging was used to assess the changes of the enamel surface after various remineralising treatments. Although SEM is usually used in the erosion studies to qualitatively assess the changes of eroded enamel and the extent of tooth wear, in the current study the enamel surface was not subjected to erosive challenge, rather, the demineralisation challenges that had been used produced enamel subsurface lesions. This was confirmed in the SEM results as the enamel surfaces that were subjected to the demineralisation challenge remained intact and minimal surface loss has been noticed comparable with sound enamel.

### **5.5.1 Results of Scanning Electron Microscopy (SEM)**

The SEM images revealed amorphous deposits on the enamel surface of both the CPP-ACP and the CPP-ACPF groups, while no deposits were seen in the enamel surface of the other groups.

The presence of calcified (amorphous) deposits on the enamel surface treated with the CPP-ACP was reported in previous studies (Ferrazzano et al., 2011, Jayarajan et al., 2011) using SEM. In the in vivo study conducted by Ferrazzano et al. (2011), reduction in the surface alteration with the presence of a diffuse and homogeneous mineral coating was found only in the demineralised enamel specimens treated with CPP-ACP Tooth Mousse.

**In summary**, in the present in vitro study, five groups were assessed for their effect on the remineralisation of enamel subsurface lesions including 0 ppm F, 1450 ppm F, 2800 ppm F, 1450 ppm F + TM and 1450 ppm F + MIplus. The remineralisation of the enamel subsurface lesions was observed in all groups. However, significantly more remineralisation was noticed in all test groups compared with the negative control group (0 ppm F). The 1450 ppm + TM and the 1450 ppm F + MIplus groups showed increased remineralisation of enamel subsurface lesions compared to the fluoride toothpaste alone, however, this difference did not reach a statistical significant level.

## **5.6 Suggestions for Future Research**

The results of the current in vitro study showed that there were no significant benefits of CPP-ACP and CPP-ACPF crème on remineralising enamel subsurface lesions when they were used supplementary to the use of fluoride toothpaste (1450 ppm F) twice daily compared to the use of the fluoride toothpaste alone over a period of 21 days.

In this study both CPP-ACP and CPP-ACPF crème were used immediately after the application of the fluoride toothpaste. Therefore further research is needed to investigate the effect of both crèmes when they are used at different times than that used in the present study. For example, using Tooth Mousse in the middle of the pH cycling regime in case of in vitro studies and at different occasion to that of the toothbrushing with fluoride toothpaste in studies which are carried out in situ or in vivo.

The CPP-ACP and CPP-ACPF crème in the present study was applied in a form of slurries. It would be interesting to investigate whether or not there would be any difference in the results if a different method of application was used such as dentifrices or as a topical application.

Due the limitation of the in vitro study, future in situ and in vivo studies comparing the remineralising agents and regimens assessed in the current study would give results that would be more directly related to the clinical use of these materials.

## **5.7 Null hypothesis**

The null hypothesis “There is no difference in the enamel remineralisation that results from Tooth Mousse, MI Paste Plus, 1450 ppm F toothpaste, 2800 ppm F toothpaste and non-fluoridated toothpaste” can be rejected as a significant differences were found in the enamel remineralisation between the test groups and the negative control group (non-fluoridated toothpaste).

## 6.0 CONCLUSION

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

- 1- A statistically significant remineralisation of enamel subsurface lesions in comparison with the baseline was found in all groups.
- 2- When remineralisation of the enamel subsurface lesions was compared between the groups, a statistically significant difference was achieved for all test groups compared with the 0 ppm F toothpaste control.
- 3- Both Tooth Mousse and MI Paste Plus were used in conjunction with 1450 ppm F and showed some increased efficacy in the remineralisation of enamel subsurface lesions, however this did not reach a significant level in our in vitro model.

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## 8.0 APPENDICES

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**Appendix 1: Approval for collection of bovine teeth.**

P.1

## Dispatch of SRM for Veterinary or Research Purposes



Approval No. 2091 Establishment Name Spenborough abattoir Date 11-03-11  
-11

**Part 1**  
To be completed by the person responsible for the handling and disposal of SRM

Name and address of research establishment  
Professor M S Duggal, Leeds Dental Institute, University of Leeds, Clarendon Way, Leeds LS29LU  
Postcode: LS29LU

Reason for application  
Collection of bovine teeth for dental research with ethical approval. All bovine enamel is sterilised using both gamma irradiation and overnight suspension in 2.5% sodium hypochlorite 20,000ppm.

