The effect of a novel alternate soaking method on enamel lesion remineralisation *in vitro*

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

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"To my parents, Lateefa and Abdulkarim, I dedicate this thesis to you."

Abstract

One goal of modern dentistry is early intervention against dental caries to preserve enamel hydroxyapatite (HAP) crystals. An alternate soaking process using aqueous calcium (Ca) and phosphate (PO_4^{3-}) solutions has been developed to form HAP on bone. However, its effect on enamel subsurface lesions has not been investigated. Therefore, this study evaluated the remineralisation potential of an alternate soaking treatment using calcium and phosphate solutions on enamel lesions *in vitro.*

Bovine enamel slabs (n = 64) with subsurface artificial lesions were randomly allocated into four groups: (1) Alternate soaking with $CaCl_2$ and Na_2HPO_4; (2) Toothpaste slurry (1450 ppm F); (3) Artificial day-time saliva (positive control); and (4) Tris-HCl (negative control). Treatments were applied twice daily for 6 minutes, and slabs were stored in artificial night-time saliva at 37 °C. After 21 days, lesions were assessed using Quantitative Light-induced Fluorescence (QLF), Micro-Computed Tomography (Micro-CT), and Energy-Dispersive X-ray spectroscopy (EDX). Data were analysed using SPSS version 29, with significance set at $p < 0.05$.

Significant reductions in lesion depth (ΔF) were observed for the alternate soaking process, toothpaste slurry, and day-time artificial saliva (Diff ± SE: 2.69 ± 1.09%, 4.57 ± 0.86%, and 0.69 ± 0.12%, respectively; $p < 0.05$). ΔF, lesion volume (ΔQ), and area indicated significantly greater remineralisation for the alternate soaking process and toothpaste slurry compared with other groups ($p < 0.05$). The results of micro-CT showed an increase in mineralisation in Ca/P, 1450 ppm F, and day-time artificial saliva (Diff ± SE) (2.00 ± 0.10; 2.30 ± 0.17; 0.90 ± 0.26, respectively) compared to Tris-HCL which showed no statistical significance. EDX confirmed increased mineral content, with statistical significance observed only for the Ca/P and toothpaste slurry (1450 ppm F) groups ($p < 0.05$).

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Abbreviation (A-Z)

ANOVA	Analysis of Variance
Ca	Calcium
Ca/P	Calcium/ Phosphate
CRT	Cathode Ray Tube
CPP-ACP	Casein Phosphopeptide-Amorphous Calcium Phosphate
CPP-ACPF	Casein Phosphopeptide–Amorphous Calcium Phosphate with Fluoride
EDX	Energy Dispersive X-ray
F	Fluoride
FAP	Fluorapatite
FDI	World Dental Federation
HAP	Hydroxyapatite
Micro-CT	Microscopy tomography
mg	Milligram
min	Minutes
mL	Millilitre
mm	Millimetre
Mol	Mole
L	Litre
PVA	Poly (Vinyl) Alcohol
SBF	Stimulated Body fluids
SE	Secondary Energy

SEM	Scanning Electron Microscope
SPSS	Statistics Package for the Social Sciences
P	Phosphorous
%	Percentage
% F	Percentage change in fluorescence
% Q	Percentage change ΔQ
ΔF	Average fluorescence loss
ΔQ	Multiplication of ΔF and Area
μm	Micrometre
nm	Nanometre
ºC	Degree of Celsius
pH	Acidity
px	Pixels
p	p-value
PO_4	Phosphate
ppm	Part per million
QLF	Quantitative Light-Induced Fluorescence
RCT	Randomised control trials
s	Second
Sig	Statistical level
SD	Standard deviation
SE	Standard error
TCP	Tricalcium Phosphate
vs	Versus

wt	Weight
β-TCP	β-Tricalcium Phosphate

Chapter 1 Introduction and Literature Review

In the following sections, the research literature on the dental caries process, including demineralisation and remineralisation, will be reviewed. In addition, the role of hydroxyapatite, fluoride, and calcium phosphate in the prevention and remineralisation of dental caries will be discussed, with a particular focus on the alternate soaking method used *in vitro* with calcium and phosphate.

1.1. Enamel caries

Enamel caries is a microbiological infectious disease that is first initiated by the acidic bacteria on the tooth's surface, forming a small subsurface lesion. The bacteria are composed of Mutans Streptococci and Lactobacillus (Al Agili, 2013). Many of these bacteria can form biofilms, which contain a thin layer of extracellular polymeric substances as a protective matrix (Shellis et al., 2002). When fermentable carbohydrates (e.g., sucrose, glucose, fructose) are present, the bacteria within the plaque biofilm metabolise these sugars through a process called glycolysis. This metabolic process produces organic acids as a byproduct (Abdul et al., 2022). These acids include formic, acetic, and lactic, which reduce the pH within the biofilm microenvironment (Abou Neel et al., 2016). Sustained consumption of carbohydrates leads to frequent acid exposure, creating a consistently acidic environment around the enamel. These acids cause a reduction in pH (pH 5.5) and shift the equilibrium between the surrounding plaque fluid with a local dissolution of enamel. As a result, the tooth then becomes softened as hydroxyapatite (HAP) crystals undergo solubilisation and become susceptible to mechanical tooth wear and tissue loss

(Fejerskov, 1997). With the continuation of this process, the dentine and then pulp will become involved (Al-Sanabani et al., 2013).

In contrast, saliva helps counteracts the process of demineralisation by delivering calcium and phosphate ions, which are secreted by the salivary glands secretory cells. Saliva also carries fluoride that was introduced from external sources such as toothpaste or drinking water, which further enhances enamel remineralisation (Abou Neel et al., 2016).

Histologically, enamel caries can be described into four zone categories in the ground section (Figure 1) (Rao, 2024). Each distinct zone has its optical properties reflecting the different degrees of demineralisation (Ricketts, 2016; Kidd and Fejerskov, 2004):
The translucent zone: This zone is situated at the advanced edge of the lesion and has a porous character, with a pore volume of 1% compared to 0.1% volume of space in sound enamel. The mineral dissolution occurs between the junction of the prism and inter-prism enamel.

The dark zone: This zone follows the theory that narrowing happens if less mineralisation occurs in rapidly advancing lesions. It has a pore volume between 2% and 4%. Some mineralisation occurs at this zone as some pores are larger, while others are smaller compared to the translucent zone. This indicates the loss of re-precipitation minerals in the previous zone (Shellis et al., 2002).

The body of the lesion: This zone contains crystals of apatite larger than those normally occurring in enamel, and it contains 5-25% by volume of pores. This indicates that the re-precipitation of minerals dissolute from deep zones. If the pH challenge continues, minerals will keep dissolving from both the core and the periphery.

The surface zone: In early lesions, there is less change in the thickness of this zone, which is 40 µm thick due to the re-precipitation of minerals that diffuse outward from the deep zones and lesion plaque.

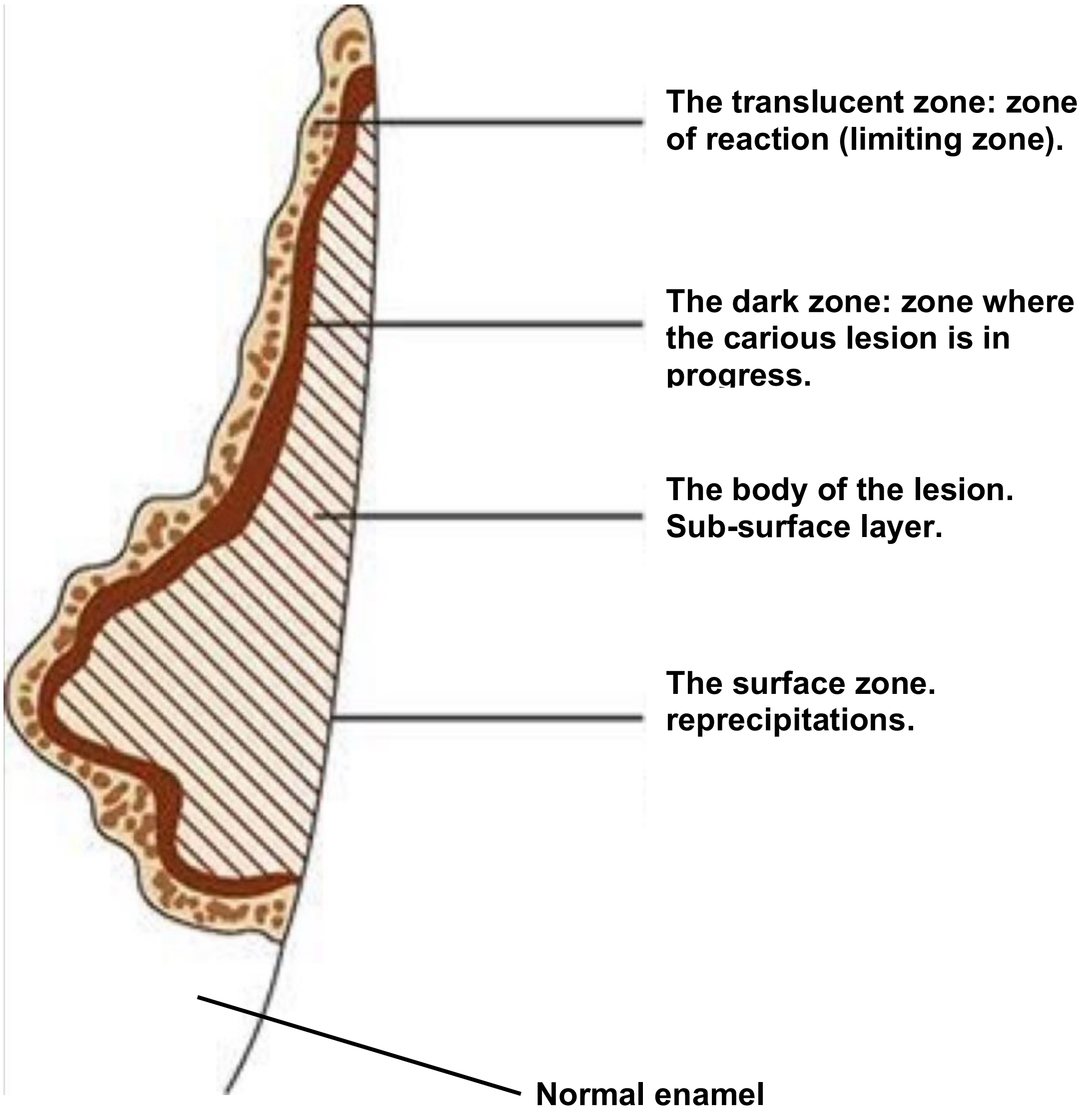


Figure 1. Zones of enamel caries in ground section. This image was modified from (Rao, 2024).

An enamel subsurface lesion is a type of demineralisation that occurs beneath the outermost layer of enamel, prior to any clinical signs of caries. These lesions are typically associated with the early stages of the carious process and can be reversed naturally through saliva (Abdul et al., 2022; Shellis et al., 2002). The lesion develops beneath a layer of intact enamel, which often remains undamaged due to its resistance to acid dissolution, despite ongoing demineralisation beneath the surface. This happens when natural remineralisation occurs, by which lost ions, primarily calcium and phosphate, are restored to demineralised enamel (Nanci and Ten Cate, 2013). When saliva is rich in these essential minerals, the process occurs more effectively, helping to repair early enamel damage. Fluoride plays a crucial role in remineralisation by attracting calcium and phosphate ions and facilitating their integration into the crystal lattice. Instead of simply rebuilding the original HAP, fluoride promotes the formation of fluorapatite (FAP) tooth structure. This helps rebuild the original HAP which is more resistant to future acid attacks (Amaechi et al., 2019). However, in persistently acidic conditions, natural remineralisation becomes inadequate, making the enamel more vulnerable to further demineralisation and caries (Kim et al., 2021).

Subsurface lesions are commonly identified as white spot lesions, characterised by white spots or areas of opacity visible under light. These features become more prominent when the enamel is dehydrated, as the removal of water from the lesion increases light scattering (Kim et al., 2021). This demineralisation is primarily driven by acidic metabolic byproducts from bacteria, predominantly *Streptococcus mutans* and species of Lactobacillus, found in dental plaque (Animireddy et al., 2014). The process requires the presence of fermentable carbohydrates, such as sugars, and a conducive environment for bacterial growth (Abou Neel et al., 2016). Early detection is crucial for the prevention and reversal of these lesions. If left untreated, subsurface lesions can progress to cavitation, necessitating restorative treatment (Arifa et al., 2019).

The subsurface enamel lesion initially develops as an incipient translucent zone. It then becomes enlarged and forms the dark zone at the centre of the lesion. When more minerals are lost, the lesion will be further enlarged, and the dark zone develops into the body of the lesion. Clinically, these lesions (known as white spot lesions) will become visible and recognisable (Nanci and Ten Cate, 2013).

In recent years, dental caries is no longer evenly distributed across populations in many countries. Instead, it has become concentrated in a smaller percentage of the population, often those from lower socio-economic backgrounds. This means that while the overall rate of caries may have decreased, certain high-risk groups still experience disproportionately important levels of disease, where access to dental care, fluoride, and proper oral hygiene practices may be limited. Poor nutrition, lack of education, and limited access to preventive measures also contribute to higher caries rates in these populations (Fayle et al., 2001). Furthermore, Inequalities, deprivation, ethnicity, and disabilities can contribute to increasing the risk of dental caries (Al Agili, 2013).

1.2. Challenges in dental caries

Dental caries continues to be a prevalent and challenging issue for dental professionals due to its complex nature and the wide range of factors influencing its development and progression (Kim et al., 2021). Detecting caries in its earliest stages remains particularly difficult. When Initial demineralisation happens, it often occurs beneath the enamel surface, making it hard to identify using standard visual or tactile examination techniques (Bishara and Ostby, 2008). Advanced tools such as laser fluorescence, digital radiography, and transillumination are proving invaluable in these situations, offering improved accuracy and earlier intervention opportunities. However, access to such technologies may be limited by practical and financial barriers (Bossù et al., 2019).

A key challenge in managing caries lies in encouraging patient compliance with preventive measures. Maintaining effective oral hygiene, making dietary changes, and attending regular dental appointments require consistent effort, which can be difficult for patients to sustain (Featherstone, 2004). Many patients are not fully aware of the long-term implications of untreated caries, leading to a lack of motivation to adhere to recommendations. Dentists play a vital role in addressing this challenge by fostering open communication, educating patients, and using motivational strategies to help them prioritise their oral health. Furthermore, the durability of restorative materials and the occurrence of secondary caries also pose a significant challenge (Watt et al., 2016).

Socioeconomic factors significantly impact oral health worldwide, causing disparities that are evident in both developing and developed nations. In the U.S., issues like dental caries are closely linked to a person's education, income, and race (Al Agili, 2013). Similarly, in the U.K., children and adults from lower social classes often suffer more from dental problems. Guangdong Province in China, despite its economic advancements, faces similar socioeconomic-related oral health issues. Research has shown that these inequalities affect both the prevalence of dental caries and the use of oral health services (Marmot, 2005). Studies have shown that disparities in dental caries were more pronounced than those in caries experience. Key factors contributing to these inequalities included household income, high levels of education, and regular dental check-ups, which significantly influenced the number of filled teeth (FT). Additionally, existing medical insurance. To address these socioeconomic oral health disparities, policies should aim to reduce the treatment burden and improve access to dental care for low-income populations (Lee and Divaris, 2014).

1.3. Composition of enamel

Enamel is the hardest calcified matrix and a translucent tissue in the human body. It consists of approximately 96% minerals, 4% organic material, and water by weight (Cai et al., 2010). Enamel crystals is organised into a series of enamel prisms (or rods) that are oriented approximately perpendicular to the surface of the tooth with some regional variation and natural curvature(Ekambaram et al., 2017). Each prism is composed of tightly packed hydroxyapatite crystals, which contribute to its hardness and durability (Abou Neel et al., 2016).

Enamel formation occurs during a process called amelogenesis, which takes place in three main stages (Cai et al., 2010):

1. Pre-secretory stage: In this stage, inner enamel epithelial cells differentiate into ameloblasts. Ameloblasts elongate and develop the organelles needed for protein secretion. Odontoblasts begins by forming predentin, which signals ameloblasts to start enamel secretion.
2. Secretory Stage: This is the active enamel mineralisation of the matrix phase. Ameloblasts (cells responsible for enamel formation) secrete enamel proteins (amelogenin, enamelin and ameloblastin) and initial organic matrix is laid down. Tomes process develops which guides the formation of enamel prisms.

3. Maturation Stage: In this stage, the protein matrix degradation and water is removed and the mineral content increases to ~96% HAP by weight. This allows HAP crystals expand laterally and vertically. This forms a fully hardened and highly crystalline tissue.

1.3.1. Demineralisation and remineralisation process of tooth enamel

In natural conditions, enamel undergoes a dynamic process between remineralisation and demineralisation, the so-called demineralisation-remineralisation cycle. Demineralisation and remineralisation are ongoing processes that occur in the oral environment, particularly on tooth surfaces. These processes are central to the development and management of dental caries. Demineralisation refers to the loss of minerals, primarily calcium and phosphate, from the tooth's hard tissues (enamel, dentine, and cementum). This process is triggered by acids produced by bacteria in dental plaque as they metabolise dietary carbohydrates (Ellwood et al., 2012).

In contrast, saliva counteracts this process of demineralisation by supplying small amount of calcium and phosphate ions. Fluoride ions are present in saliva by systemic intake or after using toothpaste, mouthrinses or other topical fluoride products (Abou Neel et al., 2016), which has a positive impact on enamel remineralisation. Remineralisation is the natural repair process where lost minerals (calcium, phosphate, and sometimes fluoride) are redeposited into demineralised areas of the tooth, restoring its structure and strength. When the pH in the mouth rises back to neutral (around 7.0), either due to saliva’s buffering action or reduced acid production, the tooth surface becomes receptive to mineral deposition (Featherstone, 2004). Calcium and phosphate ions from saliva or external sources (such as remineralising agents) are redeposited into the enamel’s demineralised areas, helping to repair the damage (Featherstone, 2000). Instead of forming entirely new crystals, the remineralisation process builds onto the existing crystal within the enamel. This repair process involves restoring lost minerals and reinforcing the tooth structure. If fluoride is present during remineralisation, the newly formed mineral will closely resemble fluorapatite, a form of mineral that is significantly more resistant to acid than the original hydroxyapatite. This remineralisation procedure can replace the lost minerals to enhance the strength of enamel and prevent tooth caries. Through this mechanism, the enamel can remineralise itself if changes are made to the diet and habits. However, it usually takes some time to repair enamel, even though the remineralisation cycle happens all day (Chou et al., 2014).

1.3.2. Role of saliva in the protection of tooth enamel

Saliva in natural conditions has a well-established protective role of arresting and reversing the caries process (Animireddy et al., 2014). The cleansing mechanism of saliva results in less accumulation of plaque on tooth enamel and protects against caries because of its supersaturation in an enriched form of calcium and phosphate with proteins (Colak et al., 2013). The oral microflora is regulated by saliva, which has a normal buffer pH of 6.75- 7.25 (Shen et al., 2011).

For the natural remineralisation process of saliva, it plays a critical role in remineralisation of enamel caries, and its protective mechanisms have been well-documented in the literature (Mandel, 1974). Saliva has four mechanisms to contribute to caries. It is well known that saliva helps in the mechanical cleansing of the oral cavity by physically washing away food particles, sugars, and bacteria from the tooth surface. This reduces the build-up of plaque, a biofilm where cariogenic bacteria thrive.

By keeping the tooth surface cleaner, saliva helps to limit the accumulation of plaque, which is a significant factor in the development of caries (Animireddy et al., 2014; Mandel, 1974). Salivary proteins play a critical role in protecting enamel by regulating mineral balance at the tooth surface (Ten Cate and Buzalaf, 2019). Proteins such as statherin and proline-rich proteins stabilise calcium and phosphate ions, preventing their spontaneous precipitation and maintaining a supersaturated environment that supports enamel remineralisation (Featherstone, 2008). Additionally, these proteins contribute to the formation of the acquired enamel pellicle, a protein-rich protective layer that reduces acid diffusion, inhibits demineralisation, and provides binding sites for beneficial antimicrobial molecules (Lendenmann et al., 2000).

Saliva is supersaturated with important minerals, this supersaturation helps to maintain the integrity of the enamel by reducing its solubility (Featherstone, 2000). The constant presence of these minerals in saliva enhances the remineralisation process, allowing them to deposit into the enamel, making it more resistant to acid attacks (Abdul et al., 2022). Moreover, saliva naturally contains calcium and phosphate, which are critical for remineralisation. In a supersaturated state, these ions are available to redeposit into the enamel, helping to fill in the porosities caused by demineralisation. Fluoride can enhance the uptake of calcium and phosphate ions into the enamel, promoting the formation of fluorapatite. (Cai et al., 2010). This makes the remineralised enamel less susceptible to future acid attacks.

The saliva buffering system is essential for maintaining a healthy oral environment by regulating pH levels and protecting teeth from acid-induced damage. Saliva contains natural buffers such as bicarbonate, phosphate, and proteins, which work to neutralise acids produced by bacteria during the breakdown of sugars. Among these, the bicarbonate buffer is particularly effective, as it converts strong acids into weaker carbonic acid, which is then expelled as carbon dioxide. This process helps to prevent enamel demineralisation, supports remineralisation, and stabilises the oral pH. The effectiveness of this system is closely linked to salivary flow rates, with higher flow improving acid clearance and enhancing its protective function (Animireddy et al., 2014).

Multiple factors, including systemic diseases, inherited disorders, certain medications, and medical interventions, can adversely affect salivary production, buffering capacity, and the availability of calcium and phosphate, preventing natural tooth remineralisation (Abdul et al., 2022).

1.4. Prevention of early enamel lesions

The prevention of enamel lesions is a fundamental aspect of maintaining dental health, focusing on arresting the early stages of tooth caries and preserving the integrity of the enamel. The primary focus should be on preventing biofilm formation and demineralisation of enamel, as well as employing methods for promoting remineralisation of lesions.

The Child of the North report highlights the urgent need to improve children's oral health by addressing issues at multiple levels (Marshman, 2024). Upstream interventions focus on policy changes, such as expanding the sugar tax, restricting junk food advertising to children, banning energy drink sales for under-16s, and introducing widespread water fluoridation to prevent cavities. Midstream efforts aim to change behaviours through school-based toothbrushing programmes, parental education on early oral care, and community awareness campaigns like Sheffield’s "Sweet Enough," while also shifting dental care towards prevention rather than just treatment. Downstream interventions focus on direct care, including improving NHS dental access, encouraging early checkups, and providing hospital treatment for severe cases. The report emphasises that a long-term solution requires a mix of policy change, community support, and accessible dental care to prevent tooth caries before it starts, rather than just treating it after it happens (Marshman, 2024).

1.4.1. Primary prevention of early enamel lesion

Primary prevention aims to stop the occurrence of early enamel lesions before they start. In general, this focuses on addressing the causes of a condition, including the application of pit and fissure sealants, in-office topical fluoride treatments, the use of fluoridated toothpaste, at-home chlorhexidine mouthwash (over 12 years), xylitol use, regular dental appointments, educating patients about saliva buffer capacity and adopting a non-cariogenic diet (Corrêa-Faria et al., 2020; Veiga et al., 2023).

To address these challenges, preventive policies should be implemented to combat early enamel lesions. These policies should aim to increase people's understanding of their oral health, encourage the adoption of healthy lifestyles, and introduce new preventive strategies and awareness campaigns to promote proper oral health (Veiga et al., 2023).

1.4.2. Secondary prevention of early enamel lesion

Secondary prevention focuses on early detection and intervention to halt the progression of early enamel lesions. This includes: early diagnosis, immediate treatment, behavioural interventions, and monitoring the effectiveness of preventive measures to minimise complications (such as pain, abscess, systemic infection, etc), and prevent the development of new lesions. The concept of caries lesion arrest is central to secondary prevention, as untreated lesions can lead to pain, tooth loss, and may act as a reservoir of cariogenic bacteria, potentially initiating new lesions. Therefore, the use of antimicrobials is a logical intervention (Horst et al., 2018).

Over the last ten years, the World Dental Federation (FDI) advocates a shift in caries management from restorative treatment to strategies that halt and prevent caries development, in line with the principles of the International Caries Classification and Management System (Ismail et al., 2015). The International Caries Classification and Management System (ICCMS™) provides a robust and comprehensive framework for the assessment, classification, and management of dental caries. This innovative system is specifically designed to promote evidence-based, patient-centred care, placing a strong emphasis on the prevention of caries, timely diagnosis, and the implementation of minimally invasive treatment strategies (Ismail et al., 2015).

Regular recall intervals and follow-up appointments are strategically determined based on the patient's specific risk level and the current status of their lesions. This tailored approach ensures that each patient receives the most appropriate care based on their unique circumstances. The systematic decision tree framework embedded within the ICCMS further assists clinicians in incorporating various factors, including caries risk, lesion activity, and individual patient preferences, into the treatment planning process (Bernd and Silvia, 2021).

All initial caries lesions should be addressed with topical fluoride application and monitored for progression. Additional options include the use of fissure sealants for occlusal lesions and potentially resin infiltration for proximal lesions extending into the outer third of the dentine (Erdwey et al., 2021).

1.5. The role of fluoride in caries prevention and enamel remineralisation

The Fluoride ion is odourless and tasteless and has a chemical formulation of F^-. It is an inorganic anion and the simplest form of fluorine (Bernd and Silvia, 2021). It is present in different forms, such as amine fluoride ($C_{27}H_{60}F_2N_2O_3$), sodium fluoride (NaF), sodium monofluorophosphate (Na_2FPO_3), stannous fluoride (SnF_2), or in combination. Fluoride was introduced into dentistry over 70 years ago, and it is now known for its dramatic decrease in the prevalence of caries worldwide (Phantumvanit et al., 2018). To date, it is widely accepted as the gold standard tool for preventing caries. Many studies have proved the regular use of fluoride toothpaste even in low levels has a profound effect in tooth enamel remineralisation (Davies and Davies, 2008). The mechanism of fluoride that increases the resistance against caries might arise from both systemic and topical applications (Erdwey et al., 2021).

1.5.1. Mechanism of action of Fluoride in caries prevention

Fluoride plays a vital role in dental health through both topical and systemic applications. Topical fluoride, delivered via toothpaste, mouth rinses, or professional treatments, directly strengthens enamel by facilitating the formation of fluorapatite, a mineral highly resistant to acid attacks. It also reduces bacterial activity and aids in the remineralisation of early enamel lesions. Systemic fluoride, typically obtained through fluoridated water or dietary supplements, becomes integrated into the developing tooth structure, enhancing its long-term resistance to demineralisation. These two approaches work synergistically, with topical fluoride providing localised protection and systemic fluoride contributing to the overall durability of teeth during formation.

Apatite minerals in human teeth are dynamic structures that can subtly change their composition in response to the surrounding environment (Amaechi et al., 2019). One of the most important of these changes is the conversion of HAP, the primary mineral in enamel, into the more stable FAP (Benson et al., 2013). This transformation occurs when fluoride ions (F^-) replace the hydroxyl ions (OH^-) that sit within the channel sites of the hydroxyapatite crystal lattice. Because fluoride has a similar ionic size but forms stronger and more stable bonds, it is readily incorporated into the structure, producing $Ca_{10}(PO_4)_6F_2$, a mineral with significantly lower solubility and greater resistance to acid attack (Philip, 2019). The presence of fluoride not only stabilises the lattice but also reduces structural imperfections, making the mineral phase more resilient to demineralisation (Chen et al., 2021). Although natural biological apatite may include additional substitutions, such as carbonate or small cations, the key process responsible for the improved durability is the direct exchange of OH^- with F^-. This single substitution plays a central role in enhancing enamel's long-term chemical stability (Featherstone, 2000).

1.5.2. Different mechanisms of fluoride in caries prevention

Fluoride plays a crucial role in preventing dental caries, primarily through topical application. It protects teeth by preventing enamel demineralisation, enhancing the remineralisation process, and reducing bacterial activity in dental plaque. Common sources of fluoride, such as drinking water, toothpaste, mouthwash, and varnishes, help maintain these protective effects. However, when fluoride is consumed systemically through supplements like drops, tablets, or lozenges, its impact on cavity prevention is limited (Featherstone, 1999).

However, the LOTUS 10-year retrospective cohort study examined the impact of water fluoridation on the oral health of adults and adolescents in England between 2010 and 2020, assessing both its effectiveness and cost-effectiveness. The findings indicate that while fluoridation was associated with a modest reduction in NHS dental treatment costs the direct health benefits, such as reductions in the need for invasive dental procedures and improvements in dental health indicators (e.g., decayed, missing, and filled teeth), were relatively small at the individual level (Moore et al., 2024). Moreover, The CATFISH study examined the impact and cost-effectiveness of water fluoridation in West Cumbria, UK. The findings indicated a modest reduction in dental caries among younger children (Goodwin et al., 2022).

1.5.3. Limitations of fluoride

While fluoride is highly effective in arresting or reversing early-stage, non-cavitated carious lesions, it has limited effectiveness once cavitation has occurred. Once a cavity forms, restorative treatment is required, as fluoride cannot rebuild the lost tooth structure (Grohe and Mittler, 2021). Also, fluoride works optimally when combined with saliva, which helps deliver calcium and phosphate for remineralisation. In individuals with xerostomia, such as those with certain medical conditions or medication-induced dry mouth, the effectiveness of fluoride in promoting remineralisation may be reduced. Fluoride treatments, whether through toothpaste, mouth rinses, or professional applications, require regular use to be effective. Individuals who do not consistently use fluoride-containing products may not experience its full protective benefits (Parnell et al., 2012).

Fluoride is a highly effective agent in preventing dental caries by enhancing enamel remineralisation and inhibiting demineralisation. However, limitations have been discovered when fluoride has been applied alone to prevent caries and remineralise enamel. These limitations indicate fluoride to be less effective below pH 4.5, and it still needs calcium and phosphate in the saliva to be effective (Elliott et al., 1985). Fluoride can only be effective in initial lesions (at the outer surface of 30 μm), and for that, full remineralisation can be difficult because fluoride does not infiltrate the lesion.

As mentioned previously, FAP is formed when fluoride ions (F^-) replace hydroxyl ions (OH^-) within the HAP lattice, where fluoride is available from saliva, toothpaste, or professional treatments (Wang et al., 2020). This ionic substitution yields a more stable crystalline phase, as F^- fits neatly into the apatite channel sites and creates a structure that is less soluble and more resistant to acidic dissolution than HAP. Despite these advantages, FAP is not without limitations. Because it is significantly less soluble, it is also less biologically dynamic, meaning it does not participate as readily in natural cycles of demineralisation and remineralisation that maintain enamel health (Ten Cate, 2008). FAP formation is largely restricted to the superficial enamel layers, since mature enamel lacks the permeability needed for deeper ionic exchange; therefore, the protective effect of fluoride is confined to only a few micrometers of the tooth surface (Ten Cate and Buzalaf, 2019). While FAP enhances enamel's resistance to acid attack, it does not make enamel immune to caries, as sufficiently low pH conditions can still dissolve even fluoride-rich mineral. These limitations highlight that, although FAP improves chemical stability, it cannot fully replicate the adaptability and biological responsiveness of natural hydroxyapatite (Nanci and Ten Cate, 2013).

1.5.4. Toxicity of Fluoride

Although fluoride efficacy increases with higher doses, however, this could increase the chance of fluorosis in younger individuals and toxicity in the older age group (Neves et al., 2002; Pro and Barthelat, 2019). Overexposure to fluoride, especially during the developmental stages of the teeth (before the age of 8), can lead to dental fluorosis, a condition characterised by the appearance of white spots, streaks, or, in severe cases, brown discolouration and pitting of the enamel (Groeneveld et al., 1990). Fluorosis occurs primarily due to excessive ingestion of fluoride from multiple sources, such as fluoridated water, toothpaste, supplements, or foods and beverages made with fluoridated water. While mild fluorosis may have a limited impact on dental function, its aesthetic effects can be a concern, particularly in severe cases (Gibson-Moore, 2009). Skeletal fluorosis is a rare case of long-term exposure to high concentrations of fluoride, particularly in areas where water contains excessive natural fluoride. Where individuals may develop skeletal fluorosis, a condition that affects bones and joints, leading to stiffness, pain, and other symptoms. This is generally not a concern in areas with controlled fluoride levels in water (Künzel, 1993).

In cases of accidental ingestion of large quantities of fluoride, particularly in young children, there is a risk of acute toxicity (Harrison, 2005). This can lead to symptoms such as nausea, vomiting, abdominal pain, and, in extreme cases, systemic toxicity requiring medical intervention. Care must be taken to ensure proper dosing and supervision of fluoride use, especially in children. For example, young children should use only a pea-sized amount of fluoride toothpaste and be supervised to avoid swallowing it (Hattab, 2013).

Fluoride alone is not sufficient to prevent dental caries in individuals with poor oral hygiene or a high-sugar diet. While fluoride can promote remineralisation and inhibit demineralisation, continuous exposure to high levels of sugar and plaque build-up may still result in caries (Hong et al., 2018). In populations with limited access to dental care, fluoride exposure may be insufficient to counteract other risk factors for caries, such as a lack of access to preventive care, education, and nutritional guidance (Horowitz, 2003).

While most of the research supports the safety of fluoride at recommended levels, some studies and activists raise concerns about potential long-term systemic effects, such as impacts on bone health, kidney function, or neurodevelopment, though these remain controversial and not conclusively proven in most populations (Buzalaf, 2011). The above-mentioned limitations of fluoride strategies justify the fact that it is better to work with new strategies that are either better or as effective as fluoride to maximise the clinical significance of remineralisation (Limeback et al., 2021), but overcome the limitations of using fluoride (Benson et al., 2013; Shahid, 2017).

Recent studies have discussions about the potential impact of fluoride exposure on children's cognitive development (Fontana, 2016). A comprehensive review by the National Toxicology Program analysed data from 74 studies and found a consistent link between higher fluoride levels and lower IQ scores, estimating a decline of 1.63 IQ points for every 1 mg/L increase in urinary fluoride concentration (Spittle, 2024).

Similarly, a meta-analysis reported that fluoride exposure above 1.5 mg/L was associated with cognitive decline, though the effect was less significant at lower concentrations (Taylor et al., 2025). It is important to note that these findings primarily relate to populations exposed to fluoride levels higher than those typically found in U.S. public water systems, where the recommended concentration is 0.7 mg/L to optimise dental health benefits while minimising potential risks.

1.6. The role of calcium and phosphate in enamel caries prevention and remineralisation

1.6.1. Effect of calcium and phosphate in enamel caries prevention

Preventing enamel caries requires maintaining a balance between demineralisation and remineralisation, with calcium and phosphate serving as key contributors to this process. As the building blocks of enamel, these minerals help safeguard teeth against acid-induced damage by strengthening the enamel structure and supporting its natural ability to repair minor mineral loss. By ensuring a consistent supply of calcium and phosphate in the oral environment, enamel remains more resilient to daily challenges, reducing the risk of lesion formation and promoting long-term dental health (Ekambaram et al., 2017).

1.6.2. Effect of calcium and phosphate on demineralisation and remineralisation of enamel

The balance of natural calcium and phosphate within plaque fluid plays a crucial role in regulating enamel demineralisation. As the primary components of enamel, these ions significantly influence the rate at which the ions structure dissolves. The outermost layer of enamel is continuously exposed to saliva and plaque fluid, creating a dynamic equilibrium between the HAP crystals and the surrounding oral environment. Enamel dissolution is not solely dependent on pH levels; the concentration of calcium and phosphate ions in the surrounding fluid also dictates the extent and speed of demineralisation (Yan et al., 2022).

Calcium and phosphate ions can be provided from extrinsic sources to form ion deposition into crystal voids in demineralisation to produce net mineral gain (Cai et al., 2010). As a result, the body forms crystalline calcium phosphate with the help of calcium to form the hard enamel that protects and surrounds the inner parts of teeth (Hornby et al., 2014). Extensive *in vitro* and clinical research has explored the efficacy of calcium delivery from oral care products containing calcium salts and their potential benefits for enamel health. Studies have demonstrated that using mouth rinses formulated with urea and calcium chloride can increase calcium concentrations in dental plaque (Yan et al., 2022). This elevation in plaque calcium levels has been associated with a reduction in enamel porosity and an increase in the hardness of acid-softened enamel surfaces.

The crucial role of calcium in enamel remineralisation is further supported by *in vitro* studies. These investigations reveal that increasing calcium concentrations in remineralisation solutions, while maintaining fluoride levels, significantly enhances the repair of subsurface enamel lesions. Additionally, materials containing tricalcium phosphate (TCP) have shown promising remineralisation effects on acid-softened enamel. For instance, pH cycling experiments indicate that β-tricalcium phosphate (β-TCP) can release calcium ions and facilitate the remineralisation of early-stage enamel lesions (Bhat et al., 2022).

Another notable remineralising agent is the casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) complex, a milk-derived protein compound with demonstrated anticariogenic properties. Research suggests that CPP-ACP reduces enamel demineralisation and enhances remineralisation by buffering free calcium and phosphate ion activity. This effect is achieved through the incorporation of amorphous calcium phosphate (ACP) into plaque and onto tooth surfaces, maintaining a state of supersaturation that favours enamel repair and protection (Xu et al., 2022).

Three calcium phosphate-based remineralisation systems have now been commercialised, where the manufacturers claim the specific form of calcium phosphate helps overcome the limited bioavailability of calcium and phosphate ions for the remineralisation process (Reynolds, 2008). Table 1 demonstrates commercially available calcium phosphate-based remineralisation technologies (Arifa et al., 2019; Chen et al., 2024; Reynolds, 2008)

Table 1: Commercially available calcium phosphate-based remineralisation technologies.

Technology	Commercial product	Remineralisation claim
Casein phosphopeptide stabilised calcium phosphate (Recaldent^{TM}, CPP-ACP)	Trident White sugar-free gum, Recaldent^{TM} sugar-free gum, Tooth Mousse, MI paste	Recaldent^{TM} (CPP-ACP) is a remineralising ingredient that strengthens teeth by delivering calcium and phosphate to the tooth surface.

Unstabilised amorphous calcium phosphate (ACP, Enamelon^{TM})	Enamel Care with liquid calcium, Nite White ACP, Day White ACP, Mentadent replenishing white	Rebuilds enamel. The deposition of hydroxyapatite onto teeth rebuilds enamel through a process called remineralisation.
The bioactive glass containing calcium sodium phosphosilicate (NovaMin^{TM})	Oravive toothpaste	Nourishes the teeth with essential calcium and phosphorous ions needed for the natural self-repair process of the teeth.
β-tricalcium Phosphate	toothpaste formulations	Protective barrier to coexist with fluoride present in saliva. This helps with releasing calcium and phosphate to help with remineralisation.
Functionalised TCP	Toothpaste formulations	Act as barrier that prevents premature TCP–fluoride interactions and facilitates delivery of TCP.
Dicalcium Phosphate Dihydrate (DCPD)	Toothpaste formulations	Increase the levels of free calcium ions in the plaque remains

		elevated for up to 12 hours after brushing.

The advantages of using commercially available calcium and phosphate products offers several advantages, including significant reduction of tooth sensitivity, remineralisation of acid erosion, promotion of fluoride uptake in plaque and inhibition of the development of dental caries (Reynolds, 2008).

1.7. Available systems for delivering calcium and phopspate in enamel remineralisation

Various delivery systems have been developed as dental products/traditional treatments in assuring effectiveness in the delivery of calcium and phosphate for enhancing enamel remineralisation and restoring the integrity of enamel (Table 2) (Ekambaram et al., 2017).

1.7.1. Gels

Gels are one of the most used forms for delivering calcium and phosphate to the teeth (Juntavee et al., 2021). They are easy to be applied and can be tailored to individual patient needs. The gel is more viscous in formulation, can promote prolonged contact with the enamel, penetrate the surface of enamel and allows for a sustained release of calcium and phosphate ions over time (Reynolds, 2008). The active compounds form a supersaturated environment around the enamel, promoting the deposition of calcium and phosphate. The penetration and retention are achieved due to the viscosity and adhesive properties of gels (Mozafari et al., 2010). Fluoride gels that incorporate calcium and phosphate can act as a reservoir of the bioavailable calcium

and phosphate to enhance the remineralisation potential, such as, CPP-ACP also known as GC Tooth Mousse MI Paste™. It is usually used with a tray, brush or applied directly to teeth which makes it good for overnight or long-term use
(Ahmed, 2015).

Gels that contain synthetic hydroxyapatite nanoparticles mimics the nature of the mineral structure in enamel. This allows the surface irregularities to be filled and enhance the smoothness of enamel to promote remineralisation. Incorporating nanoparticles into gels could enhance the precision and depth of remineralisation. Bioactive glass gels such as calcium sodium phophosilicate could release calcium and phosphate ions present with saliva, which also promote enamel remineralisation. Tricalcium phosphate (TCP) when combined with fluoride can help to stabilise calcium and neutralises the acids in the mouth which supports remineralisation of enamel (Reynolds, 2008). Studies have mentioned that combining multiple remineralising agents that contain gels in one formulation can improve the efficacy (Yan et al., 2022).

1.7.2. Solutions

Solutions are the liquid formulations that provide ions in the oral environment. It increases calcium and phosphate in saliva to allow saturation of the oral cavity and promotes remineralisation. Such as, rinses with calcium lactate and phosphate compounds; fluoride mouthwashes that include next generation remineralising agents. Typically, it is used to rinse for a few minutes ensuring mineral-rich penetration to the enamel surface (Fernando et al., 2022). These solutions are designed to maintain a high concentration of calcium and phosphate ions in a stable form to allow for the ion diffusion into demineralised areas in the enamel, where crystallisation takes place and form HAP. Such as, ACP and CPP-ACP solutions are found in a stabilised form of calcium and phosphate in a bioavailable form (Reynolds, 1997).

Solutions with calcium and phosphate ions in cluster forms can enhance enamel remineralisation by forming steady supply of ions. Such as, NovaMin based products (Ionescu et al., 2022). Furthermore, some plant extracts such as polyphenols can enhance remineralisation by stabilising the calcium and phosphate ion penetration, such as Herbal based mouth rinses (He et al., 2015).

The advantages of solution-based delivery of calcium and phosphate is the easy penetration and accessibility into enamel microstructure, the ion composition, pH and concentration of ions can be adjusted and the ease of application, for example, rinses and sprays (Reynolds, 2008). However, the stability of calcium and phosphate ions in solution can be challenging, difficulty of effective penetration into the enamel surface and preventing the penetration of unwanted penetration of calcium and phosphate when applied together such as brushite (Abou Neel et al., 2016).

1.7.3. Pastes and Toothpastes

Toothpaste plays a crucial role in everyday delivery of calcium and phosphate ions into enamel. Toothpaste are semi-solid formulations containing abrasives, fluoride, and calcium-phosphate systems (Hamba et al., 2020). Toothpaste serves as an effective vehicle for delivering calcium and phosphate ions to promote remineralisation. For further mechanical plaque removal, these pastes deposit remineralisation agents on the enamel surface whilst brushing. For example, nano-hydroxyapatite toothpaste, bioactive glass used in toothpaste (e.g., NovaMin), CPP-ACP or ACP based formulations (Schemehorn et al., 1999). Brushing daily for best results and most effective delivery. The advantages of this technique; is that it is mostly cost effective, easy to use, and can benefit populations at high risk for caries, such as children, orthodontic patients, and individuals with xerostomia. However, toothpaste is saliva dependent to act as a medium for the ion penetration. It can also have insufficient exposure time as brushing is dependent on patient compliance (Thimmaiah et al., 2019). New developments to overcome these limitations are the use of microcapsules technology to release ions gradually for prolonged efficacy. Furthermore, the use of biomimetic strategies the contain nano-hydroxyapatite to closely mimic enamel composition. Nano-hydroxyapatite toothpaste demonstrated the potential to repair surface subsurface lesions and improve enamel gloss and strength (Coelho et al., 2019).

1.7.4. Chewing Gums

Chewing gum is an effective method for delivering calcium and phosphate ions. Typically, they require chewing, which boosts salivation and the gum releases minerals slowly into saliva, so chewing gums are enriched with calcium and phosphate compounds for best utilisation. Saliva then helps these ions to penetrate the enamel. Chewing can increase the production of saliva which contains calcium and phosphate ions and helps to buffer acids in the oral microflora (Cai et al., 2009).