Type of SRM tissue  
Bovine incisor teeth from animal less than 13 months of age

Quantity of SRM required 100 teeth Frequency of collection monthly

Duration of project From: 11-10-11 To: 11-10-12

Name and address of abattoir  
Spenborough Abattoir, Headlands Road, Liversedge WF15 6PR, Dr Istvan Pocz (veterinary surgeon)  
Postcode: WF156PR

Approval number 2091

**Declaration**  
I, the undersigned, declare that the SRM collected from the above abattoir will be handled, transported and disposed of in accordance with the Animal By-Products (Enforcement) Regulations 2011 (England, Scotland, Wales)

Name in BLOCK letters: M S DUGGAL Position Prof & Head

Signature: [Signature] Date 11-03-11

Email Address: m.s.duggal@leeds.ac.uk

**Send this form to the Veterinary Manager at FSA York**

**Part 2**  
To be completed by the Veterinary Manager at FSA York.

I, the undersigned, have approved the application stated above. The OV at the named establishment may release SRM to the applicant in accordance with the SRM Regulations.

Name in BLOCK letters ASTER PROZDRAVINA Designation VETERINARY MANAGER

Signature [Signature] Date 03/10/2011

The completed form should be retained by the issuing Veterinary Manager.  
Copies should be sent to:

- the applicant via email or fax;
- the OV at the named establishment;
- the Animal Health Divisional Office in whose area the consignee establishment is situated;
- the Local Authority in whose area the recipient establishment is situated.

Part 3 overleaf...

(Rev 04/11) ABP 7/1

## Appendix 2: Normality Tests for $\Delta F$ at Baseline.

### Shapiro-Wilk and Kolmogorov-Smirnov Tests of Normality for $\Delta F$ at Baseline.

	Group	Kolmogorov-Smirnov <sup>a</sup>			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
$\Delta F$ at baseline	0 ppm	.111	24	.200*	.979	24	.882
	1450 ppm	.152	24	.158	.929	24	.090
	2800 ppm	.194	24	.020	.917	24	.050
	1450 ppm + TM	.133	24	.200*	.937	24	.137
	1450 ppm + MIplus	.133	24	.200*	.932	24	.108
*. This is a lower bound of the true significance.							
a. Lilliefors Significance Correction							

### Appendix 3: Normality Tests for Difference in $\Delta F$ .

#### Shapiro-Wilk and Kolmogorov-Smirnov Tests of Normality for Difference in $\Delta F$ .

	Group	Kolmogorov-Smirnov <sup>a</sup>			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Difference between $\Delta F$ at baseline and after treatment	0 ppm	.107	24	.200*	.988	24	.987
	1450 ppm	.115	24	.200*	.967	24	.593
	2800 ppm	.141	24	.200*	.942	24	.182
	1450 ppm+ TM	.173	24	.061	.906	24	.030
	1450 ppm + MIplus	.160	24	.113	.929	24	.092
*. This is a lower bound of the true significance.							
a. Lilliefors Significance Correction							

## Appendix 4: Normality Tests for $\Delta Q$ at Baseline.

### Shapiro-Wilk and Kolmogorov-Smirnov Tests of Normality for $\Delta Q$ at Baseline.

	Group	Kolmogorov-Smirnov <sup>a</sup>			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
$\Delta Q$ at baseline	0 ppm	.084	24	.200*	.989	24	.993
	1450 ppm	.145	24	.200*	.928	24	.090
	2800 ppm	.076	24	.200*	.982	24	.924
	1450 ppm + TM	.139	24	.200*	.958	24	.403
	1450 ppm + MIplus	.184	24	.034	.922	24	.064
*. This is a lower bound of the true significance.							
a. Lilliefors Significance Correction							