Some chewing gums are fortified with bioavailable calcium and phosphate sources, and calcium glycerophosphate. These compounds can directly deliver ions to demineralised enamel. Also, Research has shown that chewing gum containing calcium/phosphate compounds significantly enhances enamel remineralisation compared to unfortified gum or no intervention (Prestes et al., 2013). Furthermore, frequency is also important, regular use (e.g., after meals) optimises the remineralisation process. The type of chewing gum is also important. Sugar-Free gums are preferable to prevent the cariogenic effects of sugar and chewing gums with specific remineralising agents, offer superior benefits. The benefits of using gums are that it is convenient, practical, and not invasive (Lampert and Lo, 2012). It can also prevent caries by delivering calcium and phosphate, chewing gum can help arrest early enamel lesions and reduce the risk of caries. One study showed that when chewing sugar-free gum containing CPP-ACP can promote great levels or remineralisation when compared to gums that did not contain CPP-ACP (Shen et al., 2012). Another study also showed that chewing xylitol gum containing funoran and calcium hydrogen phosphate has a significant effect on the remineralisation of enamel subsurface lesions (Thaweboon et al., 2009). Some of the limitations are; not all chewing gums are equally effective; specific formulations are required for significant remineralisation and chewing gum should be used as an adjunct to other oral health measures, such as fluoride toothpaste and dietary modifications, rather than a working alone (Cai et al., 2010).

1.7.5. Tablets or Lozenges

These are the solid formulations which when put in mouth, dissolves in mouth over a period. The dissolving process of the tablet also causes a local release of calcium and phosphate ions to the saliva above background levels, to enable systemic remineralisation to enamel. For instance, calcium and phosphate lozenges, commonly suggested for xerostomia or as a remineralising agent (Elkassas and Arafa, 2014). Calcium and phosphate sources typically include bioavailable forms like calcium phosphate, or hydroxyapatite nanoparticles. The mechanism of action of tablets is by diffusion of ions into enamel subsurface lesion affected by demineralisation. It is convenient, provides prolonged exposure, compliant and portable. Tablets can be beneficial in caries prevention, erosion management, prevents demineralisation around brackets and bands, and aids in reducing sensitivity after bleaching. Tablets can be challenging in advanced enamel lesions, tablets also relay on saliva combination with probiotics or other oral health agents for multifaceted benefits (Reynolds, 1997).

1.7.6. Varnishes

Varnishes are concentrated adhesive formulations applied to the teeth. They serve as a reservoir for calcium, phosphate, and sometimes fluoride, which adhere to the enamel and are gradually released over time, examples include calcium-phosphate enriched fluoride varnishes. These products are typically used for patients at high risk for dental issues and are applied in a clinical setting by a dentist or hygienist (Cai et al., 2021).

For the varnish to effectively penetrate the enamel and maintain prolonged contact, a steady release of these ions is necessary. One study demonstrated that fluoride varnishes that contains tri-calcium phosphate can reduce the initiation and progression of subsurface enamel lesion progression and enhance remineralisation (Rirattanapong et al., 2014). Varnishes are renowned for their ability to form a protective layer that alleviates sensitivity and prevents demineralisation. They not only help prevent caries but also facilitate the remineralisation of early enamel lesions, providing a barrier against acidic challenges. Varnishes are intended to be used as a supplementary measure for remineralisation, in conjunction with regular dental check-ups and effective oral hygiene practices.

Although fluoride varnishes are widely used for caries prevention and dentin hypersensitivity management, they present several important limitations. Their effectiveness is largely dependent on patient compliance and correct clinical application, as inadequate drying or contamination with saliva can reduce fluoride uptake (Marinho et al., 2013). Fluoride varnishes also form only a superficial mineral layer, typically limited to the outer few micrometers of enamel, meaning they do not penetrate deeply into the tooth structure. This limits their long-term protective effect, especially in high-caries-risk or xerostomia patients (Rirattanapong et al., 2014). Additionally, varnishes provide only temporary fluoride availability, as the material is rapidly worn away by mastication and oral hygiene procedures. Their efficacy can also vary depending on the formulation, fluoride concentration, and carrier resin, with some products showing inconsistent fluoride release profiles (Cai et al., 2021). Finally, overuse, particularly in young children, carries a small but notable risk of excess systemic fluoride ingestion, highlighting the need for careful dosing. These limitations indicate that fluoride varnishes should be used as part of a broader preventive strategy rather than as a stand-alone solution (Marinho et al., 2013).

1.7.7. Resin-Based Systems

Calcium and phosphate delivery systems designed for enamel remineralisation represent a cutting-edge strategy in modern dentistry, particularly when utilising resin-based materials. These innovative, dual-action systems leverage the combined mechanical strengths of resin-based agents alongside the therapeutic properties of remineralising substances, specifically tailored for the repair and preservation of dental enamel. The significance of these systems lies in their ability to address the critical need for effective enamel restoration, particularly in the face of common dental issues such as caries.

Restorative dental treatments frequently employ ion-releasing materials, including glass ionomer cements and resin composites, which are engineered to slowly release essential minerals like calcium and phosphate. This gradual release not only helps to buffer the tooth but also aids in the natural remineralisation process, enhancing the overall health and integrity of the tooth structure. These materials are meticulously formulated from a resin matrix, often comprising light-cured or self-cured resins such as Bisphenol A glycidyl methacrylate (Bis-GMA), Urethane dimethacrylate (UDMA), and Triethylene glycol dimethacrylate (TEGDMA) (Li et al., 2023).

In addition to base resins, these advanced materials are incorporated with various remineralising agents, including ACP, TCP, and CPP. Such combinations ensure that the restorative material not only provides structural support but also actively contributes to the remineralisation of the enamel. To further enhance their performance, these resin-based systems typically include additives such as photo initiators, which facilitate the polymerisation process and improve the mechanical properties of the final product. A study developed a novel nanostructured resin infiltrant containing nanoparticles of amorphous calcium phosphate (NACP) to treat enamel early lesions. The study showed that the combined ICON with 30% NACP infiltrant managed to stop the progression of enamel early lesion, increase the hardness of enamel, and protect the enamel (Li et al., 2023).

The resin acts as a reservoir that gradually releases not only calcium and phosphate ions but also fluoride, when included in the formulation. This release mechanism significantly boosts remineralisation rates and fosters the formation of fluorapatite.

Resin-based delivery systems encompass a range of applications, including sealants, adhesives, fillers, composites, and protective coatings (Cai et al., 2010).

In summary, the mode of delivery is based upon; severity of enamel damage: Gels, varnishes or professional trays are more suitable for severe cases, whereas toothpastes and rinses provide an effective daily regimen system too. Chewing gum, lozenges as well as toothpastes are easy to use according to preference every day and specific needs: Targeted protection delivered by sealants and coatings provide location-specific high-risk area coverage.

Table 2. Details of products of calcium and phosphate-based systems.

Clinpro tooth creme	**3M ESPE; St Paul, MN, USA**	**0.21% sodium fluoride, tri-calcium phosphate (TCP).**
Clinpro White Varnish	3M ESPE	5% sodium fluoride, tri-calcium phosphate (TCP).
Enamel Pro Varnish	Premier Dental Products; Plymouth Meeting, PA, USA	5% sodium fluoride, amorphous calcium phosphate (ACP).
Tooth Mousse creme	GC; Tokyo, Japan	Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP).
MI Paste GC	America; Alsip, IL, USA	Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP).

Tooth Mousse Plus creme	GC; Tokyo, Japan	0.09% sodium fluoride, casein phosphopeptide-amorphous calcium phosphate (CPP-ACP).
MI Paste Plus	GC America	0.09% sodium fluoride, casein phosphopeptide-amorphous calcium phosphate (CPP-ACP).
MI Varnish	GC America	5% sodium fluoride, casein phosphopeptide-amorphous calcium phosphate (CPP-ACP).
Embrace Varnish	Pulpdent; Watertown, MA, USA	5% sodium fluoride, xylito-coated calcium and phosphate (CXP).

Resin-based systems have several limitations that may compromise their effectiveness. Their penetration into subsurface lesions is largely determined by lesion porosity and the presence of highly mineralised surface layers, which can reduce adequate infiltration and reduce treatment efficacy (Paris and Meyer-Lueckel, 2010). Polymerisation shrinkage is another challenge, as it can create interfacial gaps that increase the risk of microleakage and caries (Tjäderhane et al., 2013). Over time, resin materials may undergo hydrolytic and enzymatic degradation within the oral environment, resulting in diminished mechanical properties and reduced longevity (Breschi et al., 2018). Additionally, resin-based materials may experience discoloration from dietary and environmental factors, affecting their aesthetic stability (Van Dijken and Lindberg, 2015). Unlike biomimetic remineralisation strategies, which actively restore mineral content, resin infiltration primarily fills enamel microporosities and does not contribute to true subsurface mineral repair, limiting its regenerative potential (Paris and Meyer-Lueckel, 2010).

1.7.8. Self-assembling peptide P11-4

Self-assembling peptide P11-4 is a synthetic, amphiphilic peptide that undergoes a pH-dependent transition from a monomeric solution to a nanofibrillar scaffold when exposed to the acidic conditions characteristic of early enamel caries (Kirkham et al., 2007). This structural transformation enables the peptide to infiltrate subsurface lesions and form a three-dimensional matrix that resembles the natural enamel extracellular framework. The resulting scaffold provides nucleation sites with high affinity for calcium and phosphate ions, generating a supersaturated local environment conducive to hydroxyapatite formation within the lesion body rather than on the superficial surface (Brunton et al., 2013). Through this biomimetic mineral-templating mechanism, P11-4 has been shown to promote significant subsurface remineralisation, increase mineral density and hardness, and arrest or reverse early non-cavitated carious lesions in both *in vitro* and clinical studies (Schlee et al., 2018). These findings position P11-4 as a minimally invasive therapeutic approach aligned with contemporary caries management strategies that emphasise biological repair over operative intervention (Kirkham et al., 2007).

1.8. The role of Hydroxyapatite in tooth

1.8.1 Hydroxyapatite

Hydroxyapatite is the main inorganic mineral of calcium phosphate found in teeth and bone (Chen et al., 2021). HAP crystals are highly elongated, rod-like structures with widths of approximately 40–60 nm and lengths that can extend up to several micrometers, (Jiang et al., 2019). These crystals are densely packed into enamel rods (prisms) and interrod enamel, forming a highly ordered, hierarchical microstructure that underlies enamel's exceptional hardness and resistance to fracture (Habelitz et al., 2001).At a submicron scale, the orientation of HAP crystals within each rod is nearly parallel along the rod axis, while the rods themselves are arranged in a decussating pattern that distributes mechanical stress and enhances toughness (Amaechi et al., 2022). Additionally, the HAP crystals in enamel are only partially mineralised at the nanoscale, allowing for minimal elastic deformation under load, which contributes to enamel's unique combination of rigidity and resilience (Margolis et al., 2006). This hierarchical arrangement, spanning from the nanoscale crystal structure to the macroscale rod pattern, is crucial for preventing crack propagation and ensuring the durability of enamel under masticatory forces (Chen et al., 2021).

The balance between demineralisation and remineralisation in dental enamel is largely controlled by the pH of the oral environment (Robinson et al., 2000). Enamel is primarily composed of HAP which exists in dynamic equilibrium with calcium and phosphate ions in saliva. When the pH drops below the critical value of approximately 5.5, hydrogen ion concentration increases, promoting the dissolution of hydroxyapatite (Featherstone, 2000). Protons react with phosphate and hydroxide ions in the crystal lattice, forming soluble species that diffuse into saliva, resulting in mineral loss and enamel softening (Featherstone, 2004).

Conversely, when the pH rises above this critical threshold, the saliva becomes supersaturated with calcium and phosphate ions. This favours the redeposition of these ions into the enamel, allowing the HAP crystals to grow or repair (Palmer et al., 2008). Therefore, the pH of the oral environment directly shifts the equilibrium between mineral loss and gain, determining whether enamel undergoes demineralisation or remineralisation (Robinson et al., 2000).

Hydroxyapatite is composed of calcium (Ca^{2+}), phosphate (PO_4^{3-}) and hydroxyl (OH^-) ions. The molecular formula is $Ca_{10}(PO_4)_6(OH)_2$ (Amaechi et al., 2019) (Figure 2). Calcium and phosphate are a natural component of teeth and bones. It is highly biocompatible with human tissue, making it widely used in medical and dental applications, including bone grafts, dental implants, and coatings (Bernd and Silvia, 2021). Hydroxyapatite forms in a hexagonal crystalline structure. This orderly arrangement of calcium, phosphate and hydroxyl ions provides structural rigidity and strength, especially in tooth enamel. The crystalline structure contributes to the high hardness of hydroxyapatite, particularly in tooth enamel, which is the hardest tissue in the human body (Bossù et al., 2019). However, hydroxyapatite is brittle, meaning it can fracture under high stress. Hydroxyapatite is insoluble at neutral or slightly alkaline pH (around pH 7), making it stable in healthy oral and physiological environments. Hydroxyapatite dissolves in acidic conditions, typically when the pH drops below 5.5, which is relevant for the process of demineralisation in dental caries (Chen et al., 2021). This property is critical because it explains why hydroxyapatite is vulnerable to acid attacks in the mouth, leading to enamel breakdown. Hydroxyapatite has excellent compressive strength, making it capable of withstanding the forces exerted during chewing and biting. This property is especially important in tooth enamel.

Biological hydroxyapatite found in enamel differs significantly from pure, synthetic hydroxyapatite (Amaechi et al., 2019). Enamel crystals are not chemically ideal; instead, they contain substitutions within the hydroxyapatite lattice, including carbonate replacing phosphate or hydroxyl groups, and trace elements such as strontium, magnesium, lead, and fluoride incorporated into the crystal structure (Limeback et al., 2021). These ionic substitutions disrupt lattice regularity and increase lattice strain, which collectively enhance solubility compared to stoichiometric, laboratory-prepared hydroxyapatite. As a result, biological hydroxyapatite is inherently more reactive and more susceptible to acidic dissolution than pure HAP, an important factor governing enamel demineralisation and remineralisation dynamics (Chen et al., 2021).

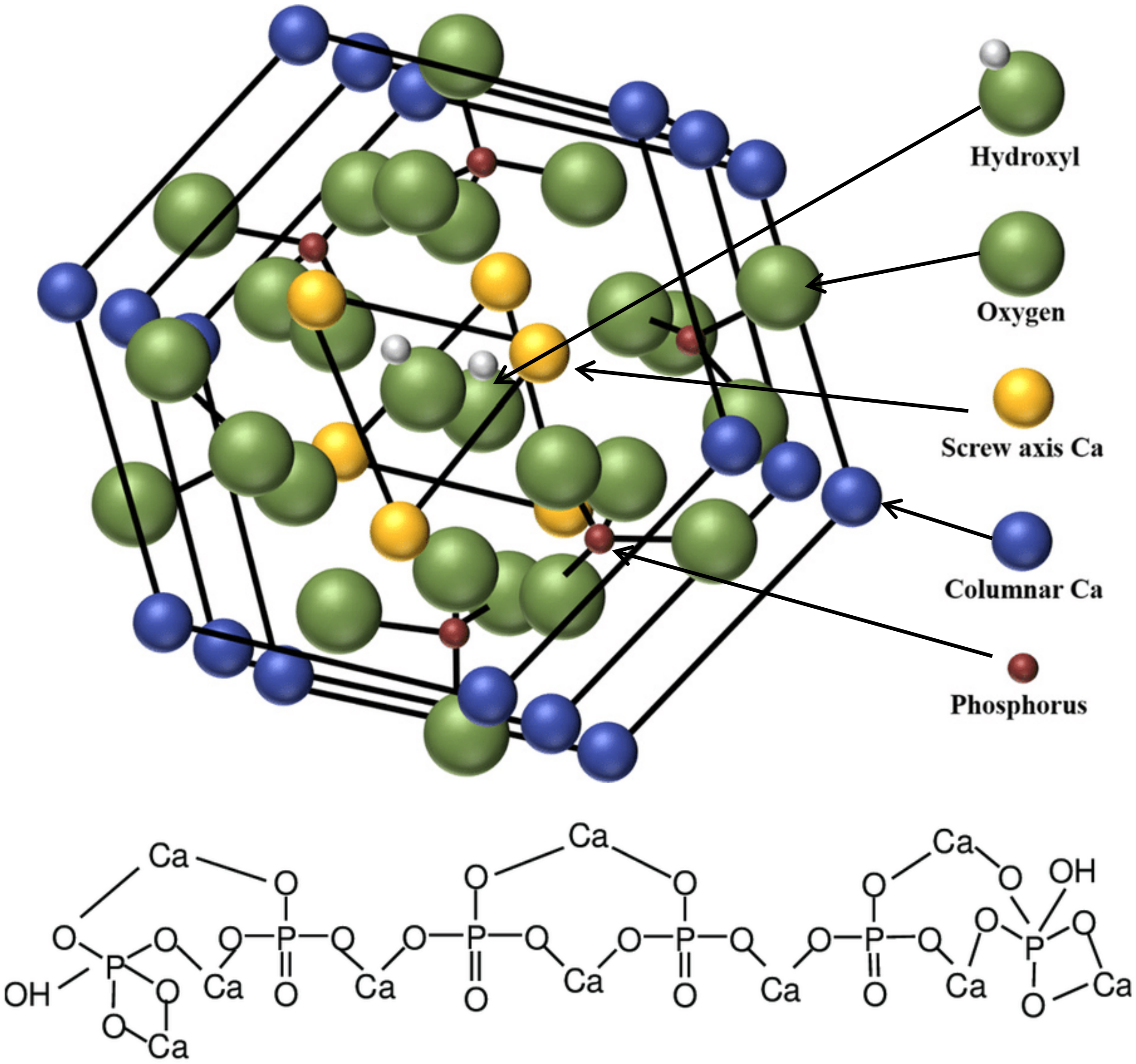


Figure 2. The atomic arrangement of HAP. This image was modified from (Brunton et al., 2013; Ylinen, 2006).

Hydroxyapatite is naturally translucent, which is an important property in tooth enamel as it contributes to the optical properties of teeth, such as their appearance and colour (Cai et al., 2010). In its pure form, hydroxyapatite is white. However, tooth enamel may take on different shades due to the presence of other substances or due to staining (Shen et al., 2012).

In biomedical applications, hydroxyapatite is known for its bioactivity, meaning it can bond with living tissues such as bone. This property makes it an ideal material for bone grafts, dental implants, and coatings on prosthetics to enhance integration with natural bone (Coelho et al., 2019). Hydroxyapatite is biodegradable under certain physiological conditions, which means it can be resorbed by the body over time. This is advantageous in applications like bone regeneration, where the hydroxyapatite scaffold is gradually replaced by natural bone tissue
(Ylinen, 2006).

Hydroxyapatite is used in a variety of dental products and treatments, including toothpastes and mouthwashes containing nano-hydroxyapatite, which help promote enamel remineralisation, restorative materials, and coatings on dental implants to improve biocompatibility and promote tissue integration. Hydroxyapatite is also widely used in orthopaedics, particularly in bone grafts and as a coating for metallic implants to enhance osseointegration and improve the implant's long-term stability (Coelho et al., 2019).

1.8.2. Hydroxyapatite properties

HAP is a bioactive, non-toxic calcium phosphate ceramic whose crystallographic structure, inorganic chemistry and physicomechanical characteristics closely resemble those of the mineral phase of enamel and bone (Bayani et al., 2017). Moreover, HAP exhibits excellent biocompatibility and osteoconductivity, enabling it to support cellular attachment, guide new bone formation, and establish stable, direct chemical bonding with surrounding biological tissues (Chen et al., 2021).

HAP is available from natural sources and has a composition of 39.68 wt% Ca, 18.45 wt% P with a pH range of 4.2 - 12.0. Stoichiometric hydroxyapatite represents the idealised form of the apatite mineral, and a precise Ca/P molar ratio of 1.67 and a weight ratio of 2.15 (Dorozhkin, 2009). This high lattice regularity confers low solubility, high crystallinity, and thermodynamic stability, features typically observed in synthetic HAP produced under controlled conditions. Consequently, stoichiometric HAP is often used as a reference material for studying mineral dissolution kinetics, precipitation behaviour, and ion substitution mechanisms in mineralised tissues (Rey et al., 2009).

In contrast, biological hydroxyapatite in enamel and bone is inherently non-stoichiometric, reflecting deviations from the ideal Ca/P ratio and the incorporation of multiple substituent ions within the crystal lattice (Palmer et al., 2008). Carbonate, the most abundant substituent, replaces either phosphate groups (B-type substitution) or hydroxyl groups (A-type substitution), typically lowering the Ca/P ratio below 1.67 (Brunton et al., 2013). Additional ions such as magnesium, sodium, strontium, and fluoride can substitute for calcium or hydroxyl sites, and vacancies commonly occur throughout the lattice. These compositional variations introduce lattice strain, reduce crystallinity, and significantly increase solubility compared with stoichiometric HAP (Bayani et al., 2017).

HAP can be synthesised using various precursors by careful control of shape, particle size, and phase (Amaechi et al., 2019). Hydroxyapatite can be synthesised from a variety of calcium and phosphate precursors, and the selection of reagents (Bayani et al., 2017). Typical calcium sources used in laboratory synthesis include calcium nitrate tetrahydrate [$Ca(NO_3)_2{\cdot}4H_2O$], calcium hydroxide [$Ca(OH)_2$], calcium chloride ($CaCl_2$), and calcium acetate, while commonly used phosphate precursors include diammonium hydrogen phosphate [$(NH_4)_2HPO_4$], phosphoric acid (H_3PO_4), and disodium hydrogen phosphate (Na_2HPO_4) (LeGeros, 2008; Watanabe and Akashi, 2009). Different synthesis strategies provide further control over the physicochemical characteristics of the final HAP material (Dorozhkin, 2009). For instance, wet chemical precipitation allows for the formation of nanoscale HAP by adjusting pH, temperature, and mixing kinetics, whereas sol–gel processing produces highly homogeneous and fine-particle HAP suitable for thin films and biomedical coatings (Rey et al., 2009). Hydrothermal synthesis, conducted under elevated temperature and pressure, enables the production of well-defined nanorods and plate-like crystals that more closely resemble biological apatite (Palmer et al., 2008). Additionally, biogenic calcium sources such as eggshells, coral, or animal bone can be converted into HAP through calcination and reprecipitation, resulting in materials that naturally incorporate trace ions similar to those found in enamel and bone. (Amaechi et al., 2019).

1.8.3. The role of hydroxyapatite in remineralisation of enamel

One of the goals of modern dentistry is early intervention of dental caries(Meyer et al., 2018). Hydroxyapatite-developed agents can be used for remineralisation of initial caries lesions. For example, hydroxyapatite toothpaste is an effective and popular alternative to fluoride as not everyone can use toothpaste containing fluoride. Some people are allergic to the ingredients of fluoride toothpaste, then hydroxyapatite-containing toothpaste is a better option (Chen et al., 2021).

In fact, demineralisation of enamel occurs when hydroxyapatite level drops and white spot lesion formation develops (Kidd and Fejerskov, 2004). However, enamel remineralisation for early lesions takes place in the natural process with the help of calcium, phosphate, salivary proteins, and other elements deposited in demineralised enamel (Meyer et al., 2018).

Hydroxyapatite remineralises enamel from within to reach the most inner part of the caries (Benson et al., 2013). Hydroxyapatite-based remineralisation relies on the ability of calcium and phosphate ions, released from nano-sized or micro-sized HAP particles, to diffuse through the porous network created during early enamel demineralisation (Dorozhkin, 2009). Initial carious lesions are characterised by subsurface mineral loss while the superficial enamel layer often remains relatively intact; however, this surface still contains nanometre-scale pores created by acid dissolution (Rey et al., 2009). These porosities act as diffusion pathways through which dissolved ions can migrate. When HAP particles contact the enamel surface, they partially dissolve in the acidic or neutral oral environment, releasing Ca^{2+} and PO_4^{3-} ions. The concentration gradient created between the particle–saliva interface and the demineralised subsurface zone drives the inward diffusion of these ions (Hannig and Hannig, 2010). As they penetrate deeper into the lesion, they precipitate within the enamel's interprismatic spaces, progressively filling voids and rebuilding the crystalline framework (Xu et al., 2022).

In addition to simple diffusion, capillary action within the microscopic channels of demineralised enamel enhances ion transport by allowing fluid movement toward the lesion's interior (Hannig and Hannig, 2010). The presence of partially dissolved HAP nanocrystals also serves as nucleation sites, enabling localised crystal growth directly within the deeper tissue rather than only on the surface. This mechanism differs from fluoride, which primarily strengthens the outer enamel layer, whereas HAP supplies minerals capable of deeply infiltrating the subsurface lesion and restoring the enamel ultrastructure from within (Dorozhkin, 2009).

Many studies showed that there is no difference between fluoride and hydroxyapatite toothpaste (Bossù et al., 2019). This study reported that both agents effectively reduce hypersensitivity, inhibit demineralisation, and support remineralisation, but HAP offers an advantage in being non-toxic, and beneficial in situations where fluoride intake needs to be controlled. Despite the mechanistic differences, clinical outcomes in terms of enamel protection, reduction of plaque accumulation, and improvement in surface microhardness were found to be similar, indicating that HAP represents a viable fluoride-free alternative in preventive dentistry (Bossù et al., 2019).

Another study compared 500 ppm of amine fluoride with 10% of hydroxyapatite for the remineralisation of caries, where both had equal efficacy (Amaechi et al., 2019). Table 3 shows the calcium and phosphate ratios for different calcium phosphate compounds (Koutsopoulos, 2002).

Table 3. The Calcium and phosphate ratios for different calcium phosphate compounds.

Compound name	Molecular formula	Ca/P ratio
Monohydrate calcium phosphate (MCPH)	$Ca(H_2PO_4)_2 \cdot H_2O$	0.50
Monocalcium phosphate (MCP) **Dicalcium phosphate dihydrate (DCPD)**	$Ca(H_2PO_4)_2$ $Ca\ (HPO4)\ \cdot 2H_2O$	0.50 1.00

Dicalcium phosphate anhydrate (DCPA)	$Ca (HPO_4)$	1.00
Octacalcium phosphate (OCP)	$Ca_8H_2(PO_4)_6 \cdot 5H_2O$	1.33
Tricalcium phosphate (TCP)	α- and β- $Ca_3(PO_4)_2$	1.50
Hydroxyapatite (HAP)	$Ca_{10}(PO_4)_6(OH)_2$	1.67

1.8.4. Current available methods for hydroxyapatite synthesis *in situ* using calcium phosphate

Methods for *in situ* HAP synthesis involved the reaction of the calcium-rich substrate with an aqueous solution of a phosphate salt, providing the PO_4^{3-} ions necessary to form HAP (Reynolds, 2008). In the literature, several methods to promote remineralisation with calcium phosphate have been developed by enhancing the delivery of calcium and phosphate ions from the environment. These methods can be subdivided into the following categories: incubation in physiological mineralisation solution, pre-soaking, alternate soaking and post-soaking (Cai et al., 2010).

One of the methods is to use the physiological mineralisation solution of Stimulated Body Fluids (SBF) which stimulates the *in vivo* mineralisation behaviour of a biomaterial. After the immersion in SBF, the apatite was developed in several hydrogel types. Calcium solutions in the pre-soaking hydrogels with SBF incubation showed an increase in the formation of apatite in the hydrogels, and this showed an increase in calcium phosphate growth (Suzawa et al., 2010). Furthermore, the alternate soaking process is used with calcium and phosphate, where a hydrogel is immersed in a solution containing one type of ion (calcium or phosphate) and subsequently immersed in a solution of the other type of ion. Repeating this increases the amount of calcium phosphate deposition within the hydrogel. Similarly, the calcium phosphate has been fabricated in cellulose, Polyvinyl Alcohol (PVA) and chitosan by the alternate soaking process. It can also change the type of calcium phosphate by post-soaking using chitosan hydrogels (Tabata et al., 2005).

1.8.5. Alternate soaking process for HAP synthesis *in situ*

There are several methods for the fabrication of the HAP (Decher, 1997; Wassell et al., 1995; Watanabe et al., 2007). A study used the biomimetic process of hydroxyapatite fabrication, which improved its characteristics such as smaller crystallite size, higher specific surface area, and greater carbonate substitution, all of which enhance its dissolution, reprecipitation behaviour and bioactivity (Brunton et al., 2013). Biomimetically synthesised HAP also demonstrates improved mechanical compatibility, enhanced protein adsorption, greater osteoconductivity, and stronger nucleation potential, enabling more effective interactions with organic matrices and promoting cell attachment and mineral deposition (LeGeros, 2008).

The main drawback is that it takes days or even months to prepare the desired thickness of hydroxyapatite layers (Kokubo, 1990; Yamamoto et al., 1998). Thus, another research group have developed a novel alternate process to improve these time restrictions (Taguchi et al., 1998).

1.8.5.1. The mechanism of alternating soaking process

In 1998, Taguchi et al reported using hydrogels as a template to form HAP using a novel alternate soaking process. This showed that apatite of bone-like can be formed on or in the hydrogel matrix using this procedure. This process became well known due to the preparation of hydroxyapatite using alternate soaking aqueous solutions of calcium and phosphate (Ca/P). Inside gels, $CaCl_2$/Tris-HCl and Na_2HPO_4/Tris-HCL ions formed hydroxyapatite which mimics bone regeneration (Taguchi et al., 1998; Taguchi et al., 1999; Taguchi et al., 1999). This method was 100 times faster than the other method on the same material's surface in which hydroxyapatite was deposited (Serizawa et al., 2001). Taguchi et al concluded that a hydroxyapatite layer was formed by the biomimetic process in alternate process on certain types of hydrogels such as agarose gel and polyvinyl alcohol (PVA) (Taguchi et al., 1999; Taguchi et al., 1999; Taguchi et al., 1999). This alternate soaking process is an effective method for preparing polymer-appetite crystals (Taguchi et al., 1999).

1.8.5.2. Application of alternate soaking process for HAP formation

Agarose gel is widely recognised as a biocompatible, inert, and structurally stable hydrogel that has been used extensively in biomedical research due to its ability to form a hydrated three-dimensional network capable of retaining and gradually releasing dissolved molecules (Ahmed, 2015). Its physicochemical characteristics particularly its high water content, adjustable porosity, and thermo-reversible gelation, have supported its use in drug delivery, cell encapsulation, and tissue engineering (Suzawa et al., 2010). These features demonstrate its suitability as a controlled-release vehicle, allowing ions such as calcium and phosphate to diffuse in a sustained and predictable manner rather than being rapidly depleted, as commonly occurs in simple aqueous systems. In medicine, agarose-based hydrogels have been used as scaffolds for bone tissue engineering, often in combination with bioactive ceramics such as HAP or bioactive glass, where they support mineral deposition and enhance tissue regeneration (Yue et al., 2020). These applications highlight the material's capacity to function as a mineralisation-supportive matrix. It was reported that HAP/agarose gel was used for repairing bone defects completely by depositing the defects (Taguchi et al., 1998). It can easily be mixed, transferred, and packed. It has a haemostatic property within Intra-osseous defects. HAP/Agarose gel can be easily resorbed within one month, and it can also stimulate soft tissue healing on or in agarose gels (Taguchi et al., 1999).

In other studies, tissue-engineered construct-hydroxyapatite composites were successfully developed to repair bone defects using the alternate soaking process of different materials that included calcium and phosphate and mesenchymal stem cells (Watanabe et al., 2007; Matsusaki et al., 2009). The alternate soaking process was used for the successful formation of apatite and calcium carbonate onto the polymer graft films. Moreover, they reported the ability to use this method to prepare 3D matrix tissue reconstitution and suggested that it is considered the fundamental method for the reconstitution of tissue matrix (Watanabe and Akashi, 2009).

Serizawa et al (2001) demonstrated hydroxyapatite deposition on polyvinyl alcohol (PVA) coating on polyethene films which was formed by adsorption and drying in a PVA solution in an aqueous solution. It was noticed that a larger number and fast-paced hydroxyapatite deposition was formed using the alternate soaking technique. PVA coating helps in the acceleration of hydroxyapatite formation through the alternate soaking process (Serizawa et al., 2001).

Further, a study used a PVA/HAP composite monolithic scaffold, which is formed through the thermal impaction of a non-solvent separated method followed by an alternate soaking process. They concluded that with the increase in the soaking cycle and soaking time, the monolith composite decreased water uptake. In addition, the PVA/ hydroxyapatite composite monolith showed outstanding application in bone engineering (Sun and Uyama, 2014).

Furthermore, they used HAP/agarose in extraction sockets in teeth, new abundant bone and new marrow tissue were also formed, and the defect was completely absorbed (Tabata et al., 2003). Additionally, the use of an alternate soaking process in extraction sockets of adult monkeys and the biological behaviour of hydroxyapatite on agarose gels was compared with a commercially available Bone Ject (bone graft material combined with bone ceramic collagen, Japan). After 12 weeks of implementation, inflammatory cells were formed in non-bone Ject particles. This concludes that HAP/agarose gel can act as a biodegradable alternative material for bone grafts applied in humans (Tabata et al., 2003).

Moreover, Taba et al, further investigated the implantation of hydroxyapatite on/ in agarose gel in periodontal defects in three dogs using the novel alternate soaking process (Tabata et al., 2005). Hydroxyapatite/agarose gel completely regenerated the periodontal ligament (PDL) and the cementum and abundant formation of new bone. They further implemented hydroxyapatite/agarose in rabbit's femoral bone defects, and regeneration of bone occurred (Tabata et al., 2005). Further to this, a study developed novel hydroxyapatite for biomineralisation by the alternate soaking process of calcium and phosphate using hydrogel and electrophoresis (Watanabe and Akashi, 2006), and in this process, hydroxyapatite was formed in a dry gel using the alternate soaking resulted in more hydroxyapatite to reach 750 µg of hydroxyapatite/ mg dry gel (Watanabe and Akashi, 2008).

1.9. Model systems for studying dental caries in enamel research

Caries demineralisation and remineralisation have been studied using different approaches. Controlled randomised trials that are well conducted are the gold standard for these types of studies in dental caries research. Major limitation of these studies is that they are expensive and time consuming. For this reason, different study models are adopted to demonstrate and mimic the oral microflora (Meyer and Enax, 2018).

1.9.1. *In vitro* Models to demonstrate demineralisation and remineralisation

These models are more frequently used in analysing subsurface enamel lesions, white spot lesion and dental caries. Further to this, the teeth specimens that are used are either from human or animal in a laboratory setting to simulate oral environment (Amaechi et al., 1999).

In the past decades, protocols of *in vitro* have evolved to study dynamics of mineral loss and gain from dental tissues. Studies have also looked at how different elements would react in the *in vitro* protocols (Arends and Ten Cate, 1981). This approach is inexpensive and performed over a short period of time (White, 1995).

In vitro models are widely used in enamel caries research to simulate the processes of demineralisation and remineralisation in a controlled environment. These models allow researchers to study various aspects of dental caries formation, prevention, and treatment without the need for clinical or animal studies in the initial stages (Dodds et al., 2015). *In vitro* studies allow researchers to observe how different conditions, such as pH, bacterial activity, or dietary sugars, affect the caries process. These models are used to test the effectiveness of preventive agents like fluoride, calcium phosphate, or other remineralising agents in halting or reversing the demineralisation process. *In vitro* caries models provide a platform for testing the performance of dental materials, such as sealants, varnishes, or fillings, in protecting enamel from acid attacks (Ellwood et al., 2012).

In vitro models allow for precise control over variables such as pH, fluoride concentration, bacterial species and time, providing valuable insights into the mechanisms of caries development and prevention. The highly controlled conditions allow for standardised testing, making it easier to replicate experiments and compare results across studies (Neves et al., 2002).

In vitro models are more cost-effective than *in vivo* studies and allow for testing of multiple treatments or conditions. *In vitro* models reduce or eliminate the need for animal or human testing in the early stages of research, addressing ethical concerns related to invasive studies. 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 occur in the oral cavity (White, 1995). *In vitro* models do not account for biological factors like immune responses, saliva flow, and overall health, which can influence the development of caries *in vivo* (Gmür et al., 2006).

1.9.2. *In vitro* model of artificial caries lesions using the pH cycling method

To create artificial caries lesions in enamel or dentine is important to study the treatment or prevention of the dental caries. This protocol has been widely used in studying the process of demineralisation and remineralisation. This protocol uses a pH cycling challenge to mimic the oral environment. Enamel subsurface lesion is characterised by having an intact surface layer with underlying subsurface demineralisation (Silverstone, 1980).

Most methods to create the artificial caries lesions that proved later that the characterises features of the model is similar to natural caries. These models included acidified gels, lactate buffers and the surface layer of the enamel remains intact (Silverstone, 1980; Stookey et al., 2011; Arends and Ten Cate, 1981).

Further to this, an *in vitro* model was developed called pH cycling model that involves the process of alteration of demineralisation and remineralisation (Featherstone, 2004). This model is formed to mimic the stimulation of the caries process. This model has become the method of choice for many caries research and has been used widely to investigate caried prevention agents on the dynamics of enamel demineralisation and remineralisation (Featherstone, 2004; Silverstone, 1980; White, 1995; Arends and Ten Cate, 1981; Nanci and Ten Cate, 2013).

1.9.3. Animal models for studing dental caries

In the past, animal caries models were used since the 1940s. This model was categorised as a valuable tool to understand the demineralisation and remineralisation process and the caries process (Silverstone, 1980). Through this model, different variables involved in the caries process that is controlled such as the oral environment by manipulating the animal's diet. Studies preferred this model over *in vitro* as it is similar to the human microflora and caries progression(Arifa et al., 2019).

The most used animal species model is rats. Studied proved that rat's caries progression resembles that of human teeth in caries microbiology and histologically (Preston et al., 2007). However, remineralisation and demineralisation are limited in animal models as it does not simulate the caries progress in clinical situation. This is due to the buffering capacity of saliva in animals are not the same as human saliva (White, 1995).

1.9.4. *In situ* Models for studying dental caries

In situ models are a methodology that involves the use of an intra-oral appliance or device that contains specimens of dental tissue and placed in the oral cavity to form an analysis of alterations in the specimens due to a modification or treatment (Pollard et al., 1996). The source of the enamel or dentine is often from bovine or human teeth (Mellberg, 1992).

In situ models have been used in many studies to analyse caries progression and prevention which involved fluoride and other remineralising agents (Zero, 1995). The main advantage of this technique is that it is performed in the human mouth, which make it more related to the actual demineralisation and remineralisation process in the oral environment. It can also act as experimental design that facilitate the control of different experimental variables in ways not achievable with clinical trials (Zero, 1995). Further to this, it has been performed in favourable costs with few ethical issues (Prestes et al., 2013).

1.10. The methods of assessing demineralisation and remineralisation of enamel

Different methods which help in demonstrating the detection of mineral loss and gain in enamel. These methods are performed in the lab and currently used to measure enamel demineralisation and remineralisation. These methods allow detection of the mineral loss and gain in enamel even with minor changes, this includes:

1.10.1. Quantitative Light-Induced Fluorescence (QLF)

Qualitative light induced Fluorescence is quantitative method of analysis that is non-destructive and sensitive. It is a camera-based technique that analyses the process of demineralisation and remineralisation of enamel slabs of subsurface enamel lesions *in situ* or *in vitro* studies (Gmür et al., 2006).

The principle of this technique is that the object is excited by a particular wavelength of light (λ) of 370 nm then 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). QLF reflects the visible light, which is in the blue region of the visible light spectrum which gives QLF the ability to reflect the fluorescence light of enamel. In 1932, Benedict et al, were the first to describe the enamel light fluorescence, and suggested its use in the detection of dental caries (Benedict and Kanthak, 1932).

When demineralisation of enamel takes place, studies have proved that reduction in the fluorescence light is reflected (Heinrich-Weltzien et al., 2003). Authors found that difference in the fluorescence light between sound and demineralised enamel which can be demonstrated by a change in the amount of light absorption and scattering (Tranæus et al., 2002). If demineralisation happens the enamel becomes porous, and saliva fills the pores which decreases the path of light in the enamel. This means when dental caries happens the scattering of the light is stronger than in sound enamel and the volume of absorption per unit of volume is small due to the less fluorescence light in caries lesion (ten Bosch and Coops, 1995).

Further to this, a software is used to analyse the images that involves using a patch to define areas of demineralised and sound enamel. This software demonstrates images as pixel values of the sound enamel to reconstruct the surface of the effect of sound enamel and gives an average fluorescence light area. The degree of fluorescence loss is estimated to 5.0% (ten Bosch and Coops, 1995). For example, if all the pixels of the lost fluorescence are greater the 5.0% is considered as part of the lesion. The software will then assign the sound enamel and the lesion part by calculating the average fluorescence loss in the lesion, known as % ΔF, and then the total Area of the lesion in mm^2. A multiplication of these two variables results in a third metric output, ΔQ (Pretty et al., 2002). 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 necessary to provide sufficient fluorescence contrast between the lesion and the sound enamel (Heinrich-Weltzien et al., 2003).

A strong correlation was found between QLF and Transverse Microradiography (TMR) (Al-Khateeb et al., 1998). By this Quantitative Light-Induced Fluorescence (QLF) has been validated as a method for measuring mineral content. The author has also thought that it has been shown to be reproducible and reliable technique (Featherstone, 2004).

In a hydrated enamel subsurface lesion, an increase in light scattering was seen in comparison to the surrounding enamel and developed a method for assessing the mineral changes in intact and demineralised tooth structure (de Josselin de Jong et al., 1995). The currently marketed systems (Inspektor Research Systems BV, Amsterdam, The Netherlands) provide three quantitative metrics:

ΔF: Percentage fluorescence loss with respect to the fluorescence of sound tooth tissue; related to lesion depth (%).

ΔQ: The ΔF times the Area. Percentage fluorescence loss with respect to the fluorescence of sound tissue times the area. Related to lesion volume (% px^2).

Area: The surface area of the lesion expressed in $pixels^2$ (px^2).

The limitation of QLF is that the confounding factors that could affect the measurements of fluorescence light such as staining, plaque and calculus (Stookey, 2004). Also, using QLF have shown to have a large standard deviation or errors for the technique used in QLF (Arango et al., 2019).

1.10.2. Transverse Micro Radiology (TMR)

This technique is the gold standard in determining the mineral gain and loss in artificial caries lesions. it is based on the comparison of the amount of X-ray absorbed from the tooth slabs with a stimulated exposed standard (White, 1995). TMR is excellent to analyse the depth of artificial caries lesions and compare the validity of other caries detection techniques. Prepare the samples for the analysis of TMR would involve cutting the samples into thin slices in a perpendicular direction of the enamel surface. Radiographic exposure of high resolution with calibrated aluminium wedges to compare and calculate the mineral gain and loss (Buzalaf et al., 2010).

The disadvantages of this technique include its potential to damage enamel samples, being time consuming and lacking reproducibility. Due to its destructive potential, its only useful in *in situ* and *in vitro* methods. Further to this, the preparation of the slices can be very technique sensitive to produce homogenous thickness (Buzalaf et al., 2010; White, 1995; Wu et al., 2010).

1.10.3. Microhardness Test

The microhardness test is commonly used in dental research to evaluate the hardness and mechanical properties of enamel, particularly in studies of enamel demineralisation and remineralisation (Deswal et al., 2022). It provides a quantifiable measurement of how resistant the enamel is to deformation, which is related to its mineral content and structural integrity. In the context of enamel caries research, microhardness testing is valuable for assessing the effects of caries, preventive treatments, and restorative materials (George et al., 2015).

This technique determines the mineralisation process of enamel due to its sensitivity to the mineral density changes (Deswal et al., 2022). This technique can demonstrate the degree of indenter penetration and porosity of superficial enamel layer that is represented as mineral loss or gain in enamel subsurface lesions. This method can also measure any resistance of the enamel surface. The measurements would involve the nano and micro indentation of Vickers and Knoop diamond tip of defined load, duration, and dimensions of geometrical patterns (Lussi et al., 2007). This technique can provide precise measurements of enamel hardness and is suitable for studying changes at the surface level and used in dental research for evaluating the effects of demineralisation, remineralisation and the protective efficacy of dental materials (Deswal et al., 2022).

The following types of microhardness tests exist:

1.10.3.1. Surface Microhardness (SMH)

Surface Microhardness (SMH) assessment involves applying a load with a diamond indenter perpendicular to a polished tissue surface. When used to evaluate de/remineralisation, SMH measurements offer qualitative insights into mineral changes, but require samples with flat surfaces (Arends and Ten Cate, 1981). Numerous factors, including lesion shape, mineral redistribution, and *in situ* protein absorption, can affect indentation length values. It is crucial to recognise that a linear correlation between indentation length and lesion depth is valid only within specific depth ranges (Arends et al., 1980; Zero et al., 1992). While this non-destructive technique enables longitudinal studies of the same specimen, it does not provide information on subsurface hardness variations or structural changes across different lesion sides (Featherstone, 2000)

1.10.3.2. Cross-Sectional Microhardness (CSMH)

Cross-Sectional Microhardness (CSMH) involves applying a load from the diamond indenter parallel to the outer anatomical surface (Arends et al., 1980). CSMH tests yield indirect indicators of mineral changes, mineral content, and profiles (the volume percentage of mineral relative to distance from the outer surface). This technique allows for quantification of mineral content, enabling estimations of mineral loss and gain. Research by Magalhães et al (2009) correlated SMH and CSMH with mineral content, surface layer, and lesion depth through transverse microradiography (TMR) in enamel lesions (de Magalhães et al., 2009). Their findings suggested that while CSMH can serve as an alternative to TMR, it does not accurately measure mineral content. However, CSMH values provide insights into the physical strength of enamel lesions—an aspect not addressed by TMR. Conversely, SMH is not recommended as a substitute for TMR in evaluating dental caries lesions.

Quantitative assessment of enamel mineralisation using microhardness testing has been widely applied to understand post-eruptive changes (Arends et al., 1980). A study investigated the superficial microhardness of human enamel across different post-eruptive ages to evaluate the process of enamel maturation and enamel samples were analysed using a Knoop indenter under a 25 g load for 5 seconds (Palti et al., 2008). Their results indicated a progressive increase in surface microhardness with age, with a statistically significant difference observed between unerupted enamel and enamel more than 10 years post-eruption. These findings support the concept of post-eruptive enamel maturation, demonstrating that enamel continues to mineralise in the oral environment following eruption (Palti et al., 2008)

In addition, the impact of clinical interventions on enamel microhardness has been evaluated in the context of orthodontic treatment (Lippert and Lynch, 2014). Another study examined the correlation between enamel surface roughness, microhardness, and depth of demineralisation following bracket bonding and debonding, the samples were analysed using micro-Vickers hardness. The study demonstrated a moderate negative correlation between enamel microhardness and demineralisation depth, indicating that lower hardness was associated with greater susceptibility to mineral loss. Additionally, increased surface roughness was weakly associated with reduced hardness, suggesting that procedural alterations to enamel surfaces can compromise enamel integrity (Zawawi and Almosa, 2025).

Microhardness has long been recognised in dentistry and has been widely used for many years. This technique is inexpensive and rapid. Smooth and flat surface of enamel slabs are needed to be effective in the measurements of microhardness (Rirattanapong et al., 2011).

The limitation of this technique is that it focuses on surface hardness and may not provide information about subsurface demineralisation or remineralisation, which is critical in the early stages of caries. Also, the test measures hardness at a microscopic level, so it may not represent the overall hardness of the entire tooth. Furthermore, it can be influenced by the quality of the enamel sample preparation, surface flatness, and the consistency of load application (George et al., 2015).

1.10.4. Microcomputed tomography (Micro-CT)

Microcomputed tomography (Micro-CT) is a high-resolution, X-ray–based imaging technique that enables three-dimensional visualisation of dental hard tissues. In enamel research, Micro-CT is widely valued for its ability to capture both external morphology and internal mineral distribution without requiring sectioning or destruction of the sample (Buzalaf et al., 2010). During scanning, multiple radiographic projections are collected as the specimen rotates, and these are reconstructed computationally into a volumetric dataset. This allows detailed assessment of enamel thickness, structural integrity, lesion architecture and mineral density gradients with micrometre-level precision. Because of its resolution capabilities, Micro-CT is especially suited for detecting early structural changes such as subsurface demineralisation, enamel porosity, and microcracks (Hoxie et al., 2023).

A key strength of Micro-CT is its capacity to quantify mineral density changes, making it a reliable tool for studying both demineralisation and remineralisation. The technique provides accurate, reproducible measurements of lesion depth, mineral volume, and mineral distribution patterns within enamel, enabling researchers to monitor progression or reversal of carious lesions over time (Hong et al., 2022; Hoxie et al., 2023). Unlike traditional methods, such as microhardness testing or histological sectioning, Micro-CT preserves the entire tooth, allowing repeated scans of the same specimen. This makes it particularly valuable for longitudinal studies investigating the effect of fluoride, bioactive materials, or remineralising agents on enamel mineral content. Its relevance has been widely demonstrated in studies assessing mineral loss and gain in enamel under controlled laboratory conditions (Buzalaf et al., 2010).

Despite its advantages, Micro-CT does present several limitations. The technique requires careful calibration to accurately convert X-ray attenuation values into mineral density measurements, and image quality may be affected by artifacts such as beam hardening or scattering, particularly when enamel is adjacent to restorative materials with different densities (Hong et al., 2022). High-resolution scans are also restricted to small sample sizes, as increasing the field of view reduces resolution. Additionally, Micro-CT systems are costly, require trained personnel, and involve lengthy reconstruction and analysis procedures. While radiation dose is not a concern for extracted teeth, it limits the feasibility of true in vivo applications (Buzalaf et al., 2010).

1.10.5. Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy (SEM) is extensively employed in dental research to examine enamel surface morphology before and after demineralisation offering micro- to nanoscale insight into structural changes associated with mineral loss or recovery. By directing a focused electron beam at a dry, conductive-coated enamel surface, SEM generates high-resolution images of enamel prisms, interprismatic regions, microcracks, surface porosities, and other fine details that cannot be resolved with conventional light microscopy (Shaik et al., 2017). This visually identify and compare morphological differences between sound enamel, demineralised enamel and enamel subjected to remineralisation. In remineralisation studies, SEM often reveals partial restoration of surface smoothness, reduction in porosity, reformation of mineral deposits or crystal-like structures, and improvement in surface integrity (Thimmaiah et al., 2019).

However, the technique has significant limitations, because specimens must be dehydrated and coated with a conductive metal layer (e.g., gold or carbon), SEM is inherently destructive. Additionally, SEM only reveals surface morphology and cannot penetrate subsurface enamel layers, so it does not provide information about internal lesion depth or volumetric mineral density changes (Margolis et al., 2006). These limitations mean SEM results should ideally be complemented with volumetric or density-based methods (e.g., micro-CT, microradiography) when a full assessment of demineralisation or remineralisation is required (Kamal et al., 2018).

In SEM, a highly focused beam of high-energy electrons is directed onto the specimen surface. As these electrons interact with the atoms in the enamel, they generate several types of signals, including secondary electrons, backscattered electrons, and characteristic X-rays. Each type of signal arises from a distinct interaction mechanism and carries information about either the surface topography, composition, or near-surface elemental distribution of the specimen (Saghiri et al., 2012).

Secondary electrons (SEs) are emitted from the outer shells of atoms near the enamel surface as a result of inelastic collisions with the primary electron beam. Because these electrons originate from a very shallow depth, only a few nanometres, SE imaging provides 3D-like highly detailed information about surface topography, such as enamel prisms, interprismatic regions, microcracks, and surface roughness (Henning and Adhikari, 2017). Backscattered electrons (BSEs), in contrast, does not provide 3D imaging and are primary electrons that are reflected from the specimen after elastic scattering. The intensity of BSE signals depends on the atomic number of the material, so regions with higher mineral content or heavier elements appear brighter in the image. This allows SEM to provide compositional contrast, which is particularly useful for distinguishing between sound and demineralised enamel or evaluating the interface between enamel and restorative materials (Saghiri et al., 2012).

Sample preparation for SEM is essential for most dental, biological, and polymer-based specimens because these materials are inherently non-conductive and often contain water (Kamal et al., 2018). Non-conductive samples such as enamel, dentin, and resin-based materials tend to accumulate electrical charge under the electron beam, leading to image distortion and artefacts. To overcome this, specimens typically undergo dehydration and coating with a thin conductive film (commonly gold, platinum, or carbon) to provide a stable pathway for electron discharge (Goldstein et al., 2018). Hydrated specimens also require preparation because water evaporates under high vacuum, causing structural damage; therefore, biological and dental hard tissues must be dried or chemically fixed before imaging (Reed, 2005)

In contrast, some materials can be examined in SEM without preparation, primarily those that are naturally conductive and vacuum stable (Saghiri et al., 2012). Metals, alloys, and certain conductive ceramics do not require coating because they are capable of dissipating charge efficiently under the electron beam. Additionally, advancements in variable-pressure and environmental SEM (ESEM) allow imaging of non-conductive or hydrated specimens without the need for dehydration or conductive coating. In ESEM, the presence of a controlled gaseous environment reduces charging and stabilises wet or uncoated biological samples, making it suitable for examining early enamel demineralisation or hydrated tissues in a more natural state (Henning and Adhikari, 2017).

1.10.6. Energy Dispersive X- ray Spectroscopy (EDX)

Energy-Dispersive X-ray Spectroscopy (EDX) is an analytical technique commonly integrated with scanning electron microscopy (SEM) to determine the elemental composition of a specimen. When the electron beam interacts with the sample surface, it transfers energy to atoms within the material and ejects inner-shell electrons (Shaik et al., 2017). The resulting vacancies are filled by electrons from higher-energy shells, and this transition releases characteristic X-rays that are specific to each element. The EDX detector measures the energy and number of these emitted X-rays, generating a spectrum that identifies the elements present and their relative abundance (Zhang et al., 2014).

EDX is widely used in dental research because it enables point analysis, line scans, and elemental mapping, providing spatially resolved information on mineral distribution. In studies of enamel demineralisation and remineralisation, EDX can quantify key mineral elements such as calcium, phosphorus, and fluoride, allowing researchers to evaluate mineral loss during acid challenge and mineral gain following remineralising treatments (Henning and Adhikari, 2017).

Limitations of this technique would include EDX cannot detect elements with atomic number less than 5 (e.g. H, He, Li, or Be), due to the absorption of low energy X-rays by the windows in front of silicon lithium detector (Nimbeni et al., 2023). Also, overlapping of peaks occurs for many elements. In addition, the nature of samples can affect the accuracy of EDX measurements. Recently, many studies have implemented the use of EDX-SEM in enamel analysis (Scholz et al., 2019; Guentsch et al., 2019).

1.11. Summary and research gap

Dental caries is a common chronic infectious disease that causes major public health problems (Reynolds, 2008). It is known as a localised destruction of tooth structure by acidic by-products of fermented carbohydrates capable of causing demineralisation (Featherstone and Lussi, 2006). Preventing the initiation of early lesions and their progression is essential for caries management (Featherstone, 2004). To date, the prevention of dental caries focuses on reducing the intake of sugar, reducing bacteria, and increasing the resistance of tooth enamel to the development of caries (Bernd and Silvia, 2021). Therefore, the use of fluoride, oral hygiene improvement, xylitol, and topical anti-microbials such as chlorhexidine or povidone-iodine are common methodologies for decreasing the chances of caries development. Moreover, parental education and improving the behaviour of children toward their diet by reducing the intake of sugars and feeding practices are also useful for preventing caries (Chou et al., 2014). It has been reported that there is a decline in dental caries, attributed to such interventions in developed countries (Petersson and Bratthall, 1996).

In current advanced dentistry, there is a huge shift from restorative approaches toward the remineralisation of early caries. Many new agents are formed that provide remineralisation management of early caries (Gomez et al., 2013). Remineralising toothpastes and mouthwashes are available in the market that can help strengthen early enamel lesions. Remineralisation, a natural repair process, has the potential to reverse or halt the advancement of early caries, thereby strengthening teeth, reducing sensitivity, and avoiding the necessity for restorative treatments, leading to improved oral health outcomes (Gordan et al., 2015; Abou Neel et al., 2016; Bishara and Ostby, 2008; Bossù et al., 2019; Colak et al., 2013; Lee and Somerman, 2018). The primary goal of enamel remineralisation is to replace the minerals that acids strip away from dental surfaces, thus preventing tooth weakening and the formation of cavities (Gordan et al., 2015).

The dynamic nature of demineralisation and remineralisation in the tooth structure involves the natural equilibrium between the loss of minerals (demineralisation) and their gain (remineralisation) (Featherstone, 2000). This balance is maintained through the presence of calcium, phosphate, and fluoride ions in the saliva, which help maintain and strengthen enamel. When factors such as a highly acidic environment (from bacterial metabolism of sugars), poor oral hygiene, or insufficient fluoride disrupt the remineralisation process, demineralisation becomes dominant (Featherstone, 2004). Over time, this leads to the breakdown of enamel and the formation of cavities. As demineralisation continues, the enamel softens, leading to visible destruction and cavitation. This is the point at which a carious lesion becomes irreversible and needs restorative treatment (Fejerskov, 1997; Featherstone and Lussi, 2006).

Despite this, clinicians and researchers are trying to maintain good oral health care for patients. Nevertheless, it remains a big challenge (Li et al., 2014). Although fluoride agents are the current gold standard among all agents, however, fluoride does not deliver a complete cure for caries, and it can cause adverse effects of fluorosis when fluoride is used in large quantities (Cai et al., 2010).

Calcium and phosphate play a fundamental role in the remineralisation of enamel, helping to reverse the effects of early demineralisation and strengthen tooth structure. The interaction of these minerals in the remineralisation process is crucial for maintaining enamel integrity and preventing dental caries (Grohe and Mittler, 2021). The advantage of using Ca and PO_4^{3-} for remineralisation is that it has a chemical similarity to bone and enamel. It is non-toxic, has excellent biocompatibility, is osteoconductive, and has crystallographic structures. Studies have shown that a combination of calcium silicate and sodium phosphate in toothpaste protected teeth from acid damage than regular fluoride toothpaste (Hornby et al., 2014). Some products add minerals directly to the enamel surface, using ingredients like hydroxyapatite, tricalcium phosphate, or amorphous calcium phosphate (ACP). Others, like casein phosphopeptide-amorphous calcium phosphate (CPP-ACP), work more gradually, creating a reservoir that slowly releases minerals to the enamel, offering longer-lasting protection. However, another study found that if calcium and phosphate are applied together, they can form a solid, interlocked crystal layer called brushite on the enamel. This suggests that using them separately might be a more effective approach, preventing premature crystal buildup (Giocondi et al., 2010).

In essence, methods like alternate soaking process is showing real promise in maximising the benefits. The alternate soaking process is a method of delivery of calcium and phosphate that enhanced bone regeneration (Taguchi et al., 1998). It involves bone samples soaked in demineralising and remineralising conditions to simulate the dynamic processes that occur in the body, such as bone resorption and deposition (Taguchi et al., 1999). Bone samples were soaked in an acidic solution that simulates the conditions leading to bone resorption. The bone was then immersed in a remineralising solution, in an alternating calcium and phosphate solutions, to simulate the bone regeneration (Taguchi et al., 1999). The process was used to study the effects of various bone regeneration treatments, such as calcium supplements, bisphosphonates (medications that slow down bone resorption), or other bone-strengthening agents, by observing how well they promote remineralisation after a period of demineralisation (Tabata et al., 2003). Calcium and phosphate ions just like with enamel, are critical for bone health. In the remineralisation phase, these ions were redeposited into the bone matrix, reinforcing its structure (Matsusaki et al., 2009).

Most studies examining the alternate soaking process have focused primarily on the applications in bone regeneration, where alternating immersion in calcium- and phosphate-rich solutions has been shown to promote controlled mineral nucleation, apatite growth, and improved mechanical properties of the treated substrates (Kono et al., 2007). These findings suggest that the technique can create conditions that closely mimic natural biomineralisation (Watanabe and Akashi, 2008). Despite this, its potential use in dental enamel repair has not yet been explored.

This absence of research is significant because enamel presents unique challenges. Unlike bone, enamel is an acellular, non-remodelling tissue that cannot undergo biological self-repair once demineralisation occurs (Driessens, 1980). As a result, early enamel lesions must rely exclusively on external remineralisation strategies to restore mineral content and halt lesion progression. However, many conventional remineralisation methods, such as fluoride treatments or calcium phosphate-based paste depend heavily on saliva composition, ion availability, pH fluctuations, and oral environmental factors. These conditions can be unpredictable, often limiting the depth and quality of mineral deposition within the lesion (Zhang et al., 2014).

A technique like the alternate soaking process may address several of these limitations. By exposing enamel to precisely controlled calcium and phosphate supersaturation cycles, the method could create a more favourable environment for consistent mineral deposition. It may also allow minerals to penetrate deeper into the lesion and support the formation of a more ordered, enamel-like apatite structure, rather than producing only superficial or loosely bound mineral layers. Moreover, because the process is chemically driven, it does not rely on biological activity, making it particularly well suited for enamel, which lacks regenerative capacity (Driessens, 1980).

Therefore, the aim of the current study is to investigate the effect of a treatment regimen containing an alternate soaking process with calcium and phosphate solutions on enamel lesion remineralisation *in vitro*. Understanding whether this controlled, biomimetic mineralisation technique can enhance enamel remineralisation has the potential to guide the development of more predictable, effective, and durable non-invasive strategies for managing early caries lesions.

Chapter 2 Research Aim, Objectives, and Hypothesis

2.1. Aim of the study

To investigate the effect of a calcium and phosphate treatment regimen, applied using an alternate soaking method, on the remineralisation of enamel lesions *in vitro*.

2.2. Objectives of the study

1. To assess the feasibility of the alternate soaking process using agarose gel as a precursor for delivering calcium and phosphate to artificial enamel subsurface lesions.
    - To determine the appropriate period of demineralisation to produce a consistent lesion of at least 300-400μm depth on each enamel slab.
2. To investigate the effect of the treatment calcium and phosphate regimen applied with an alternate soaking method on the remineralisation of enamel lesion using QLF, Micro-CT and EDX *in vitro*.

2.3. Null Hypothesis of the study

1. There is no difference in the effect of using a template or a precursor to deliver calcium and phosphate using the alternate soaking process protocol on enamel remineralisation.
2. There is no difference in the effect on enamel lesion remineralisation of treatment regimens between calcium and phosphate solutions (alternate soaking process), Fluoride toothpaste slurry (1450 ppm F), Tris Hydrochloride (Tris-HCL) and day-time artificial saliva (pH 6.8).

Chapter 3 A pilot study: Optimisation of the alternate soaking method for the delivery of calcium and phosphate on artificial enamel subsurface lesions

3.1 Aim of the study

To assess the feasibility of alternate soaking process with the use of agarose gel as precursor to deliver calcium and phosphate on artificial enamel subsurface lesions.

3.2. Material and Methods

3.2.1 Collection of Bovine teeth

The teeth were obtained from an abattoir in Leeds. The buccal surfaces of the bovine teeth were used in the present study to allow a more uniform thickness of enamel as well as a flatter surface. The enamel slabs were stored at room temperature in deionised water and 0.1% thymol (Sigma Aldrich, Germany) 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 study have shown it can affect dentine permeability (Preston et al., 2007). The teeth used in this study were obtained from a previously existing collection sourced from an abattoir. No human participants were involved, and no ethical approval was required.

3.2.2. Sample size

This pilot study was designed to support the design of the subsequent formal investigation. The primary objectives of the pilot study were not hypothesis testing, but rather to (i) assess the feasibility of the experimental protocol, (ii) evaluate measurement reliability, and (iii) obtain preliminary estimates of variability for key outcome measures, which are essential inputs for formal sample size calculation.

For this pilot study, six bovine incisors teeth were used. The sample size of this pilot study was determined following statistical advice by a qualified biostatistician at the Centre of Epidemiology and Biostatistics, University of Leeds. As this study represents a novel investigation with limited prior data available, a formal power calculation was not feasible. Therefore, a pilot sample size of 6 samples was selected following consultation with a statistician, consistent with recommendations for exploratory studies. In pilot studies, where the primary objective is to assess feasibility, small sample sizes are acceptable; it has been recommended that approximately 5–10 samples are sufficient (Kunselman, 2024)

3.2.3. Experimental Materials

1. Six enamel artificial subsurface lesion.
2. Calcium Chloride $CaCl_2$ (22.196g) (Mw=110.98), Honeywell Fluka™.
3. Di-sodium hydrogen phosphate dodecahydrate $Na_2HPO_4\ 12H_2O$ (42.98g).
4. Agarose 3 w/v% Agarose gel to 1.5 g agarose (G biosciences).

3.2.4. Preparation and selection of teeth

Before sectioning, the teeth were cleaned using a spoon excavator and a toothbrush with pumice powder and stone to remove all soft tissue remnants. To detect if there are any defects, caries, or cracks, all teeth were screened by trans-illumination and transmitted light using low-power microscopy (Leitz, Wetzlar®, Germany). Six teeth were selected for this pilot study.

3.2.5. Preparation of enamel slabs by cutting and grinding

Each tooth was mounted in the yellow 'stick' impression compound (Kerr Dental, Hayes, UK) on plates. Then, the crowns were sectioned using a wire cooled diamond water saw and cutting machine (Well@walter EBNER, CH-2400, Le Locle, Switzerland) (Figure 3). The cutting area was marked using a pencil. The buccal and palatal surfaces of each crown were removed, and the slabs were prepared from these surfaces so that each enamel slab were approximately 5 mm x 5 mm x 2 mm in size. Enamel slabs were then mounted in circular resin blocks of 3 mm thickness (Stycast; Hitek Electronic materials, Scunthorpe, UK).

Figure 3. Diamond wire saw apparatus used for teeth sectioning.

3.2.6. Storage of enamel slabs

Once the enamel slabs were prepared, the enamel slabs were kept moist in microcentrifuge tubes containing deionised water in “Sterilin” type universal tubes and left at room temperature to prevent dehydration during the experiment (Bataineh et al., 2017).

3.2.7. Preparation of the enamel artificial subsurface lesions

To obtain a subsurface enamel lesion, acid gel was prepared. Preparation of the demineralising system, acidified hydroxyethyl cellulose gel was prepared by adding 0.1 M sodium hydroxide (British Drug Houses, AnalaR Grade, UK) to 0.1 M lactic acid (Sigma Aldrich D/L GPR 87% Lactic acid) to give a pH value of 4.5 (Bataineh et al., 2017). Then 6% w/v hydroxyethyl cellulose (Sigma Aldrich) were added to the solution and stirred for one hour until a consistency like that of wallpaper paste is achieved. The mixture was left to settle for 24 hours (Bataineh et al., 2017).

Each enamel slab was mounted on a plastic rod using “sticky wax” impression compound (Kerr Dental, Hayes, UK) 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. Two coats of an acid resistant, coloured nail varnish (Max Factor “Glossfinity”, Maryland, USA) 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. An interval of 24 hours was left between the two applications to allow the nail varnish to dry completely.

Once the demineralising gel is ready for use, it was poured into universal tubes Sterilin, into which the mounted teeth were submerged. The enamel slabs were immersed in the acid gel for seven days to produce artificial enamel subsurface lesions (ThanNaing et al., 2023). The enamel slabs were removed from the acid gel and washed with deionised water. The nail varnish was removed using methanol (HPLC Gradient grade, method development, Fisher Scientific) to prepare the enamel slabs (Figure 4).

Enamel slabs were assessed after demineralisation and after remineralisation using QLF and micro-CT.

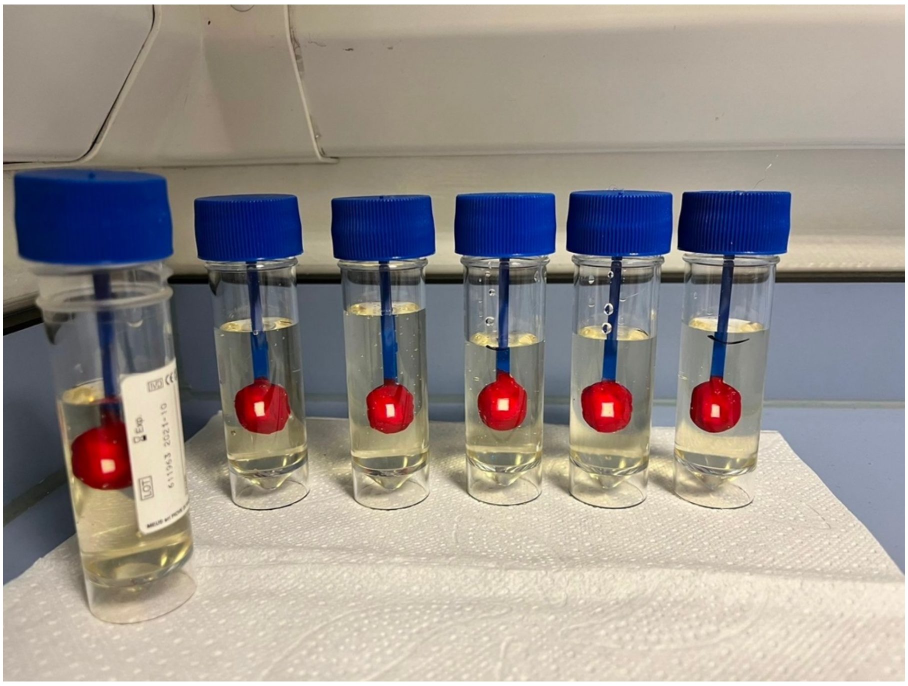


Figure 4. *In vitro* model for the development of enamel subsurface lesion.

3.2.8. Characterisation of enamel subsurface lesion

3.2.8.1. Quantitative Light-Induced fluorescence imagery (QLF)

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 (Figure 5 and 7).

To ensure that images of the enamel slabs 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.

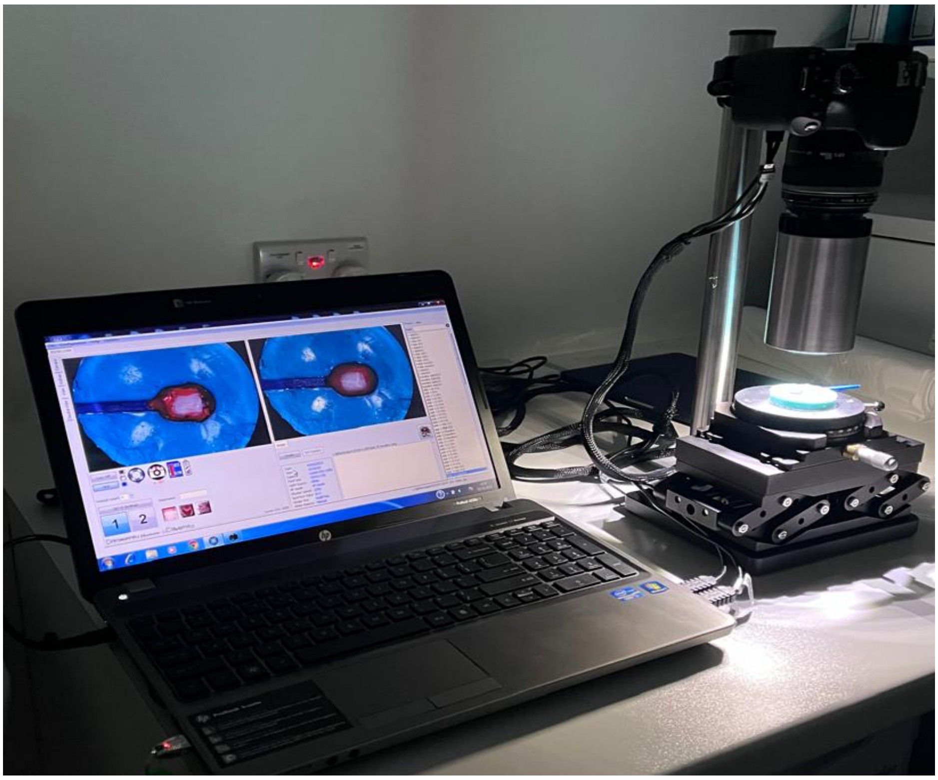
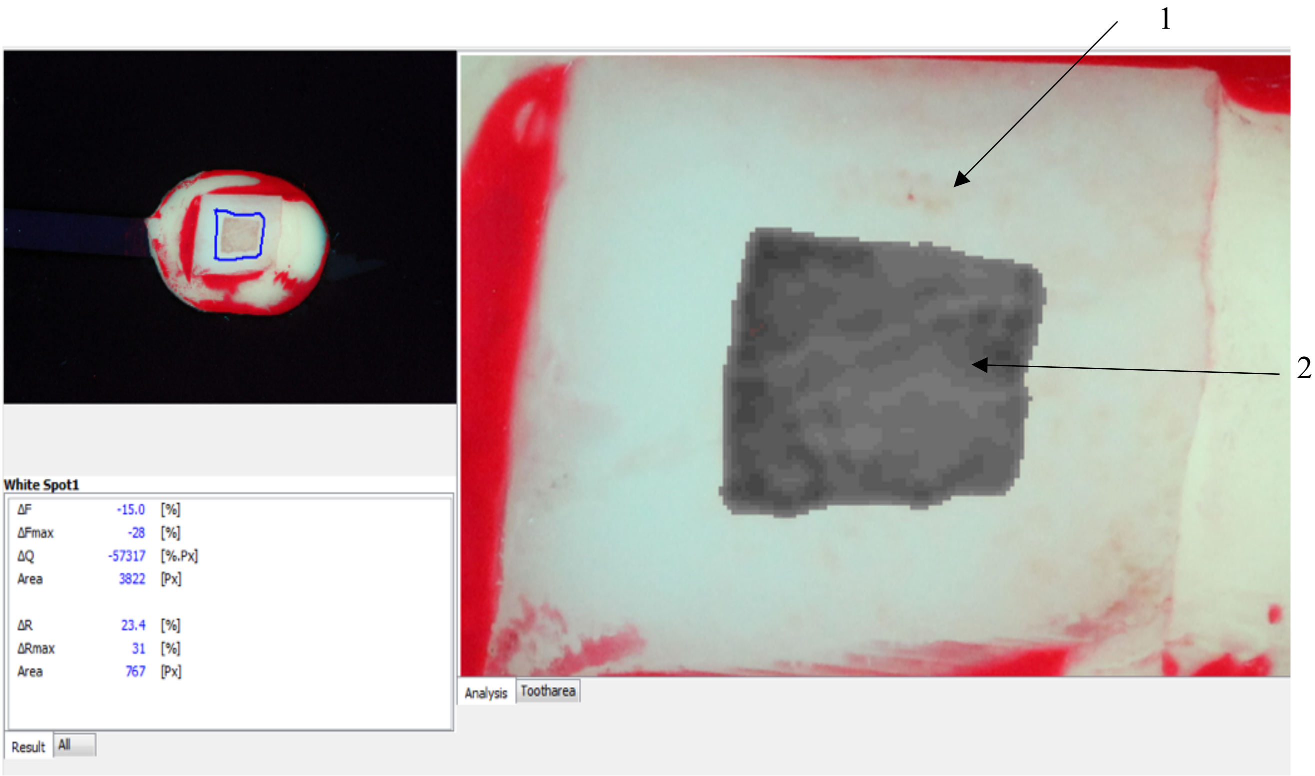


Figure 5. QLF machine, the SLR camera attached to the stand with standardised distance from the enamel slab.

A patch was drawn around the white spot lesion site by the study examiner with its borders on sound enamel (Figure 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 previously, to enable the 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 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.



White Spot1

```
ΔF        -15.0   [%]
ΔFmax       -28   [%]
ΔQ       -57317   [%.Px]
Area       3822   [Px]

ΔR         23.4   [%]
ΔRmax        31   [%]
Area        767   [Px]
```

Figure 6. Example of the blue light image analysis results including ΔF, ΔQ and Area values of the lesion.

(Arrow 1: sound enamel, arrow 2: demineralised enamel).

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

ΔF: Percentage fluorescence loss with respect to the fluorescence of sound tooth tissue; related to lesion depth (%), ΔQ: The ΔF times the Area. Percentage fluorescence loss with respect to the fluorescence of sound tissue times the area. Related to lesion volume (% px^2),

Area: The surface area of the lesion expressed in $pixels^2$ (px^2).

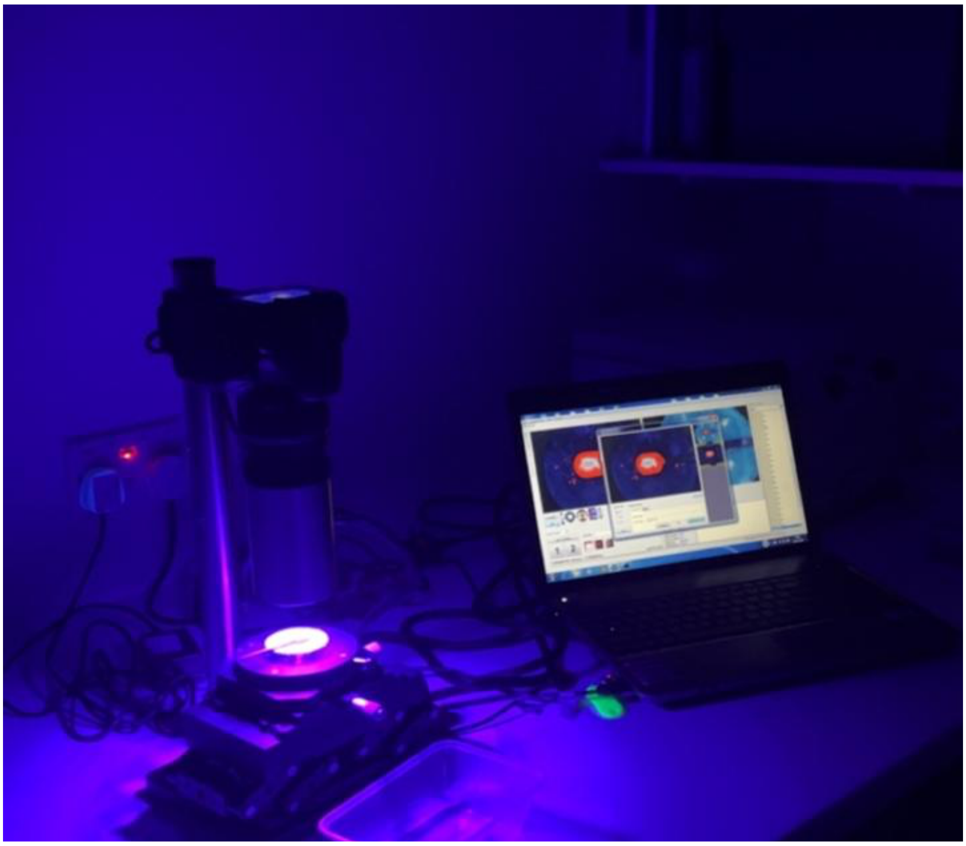


Figure 7. Quantitative Light-Induced fluorescence imagery.

This is an example of the blue light image of demineralised enamel lesions before and after the cycling regime.

3.2.8.2. Microcomputed Tomography (Micro-CT)

A phantom (Bruker, Kontich, Belgium) with known densities of 0.25 g/cm^3 which correspond to low mineral density, 0.75 g/cm^3 as medium mineral density, and high mineral density with 2.9 g/cm^3 was scanned together with each sample using a SkyScan 1172 micro-CT system (Bruker-microCT, Berlin, Germany) (Figure 8, 9). The rod was placed next to the tooth and ensured that it was stable and in the same place during the scanning. The position of the lesion was checked visually so it can be identified easily in the analysis. Scanning was performed with the following parameters: voltage of 100 kV, source current of 100 µA, with an image voxel size of

10.37 µm, an aluminium–copper filter (0.5 mm), a rotation step of 0.4° over a 180° rotation, and frame averaging of three. Flat-field correction was applied to correct for variations in camera pixel sensitivity. A filter was used for optimisation purposes, and the scan duration for each specimen was 39 minutes and 21 seconds. The configuration of each specimen was recorded and saved as a BMP file, titled according to the specimen identification and experimental group.

The acquired data were then reconstructed using NRecon software (Version 1.4.2, Skyscan N.V., Aartselaar) with the following parameters: BMP file format, 5% ring artefact correction, and 10% beam-hardening correction. A new database using DataViewer v.1.5.6 software (Bruker-microCT) was then created by removing unnecessary background data, including scans without images, partial images, or images showing only phantoms. Finally, the known mineral densities of the phantoms were used for calibration and quantification of enamel slab mineral density using ImageJ (Fiji software). Prior to analysis, image calibration was performed to enable the determination of mean grayscale values. Fifteen circular regions of interest (ROIs) were selected from the standard calibration rod, which contained materials of known mineral densities (0.25, 0.75, and 2.9 g/cm^3). These measurements were used to calibrate grayscale values to mineral density.

This software was necessary used to manipulate and analyse CT images using straight-line selection tool (yellow fine line) that is used for section of the Micro-CT images to provide the enamel mineral density at the sectioned area in plot graph form.

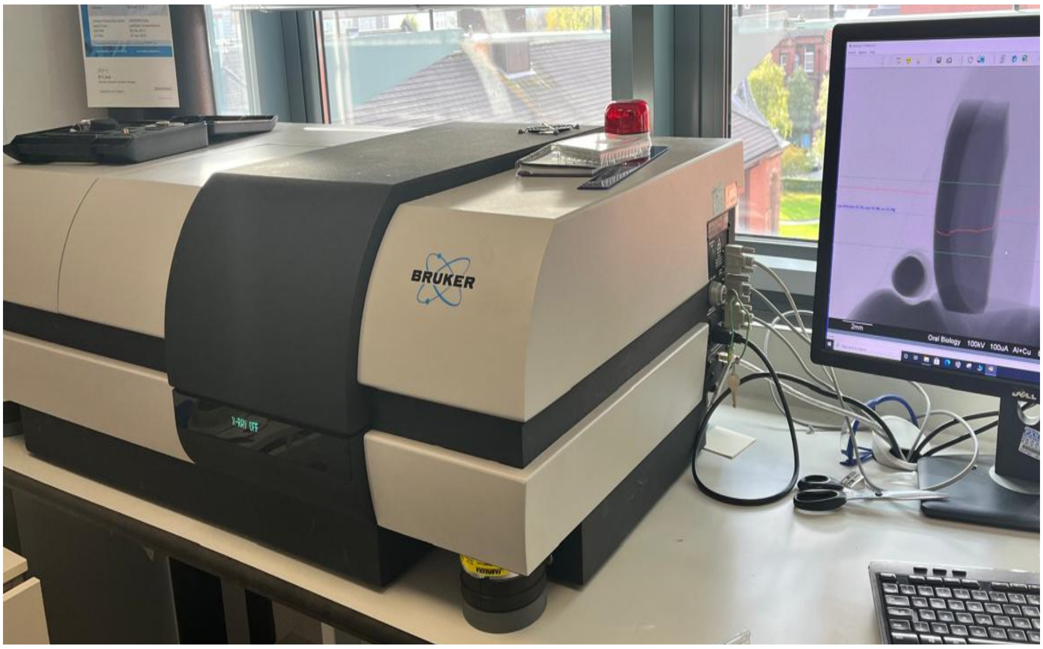


Figure 8. Microcomputed Tomography

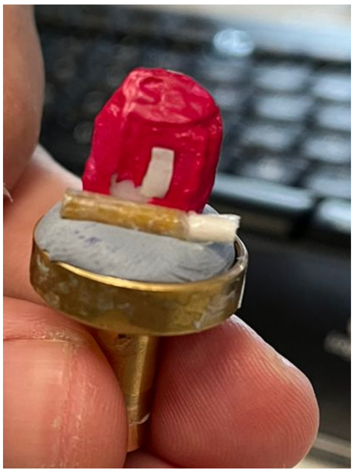


Figure 9. Represents the phantom used in calibration to the images in the Micro-CT.

3.2.9. Preparation of agarose gel

A 3% (w/v) agarose gel was prepared by adding 1.5 g of agarose (G-Biosciences, Missouri, USA) to 48.5 mL of deionised water in a 100 mL beaker, which was placed in an ice bath for 15–30 minutes. The agarose gel was added slowly. When ice was almost melted, 100 mL beaker was put on hot plate directly and aqueous solution of agarose was stirred at 80-90 °C for 15 minutes until transparency (Figure 10) (Tabata et al., 2003). Any bubbles in the aqueous solution can be popped using the ultrasonic wave. After this, the aqueous solution is warmed at 60 °C prior to be poured into metal moulds (clipped underneath to it with a glass slide) and cooling it at room temperature to obtain agarose gels. The formed 5 mm thickness of agarose gels were punched out into 1 cm diameter disks and placed in a six well plates. The formed agarose gels are clear and transparent before the application of the soaking treatment (Tabata et al., 2003) (Figure 11 and Figure 12).

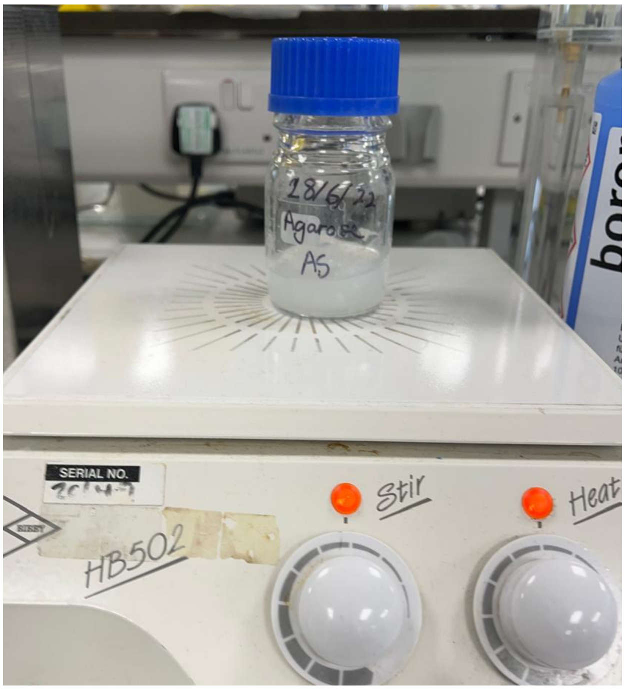


Figure 10. The beaker contains an aqueous solution of agarose gel.

Figure 11. ***In vitro*** **image of agarose gel in a six well plates.**

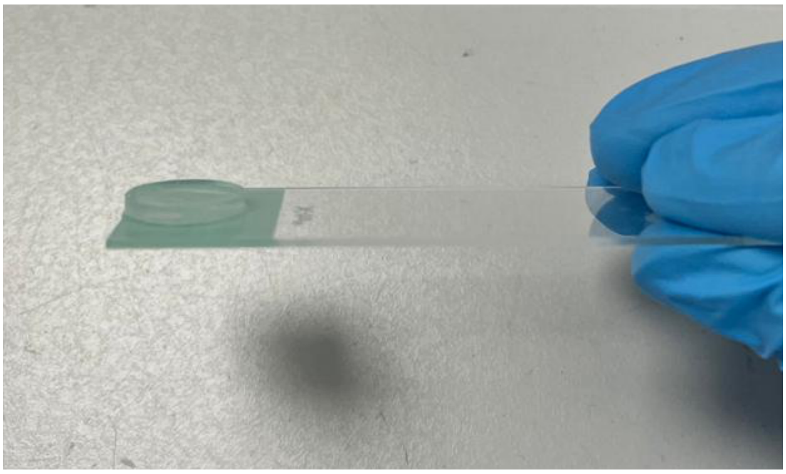


Figure 12*.* ***In vitro*** **image of agarose gel of 5 mm thickness before treatment.**

3.2.9.1. Characterisation of agarose gel

3.2.9.1.1. Freeze-drying

The freeze-drying process is a multi-step procedure designed to produce a stable, dried product while preserving the structural and functional integrity of the sample. It begins with careful preparation of the sample to ensure it is ready for the subsequent steps. This is followed by the freezing stage, where the sample is subjected to low temperatures to solidify the water content (Bellows and King, 1972).

The next phase, known as primary drying, is a critical part of the process. During this step, the frozen water in the sample begins to transfer as the temperature increases. However, the temperature must be carefully controlled. If the temperature exceeds this critical threshold, it can cause structural collapse of the sample, a phenomenon referred to as melt back or collapse (Franks, 1998). This delicate balance is essential for maintaining the quality and stability of the final product.

As the primary drying phase progresses, the ice crystals within the sample begin to separate, leaving behind a concentrated solution. This process prepares the sample for the secondary drying stage, where any remaining water molecules are removed. The secondary drying phase ensures that the sample achieves the desired level of dryness, completing the freeze-drying process (Bellows and King, 1972). Each step of this procedure plays a vital role in producing a high-quality dried product while preserving the structural and biochemical properties of the sample.

For this pilot study, the agarose gels were freeze dried for 3 days before using it on scanning electron microscope to maintain the quality and structure of the agarose gels.

3.2.9.2. Scanning Electron Microscopy (SEM)

Following freeze-drying, the agarose gels were analysed using scanning electron microscopy (SEM) to assess mineral deposition and elemental composition by energy-dispersive X-ray spectroscopy (EDX). The samples were mounted on aluminium stubs using 12 mm carbon adhesive discs (Agar Scientific). Microstructural analysis was performed using a scanning electron microscope (Hitachi S-3400N, Hitachinaka, Japan) operated at an accelerating voltage of 20 kV. Standardised scanning angles were employed, and the entire surface of each agarose gel was systematically scanned and imaged at high magnification with sample morphology of less than 0.1 µm.

Obtaining clear SEM images of HAP on the surface of the agarose gel proved challenging due to poor surface image quality. To overcome this limitation, the agarose gel was sectioned both vertically and horizontally using a blade, which enabled acquisition of diagnostically useful SEM images.