## Appendix 5: Normality Tests for Difference in $\Delta Q$ .

### Shapiro-Wilk and Kolmogorov-Smirnov Tests of Normality for Difference in $\Delta Q$ .

	Group	Kolmogorov-Smirnov <sup>a</sup>			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Difference between $\Delta Q$ at baseline and after treatment	0 ppm	.136	24	.200*	.967	24	.605
	1450 ppm	.146	24	.200*	.925	24	.077
	2800 ppm	.172	24	.064	.961	24	.449
	1450 ppm + TM	.149	24	.181	.948	24	.249
	1450 ppm + MIplus	.105	24	.200*	.944	24	.202
*. This is a lower bound of the true significance.							
a. Lilliefors Significance Correction							

## Appendix 6: Normality Tests for Area at Baseline.

### Shapiro-Wilk and Kolmogorov-Smirnov Tests of Normality for Area at Baseline.

	Group	Kolmogorov-Smirnov <sup>a</sup>			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Area at baseline	0 ppm	.127	24	.200*	.972	24	.711
	1450 ppm	.155	24	.141	.943	24	.189
	2800 ppm	.118	24	.200*	.973	24	.748
	1450 ppm + TM	.155	24	.141	.936	24	.136
	1450 ppm + MIplus	.100	24	.200*	.978	24	.863
*. This is a lower bound of the true significance.							
a. Lilliefors Significance Correction							

## Appendix 7: Normality Tests for Difference in Area.

### Shapiro-Wilk and Kolmogorov-Smirnov Tests of Normality for Difference in Area.

	Group	Kolmogorov-Smirnov <sup>a</sup>			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Difference between area at baseline and after treatment	0 ppm	.266	24	.000	.878	24	.008
	1450 ppm	.119	24	.200*	.888	24	.012
	2800 ppm	.230	24	.002	.886	24	.011
	1450 ppm + TM	.154	24	.144	.917	24	.050
	1450 ppm + MIplus	.127	24	.200*	.967	24	.604
*. This is a lower bound of the true significance.							
a. Lilliefors Significance Correction							

## Appendix 8: Abstract of the Oral Presentation at the Leeds Dental Institute Research Day ( July 2014).

Mawia Bataineh

Comparison of the newer preventive therapies on remineralisation of enamel *in vitro*

Mrs Mawia Bataineh, Dr Jinous Tahmassebi, Professor Monty Duggal

Department of Paediatric Dentistry, School of Dentistry, University of Leeds, UK

**Aim:** To investigate *in vitro* the effect of Tooth Mousse, Tooth Mousse Plus and high Fluoride concentration toothpaste (2800 ppm) in the remineralisation of demineralised enamel subsurface lesion and to compare it to the effect of 1450 ppm Fluoride toothpaste.

**Methodology:** Enamel subsurface lesions were created in bovine enamel slabs (N=120) which were then assigned randomly to five groups;(1) Fluoride free Toothpaste (control), (2) Fluoride toothpaste (1450ppm), (3) High Fluoride toothpaste (2800ppm), (4)Tooth Mousse (10% w/v CPP-ACP) and (5)Tooth Mousse Plus (10% w/v CPP-ACP, 900ppm fluoride). The enamel slabs were subjected to pH cycling regimen for 21 days, with daily exposure to the treatment solutions before and after the pH cycling. QLF images were taken at the baseline and at the endpoint of the experiment. Data analysis was done using one-way analysis of variance (One way ANOVA).

**Results:** In all five groups, both  $\Delta F$  (Average fluorescence loss) and  $\Delta Q$  (multiplication of  $\Delta F$  and area) values improved noticeably within the same group after the treatment. In addition, the mean difference in  $\Delta F$  of group 1(control) was significantly lower than the mean difference in  $\Delta F$  of the groups 2, 4 and 5 ( $p < 0.05$ ) but not group 3. Whereas, the mean difference in  $\Delta Q$  of group 1 was significantly lower when compared to all other groups. ( $p < 0.05$ ).

**Conclusion:** CPP-ACP Tooth Mousse and Tooth Mousse Plus promote the remineralisation of enamel subsurface lesion with no significant superiority in the effect compared to different concentrations of fluoride toothpastes used in this present study.