3.2.9.3. Energy Dispersive X-ray Spectroscopy (SEM-EDX)

Energy-dispersive X-ray (EDX) analysis was performed in conjunction with SEM using a Bruker XFlash® 6 detector (Berlin, Germany) (2 × 60 mm^2) to evaluate the elemental composition of the agarose gels. The elements analysed included calcium (Ca) and phosphorus (P), as principal components of tooth structure. During EDX analysis, the specimen surface was scanned with an electron beam operating at an energy of 20 kV, which induced the emission of characteristic X-rays. The energies of the emitted X-rays were detected and measured, and the electron beam was moved across the specimen surface to generate elemental distribution images and spectra for each sample (Shukla and Iravani, 2019).

3.2.10. Preparation of aqueous solution of $CaCl_2$ and Na_2HPO_4

Calcium Chloride (pH 7.4, 200 mM) was prepared by dissolving 22.196g $CaCl_2$ (Mw=110.98) (Honeywell Fluka™) in 800 mL of deionised water. Stirred well and topped up water to 1000 mL. 120mM Di-sodium hydrogen phosphate dodecahydrate were prepared by dissolving 42.98g Na_2HPOH_2O (Mw=358.14) (99% Bioserv UK limited) in 800 mL of deionised water. Stirred well and topped up to 1000 mL with water (Tabata et al., 2003).

3.2.11. The experimental Protocol/ Regime of alternate soaking process

Following the preparation of aqueous solution of $CaCl_2$ and Na_2HPO (alternate soaking process), the agarose gel disks (diameter 1 cm, thickness: 5 mm) were placed into six well plates (n=6) and immersed into 3 mL aqueous solution of $CaCl_2$ at 4 °C for 30 minutes (one disk in one well). Then the gels were rinsed with deionised water for 2-3 times. After rinsing, the gels were immersed into aqueous solution of Na_2HPO_4 at 4 °C for 30 minutes. The gels were rinsed with deionised water for 2-3 times before immersed again into aqueous solution of $CaCl_2$. This accounts for one cycle. The treatment was done for 12 cycles in total (Tabata et al., 2003) (Figure 13).

The alternate soaking protocol used in this study was performed on an agarose gel substrate and adapted from previously published *in vitro* mineralisation studies on bone. These studies commonly employed longer soaking intervals, such as 30-minute immersions with a limited number of cycles (e.g., 12 cycles), to evaluate mineral deposition under controlled laboratory conditions (Kono et al., 2007; Taguchi et al., 1998; Taguchi et al., 1999). As this pilot work progressed, it was recognised that the original alternate soaking protocol required a relatively long experimental duration. Consequently, a methodological shift was made towards using shorter soaking periods on enamel lesions. Following more recent studies done by the same authors that formulated the alternate soaking process, where they increased the number of cycles while reducing individual soaking duration (Matsusaki et al., 2009; Watanabe and Akashi, 2008).

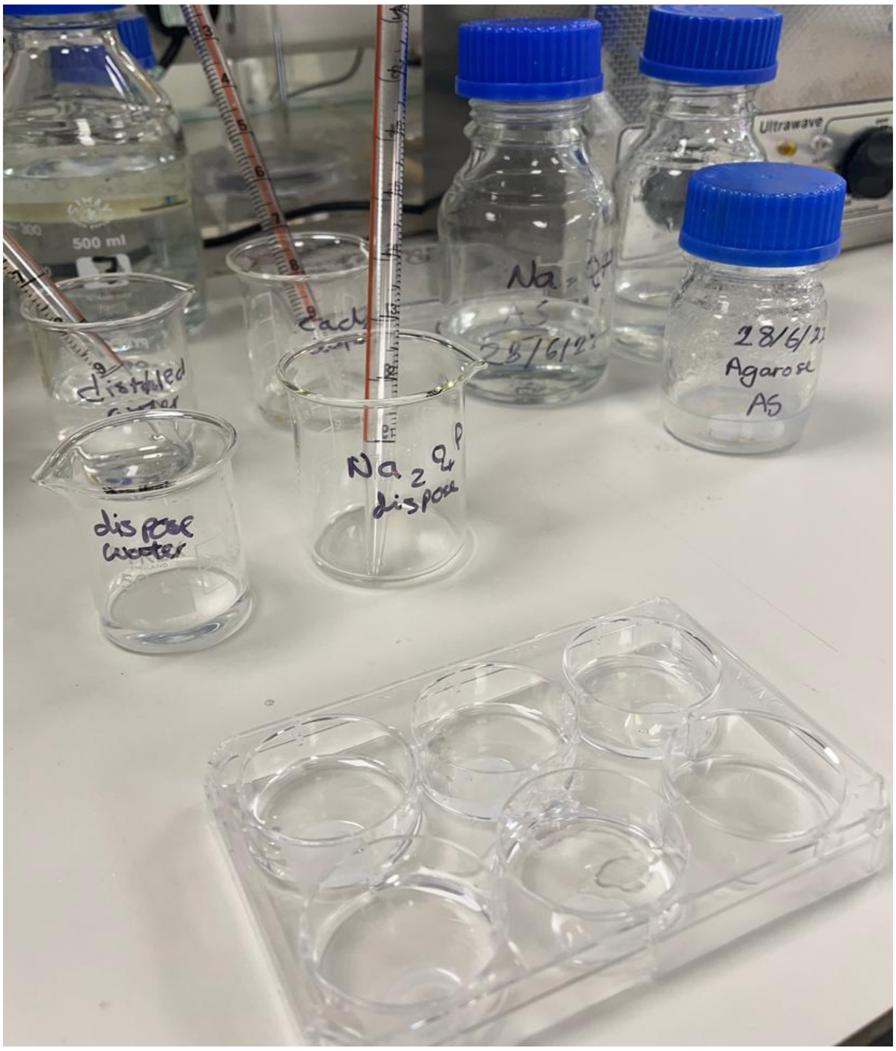


Figure 13. The agarose gel disks were placed into six well plates and immersed with aqueous solution of $CaCl_2$ for one hours.

3.2.12. Experimental protocol for the application of $CaCl_2$ and Na_2HPO_4 with alternate soaking method on enamel lesions

The protocol for alternate soaking method of the application of calcium and phosphate solution was obtained from the Matsusaki et al (2009) from Japan it has been developed and applied this protocol on bone which demonstrated bone regeneration (Matsusaki et al., 2009). In this study, the alternate soaking process group protocol started with washing enamel lesions first with deionised water. The enamel lesions were soaked in $CaCl_2$ for 10 seconds and it was soaked for 10 seconds with Tris-HCL.

After that, it was soaked with Na_2HPO_4 for 10 seconds. This was done for 12 cycles (Matsusaki et al., 2009). Then it was washed with deionised water and following this it was washed with night-time saliva. The saliva was discarded and was replaced with fresh night-time saliva to be stored for 5 hours. After 5 hours, the second cycle was done which follows the same protocol as the first cycle. After that it was stored in night-time saliva for the next day soaking. This was done for 21 days (Bataineh et al., 2017).

3.3 Statistical Analysis

SPSS statistical software package (SPSS Inc. ver.29) was used for analysing the data and measure the statistical difference. Descriptive statistics were used to calculate the mean, median, range and standard deviation. A paired-samples t-test was used to compare the difference of enamel slabs between before and after treatment of alternate soaking process (Ca/P).

3.4. Results

3.4.1. *In vitro* formation of HAP-like structure in agarose gel

The agarose gel before the treatment of alternate soaking process appeared to be soft, transparent, and partially clear (Figure 14a). However, after the exposure to calcium and phosphate solutions, all the agarose gels became whiter in colour and less transparent (Figure 14b). The agarose gels became firm to touch compared to the original agarose gel. All the agarose gels before and after treatment were stored in deionised water at a room temperature of 37 °C.

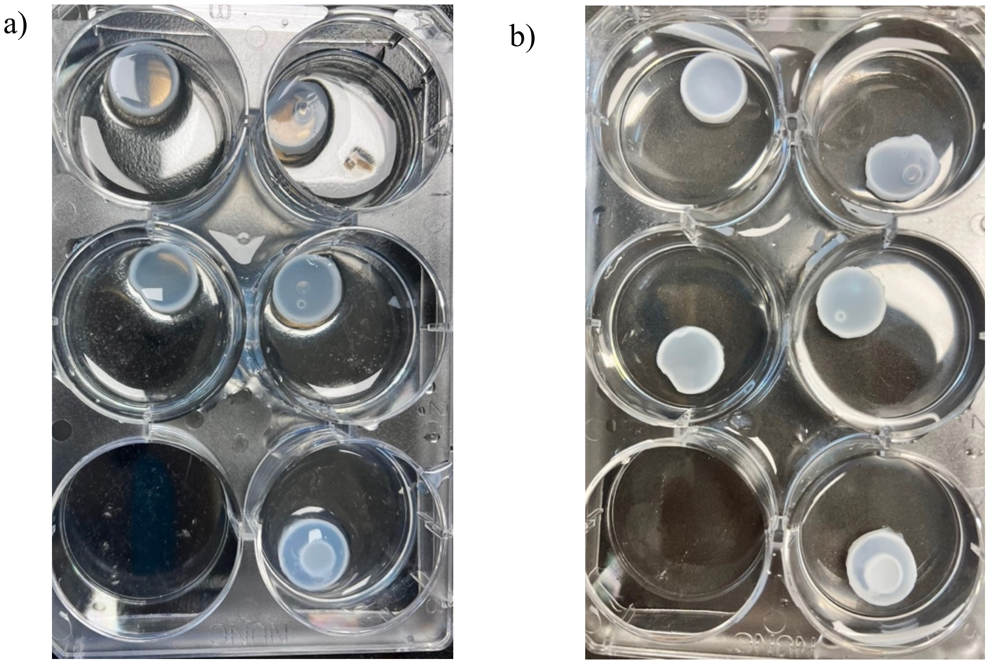


Figure 14. (a and b). ***In vitro*** **images of agarose gels.**

a) before the alternate soaking process: the agarose gel was soft and transparent; b) after 12 cycles of the alternate soaking process: white in colour and became firm to touch.

3.4.2. SEM image of HAP formation on agarose gel

Following the alternate soaking process treatment, the agarose gel was then assessed using SEM. SEM imaging was not performed on the agarose gel prior to the alternate soaking process (Calcium and Phosphate solutions), as the primary purpose of SEM and EDX analyses in this pilot study was to evaluate the surface structure and elemental composition of the agarose gel after treatment. Before Using SEM, the agarose gel was cut vertically and horizontally. Figure 15 shows SEM images of agarose gel after 12 cycles of the alternate soaking process. The white deposits on the agarose gel can be seen clearly. The high magnification of images shows the fragmented meshwork appearance of agarose gel with pores of varied sizes. The stick- like crystal appearance of HAP was not clear, however, it can be noted that there are some areas of stick-like appearance. EDX was further used to show the calcium and phosphate content as well as the Ca/P ratio of the crystals.

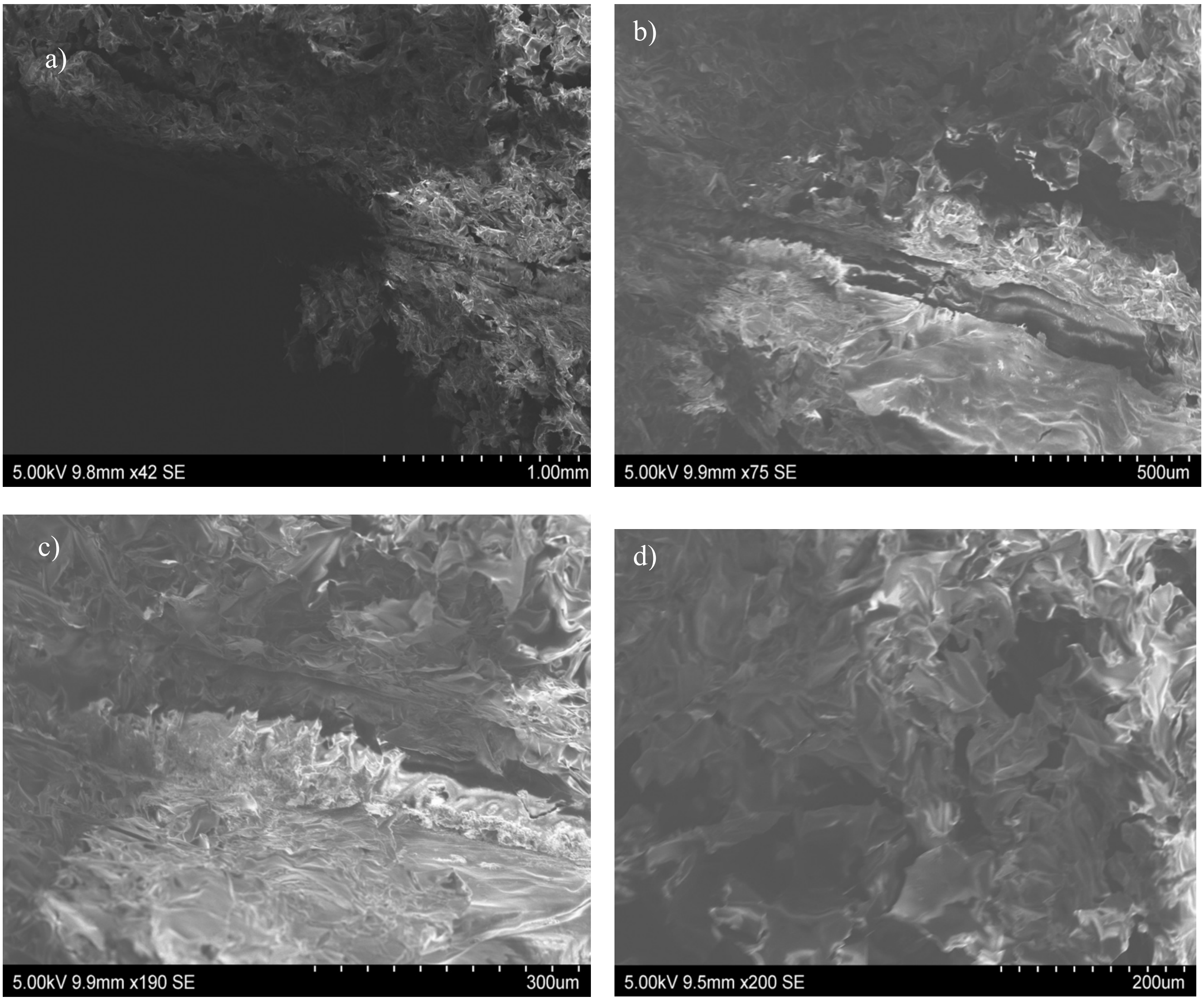


Figure 15 (a,b,c and d). SEM images of surface topography of agarose gel after 12 cycles of alternate soaking process.

a) Low magnification (scale bar: 1mm); b, c, and d) higher magnification (scale bars: 500um, 300um, and 200um).

3.4.3. EDX results to show the Ca/P ratio of crystals deposited within the agarose gel.

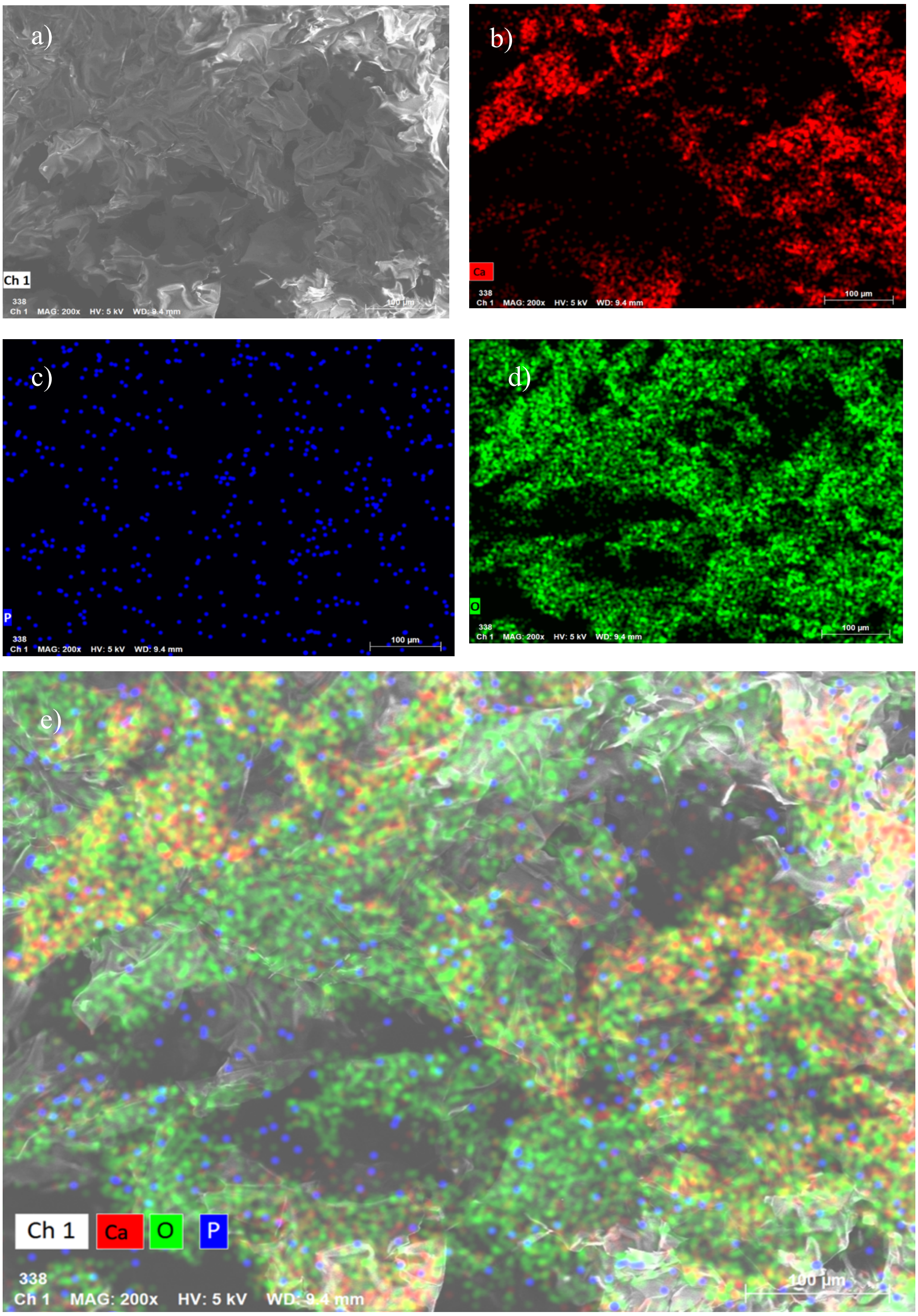


Figure 16 (a,b,c,d and e) EDX scanning of chemical composition of Ca, P, and O.

a) SEM image of agarose gel/HAP; b) EDX scanning of chemical composition of Calcium in agarose gel. c) EDX scanning of chemical composition of Phosphorous in agarose gel. d) EDX scanning of chemical composition of Oxygen in agarose gel. e) EDX mapping of Ca, P and O.

Table 4. The Ca/P ratio on the agarose gel/HAP.

Sample	Average Ca wt.%	Average P wt.%	Ca/P ratio
1	37.73	18.10	1.62
2	41.63	17.96	1.69
3	42.50	19.08	1.73

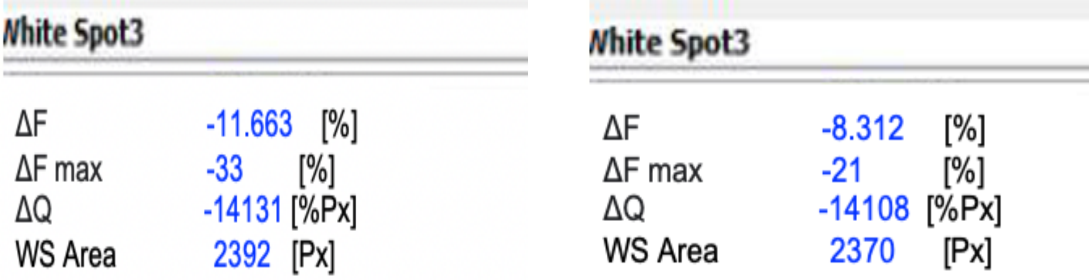
Figure 16 a. shows a selected image from one of the agarose gels using SEM. This image was further transferred to EDX for elemental scanning of the agarose gel. The presence of calcium, phosphorus, and oxygen are all expected to be present in the agarose gel due to the use of alternate soaking process. The Ca/P ratio on the agarose gel was assessed on three agarose gel randomly and was found to be 1.62, 1.69, and 1.73 (Table 4).

3.4.4. Characterisation of demineralised enamel slab using Quantitative Light-Induced Fluorescence Imagery (QLF)

All the enamel subsurface lesions were analysed using QLF before and after the alternate soaking process for 21 days to determine whether remineralisation influences the enamel demineralisation. Figure 17 shows an example of one of the enamel samples treated with alternate soaking process. The QLF lesion depth reading for this enamel slab is (ΔF%) were reduced (-8.31±1.38) compared with before the treatment (-11.66 ± 2.0) ($p<0.001$) which show significant difference. The lesion volume (ΔQ px^2) was also smaller after treatment (-14108.00 ± 613.89) compared to the control (-14131.00 ± 613.6) ($p<0.001$) which also reached significance.

Subsurface enamel lesion at baseline

Subsurface enamel lesion after alternate soaking process treatment



White Spot3

ΔF	-11.663	[%]
ΔF max	-33	[%]
ΔQ	-14131	[%Px]
WS Area	2392	[Px]

White Spot3

ΔF	-8.312	[%]
ΔF max	-21	[%]
ΔQ	-14108	[%Px]
WS Area	2370	[Px]

Figure 17. Example of the blue light image of demineralised enamel lesions before and after the cycling regime and its corresponding readings.

Table 5 shows the mean of ΔF at baseline, after treatment and the calculated mean difference of ΔF. The mean difference of ΔF was statistically significance indicating decrease in lesion depth (%). To assess whether the change in ΔF at baseline and after treatment was significantly different within the same group, paired t-test was carried out. Figure 18 shows the change in the lesion depth ΔF for all the samples at baseline and after the treatment of the alternate soaking process (Ca/P). There was a reduction in the lesion depth in all the samples when compared to baseline.

Table 5. Mean values of ΔF at baseline and after treatment for the alternate soaking process.

	ΔF at baseline (Mean± SD)	ΔF after treatment (Mean± SD)	Difference in ΔF at baseline and after treatment (Mean ± SD)	P-value
Alternate soaking process	-10.033 ± 0.94	-8.312 ± 0.72	1.72 ± 0.22	0.001*

* Significant differences at p<0.05 level.

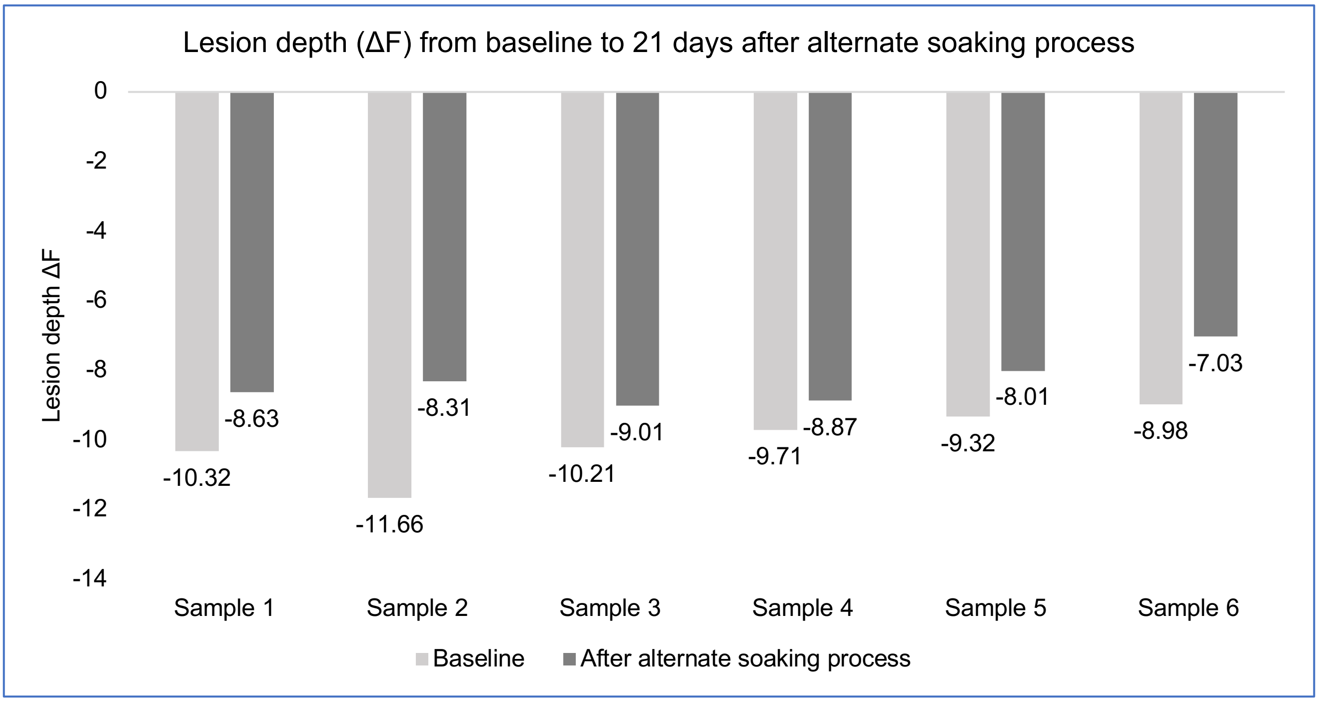


Figure 18. The change ΔF from baseline to 21 days after treatment of the alternate soaking process.

Table 6 shows the mean of ΔQ at baseline, after treatment and the calculated mean difference of ΔQ. The mean difference of ΔQ indicating decrease in lesion volume (%). To assess whether the change in ΔQ at baseline and after treatment was significantly different within the same group, paired t-test was calculated. A significant decrease in lesion volume (%) was seen in all samples of the alternate soaking process. Figure 19 shows the change in the mean of ΔQ at baseline and after treatment in the alternate soaking process. Moreover, it can be clearly stated that all samples showed decrease in the lesion volume ΔQ.

Table 6. Mean values of ΔQ at baseline and after treatment for the alternate soaking process.

	ΔQ at baseline (Mean± SD)	**ΔQ after treatment (Mean± SD)**	**Difference in ΔQ at baseline and after treatment (Mean± SD)**	**P-value**
Alternate soaking process	-1666.167 ± 796.4	-1495.2± 524.1	286.0 ± 272.3	0.04*

* Significant differences at $p<0.05$ level.

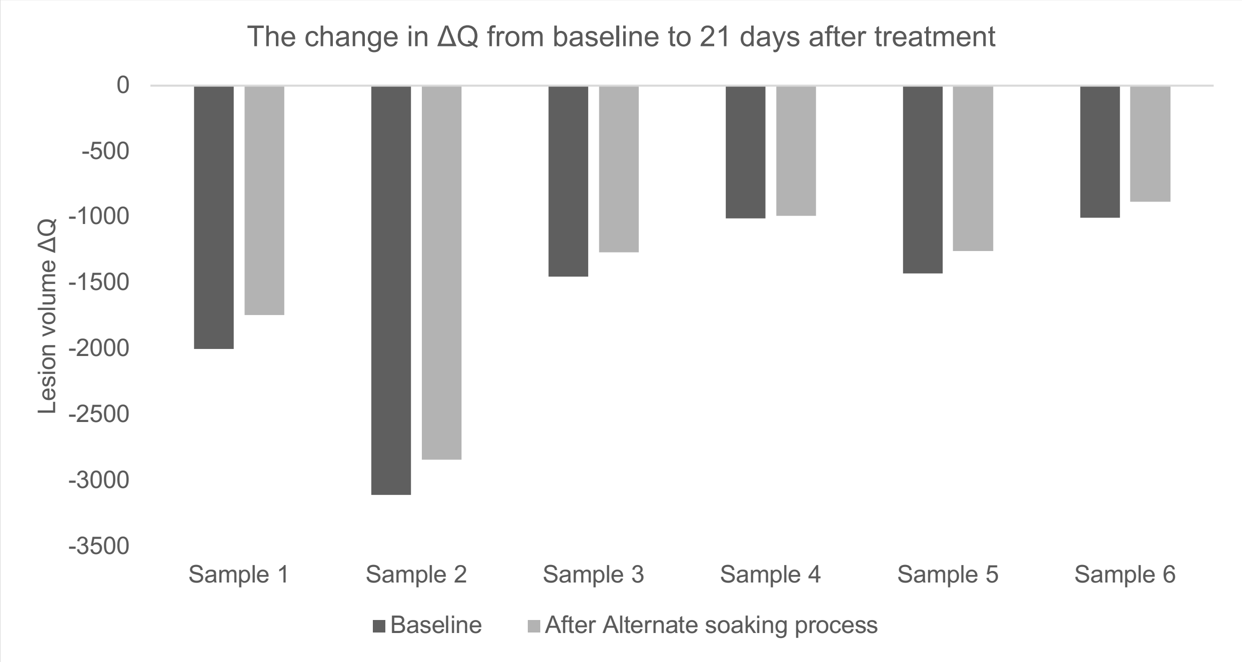


Figure 19. The change in ΔQ from baseline to 21 days after treatment for the alternate soaking process.

3.4.5. Evaluation of enamel subsurface lesions using Microcomputed tomography (Micro-CT)

Microcomputed tomography (Micro-CT) (Figure 20) presents a coronal section of enamel lesions, clearly showing a localised window area of demineralisation.. This window revealed the extension and distribution of lesion. There was no loss of continuity of enamel, only grey area along its thickness was observed. This corresponds to incipient lesion which is clearly seen. Using a straight-line profile, line plots were obtained from the coronal view of the enamel lesion at the upper, middle, and lower regions of the demineralised area. These plots were analysed using the plot file analysis to assess the mineral density within each region (Figure 21). The lower line corresponds to the region closest to the root. The upper and middle sound enamel areas were higher in mineral density compared to lower part section. It was clearly seen in the demineralised area in lower part of coronal enamel section (Figure 21 c). Furthermore, analysis of the plot files using Microsoft Excel revealed regions of reduced enamel mineral density in the lower part of the enamel, with values decreasing to approximately 1.5 g/cm^3, compared with other regions that predominantly exhibited mineral density values above 2.0 g/cm^3. These findings indicate that the areas with lower mineral density were demineralised. (Figure 22).

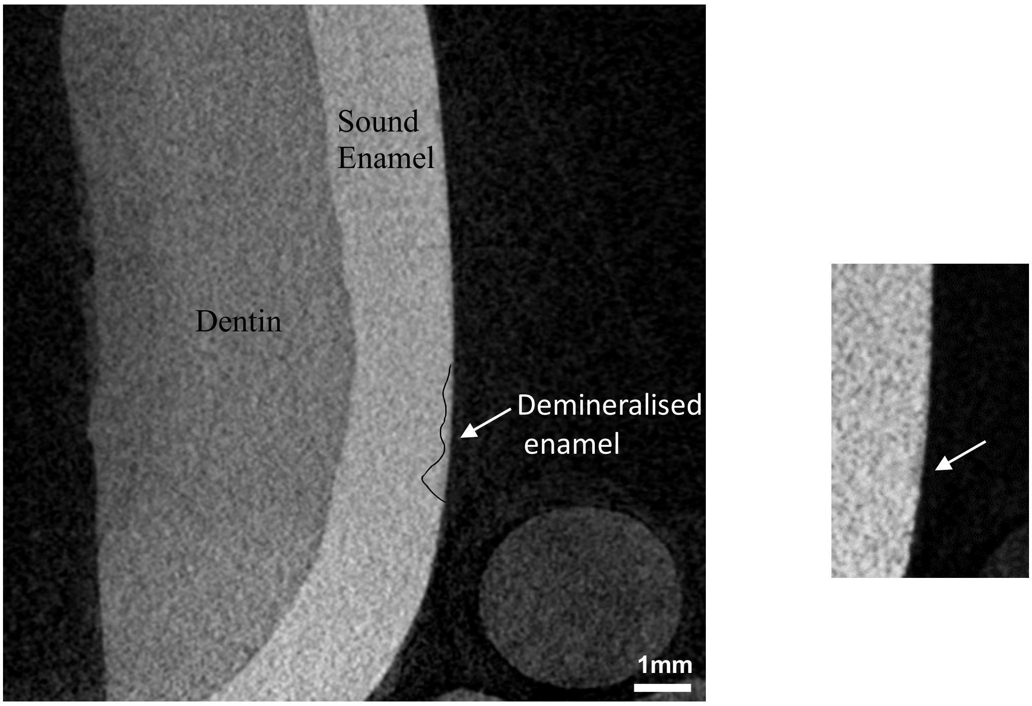


Figure 20. Represents coronal section micro-CT image of enamel slab after demineralisation. Scale bar 1mm.

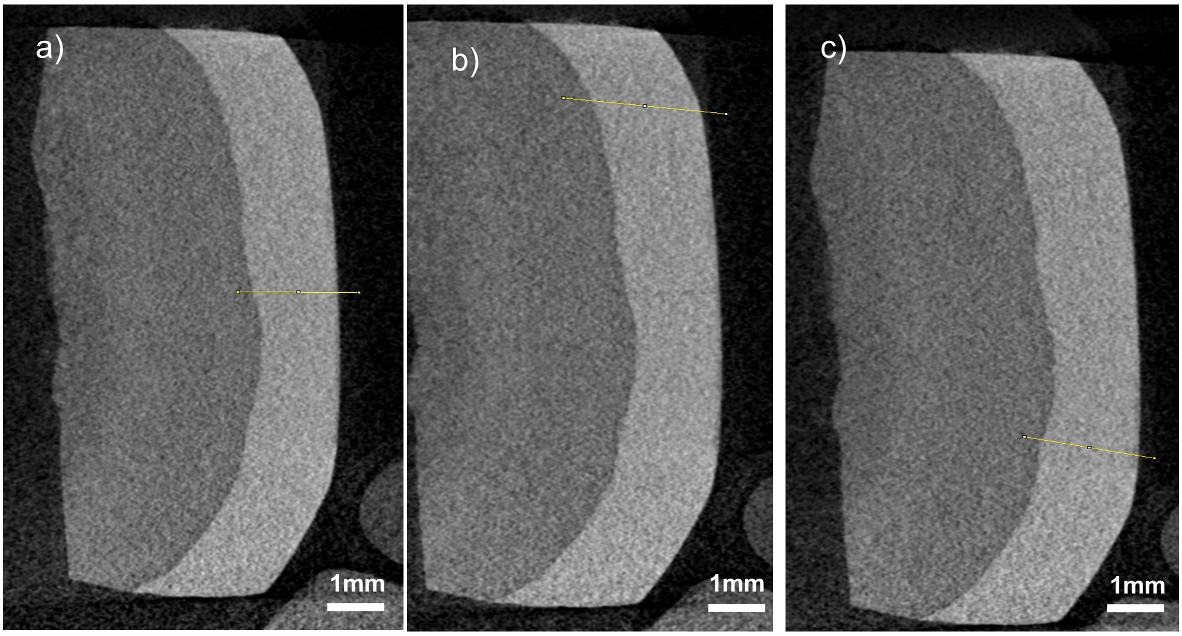


Figure 21 (a,b, and c). Representative images of coronal section of enamel slab.

Straight line selection moving between sound enamel and demineralised enamel. Sectioning was done into three parts (upper part, medium and lower part) using the

straight-line profile . a) shows upper sound enamel, b) middle sound enamel and c) lower part, demineralised window of enamel and a high magnification of the demineralised area. Scale bar 1mm.

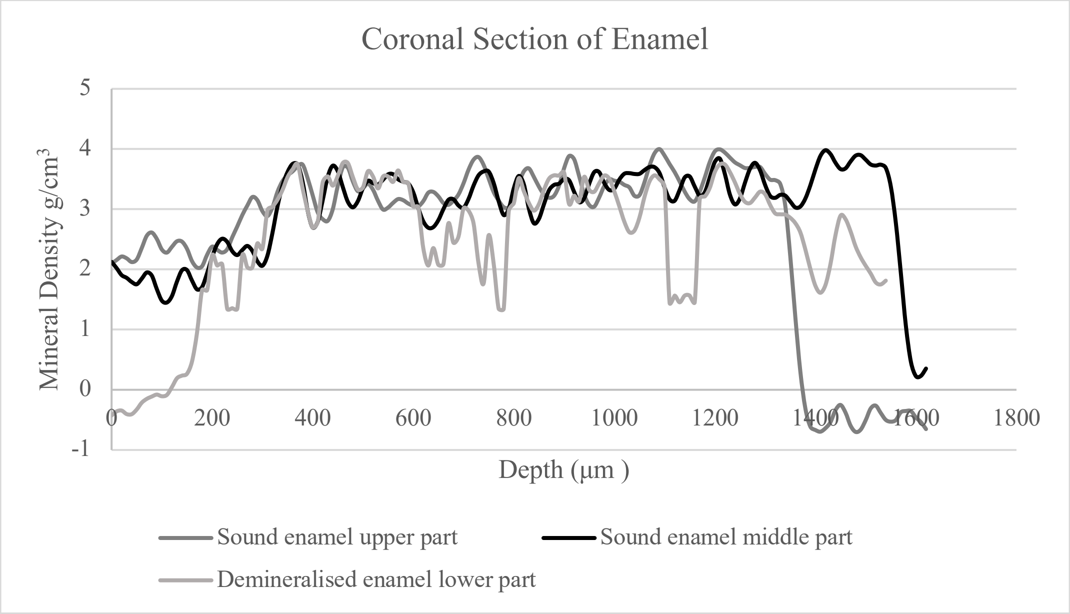


Figure 22. Difference between different sections of enamel.

Lower part of enamel slab shows areas of low mineral density indicating demineralised enamel.

Figure 23 shows transaxial sections of the enamel slab which revealed a distinct area of demineralised enamel located in the middle portion of the three analysed parts, as observed through the straight-line profile. The transaxial analysis further demonstrated a line plot of mineral density, clearly highlighting that the middle enamel region, representing the demineralised enamel, exhibited significantly reduced mineral density when compared to the adjacent parts. This reduction in mineral density underscores the extent of enamel demineralisation in the middle section, as illustrated in the line plot (Figure 24). The findings emphasise the differences in mineral content distribution across the enamel slab, providing insight into the localised impact of the demineralisation process.

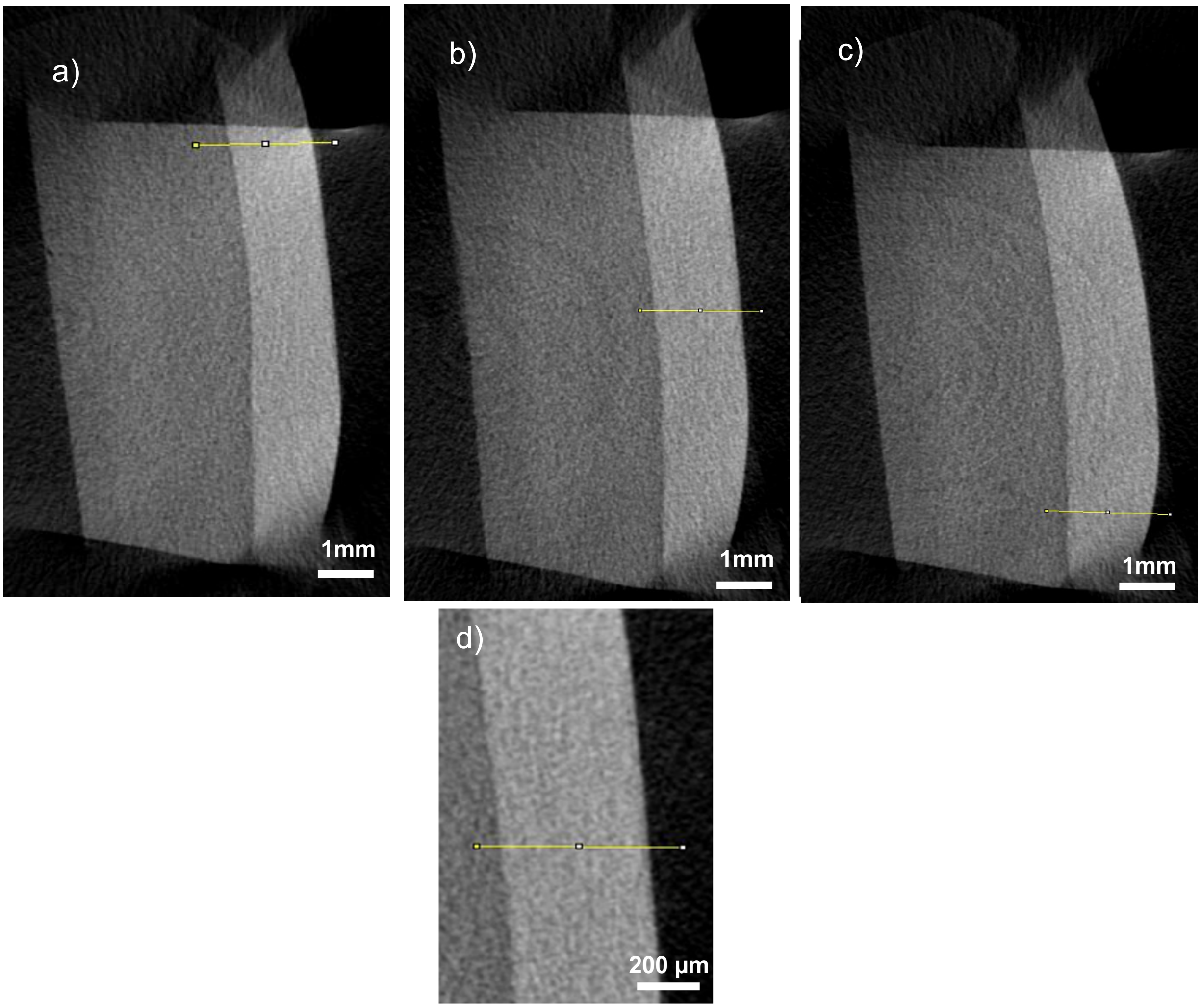


Figure 23. Representative images of transaxial section of enamel slab.

Straight line selection moving between sound enamel and demineralised enamel. Sectioning was done into three parts (upper part, middle and lower part) using the straight-line sectioning. Figure 23 a) shows upper sound enamel, b) middle demineralised enamel and d) a magnification of the middle area of the demineralised area and c) lower part, sound enamel. Images (A–C) are presented with a scale bar of 1 mm, while image (D) includes a scale bar of 200 µm.

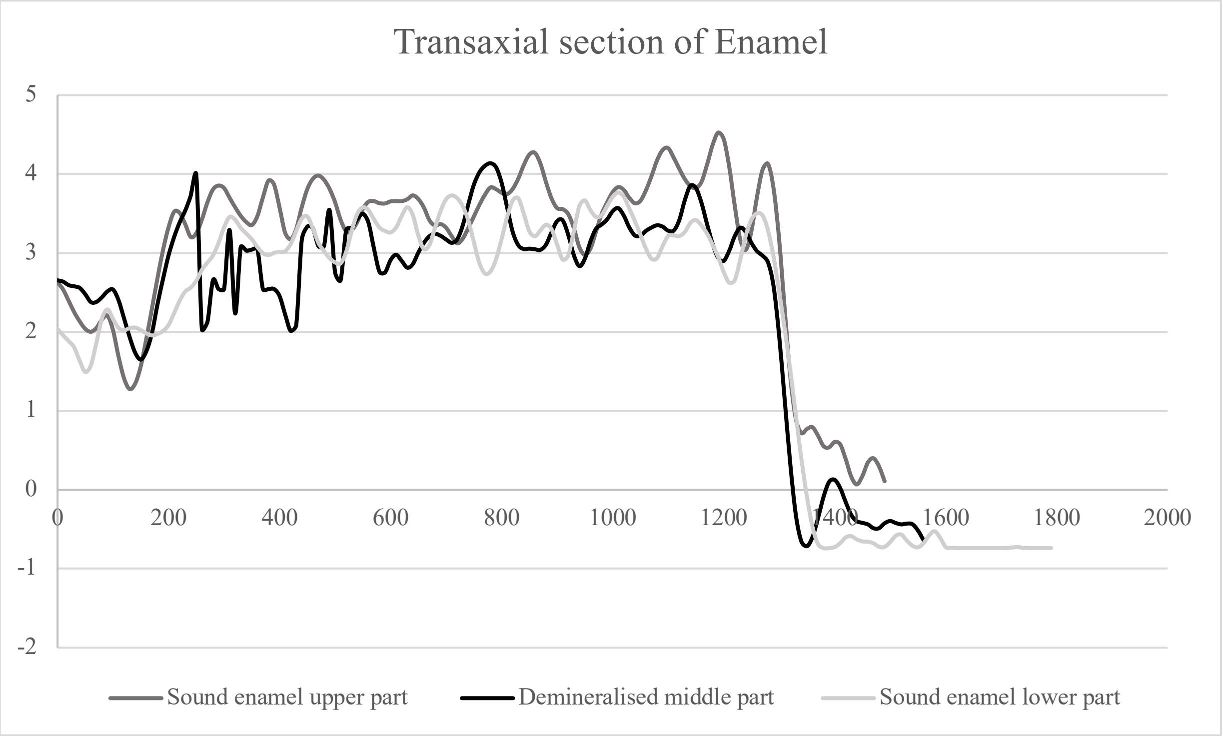


Figure 24. Difference between different transaxial sectioning parts of enamel.

Demineralised enamel middle part shows areas of low mineral density indicating demineralised enamel.

3.5. Discussion

Hydroxyapatite is used in various biomedical applications and is common due to the similarity when compared with natural bone and teeth. It has excellent properties such as osteoinduction, bioactivity and biocompatibility (Matsusaki et al., 2009). The application of HAP depends strongly on the properties of particles such as morphology, crystallinity and stoichiometry (Koutsopoulos, 2002). This pilot study identified the presence of calcium and phosphorus on/in the agarose gels using SEM with EDX analysis. SEM was employed to examine the surface morphology of the agarose gel, and the resulting images were subsequently analysed by EDX to determine the elemental composition present within the samples. The presence of calcium, phosphorus, and oxygen on/in agarose gel was all expected to be present due to the use of the alternate soaking process with Ca and P solutions. The Ca/P ratio on the agarose gels was found to be 1.62, 1.69, 1.73 which was around the Hydroxyapatite mole Ca/P ratio 1.67, which suggests the likelihood of HAP formation *in vitro* (Habibah et al., 2022).

Furthermore, in the current study, HAP was developed on the agarose gel using the alternate soaking process with calcium and phosphate for 30 minutes each and 12 cycles in total. The agarose gel became whiter in colour and less transparent. Agarose gel was soaked with calcium/Tris-HCl and phosphate/Tris-HCl buffers. This suggests that calcium phosphate was formed on the agarose gel's surface, this coincides with the description of the Taguchi’s group when they prepared agarose gel (Taguchi et al., 1999). In terms of limitation, the alternate soaking process cycle carried out for 12 cycles instead of 20 cycles. However, this suggests the likelihood of HAP formation in/on the agarose gel. Furthermore, Agarose gel after the use of alternate soaking process was assessed using SEM and EDX. It was a struggle to get images of HAP on SEM from the surface of agarose gel. It was difficult to get clear images using SEM and consequently EDX would not provide elements available without a selected image. After that, began sectioning through the agarose gel using a blade vertically and horizontally, and diagnostic images were obtained. SEM images did not show the stick-like appearance of HAP clearly. This could be due to the application of soaking for 12 cycles instead of 20 cycles that led to shortening of time. It is also possible that HAP was not formed within the agarose gel, and additional studies are necessary to verify its formation.

Further investigation was applied on six enamel slabs to assess the feasibility of alternate soaking process on enamel remineralisation. Many *in vitro* studies are investigating the effects of remineralisation using calcium and phosphate with the use of fluoride on enamel subsurface lesion remineralisation (Arifa et al., 2019). However, there is no comprehensive review article about *in vitro* articles that focuses on calcium phosphate enamel products without the use of fluoride or other remineralisation agent as biomimetic ingredients. Furthermore, no study used the alternate soaking process with calcium and phosphate solutions for remineralisation of enamel subsurface lesions and compared it against a placebo, for example, a toothpaste slurry.

The present experiment showed a reduction in enamel demineralisation, as assessed by QLF. These findings indicate that the alternate soaking process may be a feasible approach for promoting remineralisation of enamel subsurface lesions. However, this pilot study indicates that a larger sample size is required to confirm the remineralisation potential. The treatment period will last 21 days, with QLF analysis performed at baseline, 7, 14, and 21 days, while micro-CT and EDX analyses will be conducted only at baseline and after 21 days.

QLF is known to be the novel technology for the detection of the early enamel subsurface lesion. 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 (Ferreira Zandoná et al., 2010). For the lesion to be seen more clearly, the pilot study needed to be further optimised by increasing the demineralisation period to 10 days instead of 7 days to increase the window of demineralisation and to follow the protocols of demineralisation in previous studies(White, 1995; Wu et al., 2010; Yassen et al., 2011; Zero, 1995; Zou et al., 2011). The enamel lesions from the demineralisation trial were noticed to be small and not clear. Future investigation is needed to develop more profound enamel lesions to be easily visualised using the instruments and to visualise the remineralisation.

Further to this, enamel demineralisation was assessed by using Micro-CT at baseline (after 7 days of demineralisation) and after treatment. For this study, enamel lesion has been successfully created. Micro-CT can provide precise measurements, not destructive to the tooth, shows any change in the mineral with time and position, and can provide longitudinal changes in the dental tissue's mineral content (Buzalaf, 2011). For Micro-CT it was thought to focus on the depth of 0-300 μm instead of a broad range of depth as the aim of this study is to investigate subsurface enamel lesion (Zou et al., 2011). This pilot study involved only baseline imaging using EDX and micro-CT.

The primary aim was to assess the feasibility of applying EDX to agarose gel in order to characterise the surface composition of the gel, and to evaluate the suitability of micro-CT for detecting mineral changes and determining whether modifications to the demineralisation protocol were required prior to the main study.

In terms of future experimentation, more investigations is needed to be conducted on the use of the alternate soaking process for remineralisation. Agarose gel was noticed, from this pilot study, is a solid material and is hard to be applied on enamel lesion. This will make it challenging for the agarose gel to function as carrier for Ca/P to be delivered to the enamel lesion. As bone is different from teeth, teeth do not require a scaffold for the delivery of calcium and phosphate using the alternate soaking process. The pilot study did not provide any definitive conclusions but the interim report for a change in the design of the methods. Therefore, it was noted that the alternate soaking process protocol approved to achieve enamel remineralisation without a carrier or template.

Training has been received before undergoing any experiment, including creating enamel slides and lesions, using a pH meter, mixing reagents in a safe and correct way, and applying the alternate soaking process method. Also, training was received for all the use of the instruments in this study, which includes QLF, Micro-CT, EDX, and freeze-drying.

3.6 Conclusion

The evidence from this pilot study suggests that the alternate soaking process protocol managed to achieve enamel remineralisation in all the enamel samples. Furthermore, the use of alternate soaking process (Ca/P) to achieve this remineralisation does not require the use of a carrier or a template on enamel remineralisation.

3.7 Outcome of null hypothesis

There is no feasibility in using a template or a precursor to deliver calcium and phosphate using the alternate soaking process protocol on enamel remineralisation.

Chapter 4 The effect of a novel alternate soaking method on enamel lesion remineralisation *in vitro*

4.1. Research aim, and null hypothesis

4.1.1. Aim

To investigate the effect of a treatment regimen involving an alternate soaking process with calcium and phosphate solutions on enamel lesion remineralisation *in vitro*.

4.1.2. Null Hypothesis

There is no difference in the effect of treatment regimens involving an alternate calcium and phosphate solutions (alternate soaking process), fluoride toothpaste slurry (1450 ppm F), Tris hydrochloride (Tris-HCL) and day-time artificial saliva (pH 6.8) on enamel lesion remineralisation *in vitro*.

4.2. General Materials and Methods

This was an *in vitro* study designed to investigate the remineralisation of the enamel subsurface lesions using different remineralising agents. The methodology adopted in the main study including preparation of tissue samples and the cycling protocol as well as the materials and equipment used will be described in this section. Finalisation of the main materials and methods is adopted after the results of the pilot study.

4.2.1. Bovine enamel selection, preparation and storage

In this study, a total of 64 bovine teeth were used and allocated equally into four experimental groups (n = 16 per group). The larger sample size relative to the pilot study was required to enable meaningful statistical comparisons between experimental conditions and to improve the precision and robustness of the outcome estimates.

The sample size for the main study was determined based on a combination of preliminary variability estimates obtained from the pilot study and evidence from the published literature reporting similar *in vitro* experimental designs and outcome measures. The pilot study provided estimates of experimental and biological variability, which are key parameters for informing sample size decisions when prior data are limited (Julious, 2005).

Following statistical advice from a qualified biostatistician at the Centre of Epidemiology and Biostatistics, University of Leeds, a group size of 16 specimens per condition was considered sufficient to detect clinically and experimentally meaningful differences between groups while accounting for inherent specimen variability and potential experimental loss. Equal allocation across groups ensured balanced experimental conditions and minimised bias.

Although the study represents a novel investigation, the combined use of pilot-derived variability estimates and published methodological norms allowed for a justified and evidence-informed determination of sample size for this study, rather than reliance on arbitrary selection (Julious, 2005).The selection and preparation of bovine enamel teeth followed the procedures described in sections 3.2.3 to 3.2.5; please refer to these sections for further details (Figure 25).

4.2.2. Preparation of the enamel lesion

The preparation of enamel lesion followed the procedure described in section 3.2.7 (Figure 25 and 26).

As mentioned previously, a pilot study was carried out in chapter three to determine the appropriate period of demineralisation to produce a consistent lesion of at least 300-400µm depth on each enamel slab. The demineralisation period was increased to 10 days. Once the demineralising gel was ready for use, it was poured into universal tubes Sterilin, into which the mounted teeth will then be submerged. The enamel slabs were immersed in the acid gel for 10 days to produce artificial enamel subsurface lesions. The enamel slabs were removed from the acid gel and washed with deionised water.

4.2.3. Experimental and control groups

The enamel slabs with artificial subsurface lesion were randomly assigned according to a randomisation table to four groups with 16 enamel lesions each. The study period for all groups were applied for 21 days. The groups are;

Group 1: Enamel artificial subsurface lesions treated with alternate soaking protocol of Calcium and Phosphate solutions (Ca/P) (Section 4.2.4.2), applied twice daily.

Group 2: Enamel artificial subsurface lesions treated with toothpaste slurry of 1450 ppm of Fluoride (Section 4.2.4.4), applied twice daily.

Group 3: Enamel artificial subsurface lesions treated with Day-time artificial saliva (Table 6), applied twice daily as positive control (Section 4.2.4.5).

Group 4: Enamel artificial subsurface lesions treated with Tris-Hydrochloride (Tris-HCL) (section 4.2.4.1), applied twice daily as negative control.

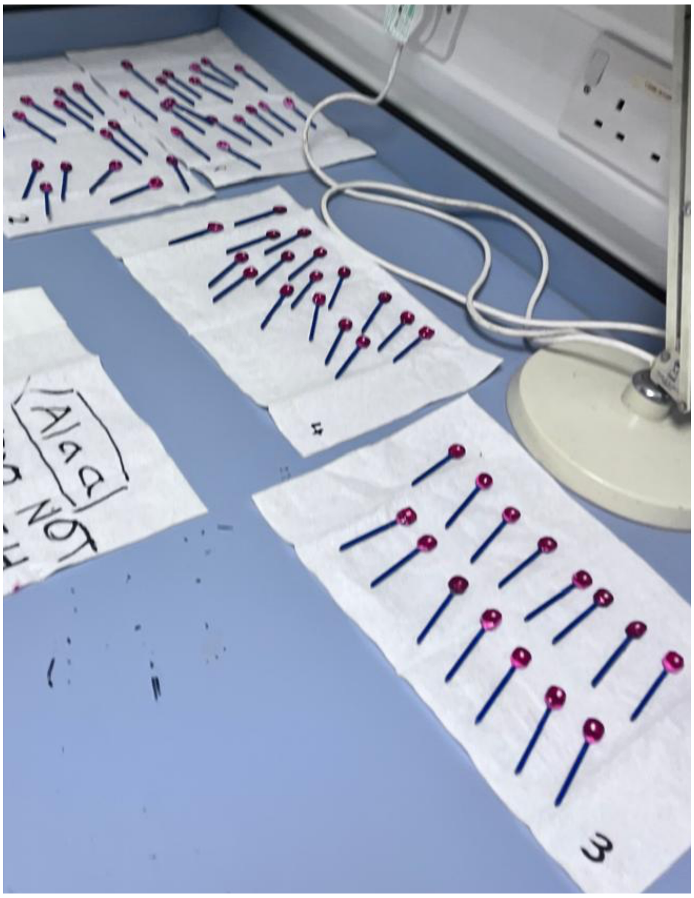


Figure 25. Representative enamel slabs illustrating the four groups before demineralised gel application.

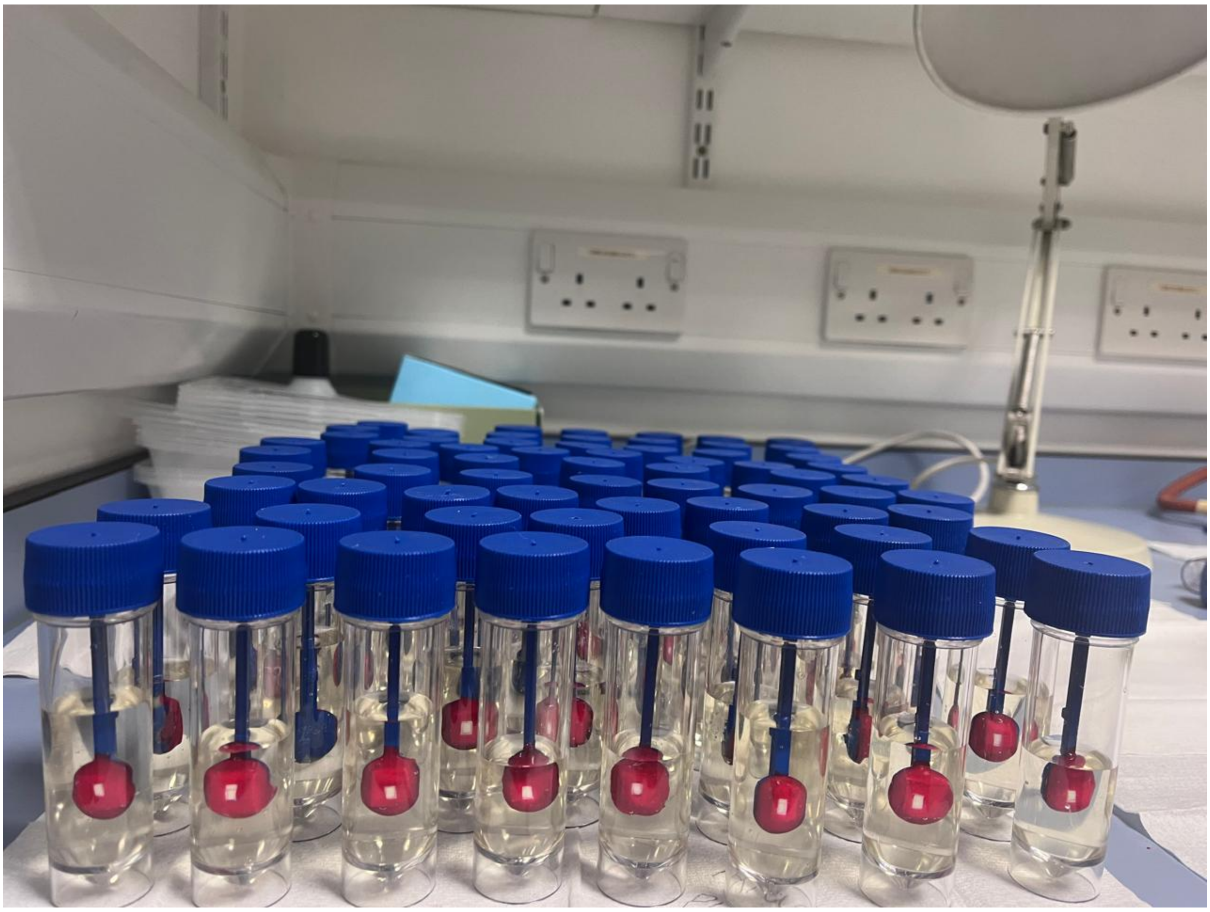


Figure 26. Representative image of 64 enamel subsurface lesions following 10 days of demineralised gel application.

4.2.4. Preparations of solutions used in the study

4.2.4.1. Preparation of aqueous solution of Tris Hydrochloride (Tris-HCl) (50mM-pH 7.4)

2-Amino-2-(hydroxymethyl)-1.3-propanediol-hydrochloride $C_4H_{11}NO_3$ x HCL (Mw=157.56) (Roche Diagnostics GmbH, Mannheim, Germany) were dissolved in 1000 mL of deionised water (Matsusaki et al., 2009).

4.2.4.2. Preparation of aqueous solution of Calcium Chloride ($CaCl_2$) and Di-sodium hydrogen phosphate dodecahydrate (Na_2HPO_4)

Calcium Chloride (pH 7.4, 200 mM) was prepared by dissolving $CaCl_2$ (Mw=110.98) (Honeywell Fluka™ Seelze, Germany) in 1000 mL of deionised water with Tris hydrochloride (Tris-HCl) (50mM pH 7.4) (Matsusaki et al., 2009).

4.2.4.3. Preparation of Di-sodium hydrogen phosphate dodecahydrate (pH 7.4, 200 mM).

Na_2HPO_4 $12H_2O$ (Mw=358.14) (Bioserv UK limited, Catcliffe, England) were dissolved in 1000 mL of deionised water with Tris hydrochloride (Tris-HCl) (50mM-pH 7.4) (Matsusaki et al., 2009).

4.2.4.4. Toothpaste 1450 ppm F slurry

Toothpaste slurries were prepared by mixing the toothpaste (Aquafresh complete care, Surrey, UK) with deionised water in a volume ratio of 1:4 (toothpaste: deionised water), using a WhirliMixer® (Fisons, Glasgow, UK) for 1 minute (Figure 27). This toothpaste was selected because it contains 1450 ppm of sodium fluoride and does not contain calcium or phosphate.

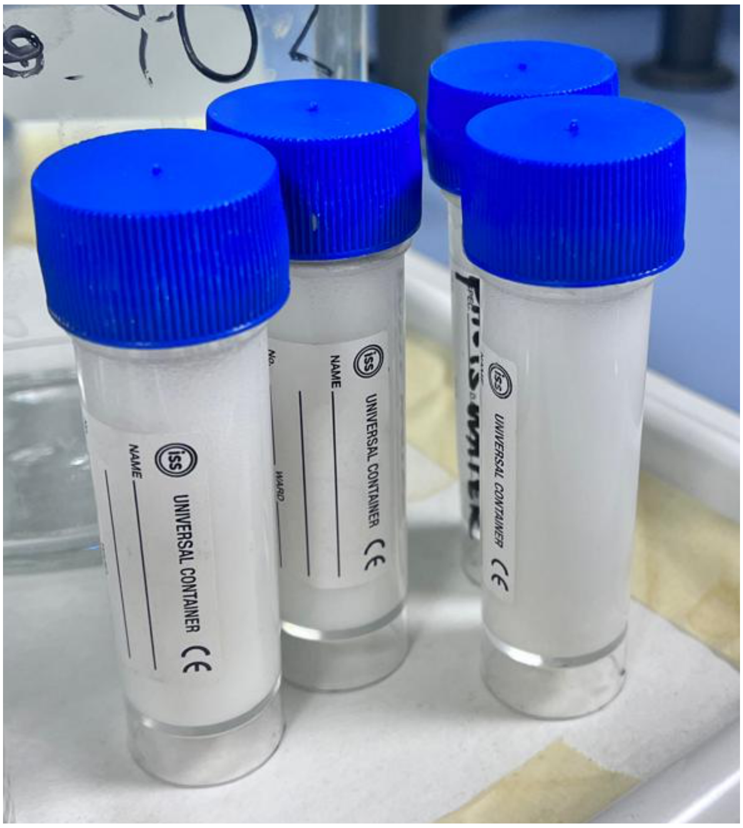


Figure 27. Demonstrates the toothpaste slurries after the mixture with deionised water in a volume ratio of 1:4 (toothpaste: deionised water).

4.2.4.5. Artificial saliva preparation

In this study, two distinct types of artificial saliva were employed: artificial day-time saliva and artificial night-time saliva, as detailed in Table 7 and Table 8. Each solution played a specific role within the experimental framework. The artificial day-time saliva was utilised as a positive control group of the cycling protocol. Its supersaturated composition allowed it to support the remineralisation of the enamel lesions, promoting the deposition of minerals and aiding in the repair of demineralised areas. This property made the day-time saliva a critical component in simulating the natural remineralisation process that occurs during active periods of the day (as provided by Dr RP Shellis, Department of Oral and Dental Science, University of Bristol, Bristol, UK) (Bataineh et al., 2017).

In contrast, the artificial night-time saliva was used during the night time phase of the study, serving as a storage medium for the enamel slabs. Unlike the day saliva, the night saliva was a saturated solution, meaning it lacked the mineral content necessary to contribute to remineralisation. As a result, the night saliva did not induce any mineral changes or alterations in the enamel lesions, ensuring that no remineralisation occurred during the storage period (Bataineh et al., 2017).

The formulations of the saliva shown in Table 7 were recommended by Dr RP Shellis (Department of Oral and Dental Science, University of Bristol, UK) (Bataineh et al., 2017).

Table 7. The content of the day-time artificial saliva used for the study.

Salt	Concentration Mol
Calcium carbonate	0.07
Magnesium carbonate (hydrated basic)	0.019
Potassium di-hydrogen phosphate	0.554
HEPES buffer (acid form)	4.77
Potassium chloride	2.24

Briefly, the above components were added into 900 mL deionised water, and 1.8 mL 1 mol/L HCL and stirred on a shaker until it all dissolved.

4.2.4.6. The preparation of night-time artificial saliva

The formulation of the night-time saliva is shown in Table 8 (Bataineh et al., 2017).

Table 8. The formulation of the night-time saliva.

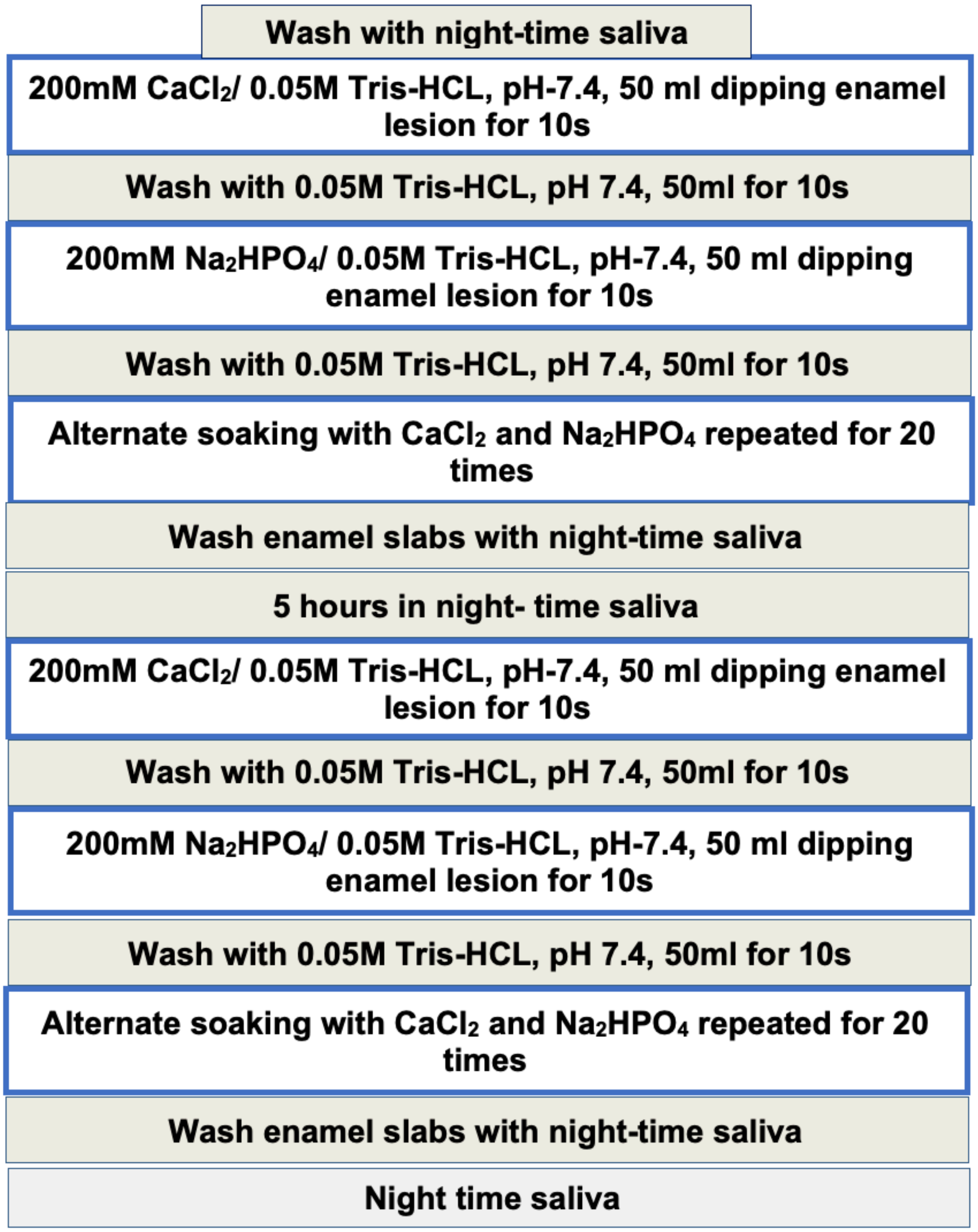
Salt	Concentration g/L
Calcium carbonate	0.05
Magnesium carbonate (hydrated basic)	0.019
Potassium di-hydrogen phosphate	0.068
HEPES buffer (acid form)	4.77
Potassium chloride	2.24

4.2.5. Use of cycling regime for the treatments on bovine enamel lesions

The enamel slabs were stored in artificial night-time saliva at 37˚C for 21 days. Enamel slabs were then placed in night-time artificial saliva. The night-time saliva was changed every day. The artificial saliva has been changed every day throughout the cycling regime. The enamel slabs were kept in the incubator at 37˚C at all times except during the dipping in the solutions.

4.2.5.1. Application of $CaCl_2$ and Na_2HPO_4 with alternate soaking method on enamel lesions

In this study, the alternate soaking process group protocol started with washing enamel lesions first with deionised water. The enamel lesions were soaked in $CaCl_2$ for 10 seconds and it was soaked for 10 seconds with Tris-HCL. After that, it was soaked with Na_2HPO_4 for 10 seconds. This was done for 20 cycles which account for 6 minutes (Matsusaki et al., 2009). Then it was washed with deionised water and following this it was washed with night-time saliva. The saliva was discarded and was replaced with fresh night-time saliva to be stored for 5 hours. After 5 hours, the second cycle was done which follows the same protocol as the first cycle. After that it was stored in night-time saliva for the next day soaking. This was done for 21 days (Bataineh et al., 2017). (Figure 28).



Wash with night-time saliva

200mM $CaCl_2$/ 0.05M Tris-HCL, pH-7.4, 50 ml dipping enamel lesion for 10s

Wash with 0.05M Tris-HCL, pH 7.4, 50ml for 10s

200mM Na_2HPO_4/ 0.05M Tris-HCL, pH-7.4, 50 ml dipping enamel lesion for 10s

Wash with 0.05M Tris-HCL, pH 7.4, 50ml for 10s

Alternate soaking with $CaCl_2$ and Na_2HPO_4 repeated for 20 times

Wash enamel slabs with night-time saliva

5 hours in night- time saliva

200mM $CaCl_2$/ 0.05M Tris-HCL, pH-7.4, 50 ml dipping enamel lesion for 10s

Wash with 0.05M Tris-HCL, pH 7.4, 50ml for 10s

200mM Na_2HPO_4/ 0.05M Tris-HCL, pH-7.4, 50 ml dipping enamel lesion for 10s

Wash with 0.05M Tris-HCL, pH 7.4, 50ml for 10s

Alternate soaking with $CaCl_2$ and Na_2HPO_4 repeated for 20 times

Wash enamel slabs with night-time saliva

Night time saliva

Figure 28. Flow chart for the application of Ca+ and P solutions with alternate soaking method on lesion.

4.2.5.2. Application of toothpaste slurry (1450 ppm F) on enamel lesions

The enamel lesions for the toothpaste slurry 1450 ppm F group were first washed with deionised water. Then the samples were soaked in the toothpaste slurry for a total of 6 minutes. The fluoride treatment was applied for 6 minutes to allow comparison with the alternating soaking process and to standardise the protocol across all experimental groups. The samples then was washed with deionised water. Following this, it was washed with night-time saliva. The night saliva was discarded and was replaced with night-time saliva to be stored for 5 hours. After that, the second cycle was done, and it was the same as the first cycle. Then the samples were stored in night-time saliva for soaking on the next day. This was done for 21 days (Bataineh et al., 2017). (Figure 29).

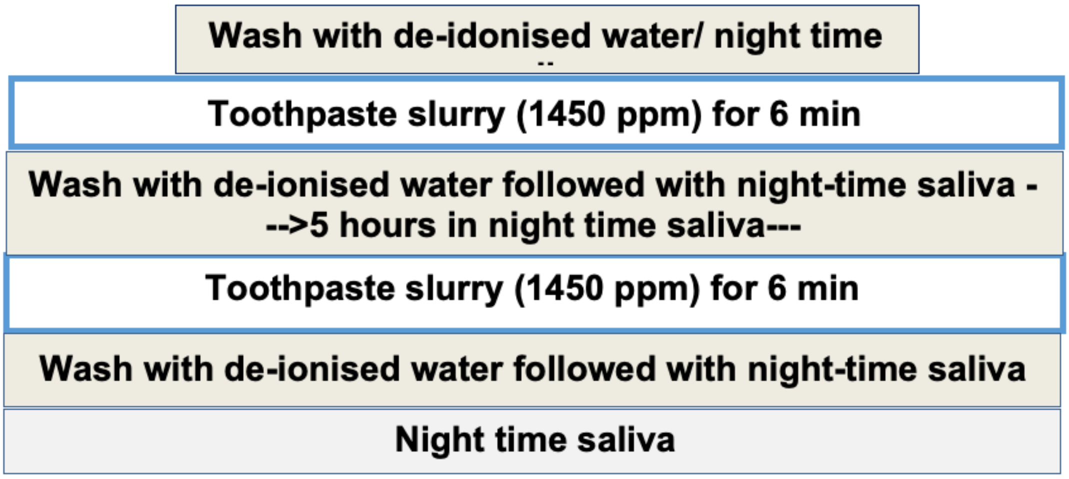


Figure 29. Flow chart for toothpaste slurry 1450 ppm F group.

4.2.5.3. Application of Day-time artificial saliva on enamel lesions

The enamel lesions in the day-time artificial saliva group were first washed with deionised water. The enamel lesions in this group were soaked in the day-time artificial saliva for 6 minutes. Then it was washed with deionised water and following this it was washed with night-time saliva. The night saliva was discarded and was replaced with night-time saliva to be stored for 5 hours. After 5 hours the second cycle was done, and it followed the same protocol as the first cycle. Last step, the enamel lesions were stored in night-time saliva for the next day. This was done for 21 days (Bataineh et al., 2017) (Figure 30).

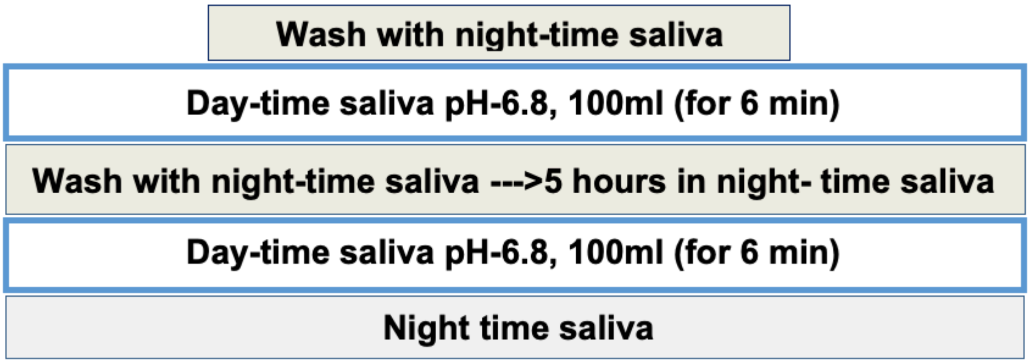


Figure 30. Flow chart for day-time artificial saliva group.

4.2.5.4. Application of Tris-HCL method on enamel lesions

The enamel lesions for the Tris-HCL group were first washed with deionised water. After that, the enamel lesions were soaked in Tris-HCL for 10 seconds, then soaked with Tris-HCL for another 10 seconds. Then soaked again with Tris-HCL for 10 seconds. It was done for 20 cycles which account for 6 minutes(Matsusaki et al., 2009). Then it was washed with deionised water following this it was washed with night-time saliva. The night saliva was discarded and was replaced with night-time saliva to be stored for 5 hours. The second cycle was done the same as the first cycle.

Following this, it was stored in night-time saliva for the next day. This was done for 21 days (Bataineh et al., 2017). (Figure 31).

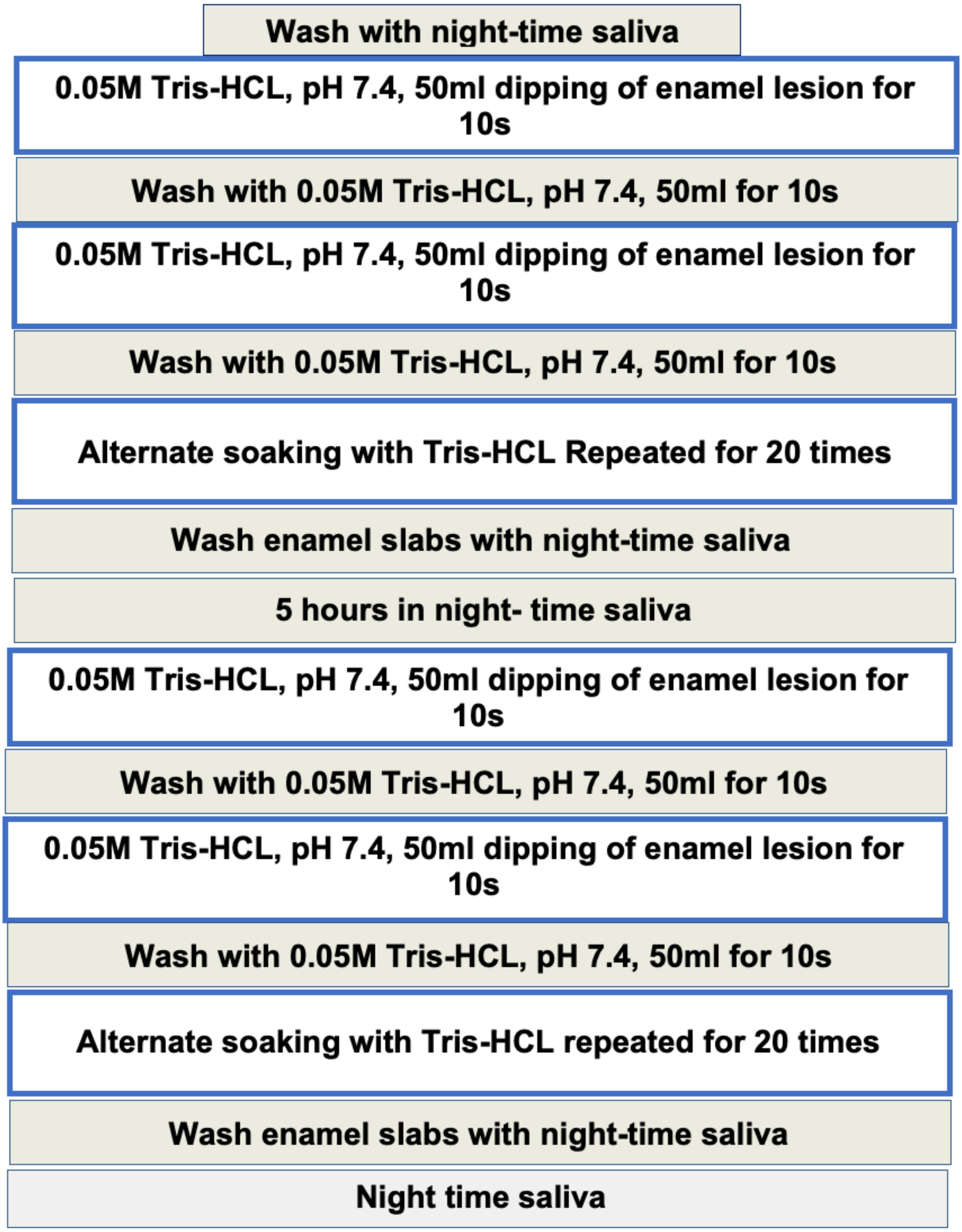


Figure 31. Flow chart for Tris-HCL group.

4.2.6. Characterisation of enamel slabs

4.2.6.1. Quantitative Light-Induced Fluorescence (QLF) Measurements

For each enamel slab, QLF measurements were taken after the creation of the enamel subsurface lesion and at day 7, day 14, and at the end of the 21-day experiment period using the QLF machine (QLF-D Biluminator™ 2) (Inspektor Research Systems BV, Amsterdam, The Netherlands). The characterisation of enamel slabs followed the procedures described in sections 3.2.8.1; please refer to this section for further details.

4.2.6.1.1. The ΔF range analysis of the artificial enamel lesions

After performing the QLF baseline analysis for all enamel slabs, the range of ΔF values was found to vary between -6.21 and -19.61. The enamel slabs with the ΔF range (-7.32) to (-12.64) with an average of (-10.07) were selected to be involved in the experiment to pick up the differences in ΔF after treatment.

4.2.6.2. Microcomputed Tomography analysis of enamel lesions

Three enamel slabs from each group were selected randomly for the Micro-CT (Skyscan 1172 Bruker, Belgium) analysis after demineralisation and after treatment for each group. The characterisation of enamel slabs followed the procedures described in sections 3.2.8.2; please refer to this section for further details.

The image stacks were then scrolled to identify the slice corresponding to the deepest extent of the subsurface enamel lesion, which was selected for analysis. Using the “Straight Line” tool in ImageJ, a line was drawn perpendicular to the tooth surface, passing through the centre of the deepest portion of the lesion, extending from the enamel surface to the enamel–dentin junction. The calibration phantom was positioned beneath the enamel subsurface lesion to serve also as a reference, and the straight line tool was drawn through the centre of this demineralised lesion (Figure 33 and 34).

Mean mineral density values were obtained by manually aligning the post-treatment images (after remineralisation) with the corresponding baseline images (demineralisation), The mean mineral density for each slab was calculated by averaging the mineral density across the line profile values recorded within the selected deepest slice. Subsequently, the group mean mineral density was determined by averaging the individual slab mean values within each experimental group.

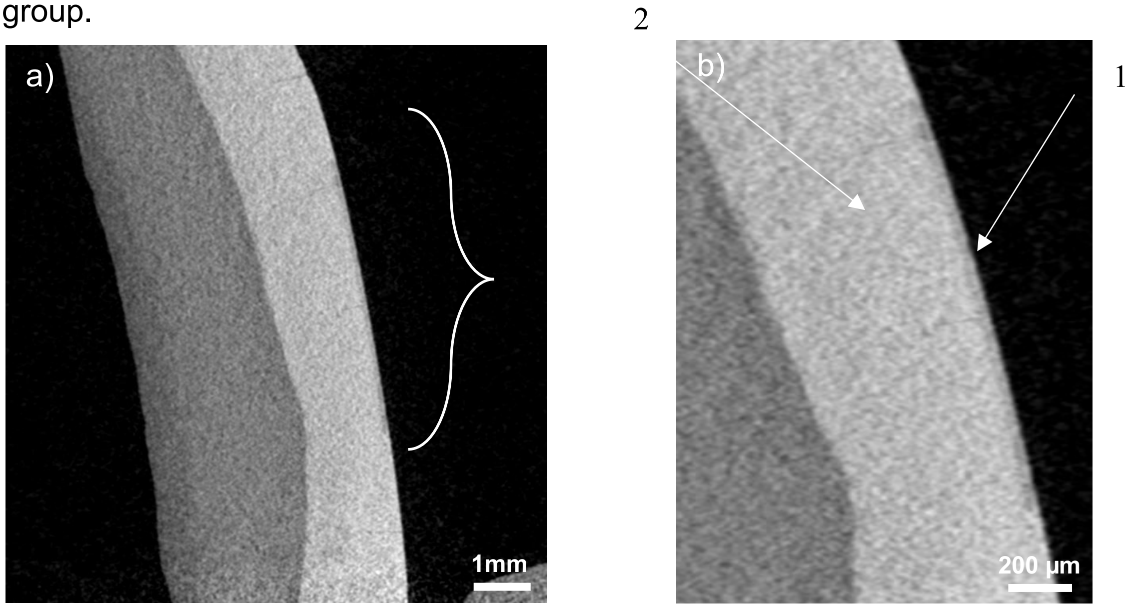


Figure 32. Represents coronal section micro-CT image of enamel slab after demineralisation.

(Arrow 1: demineralised enamel, arrow 2: sound enamel). Image (a) presented with a scale bar of 1 mm, while image (b) includes a scale bar of 200 µm.

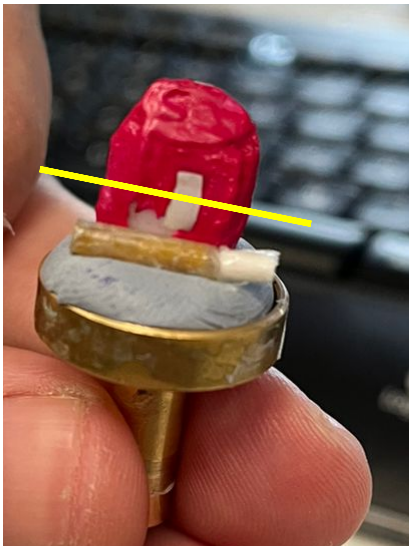


Figure 33. Represents an enamel lesion with the calibration phantom positioned beneath the enamel subsurface lesion to serve as a reference. Imaginary straight-line profile was drawn through the centre of the enamel lesion to the reference region.

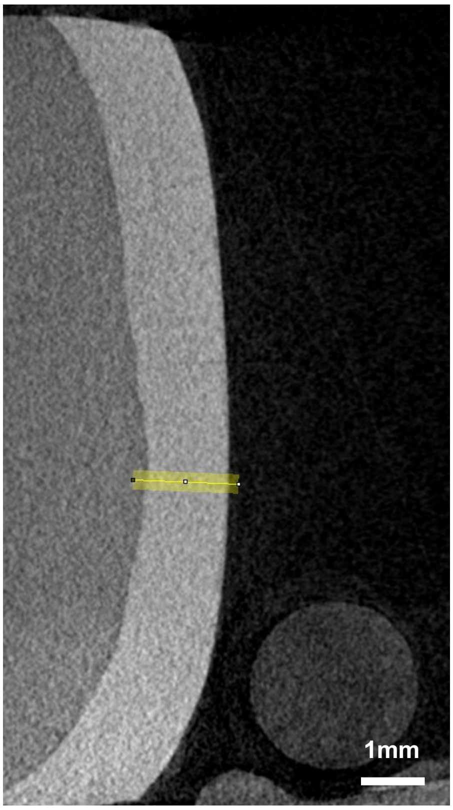
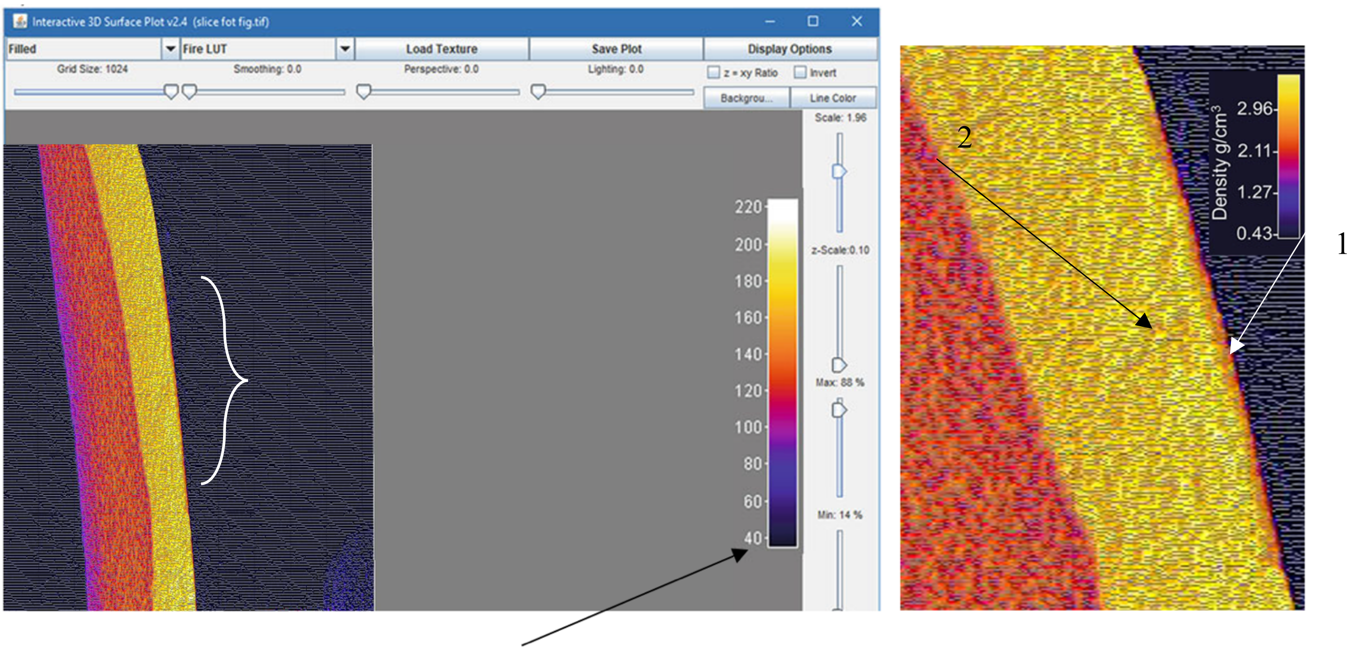


Figure 34. Represents coronal section micro-CT image of enamel lesion after demineralisation. Straight line profile was placed above the reference towards the center of the lesion. Scale bar is 1 mm.

Figure 32 represents a Micro-CT coronal section image of an enamel slab that has a window area of demineralisation. This window area revealed the extension and distribution of the lesion. There was no loss of continuity of enamel, only a grey area along its thickness was observed. This corresponds to an incipient lesion which is clearly seen.

The tool 3D interceptive surface plot can be carried out with Fiji for the generation of colour-coded contour maps (heat maps) to quantify mineral density (Figure 35). The interactive 3D Surface Plot plugin can assign distinct colours to different grayscale values (using a lookup table LUT) which allows users to generate coloured mineral maps which can visually emphasise changes in mineral density. The image has been calibrated against hydroxyapatite phantoms, the grayscale values can be replaced with corresponding values for mineral density.



Calibration bar – greyscale values

Figure 35. The tool interceptive 3D surface plot after demineralisation. (Arrow 1: demineralised enamel, arrow 2: sound enamel).

4.2.6.3. Energy Dispersive X-ray (EDX) assessment of enamel lesions

Three enamel lesions from each group were selected randomly for the EDX analysis EDX analysis was performed in conjunction with scanning electron microscopy (SEM) using a Bruker XFlash® 6 detector (2 × 60 mm²) to evaluate the elemental composition tooth structure. The elements analysed included calcium (Ca) and phosphorus (P), which are principal components of dental hard tissues. During EDX analysis, the specimen surface was scanned with an electron beam operating at an energy of 10–20 keV, inducing the emission of characteristic X-rays.

Before examination, enamel slabs were gold-coated using an Agar Auto Sputter Coater (Agar Scientific, Sussex, UK), with a probe current of 50. SEM analysis was conducted using a Hitachi S-3400N scanning electron microscope equipped with a tungsten electron source and a secondary electron detector. The instrument was operated at an accelerating voltage of 10 kV under high-vacuum conditions, with a nominal working distance of 10 mm. Specimens were mounted on 10 mm diameter pinned stubs using conductive adhesive tape.

Measurements were performed at baseline (after demineralisation) and after application of the remineralising agents. The elemental composition of calcium (Ca) and phosphorus (P) was obtained for each sample.

The percentage mineral gain for each sample was calculated using the following formula:

Mineral gain (%)= ((Ca+P) after treatment – (Ca+P) baseline /(Ca+P) baseline)) x 100

This approach quantifies the increase in mineral content relative to baseline and has been widely applied in studies evaluating remineralisation potential using SEM-EDX measurements (Konagala et al., 2020; Shaik et al., 2017)

4.2.7. Randomisation and Blindness

All enamel slabs were randomly assigned to one of the four groups using a table of randomisation numbers using the random number generator website https://stattrek.com/statistics/random-number-generator.

4.2.8. Statistical analysis

The data were analysed using SPSS statistical software package for windows version 29.0. Descriptive statistics were used to calculate the mean and standard deviation. The normality of the data distribution was assessed using the Shapiro-Wilk test.
Shapiro-Wilk test was carried out to choose between the parametric tests paired t-test, independent t-test and its nonparametric alternative Wilcoxon signed rank test. The Mann-Whitney U test was used to test the significance of changes from baseline and between treatment groups.

In case of normally distributed differences, the paired t-test was used to test the significance of changes from baseline in ΔF, ΔQ and Area, otherwise, the nonparametric alternative Wilcoxon signed rank test was used. The independent t-test was used to test differences in ΔF, ΔQ and Area between two groups both with normally distributed values, otherwise the nonparametric alternative Mann-Whitney U test was used. Furthermore, the Bonferroni test was used to assess if there was any significant difference between each of the groups. The significance level was set at (p <0.05).

Chapter 5 Results

5.1. Quantitative Light-Induced Fluorescence (QLF): Results for different treatment groups (Toothpaste slurry 1450 ppm F, Alternate calcium and phosphate solutions, Tris-HCL and Day-time artificial saliva)

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

ΔF: Percentage fluorescence loss in the enamel lesion area with respect to the fluorescence of sound tooth tissue. Related to lesion depth (%).

ΔQ: ΔF times the Area. Percentage fluorescence loss in the enamel lesion area with respect to the fluorescence of sound tissue times the area. This is related to lesion volume (%px^2).

Area: The surface area of the lesion expressed in pixels^2 (px^2).

5.1.1. The mean of Lesion depth (ΔF) of enamel lesions

5.1.1.1. Test Normality

The values of ΔF after treatment and the difference between baseline and after treatment were checked for all groups to see if there was a difference within and between the groups. The normality tests (Shapiro-Wilk 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 the Tris-HCL group were not statistically different therefore data was not considered to be normally distributed and required a non-parametric test to assess the distribution of the enamel slabs. Table 9 tests the significance of changes from baseline and between treatment groups. In the table (treatment) means after 21 days of treatment and (difference) means between baseline and the end of treatment.

Table 9. Testing normality of values of ΔF for all groups.

	Test Statistics	**Degree of freedom**	**Significance P- value**
Tris-HCL (treatment)	0.956	16	0.586
Day-time saliva (treatment)	0.954	16	0.560
Toothpaste slurry F(treatment)	0.923	16	0.188
Ca/P (treatment)	0.910	16	0.116
Tris-HCL (difference)	0.593	16	0.000*
Day-time saliva (difference)	0.910	16	0.116
Toothpaste slurry F (difference)	0.936	16	0.301
Ca/P (difference)	0.972	16	0.864

* Significant differences at $p<0.05$ level

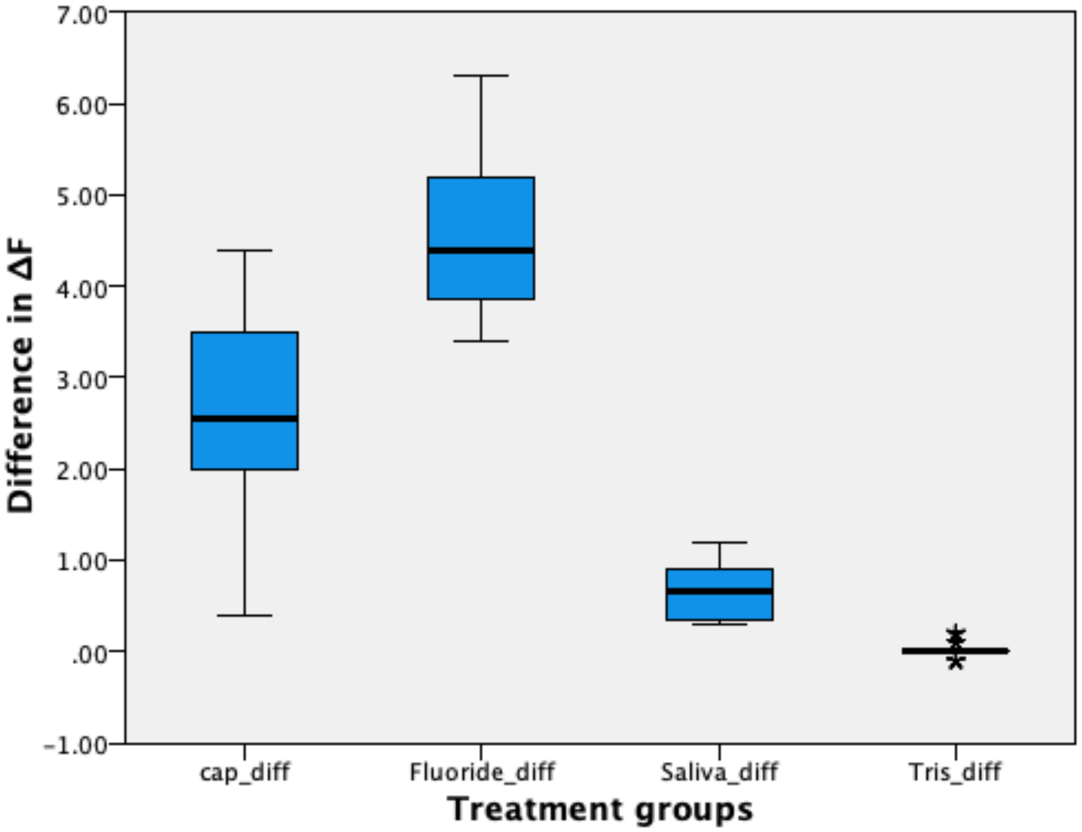


Figure 36. Boxplot for the distribution of the ΔF values (lesion depth) at baseline and after treatment for all groups.

The line in the box of Box-and-whisker plot is the median value of the data. The error bars represent SD. Cap (Calcium and phosphate group), Fluoride (Toothpaste slurry group), Saliva (day-time artificial saliva group) and Tris (Tris-HCL group).

The boxplot (Figure 36) demonstrates ΔF values difference between groups where Ca/P and toothpaste slurry F were similar in the range. However, Tris-HCL has the most variation compared to the other groups and contains a multiple extreme outlier.

5.1.1.2. Difference in ΔF within each group

The ΔF mean values both at baseline and after treatment are shown in Table 10. Overall, there was an improvement in ΔF values for all the groups in the study except

Tris-HCL group. To assess whether the change in ΔF at baseline and after treatment was significantly different within the same group, paired t-test and Wilcoxon signed rank test were used. There was a statistically significant improvement in the ΔF values after treatment compared with baseline in toothpaste slurry 1450 ppm of F, Ca/P and day-time artificial saliva groups ($p<0.05$). Figure 37 shows the change in the mean of ΔF at baseline and after treatment for all groups. The graph shows clear difference between baseline and after treatment in both Ca/P (2.69 ± 1.09) and 1450 ppm of F (4.57 ± 0.86) groups compared to the day-time saliva group. Toothpaste slurry F being the highest significance.

Table 10. Mean values of ΔF at baseline and after treatment for all groups.

Group	Baseline		Treatment		Difference		P-value
	Mean	SD	Mean	SD	Mean	SD	
Tris-HCL	-10.19	1.39	-10.18	1.37	0.01	0.02	0.515
Day-time Saliva	-10.31	1.35	-9.62	1.23	0.69	0.12	0.042*
Toothpaste slurry F	-11.19	0.81	-6.63	0.58	4.57	0.86	0.000*
Ca/P	-10.32	1.14	-7.63	0.42	2.69	1.09	0.000*

Using Wilcoxon signed rank test and using paired t-test. * Significant differences at $p< 0.05$ level.

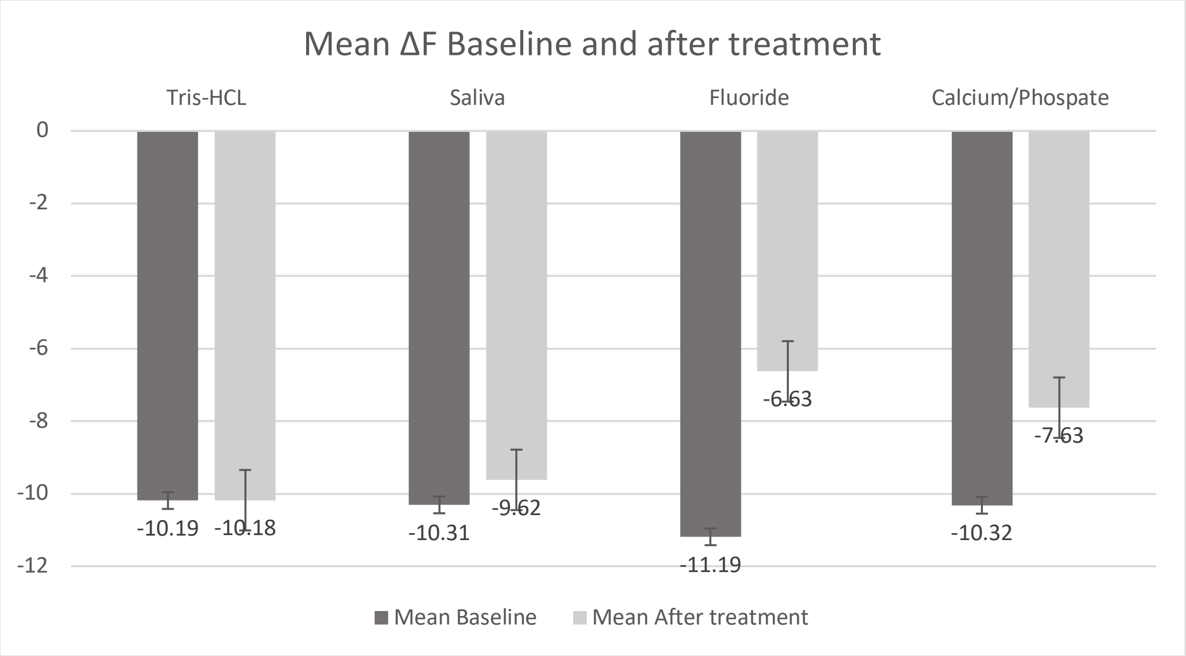


Figure 37. ΔF mean values at baseline and after treatment for all groups.

5.1.1.3. Difference in ΔF between all groups

Table 11 shows the cross-comparison between different treatment groups after treatment. There was no significant difference between day-time saliva and Tris-HCL ($p>0.05$). However, both toothpaste slurry F and Ca/P groups were significantly increased in the ΔF compared to the Tris-HCL group ($p<0.05$). Both toothpaste slurry F and Ca/P groups were significantly increased in the ΔF compared to the day-time saliva group ($p<0.05$). Interestingly, the toothpaste slurry F group was significantly increased compared to that of the Ca/P groups ($p<0.05$). Figure 38 shows the highest mean difference in ΔF was seen in the toothpaste slurry F vs. Tris-HCL (3.56 ± 1.49) followed by toothpaste slurry F vs. day-time saliva (3.00 ± 1.48). The group with the least amount of reduction in mean difference ΔF and highest standard deviation was the day-time saliva vs. Tris-HCL and toothpaste slurry F vs. Ca/P with a mean difference of (0.56 ± 1.33) and (1.00 ± 0.58) respectively (Table 11).

Table 11. ΔF multiple comparisons between treatment groups.

Groups	Mean Difference	SD Difference	P-value
Day-time Saliva vs. Tris-HCL	0.58	1.33	0.112
Toothpaste slurry vs. Tris-HCL	3.53	1.49	0.000*
Ca/P vs. Tris-HCL	2.61	1.38	0.000*
Toothpaste slurry vs. Day-time Saliva	3.01	1.48	0.000*
Ca/P vs. Day-time Saliva	2.32	1.35	0.000*
Toothpaste slurry vs. Ca/P	1.05	0.58	0.001*

All comparisons using independent t-test. * Bonferroni correction p <0.008.

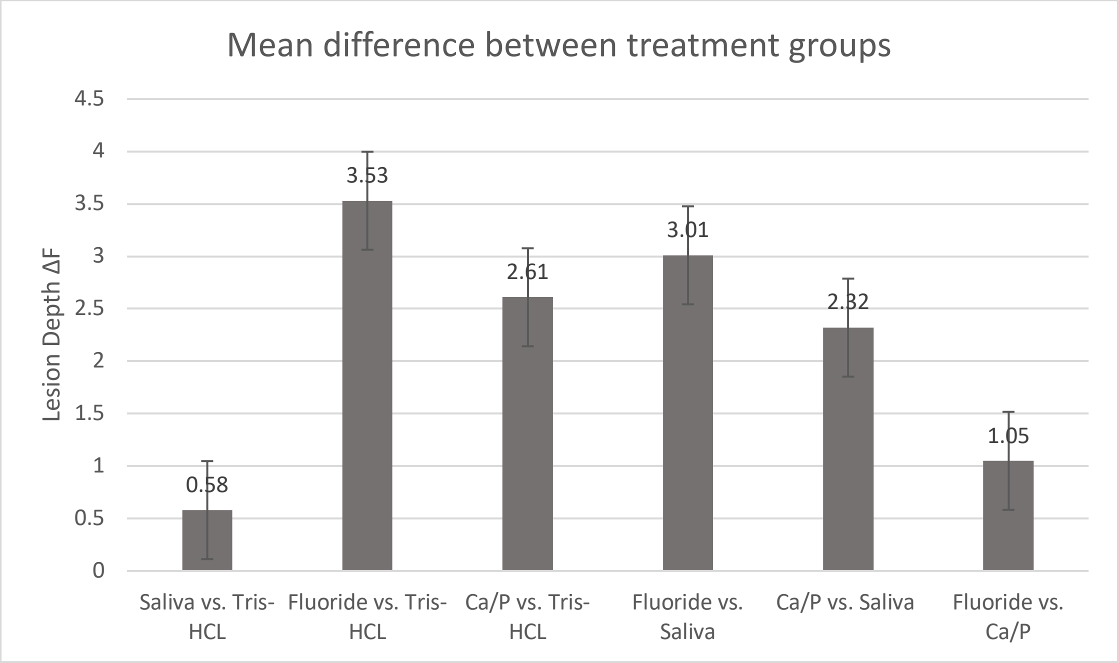


Figure 38. Mean difference between treatment groups

5.1.2. ΔQ: Lesion volume: Percentage fluorescence loss with respect to the fluorescence of sound tissue times the Area

5.1.2.1. Test Normality

Shapiro-Wilk test was carried out to choose between the parametric tests; paired t-test, independent t-test and its non-parametric alternative Wilcoxon signed rank test. Also, Mann-Whitney U test to test the significance of changes from baseline and between treatment groups (Table 12). The data were considered normally distributed if the p values from these tests were not statistically significant (p-value > 0.05). P values for all groups except for the Ca/P group were statistically significant, therefore data was not considered to be normally distributed and required a non-parametric test to assess the distribution of samples. Table 12 show the significance of changes from baseline and between treatment groups. In the table (treatment) means after 21 days of treatment application and difference means the (difference) between baseline and end of treatment.

Table 12. Testing normality of values of ΔQ for all groups.

	Statistic	Degree of Freedom	P-value
Tris-HCL (treatment)	0.750	16	0.001*
Day-time saliva (treatment)	0.864	16	0.022*
Toothpaste slurry F (treatment)	0.606	16	0.000*
Ca/P (treatment)	0.730	16	0.001*
Tris-HCL (difference)	0.884	16	0.046*
Day-time saliva (difference)	0.721	16	0.003*
Toothpaste slurry F (difference)	0.625	16	0.000*
Ca/P (difference)	0.936	16	0.304

* Significant differences at p<0.05 level.

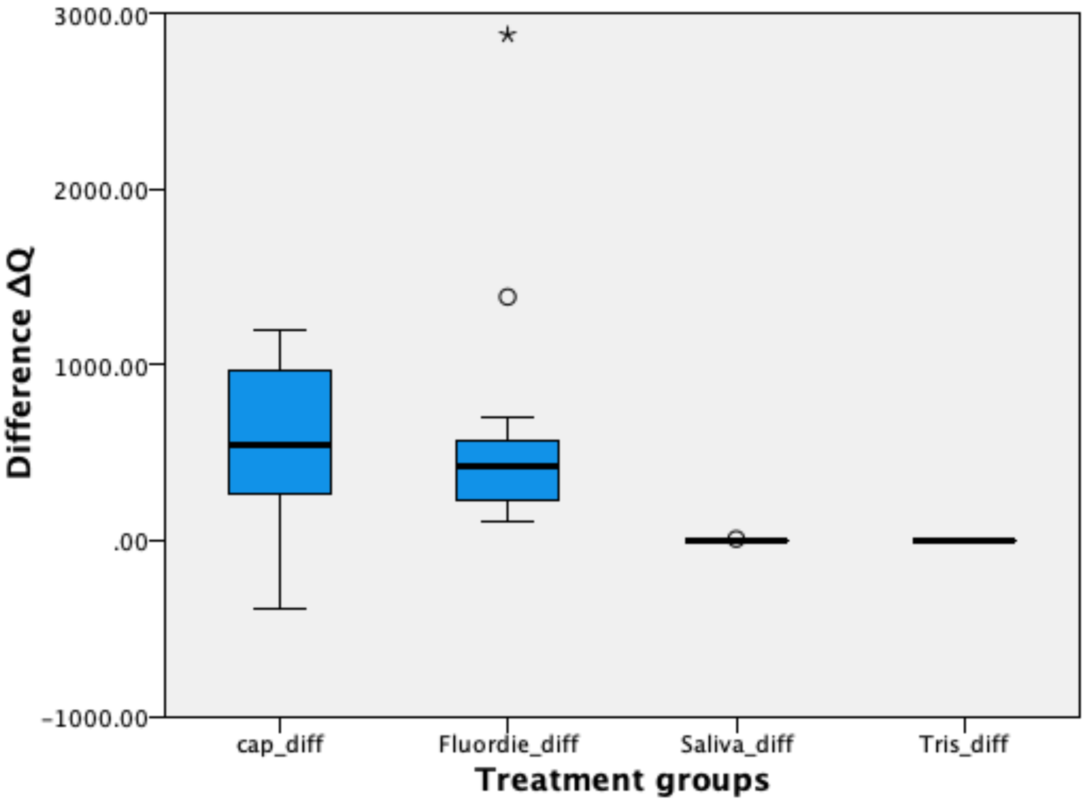


Figure 39. Boxplot for the distribution of the ΔQ values (%px^2) at baseline and after treatment for all groups.

Error bars represent SD, the line in the box of Box-and-whisker plot is the median value of the data. Cap (Calcium and phosphate group), Fluoride (Toothpaste slurry group), Saliva (artificial saliva group) and Tris (Tris-HCL group).

The boxplot (Figure 39) shows the distribution of ΔQ at baseline and the difference between groups where Ca/P has a wider range than toothpaste slurry F. However, Tris-HCL and day-time saliva have no range with outliers seen in toothpaste slurry F and day-time saliva.

5.1.2.2. Difference in ΔQ within each group

The ΔQ mean values both at baseline and after treatment are shown in (Table 13). There were improvements in ΔQ values for all the groups in the study except Tris-HCL group.

Table 13. Mean values of ΔQ at baseline and after treatment for all groups.

Group	Baseline		Treatment		Difference		P value
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	
Tris-HCL	-2867.25	1374.29	-2866.94	1374.39	0.31	0.95	0.273
Day-time Saliva	-2854.50	1582.94	-2852.69	1582.77	1.81	0.68	0.001*
Toothpaste slurry F	-2002.88	1313.84	-1395.44	772.59	607.44	541.25	0.000*
Ca/P	-1996.81	895.61	-1440.94	717.40	555.87	178.21	0.000*

Using Wilcoxon signed rank test and paired t-test. * Significant differences $p < 0.05$ level.

To assess whether the change in ΔQ at baseline and after treatment was significantly different within the same group, paired t-test and Wilcoxon signed rank test were used. The results of the significance are shown in (Table 13). There was a statistically significant improvement in the ΔQ values after treatment compared with that at baseline in toothpaste slurry F, Ca/P groups and day-time saliva ($p <0.05$) (Figure 40).

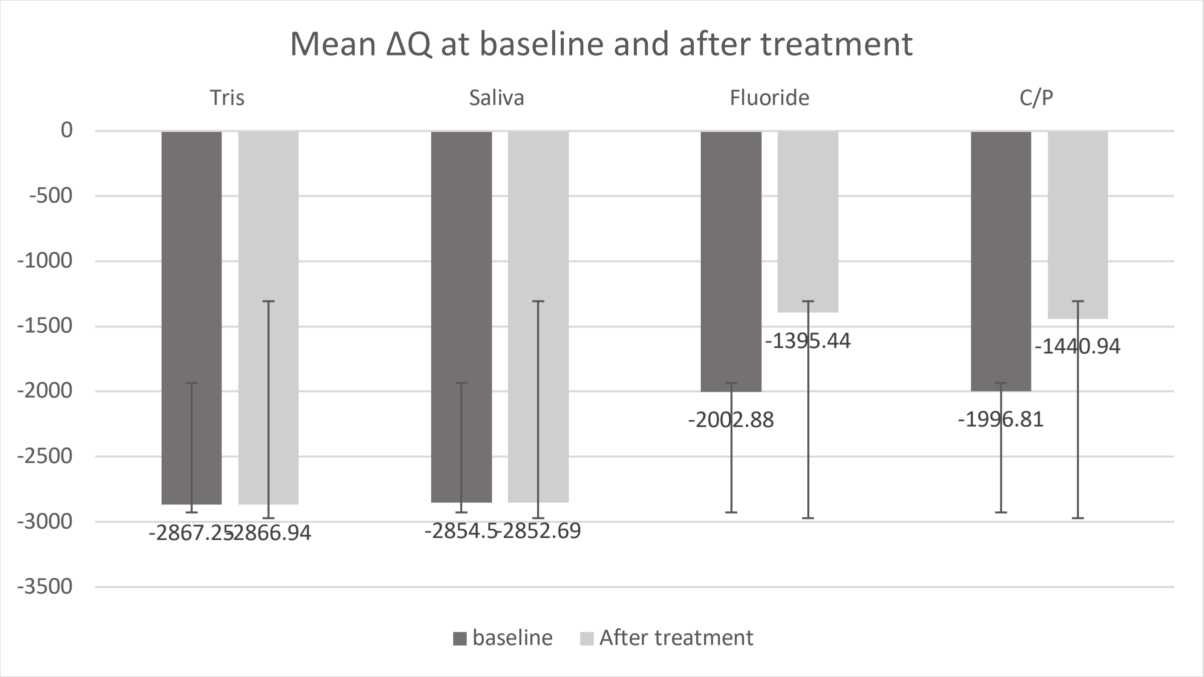


Figure 40. The change in the mean of ΔQ at baseline and after treatment

5.1.2.3. Difference in ΔQ between all groups

Table 14. ΔQ multiple comparisons between groups.

Groups	Mean Difference	SD Difference	P value
Day-time saliva vs. Tris-HCL	14.25	1533.07	0.970
Toothpaste slurry F vs. Tris-HCL	1471.50	1608.06	0.000*
Ca/P vs. Tris-HCL	1426.00	1548.28	0.000*
Toothpaste slurry F vs. day-time saliva	1457.25	1479.92	0.000*
Ca/P vs. day-time saliva	1411.75	1741.78	0.000*
Toothpaste slurry F vs. Ca/P	45.50	1184.41	0.879

Comparisons using the Mann–Whitney U test. * Bonferroni correction $p < 0.008$.

The cross-comparison between different treatment groups after treatment, there was not significant difference between day-time saliva vs. Tris-HCL and between toothpaste slurry F vs. Ca/P ($p>0.05$) (Table 14). However, both toothpaste slurry F and Ca/P groups were significantly increased in the ΔQ compared to the Tris-HCL group ($p<0.001$). Both toothpaste slurry F and Ca/P groups were significantly increased in the ΔQ compared to the day-time saliva group ($p<0.001$). In this section, all comparisons between groups were assessed using the Mann-Whitney U test (Bonferroni correction $p < 0.008$).

The highest difference can be seen in toothpaste slurry F vs. day-time saliva and the lowest difference is toothpaste slurry F vs. Ca/P followed by toothpaste slurry F vs. day-time saliva (1457.25 ± 1479.92). There was a small difference between toothpaste slurry F vs. Ca/P and day-time saliva vs. Tris-HCL (45.5 ± 1184.41) and (14.25 ±1533.07) respectively (Figure 41).

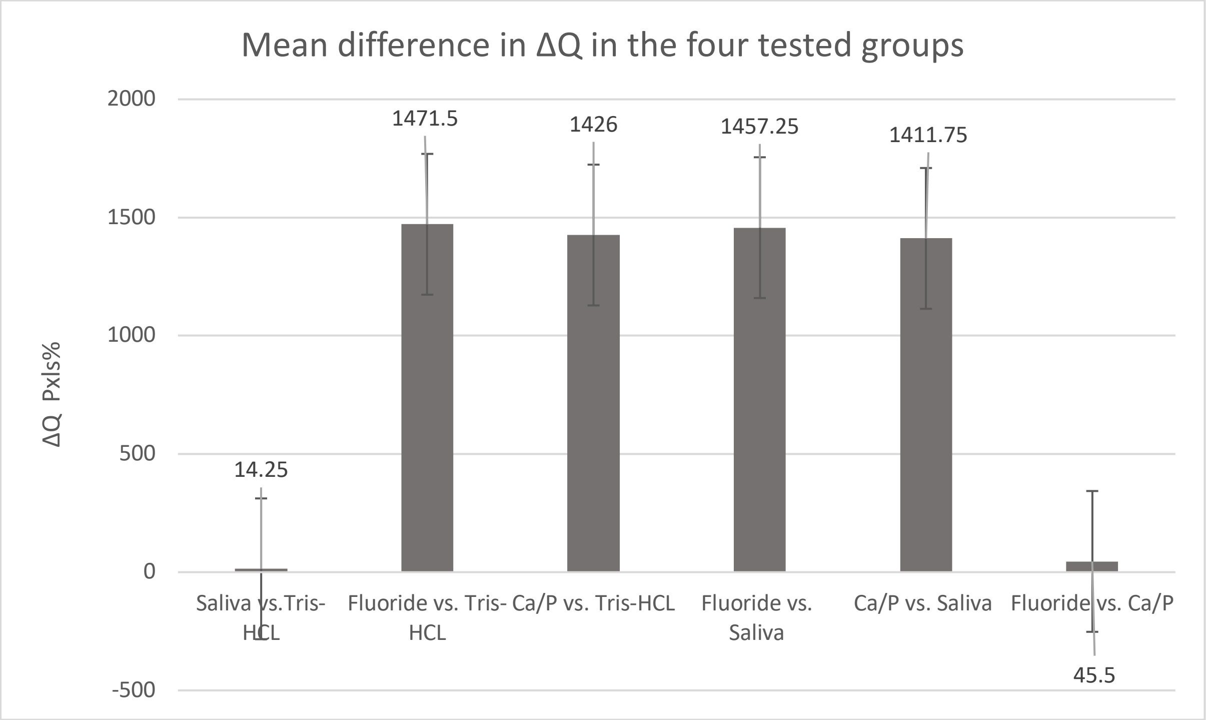


Figure 41. The difference in ΔQ between the four tested groups.

5.1.3. QLF: Area of the enamel subsurface lesion before and after treatment

5.1.3.1. Test normality of the Area of the enamel subsurface lesion

To check if the area of the lesion between baseline and after was normally distributed, a data normality test was carried out by Shapiro-Wilk test. If the p-value was statistically significant that means data is not normally distributed. However, if the p-value was not statistically significant, therefore, data was normally distributed ($p > 0.05$). P-values that showed statistical significance were toothpaste slurry F and Ca/P groups (treatment) and Tris-HCL, day-time saliva, and Ca/P (difference). Therefore, data was not considered to be normally distributed and required a non-parametric test to assess the distribution of the enamel slabs. Table 15 tests the significance of changes from baseline and between treatment groups. In the table (treatment) means after 21 days of treatment and (difference) means the difference between baseline and end of treatment.

Table 15. Tests of Normality of the area of the enamel subsurface lesion for all groups.

	Statistic	Degree of Freedom	Sig.
Tris-HCL (Treatment)	0.950	16	0.489
Day-time Saliva (Treatment)	0.922	16	0.184
Toothpaste Slurry F (Treatment)	0.782	16	0.002*
Ca/P (Treatment)	0.548	16	0.000*
Tris-HCL (Difference)	0.833	16	0.008*
Day-time Saliva (Difference)	0.424	16	0.009*
Toothpaste Slurry F (Difference)	0.912	16	0.127
Ca/P (Difference)	0.874	16	0.032*

*Statistical significance at $p < 0.05$ level.

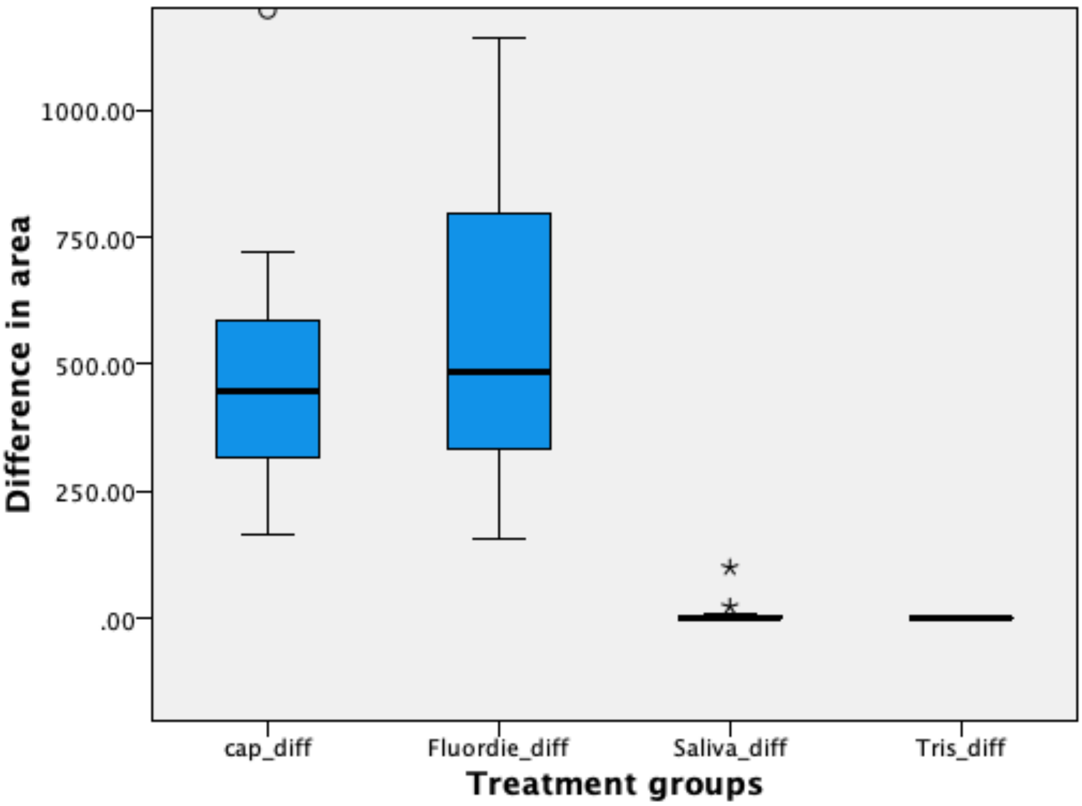


Figure 42. Boxplot for the distribution of the area values enamel subsurface lesion at baseline and after treatment for all groups.

Error bars represent SD, the line in the box of Box-and-whisker plot is the median value of the data. Cap (alternate soaking process Ca/P group), Fluoride (Toothpaste slurry group), Saliva (artificial saliva group) and Tris (Tris-HCL group)

Figure 42 shows the difference in the Area of enamel subsurface lesions for different groups. Toothpaste slurry 1450 ppm F showed wider range than Ca/P. However, day-time saliva and Tris-HCL shows no range at all. Also, day-time saliva showed a multiple extreme outlier.

5.1.3.2. Mean Area of the enamel subsurface lesions

The area of the enamel subsurface lesion at baseline for all groups were checked to see if there was a difference between the groups. The change in the mean of the area at baseline and after treatment can be seen and all four groups showed statistical significance (Table 16). The change in the area was assessed using the Wilcoxon signed rank test and paired t-test.

Table 16. Mean area changes at baseline and after treatment.

Group	Baseline		Treatment		Difference		P value
	Mean	SD	Mean	SD	Mean	SD	
Tris-HCL	1815.25	535.81	1815.13	535.78	0.12	0.03	0.666
Day-time Saliva	1444.56	486.01	1435.00	476.33	9.56	9.68	0.005*
Toothpaste slurry F	1465.75	343.55	884.69	116.76	581.06	226.79	0.000*
Ca/P	1381.94	394.75	898.31	258.57	483.63	136.18	0.000*

Using Wilcoxon signed rank test and paired t-test. * Significant differences $p < 0.05$ level.

There was a high significant improvement in the toothpaste slurry F and Ca/P group ($p<0.001$) and there was a lower significant difference ($p<0.05$) in the day-time saliva and no statistical significance for the Tris-HCL group (Figure 43).

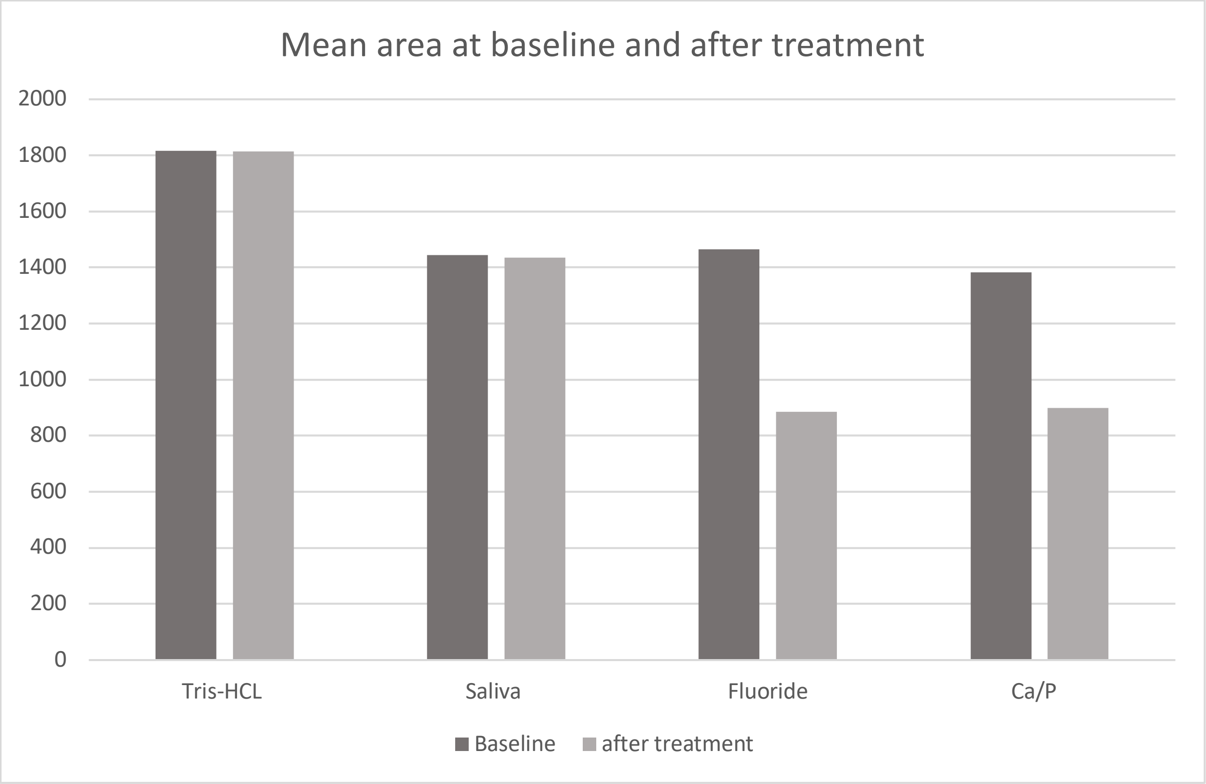


Figure 43. The mean area of enamel subsurface lesion within groups at baseline and after treatment.

5.1.3.3. Difference in the Area of enamel subsurface lesion between groups before and after treatment

The difference between groups was assessed using an independent t-test and Mann-Whitney U test (Bonferroni correction $p < 0.008$). Table 17 demonstrated that all groups showed significant differences ($p<0.001$) except for Tris-HCL vs. day-time saliva and toothpaste slurry F vs. Ca/P. The lowest difference is toothpaste slurry F vs. Ca/P.

Table 17. Area comparison of enamel subsurface lesion between separate groups before and after treatment.

Group	Mean Difference	Sd Difference	P-value
Day-time saliva vs. Tris-HCL	379.63	606.29	0.043
Toothpaste slurry F vs. Tris-HCL	929.94	593.24	0.000*
Ca/P vs. Tris-HCL	916.31	606.04	0.000*
Toothpaste slurry F vs. Day-time saliva	550.31	529.54	0.002*
Ca/P vs. Day-time saliva	536.69	436.34	0.001*
Toothpaste slurry F vs. Ca/P	362.41	295.86	0.752

Using independent t-test and using Mann–Whitney U test. * Bonferroni correction $p < 0.008$.

The highest difference can be seen in toothpaste slurry F vs. Tris-HCL (929.94± 593.24) and the lowest difference is toothpaste slurry F vs. Ca/P followed by Tris-HCL vs. day-time saliva (362.41± 295.86) (379.63 ± 606.29), respectively (Table 17) (Figure 44).

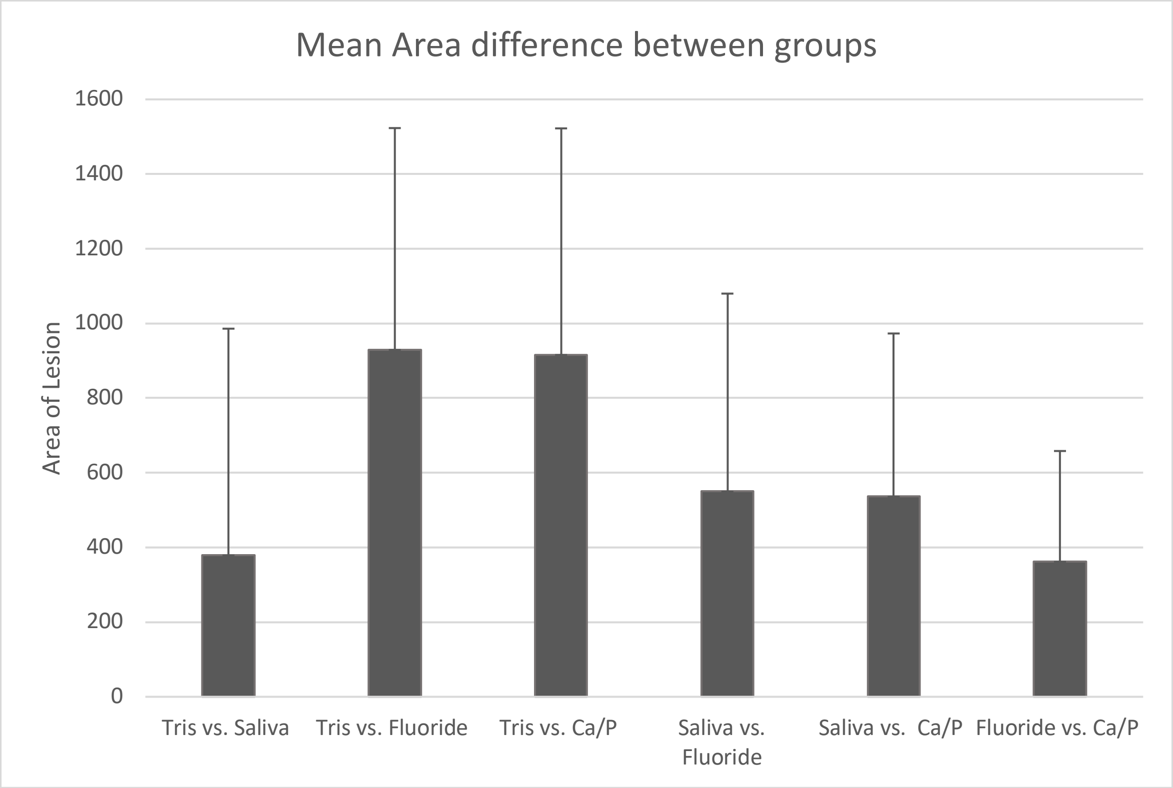


Figure 44. Means of the difference in enamel subsurface lesion between groups.

5.1.4. Lesion depth ΔF comparison within the same group over time

5.1.4.1. Comparison of ΔF mean at different time points for the Ca/P group

To assess the comparisons within the same group for ΔF (for every individual group) at different time points: at baseline, 7, 14 and 21 days. Repeated measure ANOVA was used for this comparison. The mean lesion depth ΔF at baseline (-10.32 ± 1.14) for the Ca/P group has reduced gradually after 7, 14, and 21 days to reach (-7.63 ± 0.42) at day 21 (Table 18).

Table 18. Comparison of ΔF mean at different time points for the Ca/P group.

Ca/P	Mean	SD	F-value	Sig.
Baseline	-10.32	1.14	58.88	0.000*
After 7 Days	-9.24	0.92		
After 14 Days	-8.59	0.69		
After 21 Days	-7.63	0.42		

* Significant differences p<0.05 level.

The comparison of ΔF mean at different time points (baseline, 7, 14 and 21) shows that there is a reduction in the mean between different points (Table 18). This indicates that there is a significant difference between different time frames for the alternate soaking process of Ca/P group (p<0.001).

5.1.4.2. Comparison of ΔF mean at different time points for Tris-HCL group

In the Tris-HCl group, intra-group comparisons of ΔF across time points (baseline, and days 7, 14, and 21) showed that the mean value at baseline (-10.19 ± 1.39) was essentially unchanged at day 21 (-10.18 ± 1.37). Accordingly, no statistically significant differences were observed between any of the time points (Table 19).

Table 19. Comparison of ΔF mean at different time points for the Tris-HCL group.

Tris-HCL	Mean	SD	F-value	p-value
Baseline	-10.19	1.39	0.82	0.991
After 7 Days	-10.20	1.39		
After 14 Days	-10.19	1.39		
After 21 Days	-10.18	1.37		

*Significant differences at p<0.05 level.

5.1.4.3. Comparison of ΔF mean at different time points for toothpaste slurry (1450 ppm F) group

In the toothpaste slurry group (1450 ppm F), intra-group comparisons of ΔF at different time points (baseline, and days 7, 14, and 21) revealed a significant reduction over time. The mean ΔF decreased from (-11.19 ± 0.81) at baseline to (-9.36 ± 1.28) at day 7, (-8.03 ± 0.79) at day 14, and (-6.63 ± 0.58) at day 21. This progressive decline was statistically significant ($p < 0.001$), confirming a consistent reduction in ΔF across the evaluation period (Table 20).

Table 20. Comparison of ΔF mean at different time points for the toothpaste slurry of 1450 ppm of Fluoride group.

Fluoride	Mean	SD	F-value	P-value
Baseline	-11.19	0.81	160.76	0.000*
After 7 Days	-9.36	1.28		
After 14 Days	-8.03	0.79		
After 21 Days	-6.63	0.58		

* Significant differences $p<0.05$ level.

5.1.4.4. Comparison of ΔF mean at different time points for artificial day-time saliva

To assess the comparison between different times in artificial day-time saliva, a comparison was done on ΔF at different time points: at baseline, 7, 14 and 21 days. The mean at baseline (-10.31 ± 1.35) which reduced gradually to 21 days (-9.62 ± 1.23), where day 7 (-10.03 ± 1.40) and after 14 days (-9.83 ± 1.29) (Table 21). This confirms statistical significance in the saliva group at different time points.

Table 21. Comparison of ΔF mean at different time points for artificial day-time saliva group.

Day-time saliva	Mean	SD	F-value	p-value
Baseline	-10.31	1.35	38.82	0.000*
After 7 Days	-10.03	1.40		
After 14 Days	-9.83	1.29		
After 21 Days	-9.62	1.23		

*

Significant differences p<0.05 level

Figure 45 shows the mean ΔF values of all treatment groups at baseline and after 7, 14, and 21 days. Overall, the Ca/P and toothpaste slurry F groups demonstrated a gradual improvement in ΔF values over time, with the toothpaste slurry F group showing the greatest change by day 21. In comparison, the day-time saliva group showed only a slight improvement, while the Tris-HCl group remained almost unchanged throughout the study period. These findings suggest that the treatment groups had a greater remineralisation effect than the control groups over 21 days.

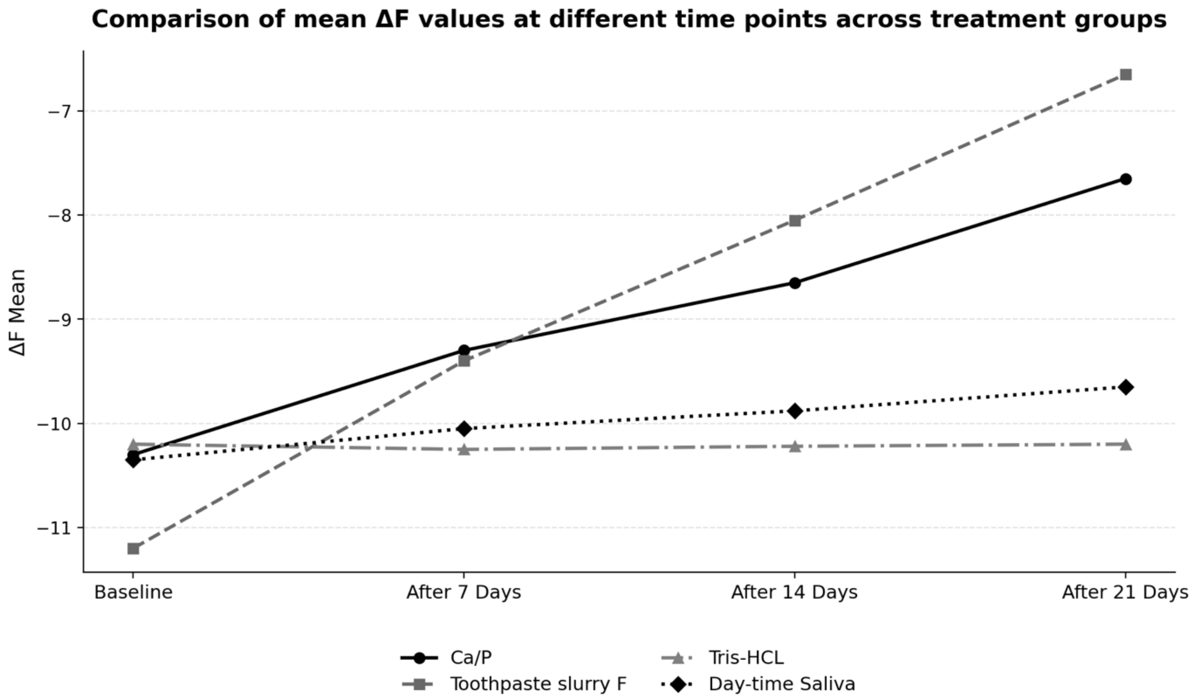


Figure 45. Comparison of mean ΔF values at different time points across treatment groups

5.1.5. ΔQ change comparison within the same treatment group

5.1.5.1. Comparison of ΔQ mean at different time points for Ca/P

Table 23 shows significant reduction in the mean values of the alternate soaking process Ca/P group from baseline to 21 days. The reduction in the ΔQ continued to reduce since the application of the calcium and phosphate solutions and the reduction has increased from (-1996.81 ± 895.61) until after 21 days (-1440.94 ± 717.40). It showed that the mean difference between different time points shows that ΔQ has statistical significance ($p < 0.001$) (Table 22).

Table 22. Comparison ΔQ (% pixels) mean at different time points for Ca/P.

Ca/P	Mean	SD	F-value	p-value
Baseline	-1996.81	895.61	14.24	0.000*
After 7 Days	-1842.88	916.58		
After 14 Days	-1643.50	837.48		
After 21 Days	-1440.94	717.40		

Significant difference at $p<0.05$ level.

5.1.5.2. Comparison of ΔQ (% pixels) mean at different time points for Tris-HCL

Table 23 shows values of ΔQ mean at different time points for the Tris-HCL group. The reduction in the mean is minimal and this correlates with the finding of no statistical significance at different time points.

Table 23. Comparison of ΔQ mean at different time points for Tris-HCL.

Tris-HCL	Mean	SD	F-value	p-value
Baseline	-2867.25	1374.29	0.84	0.479
After 7 Days	-2867.00	1374.67		
After 14 Days	-2867.06	1374.31		
After 21 Days	-2866.94	1374.39		

*Significant differences at $p<0.05$ level.

5.1.5.3. Comparison of ΔQ (%) mean at different time points for artificial day-time Saliva

The mean difference in ΔQ of the day-time saliva group at different time points (baseline, 7, 14 and 21 days) was statistically significantly lower compared to Ca/P and toothpaste slurry F groups ($p < 0.05$). The mean and standard deviation at baseline was (-2854.5 ± 1582.94) and after 21 days with a minimal reduction of (-2852.69 ± 1582.77) (Table 24).

Table 24. Comparison of ΔQ mean (%) at different time points for artificial day-time saliva.

Day-time Saliva	Mean	SD	F-value	p-value
Baseline	-2854.5	1582.94	5.35	0.019*
After 7 Days	-2853.5	1582.56		
After 14 Days	-2853.44	1582.58		
After 21 Days	-2852.69	1582.77		

* Significant differences at p<0.05 level

5.1.5.4. Comparison of ΔQ (%) mean at different time points for Toothpaste slurry 1450 ppm of Fluoride

When ΔQ mean was compared at different time points within the same group, it showed significant reduction in the ΔQ mean indicating statistical significance in this group. The baseline was (-2002.88 ± 1313.84) and after 21 days of treatment (-1395.44 ± 772.59) which shows a higher reduction then all the other groups (Table 25).

Table 25. Comparison of ΔQ mean (%) at different time points for toothpaste slurry 1450 ppm of fluoride.

Toothpaste slurry F	Mean	SD	F-value	p-value
Baseline	-2002.88	1313.84	8.78	0.000*
After 7 Days	-1782.25	1321.58		
After 14 Days	-1545.13	908.68		
After 21 Days	-1395.44	772.59		

* Significant differences p<0.05 level.

Figure 46 shows the mean ΔQ values of all treatment groups at baseline and after 7, 14, and 21 days. Overall, the Ca/P and toothpaste slurry F groups demonstrated a progressive improvement over time, with the toothpaste slurry F group showing the greatest change by day 21, followed by the Ca/P group. In contrast, the day-time saliva and Tris-HCL groups remained relatively stable throughout the study period, showing only minimal change from baseline. These findings indicate that the active treatment groups had a greater effect on improving ΔQ values over time compared with the control groups.

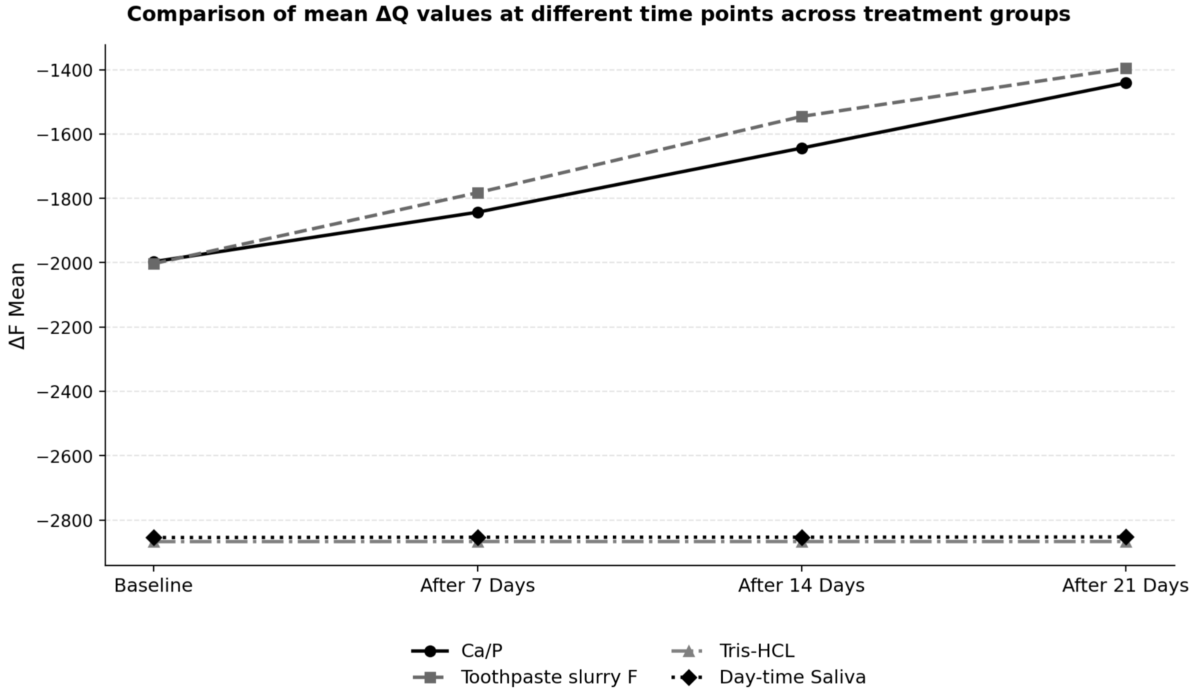


Figure 46. Comparison of mean ΔQ values at different time points across treatment groups.

5.1.6. Lesion Area comparison within the same group

5.1.6.1. Comparison of Area mean (%) at different time points for alternate soaking process Ca/P

The lesion area means values at different time points (baseline, day 7, day 14, and day 21) are shown in table 26. It can be noted that there is a significant decrease in the lesion area in this group (Ca/P). This means there is statistical significance in this group.

Table 26. Comparison of the lesion Area at different time points for Ca/P.

Ca/P	Mean	SD	F-value	P-value
Baseline	1381.94	394.75	48.05	0.000*
After 7 Days	1130.5	363.37		
After 14 Days	1014.81	288.36		
After 21 Days	898.31	258.57		

* Significant difference at p< 0.05 level.

5.1.6.2. Comparison of the Area mean (%) of enamel subsurface lesion at different time points for Tris-HCL treatment

For the Tris-HCL comparison group at different time points from baseline to after 21 days shows same mean and standard deviation at different time points. This demonstrates no change in the lesion which indicates no statistical significance ($p<0.05$) (Table 27).

Table 27. Comparison of the Area of the lesion mean (%) at different time points for Tris-HCL.

Tris-HCL	Mean	SD	F-value	P-value
Baseline	1815.25	535.81	4.00	0.081
After 7 Days	1814.75	535.85		
After 14 Days	1814.75	535.93		
After 21 Days	1814.63	535.78		

* Significant differences p<0.05 level.

5.1.6.3. Comparison of Area mean (%) at different time points for artificial day-time saliva

Although lesion area measurements in the day-time artificial saliva group showed statistically significant differences across time points, the reduction from baseline to day 21 was minimal. (Table 28).

Table 28. Comparison of Area mean at different time points for artificial day-time saliva.

Day-time saliva	Mean	SD	F-value	P-value
Baseline	1444.56	486.01	2.34	0.042*
After 7 Days	1444.56	486.01		
After 14 Days	1444.37	486.24		
After 21 Days	1442.00	486.33		

*

Significant differences at p<0.05 level

5.1.6.4 Comparison of Area mean at different time points for toothpaste slurry of 1450 ppm of Fluoride

The enamel subsurface lesion for the toothpaste slurry group shows a significant reduction in the mean throughout the period of treatment. The reduction can be noted clearly from baseline (1465.75 ± 343.55), day 7 (1156.88 ± 337.12), day 14 (988.56 ± 199.63) and after 21 days (884.69 ± 116.76). The highest reduction in the period of treatment would be from baseline to day 7 compared to the other days (Table 29

Table 29. Comparison of Area mean (%) at different time points for toothpaste slurry F.

toothpaste slurry F	Mean	SD	F-value	P-value
Baseline	1465.75	343.55	28.43	0.001*
After 7 Days	1156.88	337.12		
After 14 Days	988.56	199.63		
After 21 Days	884.69	116.76		

* Significant differences at $p<0.05$ level.

5.2. Micro-computed Tomography (Micro-CT) results for different solutions (toothpaste slurry 1450 F, alternating calcium and phosphate sloutions, Tris-HCL, and day-time artificial saliva)

5.2.1. Comparison of the mineral density within the same group at baseline and after 21 days using Micro-CT.

To further investigate the results seen in the previous section, especially at baseline and after 21 days, paired sample t-tests were performed to compare the changes in mineralisation from baseline and after treatment within the same group. Table 31 shows that the alternate soaking process Ca/P group mineral density has increased from (0.90 ± 0.20) to (2.00 ± 0.10) after treatment which proved statistical significance in this group ($p < 0.05$). Furthermore, the Toothpaste slurry F group has also had an increase in mineral density from baseline (0.87 ± 0.15) to (2.30. ± 0.17) and showed higher statistical significance then Ca/P ($p < 0.05$). The artificial day-time saliva also showed statistical significance (0.79 ± 0.17) to (0.90 ± 0.26), however, it is lower than then Ca/P significance. In the other hand, Tris-HCL showed no increase in the mineral density, and this correlates with no statistical significance. The results of the paired T-Test are shown in Table 31.

Table 30. Comparison of the mineral density of the enamel subsurface lesion for individual groups at baseline and after treatment (after 21 days) using micro-CT mean unit g/cm^{3}.

Micro-CT		**Mean**	**SD**	**t-value**	**p-value**
Ca/P	Baseline	0.90	0.20	20.82	0.000*
	After 21 days	2.00	0.10		
Tris-HCL	Baseline	0.93	0.23	0.36	0.724
	After 21 days	0.95	0.24		
Day-time Saliva	Baseline	0.79	0.17	2.18	0.045*
	After 21 days	0.90	0.26		
Toothpaste slurry F	Baseline	0.87	0.15	35.68	0.000*
	After 21 days	2.30	0.17		

* Significant differences at $p<0.05$ level.

5.2.2. Comparison of the mineral density between the groups using Micro-CT

Table 31. Comparison of the mineral density in the enamel subsurface lesion between groups using micro-CT, mean unit g/cm^3.

Groups	Mean Difference	SD Difference	P-value
Day-time Saliva vs. Tris-HCL	0.10	0.21	0.247
Toothpaste slurry vs. Tris-hcl	1.37	0.22	0.000*
Ca/P vs. Tris-HCL	1.07	0.23	0.000*
Toothpaste slurry vs. Day-time saliva	1.27	0.20	0.000*
Ca/P vs. Day-time Saliva	0.97	0.21	0.000*
Toothpaste slurry vs. Ca/P	0.30	0.19	0.000*

* Significant differences at $p<0.05$ level.

Table 31 shows the cross-comparison between different treatment groups after treatment. There was no significant difference between day-time saliva and Tris-HCL ($p<0.05$). However, both toothpaste slurry F and Ca/P groups were significantly increased in the mineral density compared to the Tris-HCL group ($p<0.001$). Both toothpaste slurry and Ca/P groups were significantly increased in mineral density compared to the day-time saliva group ($p<0.001$). Interestingly, the toothpaste slurry group was significantly increased compared to Ca/P group ($p<0.001$). Figure 53 show that the mean difference (mineral density) has increased in both Ca/P and toothpaste slurry F. However, the mean difference for toothpaste slurry is higher than that of the alternate soaking process of Ca/P by around 0.3g/cm^3.

Figure 47 illustrates a coronal section of an enamel lesion at baseline and after treatment using the Ca/P alternate soaking process. Figure 47a clearly demonstrates the dark demineralised window previously described in the Materials and Methods section. Following 21 days of treatment, Figure 47b shows a marked reduction in the thickness of the demineralised zone. In addition, the enamel appears more uniform in colour after treatment.

A similar effect is observed in Figure 50, which represents the toothpaste slurry group containing 1450 ppm fluoride. Figure 50b clearly demonstrates a reduction in the demineralised area following treatment when compared with baseline.

In contrast, Figures 48 and 49 show coronal sections of enamel lesions before and after treatment with daytime artificial saliva and Tris-HCL, respectively. In both groups, no noticeable reduction in the thickness of the demineralised lesion was observed after the 21-day treatment period.

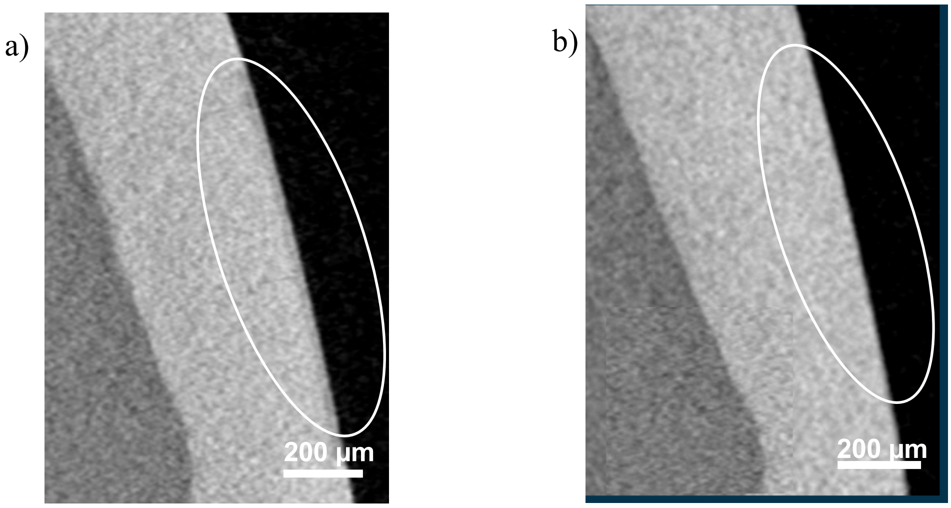


Figure 47(a and b). Represents coronal section micro-CT image of enamel lesion before and after remineralisation.

a) At baseline (subsurface enamel lesion) b) after treatment using alternate soaking process Ca/P.

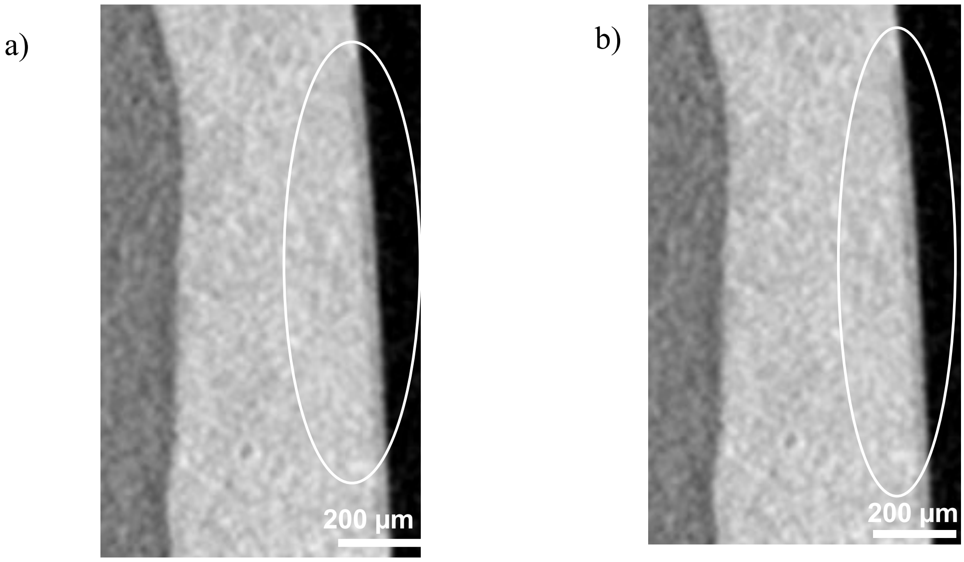


Figure 48 (a and b). Represents coronal section micro-CT image of enamel lesion before and after remineralisation.

a)At baseline (subsurface enamel lesion) b) after treatment using artificial day time saliva

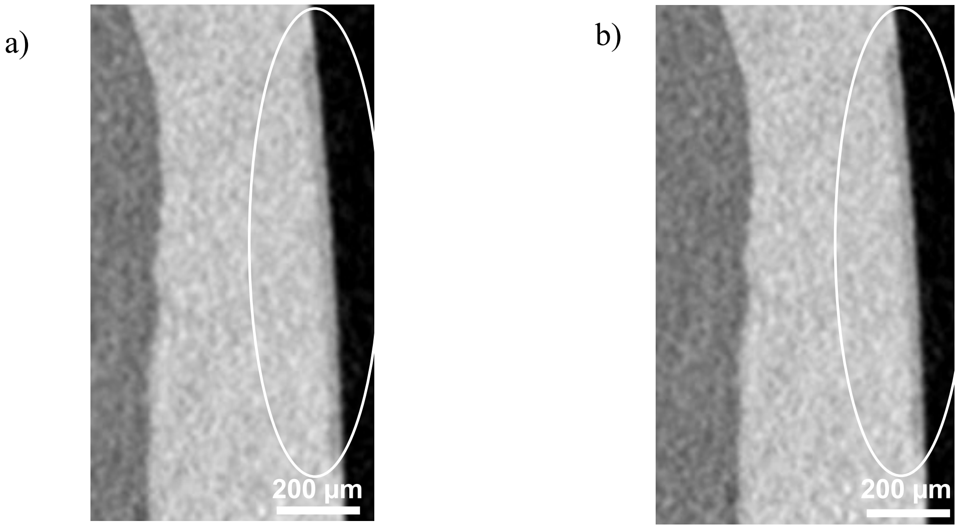


Figure 49. (a and b). Represents coronal section micro-CT image of enamel lesion before and after remineralisation.

a)At baseline (subsurface enamel lesion) b) after treatment using Tris-HCL.

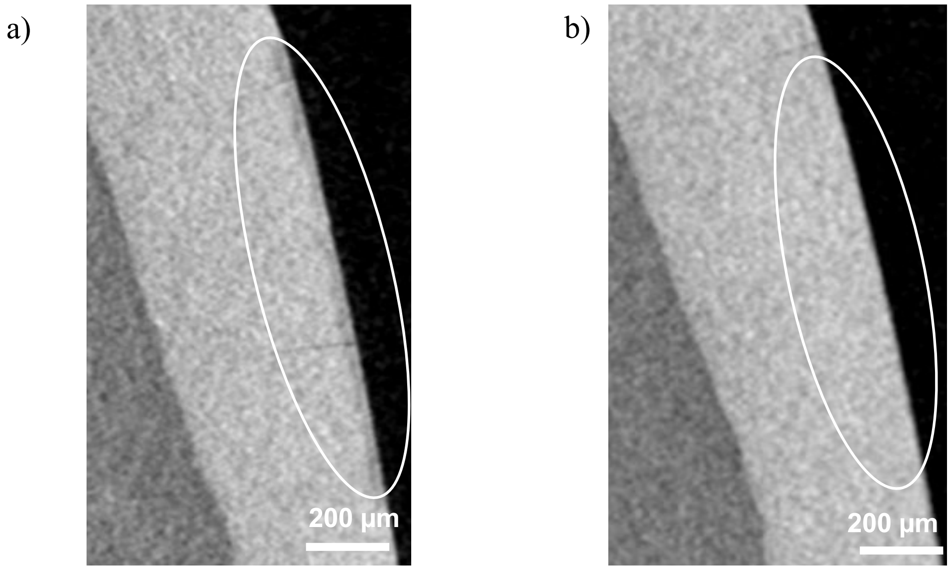


Figure 50 (a and b). Represents coronal section micro-CT image of enamel lesion before and after remineralisation.

a)At baseline (subsurface enamel lesion) b) after treatment using toothpaste slurry 1450 PPM F

Figure 43 to 46 is plugins used to visually demonstrate the demineralisation and remineralisation window of the enamel slabs. For the alternate soaking process (Ca/P) and toothpaste slurry F groups, the initial image (Figure 51 to 54 (a)) shows the demineralised window, indicated by a reduction in mineral density within the defined region according to the colour scale. After 21 days of treatment with Ca/P or toothpaste slurry F (Figure 51 b and 54 b) , a clear increase in mineral density was observed, reflecting a reduction in the thickness and extent of the demineralised zone and indicating effective remineralisation. In contrast, the Tris-HCL and day-time artificial saliva groups (Figure 52 b and 53 b) showed no visible change in the demineralisation window, indicating minimal or no remineralisation over the same period.

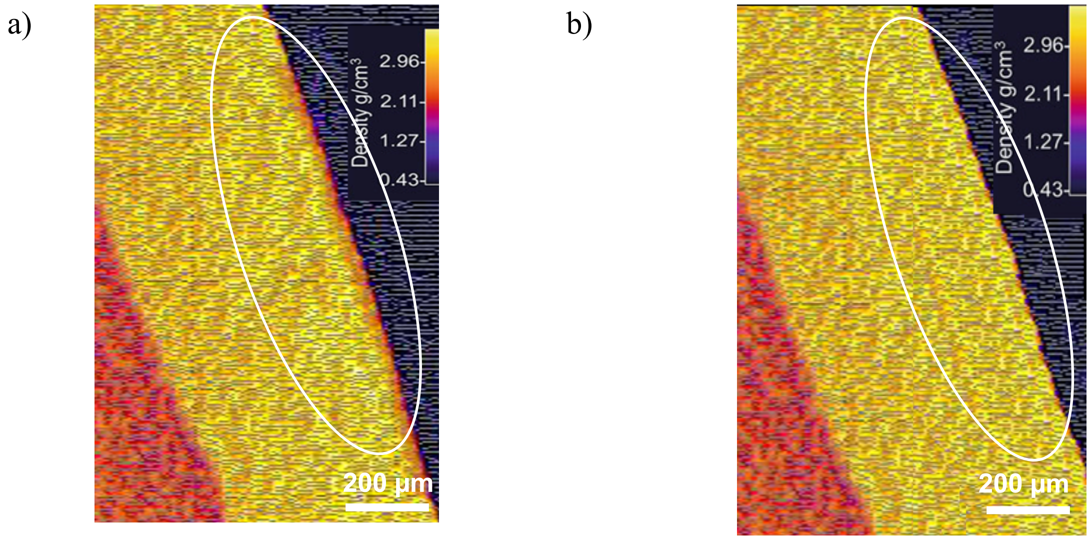


Figure 51 (a and b) The tool interceptive 3D surface plot before and after treatment in the alternate soaking process group.

a) At baseline (subsurface enamel lesion b) after treatment using alternate soaking process Ca/P.

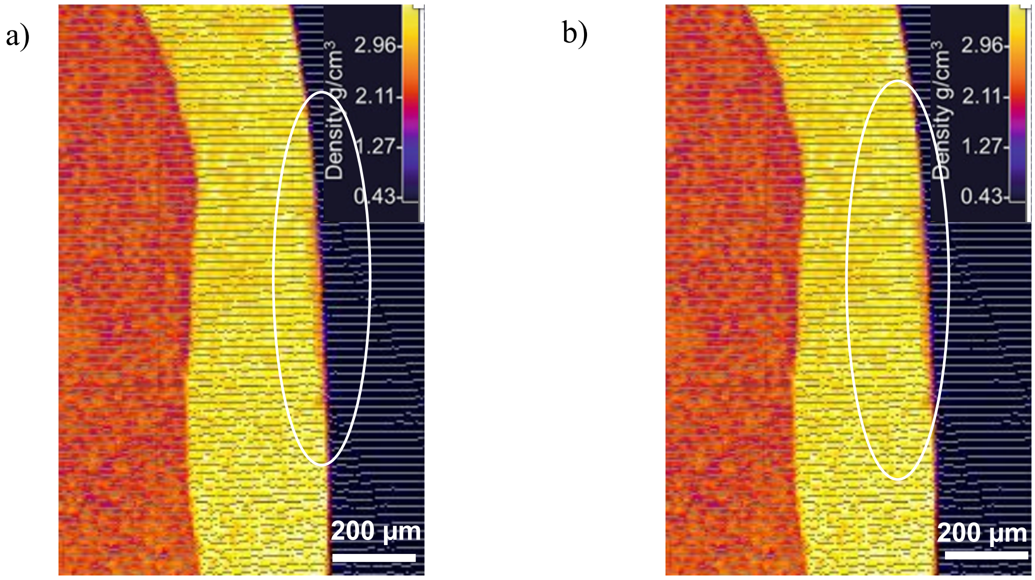


Figure 52 (a and b) The tool interceptive 3D surface plot before and after treatment in day time artificial saliva group.

a) At baseline (subsurface enamel lesion b) after treatment using day time artificial saliva

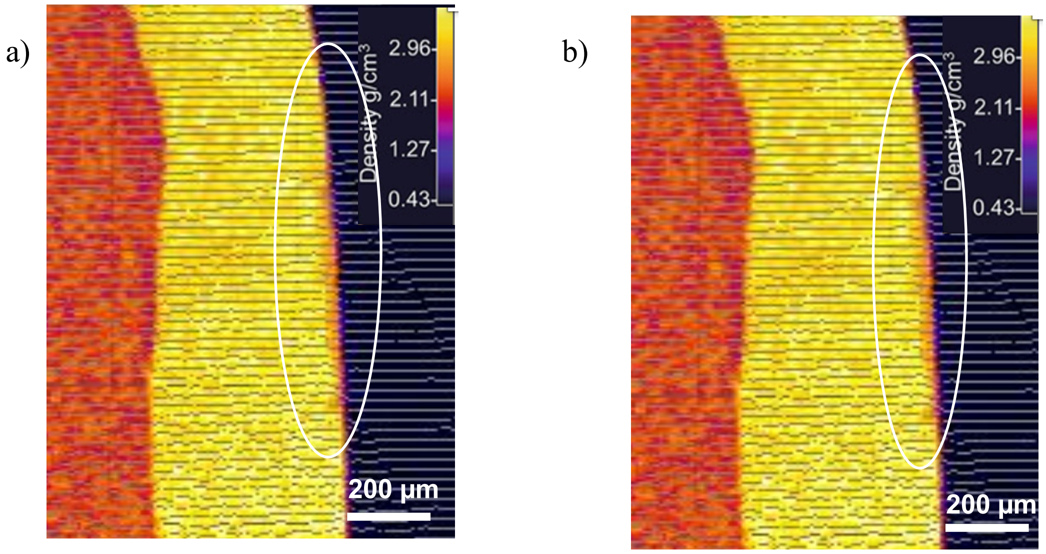


Figure 53 (a and b) The tool interceptive 3D surface plot before and after treatment in tris-HCL

a) At baseline (subsurface enamel lesion b) after treatment using tris-HCL.

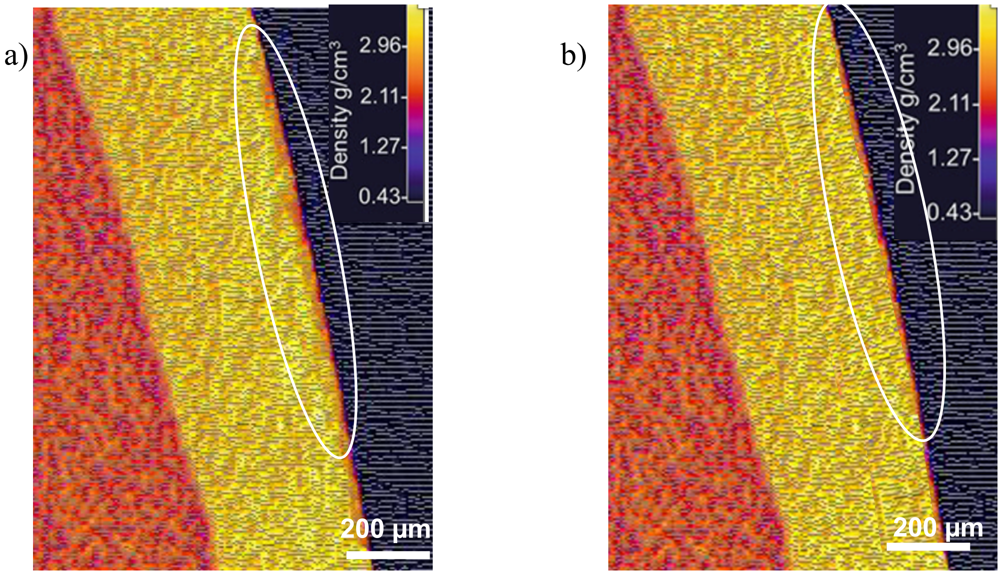


Figure 54 (a and b) The tool interceptive 3D surface plot before and after treatment in toothpaste slurry F

a) At baseline (subsurface enamel lesion b) after treatment using toothpaste slurry F

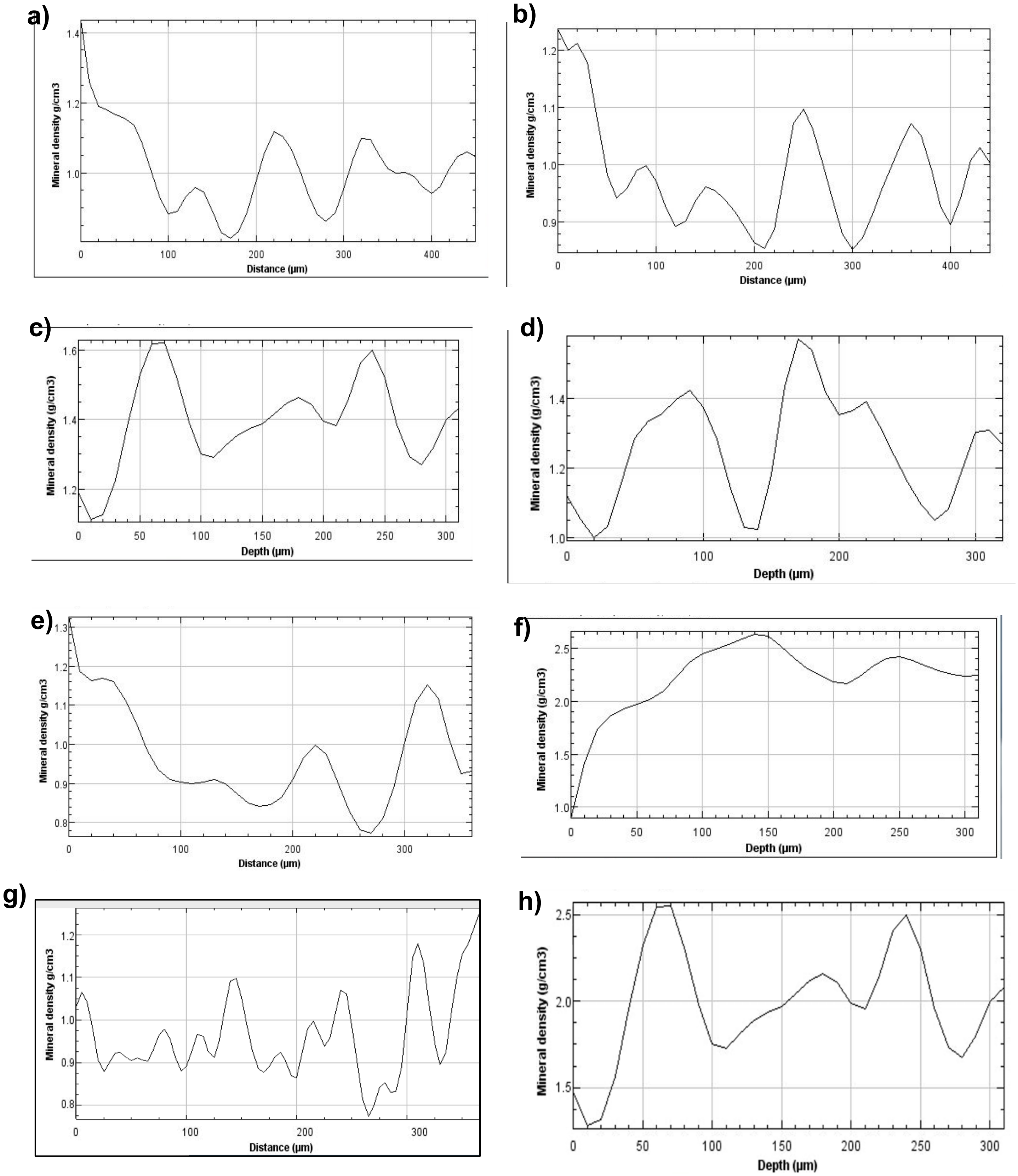


Figure 55. Mineral density of groups at baseline and after treatment using Micro-CT. All the graphs begins at the enamel surface and extends toward the subsurface enamel region. a) Baseline tris-HCL, b) After treatment of tris-HCL, c) Baseline day-time artificial saliva, d) After treatment of day-time artificial saliva, e) Baseline toothpaste slurry F, f)

After treatment toothpaste slurry F, g) Baseline alternate soaking process, h) After treatment with alternate soaking process.

The mineral density (MD) values demonstrated an increase after treatment in both the alternate soaking and toothpaste slurry groups compared to their respective baseline values. In contrast, the day-time saliva group showed only a minimal increase in mineral density, while the Tris-HCl group exhibited no measurable change relative to baseline (Figure 47).

5.3. Energy dispersive X-ray (EDX) results for different solutions (toothpaste slurry 1450 F, alternating calcium and phosphate solutions, Tris-HCL and day-time artificial saliva)

SEM-EDX analysis was undertaken for the evaluation of mineral gain of three enamel samples. Analysis was completed before the cycling regime (after demineralisation/ baseline) and after the application of the respective treatment for each group. EDX demonstrated the mineral gain of different minerals by analysing the weight percentage. Mineral gain was analysed for all the groups.

5.3.1. EDX comparison of the mineral gain within the same group

Table 32 shows the mean calcium and phosphorus weight percentages (Ca + P wt%) and their corresponding standard deviations for all groups at baseline and after treatment and from this table the mean mineral gain was calculated. Toothpaste slurry and alternate soaking process groups exhibited the greatest increases in mean Ca+P wt% from baseline to after treatment, indicating a substantial enhancement in mineral content following intervention. Specifically, the toothpaste slurry group increased from (32.1 ± 1.662 wt% to 43.1 ± 2.092 wt%), while the alternate soaking process group increased from (34.3 ± 2.753 wt% to 39.5 ± 3.251 wt%). In contrast, the day-time saliva group demonstrated a more modest increase (32.1 ± 1.951 wt% to 34.6 ± 3.204 wt%), and the Tris-HCL control group showed only a minimal change (36.3 ± 3.512 wt% to 37.1 ± 3.669 wt%). Overall, these findings suggest that both the toothpaste slurry and alternate soaking process were more effective in promoting mineral gain compared with artificial day-time saliva and Tris-HCL,

Table 32. **Mean calcium and phosphorus weight percentages (Ca + P wt%) and their corresponding standard deviations for all groups at baseline and after treatment.**

	After demineralisation (Baseline)		**After treatment**	
	Mean Ca+P wt%	**SD**	**Mean Ca+P wt%**	**SD**
Tris-HCL	36.3	3.512	37.1	3.669
Toothpaste Slurry	32.1	1.662	43.1	2.092
Alternate soaking process	34.3	2.753	39.5	3.251
Day-time Saliva	32.1	1.951	34.6	3.204

The EDX results demonstrate marked differences in mean mineral gain (Ca+P%) among the tested groups. The Toothpaste slurry (F) group showed the highest mineral gain (31.6 ± 0.40%), followed by the Ca/P alternate soaking group (22.7 ± 5.68%), with both groups exhibiting highly significant increases compared with baseline (p =

0.000). In contrast, the day-time saliva group displayed a lower mean mineral gain (8.6 ± 1.87%) that did not reach statistical significance (p = 0.073), while the Tris-HCL control group demonstrated only minimal mineral gain (4.9 ± 0.52%) with no statistically significant difference (p = 0.234) (table 33) (Figure 48),

Table 33. Comparison of the percentage of mean mineral gain for all the groups (EDX scores).

EDX	Mean mineral gain (Ca+P%)	SD	t-value	P-value
Ca/P	22.7	5.68	18.74	0.000*
Tris-HCL	4.9	0.52	1.23	0.234
Day-time Saliva	8.6	1.87	2.36	0.073
Toothpaste slurry F	31.6	0.40	77.70	0.000*

* Significant differences at $p<0.05$ level.

5.3.2. Comparison of the mineral density between the groups using EDX

Table 34. Comparison of the mineral gain in the enamel subsurface lesion between groups using EDX.

Groups	Mean Difference	SD Difference	P-value
Day-time saliva vs. Tris-HCL	0.08	0.256	0.230
Toothpaste slurry F vs. Tris-HCL	0.67	0.163	0.000*
Ca/P vs. Tris-HCL	0.61	0.171	0.000*
Toothpaste slurry F vs. Day-time saliva	0.59	0.202	0.000*
Ca/P vs. Day-time saliva	0.53	0.209	0.000*
Toothpaste slurry F vs. Ca/P	0.06	0.067	0.002*

* Significant differences at p< 0.05 level.

Table 36 shows the cross-comparison between different treatment groups after treatment. There was not significant difference between day-time saliva and Tris-HCL (p <0.05). However, both toothpaste slurry F and Ca/P groups showed significance compared to the Tris-HCL group and day-time saliva (p<0.001). Also, both toothpaste slurry F and Ca/P groups had an increase in the percentage of the mineral gain compared to the day-time saliva group and Tris-HCL (p<0.001). Interestingly, the toothpaste slurry F group showed highest mineral gain when compared to Ca/P groups (p<0.001).

Chapter 6 Discussion

In this study, the null hypothesis was rejected, as statistically significant differences in enamel remineralisation were observed for the parameters ΔF, ΔQ, and lesion area assessed by QLF, as well as measurements obtained from micro-CT and EDX, between the test groups (alternate soaking process Ca/P and toothpaste slurry containing 1450 ppm fluoride) and the control groups (day-time artificial saliva and Tris-HCl). This suggests that the test groups were effective in promoting remineralisation in comparison with the control groups. By using multiple evaluations of remineralisation, it allows triangulation with each evaluation showing remineralisation for the test groups. The results seen in all enamel lesions showed similar results within each group and there were no super responders.

A search of available literature showed that many studies including *in vitro, in situ,* and *in vivo* investigated the effect of calcium and phosphate on enamel subsurface lesion remineralisation which is available in literature (Amaechi et al., 2022; Konagala et al., 2020; Shaik et al., 2017). However, there appears to be shortage of literature investigating the effect of soaking calcium and phosphate separately and in solution form. The majority of studies demonstrated the effect of calcium and phosphate together (Bernd and Silvia, 2021; Bhat et al., 2022; Brunton et al., 2013). However, another study found that if calcium and phosphate are applied together, they can form a solid, interlocked crystal layer called brushite on the enamel (Abou Neel et al., 2016). This suggests that using them separately might be a more effective approach, allowing for better integration into the enamel without premature crystal buildup (Giocondi et al., 2010). Further to this, studies have demonstrated the use of the effect of alternate soaking process Ca/P on bone only (Matsusaki et al., 2009). However, no study used this method on enamel subsurface lesion and compared it with toothpaste slurry containing 1450 ppm F, artificial saliva and Tris-HCL.

Therefore, the current *in vitro* study provide evidence to show the positive effect of a novel treatment regimen containing an alternate soaking process with calcium and phosphate solutions on enamel lesion remineralisation *in vitro.*

6.1. *In vitro* model

The present study used an *in vitro* model to study enamel remineralisation. In this model demineralisation of the enamel slabs was applied by creating the subsurface enamel lesion using demineralisation gels discussed previously in the material and methods (refer to section 4.2.2.4). This model that studied enamel demineralisation and remineralisation is widely accepted and has been adopted in numerous studies (Nanci and Ten Cate, 2013). The key advantage of the *in vitro* model is that it provides the ability to carry out single variable experiments under highly controlled conditions (Bataineh et al., 2017; White, 1995). It also enables 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 requires less time to undertake compared to *in vivo* and human studies (White, 1995).

Despite this, 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(Shellis, 1984; White, 1995). The salivary pellicle, a proteinaceous layer on the tooth surface, impacts how minerals interact with enamel. *In vitro* models lack this dynamic, affecting how closely they simulate the real-life conditions of demineralisation and remineralisation cycles. It is essential to recognise that lesions created in a laboratory setting are not identical to those that develop naturally in the human oral environment and should instead be referred to as caries-like lesions (Robinson et al., 2000) The *in vitro* model can be adjusted to align with the specific hypotheses, objectives, methods, and conclusions of different studies. Another limitation is lack of pH cycling in this study and *in vivo* conditions that naturally influence the balance between demineralisation and remineralisation. The natural oral pH varies due to eating, drinking, and bacterial metabolism. In pH cycling models, this fluctuation is artificially created to mimic *in vivo* conditions, providing a more accurate picture of how enamel is affected over time.

6.2. Study design

Four groups were used in this study to investigate the remineralisation of enamel subsurface lesion. The alternate soaking process (Ca/P), Fluoride (sodium fluoride) 1450 ppm toothpaste slurry, artificial day-time saliva (positive control), and Tris-Hydrochloride (Tris-HCL) (negative control). This study is a one-phase *in vitro* study design. The enamel lesion was distributed randomly in each group to minimise the introduction of bias in the study results.

6.3. Bovine teeth

Preferably, human teeth are preferred in a study of caries process. However, their composition is variable, due to genetic influences, and environmental conditions. Furthermore, sources of human teeth are becoming more increasingly limited and there is a significant increase in difficulty of obtaining human teeth for research purposes (Stookey et al., 2011).

For these reasons bovine teeth were used in this study. There is an increase in the use of bovine teeth in research as they provide a greater surface area to investigate the effect of treatment (Yassen et al., 2011). Bovine teeth are easier to obtain and have been demonstrated to perform similarly to human teeth. Furthermore, in literature due to the similarity of characteristics of bovine teeth and human teeth, bovine can make a good substitute for human teeth in caries research (Yassen et al., 2011; Tanaka et al., 2008). In addition, 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). This greater uniformity means that differences are less likely to be hidden by the inherent variability of the substrate. Caries lesions in bovine teeth have mineral distribution and structural changes comparable to human teeth (Arango et al., 2019). I*n vitro* studies have successfully used bovine enamel to evaluate the effect of different anti-cariogenic agents on enhancing enamel remineralisation and inhibiting enamel demineralisation (Amaechi et al., 1999; Arango et al., 2019; Busch et al., 2003).

Other researchers have questioned the value of using bovine enamel. They argue that bovine enamel is softer than human enamel and has higher rate of lesion progression and lesion depth (Amaechi et al., 1999). Moreover, some dental researchers have questioned their use in research as bovine enamel does not have the exact structure and chemistry of human enamel (Wennberg and Orstavik, 1990; Buzalaf et al., 2010).

6.4. Enamel slabs preparation and storage

The enamel thickness was found to influence the fluorescence, while this confounding factor could not be absolutely standardised in the present study, randomising the enamel slabs to separate groups attempted to mitigate this potential for bias. Moreover, only profound differences in the total thickness of enamel tissue have been shown to influence the quantification of remineralisation with the QLF (Wu et al., 2010). The impact of this variable was likely to be minimal on the results of the current study.

6.5. Artificial caries lesions

Lesions created by acid buffer solutions have been shown to produce larger and deeper lesions than acidified gels (including Acidified hydroxyethyl cellulose gel). The rapid diffusion rate of acid buffer solutions does not allow re-precipitation of minerals and prevents the formation of an intact surface layer over the lesion (produces an erosion-like lesion). Acidified gels on the other hand create more controlled demineralisation process and allow re-precipitation of dissolved mineral ions to create an intact surface layer of the lesion that mimics the caries process (Amaechi et al., 1999).

6.7. Alternating soaking process

The same protocol was applied on enamel to assess the remineralising potential. The main reason for soaking the calcium first then rinsing it with Tris-HCL was to remove any excess of calcium ions and then soaking the samples in phosphate. Twenty cycles were completed with each step lasting for 10 seconds (Matsusaki et al., 2009). It was hypothesised that the cycling protocol would allow penetration of calcium and then phosphate thereby this mechanism prevents blockage of surface pores of the lesion (Abou Neel et al., 2016). Allowing the penetration of calcium first prevents the formation of brushite on the surface layer and allowing hydroxyapatite-like crystals to form and prevent diffusion of calcium and phosphate ions (Al-Sanabani et al., 2013). Giocondi et al (2010) reported the formation of brushite on the surface of enamel slabs when calcium and phosphate were applied together producing solid interlocked crystal (Giocondi et al., 2010). Furthermore, when demineralisation happens calcium release precedes phosphate release from enamel, dentin, and cementum. By reversing the mechanism of demineralisation, studies have suggested that remineralisation is possible and prevent precipitation of calcium and phosphate on the surface of enamel which will prevent penetration of mineral ions. (Abou Neel et al., 2016; Al-Sanabani et al., 2013). Although bone and enamel are distinct in structure and function, their shared use of hydroxyapatite suggests that similar principles of calcium and phosphate ion-driven mineralisation apply to both tissues (Driessens, 1980).

The deposition of calcium and phosphate ions from a supersaturated environment (such as saliva in enamel or bodily fluids in bone) facilitates the formation of hydroxyapatite crystals. While this process is fundamentally similar, the way mineralisation takes place is influenced by each tissue's unique structural and organic environment. Enamel is a non-regenerative tissue, meaning once it is fully formed, it cannot be remodeled through cellular processes. Mineralisation in enamel occurs primarily during development and is later maintained by external remineralisation processes, often with the aid of fluoride in saliva. Bone is a dynamic and regenerative tissue that constantly remodels in response to mechanical stress, injury, and metabolic needs. Cells, such as osteoblasts and osteoclasts, regulate bone deposition and resorption, allowing it to adapt to changes throughout life. This cellular remodeling is absent in enamel (Roberts et al., 2022).

6.7.1. Alternate soaking process in solution form

The alternate soaking process for bones is conducted in solution form because this method allows treatment agents to penetrate the bone's structure deeply and uniformly. This approach closely resembles how bones naturally interact with bodily fluids, which are essential for processes like mineralisation, demineralisation, and overall bone maintenance (Driessens, 1980). Using solutions provides several advantages. First, it ensures the even distribution of ions or molecules, such as calcium and phosphate, throughout the bone matrix. This even distribution is crucial for processes like remineralisation, where minerals are redeposited into the bone. Second, solutions allow to precisely control key variables, such as ion concentration, pH, temperature, and soaking duration, which are critical for promoting the stepwise formation of minerals in a controlled manner. Such precision enables the replication of physiological conditions that occur in the human body.

Alternating between solutions with different compositions, such as calcium-rich and phosphate-rich solutions, enhances the dynamic interaction between the bone and the treatment agents. This stepwise alternation encourages gradual mineral build-up, similar to the way bones naturally regenerate or strengthen in living organisms (Palmer et al., 2008). Moreover, this method is versatile and adaptable, to modify the solutions and conditions to suit the specific objectives.

In the current study the alternate soaking process was conducted in solution form because it also closely mimics the natural cycles of demineralisation and remineralisation that occur in the oral environment. In the mouth, teeth are constantly bathed in saliva that helps counteract the effects of acids from food, drinks, or bacterial activity. By alternating between solutions, after the application of demineralising solution that simulates acid attacks remineralising solutions containing minerals like calcium, phosphate, can replicate the natural processes under controlled conditions. The liquid form allows minerals to penetrate deeply and uniformly into the tooth structure, targeting damaged areas such as early caries lesions, as seen in the current study.

Solutions offer additional advantages, such as ensuring even distribution of ions across the tooth surface, making it easier to observe and measure changes in mineral content (Abou Neel et al., 2016). Researchers can also precisely control variables like pH, ion concentration, and soaking time, which are crucial for understanding how different treatments or conditions affect enamel. The flexibility of solutions allowed for the testing of various products to determine their effectiveness in strengthening teeth and repairing early damage.

6.8. Toothpaste slurry 1450 ppm of Fluoride

The toothpaste used in this study included sodium fluoride toothpaste which was used to assess the comparison of remineralisation. 1450 ppm F is the most common fluoride concentration in adult toothpaste preparations in the United Kingdom. The idea of using the toothpaste in slurry form instead of toothpaste in full concentration is to mimics how toothpaste is diluted with saliva during actual brushing. This replicates realistic oral exposure rather than testing the undiluted toothpaste, which may not reflect in-mouth conditions (Bataineh et al., 2017).

6.9. Quantitative Light-Induced Fluorescence (QLF)

QLF is a novel technique for the detection of early demineralisation of enamel (Bataineh et al., 2017). In the current study, QLF was used to assess the enamel demineralisation and remineralisation at baseline and following the treatment. It is a system used to measure the fluorescence loss after demineralisation and to detect and quantify the analysis of early caries as strong correlation was found between the mineral loss and fluorescence loss in enamel after demineralisation (Hattab, 2013; Pretty et al., 2002). The advantage of this technique is being non-destructive which allows the longitudinal monitoring of early carious lesions. In this study QLF was used to quantify the enamel subsurface lesion at baseline (after 10 days of demineralisation gel), and enamel lesion remineralisation after 7, 14, 21 days following the treatment for all the four groups. Pretty et al (2002) analysed the intra-examiner and inter-examiner reliability of QLF, reported high reproducibility of QLF for the *in vitro* caries lesions (Pretty et al., 2002).

6.9.1. Results of Quantitative Light-Induced Fluorescence (QLF)

As mentioned before, QLF produces three parameters that include; the ΔF which represents the percentage fluorescence loss and related to lesion depth, Area represents the surface area of the lesion as well as ΔQ which is the ΔF times the area and represent the lesion volume. All these values were calculated in this study; however, the ΔF value was considered as the main indicator for the mineral loss and the lesion progression or regression in the present study.

6.9.1.1 Effect of alternating soaking process of calcium and phosphate on remineralising enamel subsurface lesions

In the current study, the treatment with alternate soaking process (calcium and phosphate solutions) was applied twice daily for 21 days on enamel lesions. The results showed that the effect of alternate soaking process on all QLF parameters (ΔF, ΔQ and lesion Area) showed statistically significant remineralisation of the subsurface lesionsThe value of ΔF, decreased at all time points compared with baseline, and this reduction was statistically significant across all QLF parameters.. Statistical significance was seen between alternate soaking process and Tris-HCL, day-time artificial saliva, and toothpaste slurry groups. statistical significance was also noted in ΔQ lesion volume which reflects the total demineralisation that considers the area of the lesion and the depth. However, when ΔQ was compared between toothpaste slurry of fluoride and alternate Ca/P, no statistical significance was noted. Additionally, comparison of lesion area between the toothpaste slurry and alternate Ca/P groups revealed no statistically significant difference.

The remineralisation potential due to the alternate soaking process was lower in comparison with toothpaste slurry group but higher than day-time artificial saliva and Tris-HCL. Although comparing the results of this *in vitro* study on enamel subsurface lesion to a study done on bone may not be valid, however the same trend was noticed on bone (Matsusaki et al., 2009) where they manged to demonstrate bone regeneration. Further to this, bone and enamel do share the similar mineral composition, which is hydroxyapatite. However, bone and teeth have different tissue morphologies and organic content. Furthermore, enamel is mainly inorganic in composition, and bone has a higher organic composition (Palmer et al., 2008). In this study, the main aim was to demonstrate remineralisation potential of alternate soaking process and to test the theory of it on enamel subsurface lesion.

The described alternate soaking process for enamel remineralisation is applied by soaking calcium first, followed by rinsing with Tris-HCl, then exposing the enamel lesions to phosphate to allow for ion delivery to optimise remineralisation without surface clogging. This process prevents premature precipitation of calcium phosphate compounds like brushite ($CaHPO_42H_2O$), which can obstruct enamel surface pores and reduce the effectiveness of remineralisation treatments. The proposed mechanism of soaking in a calcium solution first is that calcium ions can penetrate the porous subsurface enamel lesions without immediately reacting with phosphate to form precipitates on the surface. Tris-HCl rinse as mentioned previously acts as a buffer rinse helps maintain pH stability, enhancing the controlled delivery of ions and further preparing the enamel surface for phosphate application. Applying phosphate afterward promotes remineralisation within the enamel structure rather than on the surface. This correlates with a study that discussed brushite can form rapidly when calcium and phosphate ions are applied together in acidic to neutral conditions and precipitation can form and block enamel pores, limiting further ion penetration and hindering subsurface enamel lesion remineralisation. Separating calcium and phosphate applications allows for the gradual build up crystals, mimicking natural enamel formation (Abou Neel et al., 2016). This suggests that this approach could promotes better integration into the enamel matrix, as opposed to creating a surface coating that lacks structural depth (Abou Neel et al., 2016).

The sequence of natural demineralisation in the early stages of caries is calcium ions are generally lost from enamel before phosphate ions, leading to a gradual breakdown of the enamel structure (Rujiraprasert et al., 2023). This loss creates a porous, demineralised subsurface region beneath a relatively intact surface layer. For remineralisation to take place, the sequence needs to be reversed. In this study the aim was to deliver calcium ions first; this protocol mirrors the natural mineral loss process and encourages the ions to penetrate deeply into the subsurface lesion. A controlled phosphate application could lead to the formation hydroxyapatite-like crystals within enamel (Zhao et al., 2012). This sequential approach is key to avoiding the formation of superficial mineral layers, such as brushite. Traditional remineralisation treatments that deliver calcium and phosphate simultaneously can result in surface precipitation that limits mineral uptake in deeper layers (Al-Sanabani et al., 2013).

6.9.1.2. Effect of toothpaste slurry 1450 ppm F on remineralising enamel subsurface lesions

Overall, in the present study it was noted that the highest remineralisation was toothpaste slurry 1450 ppm F, then alternate soaking process, followed by day-time artificial saliva and then least was Tris-HCL.

The toothpaste slurry with 1450 ppm fluoride used in this study serves as a treatment group to compare with the alternate soaking process Ca/P. This concentration is a standard in toothpaste formulations in the UK and is recognised for its efficacy in promoting remineralisation and inhibiting demineralisation (Rujiraprasert et al., 2023). This fluoride concentration has been shown to be effective in both remineralising enamel lesions and preventing further demineralisation (Davies and Davies, 2008).

Sodium Fluoride (NaF) is a widely studied and reliable form of fluoride for enamel remineralisation (Arifa et al., 2019). It releases fluoride ions upon dissolution, which then integrate with calcium and phosphate to promote the formation of fluorapatite in demineralised areas. The ability of NaF to facilitate this process is well-supported in the literature and contributes to its inclusion in many toothpaste formulations as a key remineralising agent (Bataineh et al., 2017). According to an *in vitro* experiment that evaluated the effect of remineralisation of carious lesions and fluoride uptake by enamel, found that enamel remineralisation and fluoride uptake was significantly greater when using NaF compared to any other fluoride dentifrice (Hattab, 2013).

The effect of toothpaste slurry group on enamel subsurface lesion remineralisation showed the highest remineralisation that was achieved when compared to all other groups. In addition, statistical significance was found between baseline, at 7, 14 and

21 days after treatment in all QLF parameters. Statistical significance was noted between toothpaste slurry and all other groups. The only parameter ΔQ lesion (lesion volume) and area of the lesion did not show statistical significance between toothpaste slurry and alternate soaking process Ca/P. This correlates with the ability of alternate soaking process to remineralising artificial lesions but not as fluoride.

The effect of fluoride in remineralisation is well established, longitudinal epidemiological studies showed that fluoride might inhibit complete lesion remineralisation (Dirks, 1966; Groeneveld et al., 1990). However, one of limitation of fluoride toothpaste according to ten Cate et al., (1981) discussed the topical application of high concentration of fluoride can result in only initial high deposition of minerals followed by lower deposition in the enamel lesion which means that a high mineral deposition in the body of the lesion was found when no fluoride was added to the remineralising solution (Arends and Ten Cate, 1981). In this study, alternate soaking process allowed gradual deposition of calcium ions first and then followed by phosphate ions, this prevents the initial high deposition of minerals. One study confirmed that when calcium is applied followed by phosphate (not together) will allow high depositions of minerals (Abou Neel et al., 2016).

To compare, CPP-ACP uses calcium and phosphate to promote remineralisation and act as a reservoir for calcium and phosphate, which is similar to the current study in terms of the use of Ca/P. It also prevents ion dissolution and maintains solution supersaturation, thus promoted deeper enamel remineralisation. CPP-ACP has been introduced into various oral health products, including Tooth Mousse and its fluoride-containing counterpart, Tooth Mousse Plus (CPP-ACPF), both manufactured by GC in Tokyo, Japan (Bataineh et al., 2017; Hamba et al., 2020). Over time, these products have found their place in clinical settings and have been evaluated across several clinical trials (Sitthisettapong et al., 2015; Thierens et al., 2019). Despite their growing use, the extent to which CPP-ACP and Casein Phosphopeptide–Amorphous Calcium Phosphate with Fluoride (CPP-ACPF) contribute to the remineralisation of white spot lesions (WSLs) remains uncertain. Although some researchers have suggested potential benefits in preventing and managing WSLs, the evidence indicates that their effects may not be significantly more effective than fluoride-based treatments alone (Pithon et al., 2015; Wang et al., 2020). However, one study demonstrated that both the 1450 ppm fluoride combined with CPP-ACP (Tooth Mousse) and CPP-ACFP (MI Paste Plus) significantly promoted remineralisation of enamel subsurface lesions, as reflected by improvements in all QLF parameters (ΔF, ΔQ, and lesion area), when compared to the baseline and the fluoride-free control group. Although these groups also showed greater remineralisation than the groups treated with 1450 ppm or 2800 ppm fluoride alone, the differences were not statistically significant. This lack of significance may be attributed to the application of fluoride toothpaste prior to CPP-ACP or CPP-ACPF may have contributed to the formation of a fluorapatite-rich surface layer, potentially blocking lesion pores and restricting the diffusion of calcium and phosphate ions deeper into the enamel to support further remineralisation (Bataineh et al., 2017). However, in the current study all QLF parameters for the alternate soaking process did show statistical significance when compared to all the other groups and throughout the period. This suggests that the method of delivery of calcium followed by phosphate has promising results.

Studies reported that using CPP-ACP or CPP-ACFP creams on their own significantly enhanced enamel remineralisation compared to both fluoride treatments and negative controls (Jayarajan et al., 2011; Zhang et al., 2011). In contrast, another study explored the effects of these creams when used alongside 1450 ppm fluoride toothpaste (Bataineh et al., 2017). Supporting this approach, Kumar et al (2008) observed an additive remineralising effect when CPP-ACP was applied following fluoride toothpaste, although the statistical significance of this effect was not clearly reported (Kumar et al., 2008). The ability of CPP-ACP to support enamel repair has been attributed to its localisation within the dental plaque matrix, where it increases calcium and phosphate availability, as well as its ability to bind to bacterial surfaces, creating a bioavailable reservoir of calcium ions (Reynolds, 2008).

6.9.1.3. Effect of Tris-Hydrochloride on remineralising enamel subsurface lesions

The Tris-HCL group in this study functioned as a negative control. This is because Tris-HCL was part of alternate soaking process reagent mixture and to understand whether it can remineralise enamel lesions if applied alone. In the alternate soaking model, Tris-HCL was used to remove any excess of calcium ions and then soaking the enamel slabs in phosphate to remove any excess before the start of the second cycle. Moreover, this confirms that Tris-HCL acted as a buffering agent, and it does not contribute directly to enamel remineralisation. Its primary role lies is stabilising the solution of pH, creating a controlled environment necessary for the alternate soaking process (Matsusaki et al., 2009). This will prevent conditions that might otherwise lead to calcium-phosphate precipitation on the enamel surface (Abou Neel et al., 2016).

No remineralisation was achieved in Tris-HCL group throughout the period and Tris-HCL failed to produce remineralisation when compared with other groups. Also. It did not reach significant level.

6.9.1.4. Effect of day-time artificial saliva on remineralising enamel subsurface lesions

In this study, day-time artificial saliva showed minimal statistical significance in ΔF, ΔQ and Area parameters. Furthermore, when the day-time saliva was compared between baseline, 7, 14, and 21 days, it did show statistical significance in ΔF, ΔQ and Area parameters.

Statistical significances were seen when alternate soaking process Ca/P and toothpaste slurry F were compared to day-time saliva. This means day-time artificial saliva did not perform as well as alternate soaking process and toothpaste slurry F. This could be due to the fact day-time artificial saliva is supersaturated with different minerals that can cause remineralisation but not as the high content of calcium and phosphate as in the alternate soaking process and sodium fluoride in toothpaste slurry. However, no statistical significance when day-time saliva was compared to tris-HCL which explains that there is no difference in remineralisation noted.

6.10. Micro-computed tomography (micro-CT)

Micro-computed tomography (Micro-CT) have been recently used as a visualisation method to demonstrate three dimensional (3D) images (Zou et al., 2011). As mentioned previously, micro-CT is excellent as its non-destructive and can provide qualitative and quantitative analysis. Micro-CT can be used to analyse mineralised structures like enamel and bone which can evaluate the morphology and mineral density (Palmer et al., 2008). Robust studies have used Micro-CT due to its high resolution to provide accurate analysis (Shen et al., 2011).

In this study, comparison of enamel lesions at baseline and after treatment at 21 days to evaluate the effect of different treatment remineralisation. Micro-CT can obtain steady virtual cutting/ sectioning along its thickness where disarray induced by physical cutting. Studies showed that Micro-CT cutting is thinner than actual cutting device (Davis and Wong, 1996). Using Micro-CT made it possible to measure and visualise longitudinal mineral changes during demineralisation and remineralisation in the same lesion using a technique called 3D registration (Konagala et al., 2020).

6.10.1. Results of Micro-computed tomography (micro-CT)

In the present study, the demineralised window of the enamel lesions was observed to extend across approximately 200–300 μm of enamel thickness. This can be compared with the study by Hoxie et al., in which micro-CT mineral density profiling was performed within the most superficial 250 μm of enamel (Hoxie et al., 2023). Therefore, the lesion depth range assessed in the present study is broadly comparable to the depth used by Hoxie et al., as both focus on the superficial enamel region where early subsurface demineralisation is typically identified. It should be noted that both studies refer to the depth range selected for mineral density analysis rather than the exact lesion depth itself, where both describes the observed extent of the demineralised area. This has been reduced in the alternate soaking process Ca/P and toothpaste slurry F groups. The mineral density has also increased in toothpaste slurry. There is an average increase above 2 g/cm^3 from 0-300 μm in toothpaste slurry group. In the other hand, the alternate soaking process group showed an increase of approximately above 1 g/cm^3 from 0-300 μm. This indicate that both groups had remineralisation potential, however, toothpaste slurry group was higher.

Studies have explained the ability of the toothpaste containing fluoride to remineralise enamel subsurface lesion, however, recent studies explained the need of calcium phosphate to increase the remineralisation potential (Bhat et al., 2022; Hamba et al., 2020; Thimmaiah et al., 2019). Using the alternate soaking method that was explained previously can be an excellent method of delivery of calcium and phosphate as it reverses the mechanism of demineralisation. A study that explained the phenomena of demineralisation suggested the best way to improve remineralisation is to reverse demineralisation by allowing calcium penetration first followed by phosphate (Abou Neel et al., 2016). Although fluoride enhances enamel resistance by promoting fluorohydroxyapatite formation at the surface, this mechanism restricts deeper ion penetration and therefore offers limited effectiveness in remineralising deeper subsurface lesions (Amaechi et al., 2019).

A limitation of the micro-CT mineral density analysis is that the change observed in the groups, may not fully represent the true biological pattern of enamel remineralisation. In enamel lesions, mineral deposition is generally expected to occur in a surface-dominant or subsurface gradient. Therefore, the large apparent increase in mineral density may have been influenced by image or calibration artefacts.

6.11. Energy dispersive x-ray (EDX)

The scanning electron microscope produces highly magnified images through the interaction between the electron beams with the specimen surface. EDX is a detector on SEM that is commonly used to demonstrate the chemical and elemental analysis of a sample to determine the weight percentage ratio and composition. It can act as a semi-quantitative as it provides elemental ratios (Hegde and Moany, 2012).

6.11.1. Results of Energy dispersive x-ray Spectroscopy (EDX)

In the current study, EDX imaging was used to assess the changes of the enamel subsurface lesion to various remineralising treatments.

Studies have shown that low mineral content in enamel is susceptible to dental caries(Shellis, 1984; Targino et al., 2011). Further to this, the degree of chemical composition and the porosity determines the solubility of the enamel mineral content with demineralisation. A study discussed that increase in mineral content can only be seen in *in vitro* conditions, thus this overlooked the physiology of the human oral cavity (Shaik et al., 2017). However, in this study, no pH cycling was added to the protocol as this study aimed to see the feasibility of the alternate soaking process. EDX measurements (element weight percentage and the weight gain) of the enamel lesions demonstrated to have increased percentage in the element weight in both alternate soaking process Ca/P and toothpaste slurry F group.

The mineral gain for both alternate soaking process Ca/P and toothpaste slurry F are higher than artificial day-time saliva and Tris-HCL. These finding are good in agreement with results of the QLF and Micro-CT except for day-time saliva.

From this results EDX suggests that in this present study, penetration of calcium, phosphate and fluoride into the subsurface enamel lesion. This confirms that remineralisation and penetration of calcium and phosphate using alternate soaking process (at least superficial) is observed.

There was statistical significance between all the groups except for tris-HCL versus day-time saliva which explains that there is no difference in remineralisation noted. The Tris-HCL had the lowest percentage of mineral gain to all other groups. In contrast, toothpaste slurry F showed to have the highest mineral gain percentage compared to all other groups followed by alternate soaking process. This explains that remineralisation using the alternate soaking process has been achieved alongside the toothpaste slurry F.

In summary, in the present *in vitro* study, four groups were assessed for their effect on the remineralisation of enamel subsurface lesions including alternate soaking process (calcium and phosphate dissolutions, toothpaste slurry 1450 ppm F, day-time artificial saliva, and Tris-hydrochloride). The remineralisation of the enamel subsurface lesions was observed in alternate soaking process, toothpaste slurry 1450 ppm F and followed by minimal remineralisation in day-time artificial saliva. However, no remineralisation was noted in Tris-hydrochloride group.

6.13. Suggestions for Future Research

The results of the current *in vitro* study showed that there were significant benefits of the alternate soaking process of calcium and phosphate solutions on remineralising enamel subsurface lesions for a period of 21 days.

In this study alternate soaking process were applied on enamel subsurface lesion without pH cycling. It would be interesting to see the same study was repeated, but with a longer period of cycling e.g. 60, 90 and 120 days to see if further remineralisation is possible. Alternatively, the sample size could be increased, and this may show difference between groups. Increasing the sample size may enhance statistical power, improve the precision and reliability of the results, and provide a more accurate representation of biological variability, thereby strengthening the validity between-group comparisons. Studies are needed to assess the achievement of similar findings with fewer cycles and whether this would reach clinical application. Further studies are needed to investigate the effect of alternate soaking process under pH cycling regime conditions as pH cycling in enamel studies is used to simulate what happens in the mouth and to test interventions for caries prevention (Bataineh et al., 2017). It would be also interesting to demonstrate the alternate soaking process in an *in vivo* or *in situ* conditions.

Despite the superior remineralisation performance observed with the toothpaste slurry, the alternate soaking technique remains a valuable approach with potential for further product development. Fluoride-based systems are well established for enhancing surface remineralisation; however, their action is largely associated with the formation of a highly mineralised surface layer, which may restrict deeper ion diffusion into subsurface lesions (Al-Sanabani et al., 2013).

In contrast, the alternate soaking approach promotes repeated exposure to calcium- and phosphate-rich environments, which may facilitate gradual mineral deposition and potentially support more homogeneous remineralisation within subsurface regions, although this requires further investigation. Therefore, while fluoride remains the gold standard for rapid and robust remineralisation, alternate soaking systems may offer complementary benefits by targeting different mineralisation dynamics.

The alternate soaking process was compared to 1450 ppm F toothpaste slurry, artificial saliva, and tris-HCL. Suggestion of further studies to investigate the effect of commercially available calcium and phosphate solutions compared to alternate soaking process. It would be interesting to investigate whether there would be any difference in the results. The comparison may reveal which approach deposits more minerals or produces better enamel remineralisation. When compared with existing CPP-ACP products, the alternate soaking system demonstrates both similarities and potential advantages. CPP-ACP products function by stabilising calcium and phosphate ions and maintaining them in a bioavailable state at the tooth surface, enabling remineralisation under favourable conditions (Thierens et al., 2019). Similarly, the alternate soaking approach provides controlled delivery of mineral ions; however, it does so through repeated, timed exposures rather than continuous presence (Watanabe and Akashi, 2008).

Furthermore, different studies are needed to see if different use of scaffolds like hydrogels as a method of delivery of calcium and phosphate would change the results. Also, future research may incorporate the use of human saliva to assess whether it influences the action alternate soaking process.

It would be valuable for future studies to apply the same protocol using human enamel substrates, whether from primary or permanent teeth as opposed to the bovine samples utilised in the current study. Using human enamel would increase the clinical relevance, accuracy, and applicability of this study.

From a clinical perspective, patient compliance is a critical factor influencing the success of remineralising products (Bhat et al., 2022). While the experimental alternate soaking protocol involves multiple cycles that may not be directly translatable to routine patient use, the underlying concept could be adapted into simplified, time-efficient application methods, such as short-duration rinses. However, this requires further investigation.

Furthermore, there is considerable potential for enhancing the clinical applicability of the alternate soaking system through incorporation of fluoride. Combining fluoride with calcium- and phosphate-based alternate soaking solutions could synergistically enhance remineralisation by promoting fluorohydroxyapatite formation while maintaining ion availability for subsurface mineral uptake (Amaechi et al., 2019). Such a hybrid approach may overcome the limitations of fluoride-only systems by supporting both surface strengthening and deeper lesion remineralisation. Future studies should therefore investigate fluoride-enriched alternate soaking protocols to optimise remineralisation efficacy while maintaining practicality for clinical and at-home use.

6.14. Outcome of the null hypothesis

The null hypothesis "There is no difference in the enamel remineralisation that results from alternate soaking process, toothpaste slurry 1450 ppm F, day-time artificial saliva, and Tris-HCL" should be rejected as significant differences were found in the enamel remineralisation between the toothpaste slurry 1450ppm F and the alternate soaking process, day-time artificial saliva and Tris-hydrochloride groups.

Chapter 7 Conclusion

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

1. A significant remineralisation of enamel subsurface lesions was found after treatment in groups: alternate soaking process Ca/P, toothpaste slurry 1450 ppm F, and day-time artificial saliva using QLF analysis.
2. A significant remineralisation of enamel subsurface lesions was found after treatment in groups: alternate soaking process Ca/P and toothpaste slurry 1450 ppm F in Micro-CT and EDX analysis.
3. In comparison between the groups a significant remineralisation was achieved for alternate soaking process Ca/P and toothpaste slurry 1450 F groups compared with Tris-HCL and day-time artificial saliva groups.
4. The novel alternate soaking process Ca/P treatment resulted in greater remineralisation than saliva alone *in vitro*.
5. Alternate soaking process with Ca/P as a treatment could offer an alternative or enhancement to current prophylactics.

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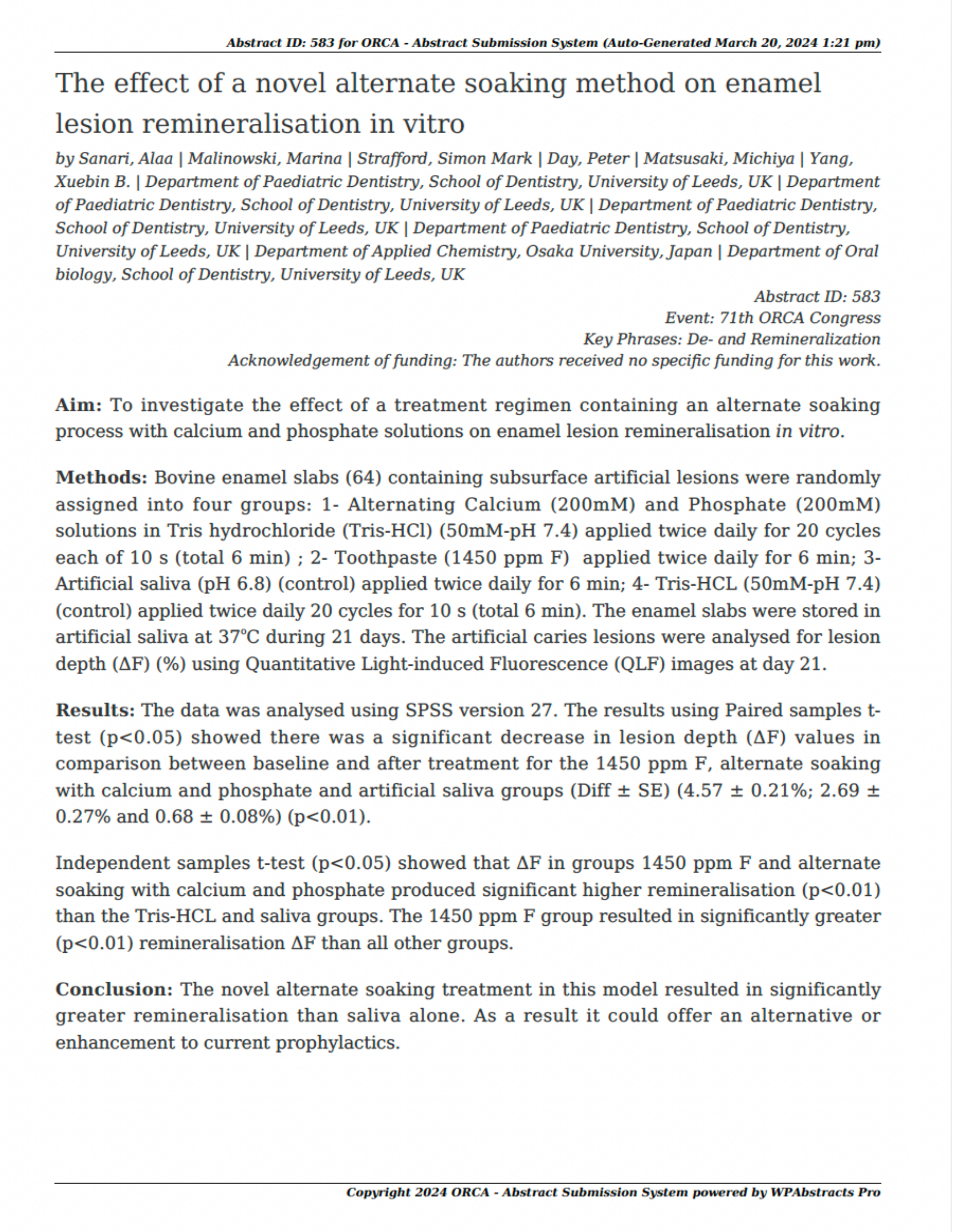
Appendix 1:Abstract of the Oral Presentation at ORCA conference (July 2024)



Abstract ID: 583 for ORCA - Abstract Submission System (Auto-Generated March 20, 2024 1:21 pm)

The effect of a novel alternate soaking method on enamel lesion remineralisation in vitro

by Sanari, Alaa | Malinowski, Marina | Strafford, Simon Mark | Day, Peter | Matsusaki, Michiya | Yang, Xuebin B. | Department of Paediatric Dentistry, School of Dentistry, University of Leeds, UK | Department of Paediatric Dentistry, School of Dentistry, University of Leeds, UK | Department of Paediatric Dentistry, School of Dentistry, University of Leeds, UK | Department of Paediatric Dentistry, School of Dentistry, University of Leeds, UK | Department of Applied Chemistry, Osaka University, Japan | Department of Oral biology, School of Dentistry, University of Leeds, UK

Abstract ID: 583
Event: 71th ORCA Congress
Key Phrases: De- and Remineralization
Acknowledgement of funding: The authors received no specific funding for this work.

Aim: To investigate the effect of a treatment regimen containing an alternate soaking process with calcium and phosphate solutions on enamel lesion remineralisation *in vitro*.

Methods: Bovine enamel slabs (64) containing subsurface artificial lesions were randomly assigned into four groups: 1- Alternating Calcium (200mM) and Phosphate (200mM) solutions in Tris hydrochloride (Tris-HCl) (50mM-pH 7.4) applied twice daily for 20 cycles each of 10 s (total 6 min) ; 2- Toothpaste (1450 ppm F) applied twice daily for 6 min; 3- Artificial saliva (pH 6.8) (control) applied twice daily for 6 min; 4- Tris-HCL (50mM-pH 7.4) (control) applied twice daily 20 cycles for 10 s (total 6 min). The enamel slabs were stored in artificial saliva at 37°C during 21 days. The artificial caries lesions were analysed for lesion depth (ΔF) (%) using Quantitative Light-induced Fluorescence (QLF) images at day 21.

Results: The data was analysed using SPSS version 27. The results using Paired samples t-test ($p<0.05$) showed there was a significant decrease in lesion depth (ΔF) values in comparison between baseline and after treatment for the 1450 ppm F, alternate soaking with calcium and phosphate and artificial saliva groups (Diff ± SE) (4.57 ± 0.21%; 2.69 ± 0.27% and 0.68 ± 0.08%) ($p<0.01$).

Independent samples t-test ($p<0.05$) showed that ΔF in groups 1450 ppm F and alternate soaking with calcium and phosphate produced significant higher remineralisation ($p<0.01$) than the Tris-HCL and saliva groups. The 1450 ppm F group resulted in significantly greater ($p<0.01$) remineralisation ΔF than all other groups.

Conclusion: The novel alternate soaking treatment in this model resulted in significantly greater remineralisation than saliva alone. As a result it could offer an alternative or enhancement to current prophylactics.

Appendix 2: Abstract of the Oral Presentation at the Leeds Dental Institute Research Day (June 2024).

#1 Verbal Presentation

Alaa Sanaari

The Effect of a Novel Alternate Soaking Method on Enamel Lesion Remineralisation in vitro

Aim: To investigate the effect of a treatment regimen containing an alternate soaking

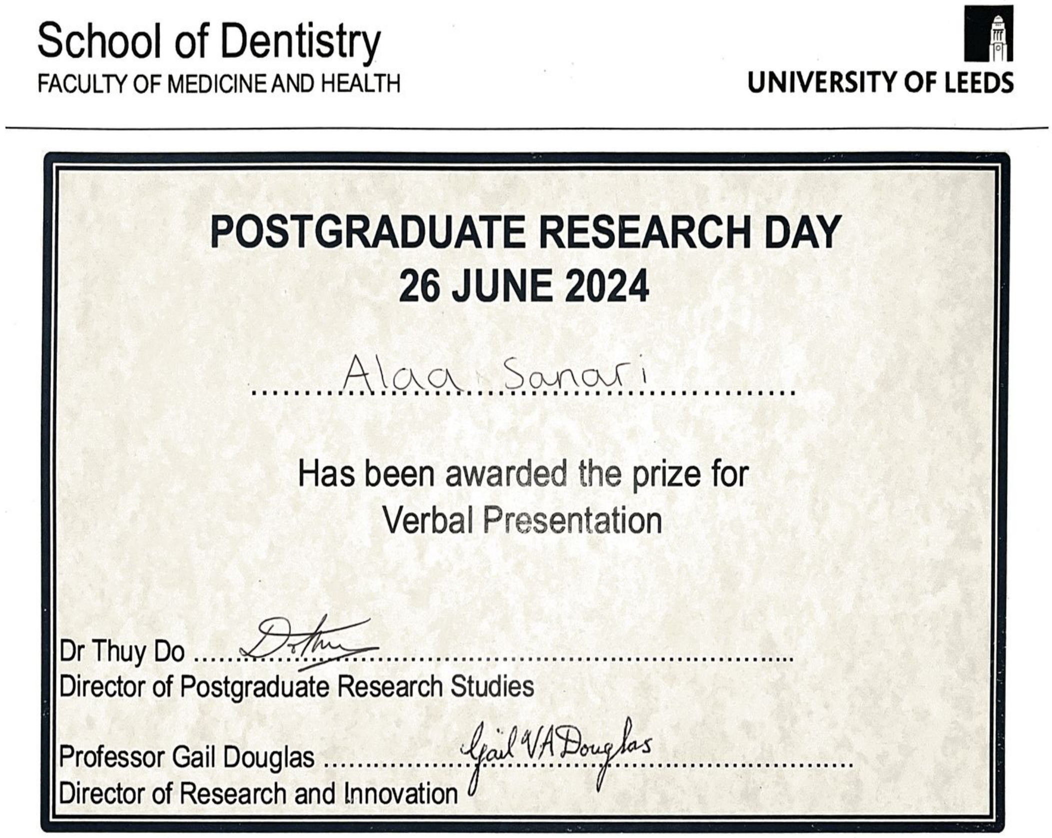
process with calcium and phosphate solutions on enamel lesion remineralisation in vitro.

Methods: Bovine enamel slabs (64) containing subsurface artificial lesions were randomly assigned into four groups: 1- Alternating Calcium (CaCl2) and Phosphate (Na2HPO4) solutions; 2- Toothpaste slurry (1450 ppm F); 3- Artificial daytime saliva; 4- Tris-HCL. Solutions were applied twice daily (total 6 min) for all groups. The enamel slabs were stored in artificial night-time saliva at 37oC during 21 days and were analysed for lesion depth (ΔF%), ΔQ and Area using Quantitative Light-induced Fluorescence (QLF) images.

Results: Using paired samples t-test ($p<0.05$) showed there was a significant decrease in lesion depth (ΔF) values in comparison between baseline and after treatment for the 1450 ppm F, alternate soaking with calcium and phosphate and artificial saliva (Diff ± SE) (4.57±0.21; 2.69±0.27 and 0.68±0.08) ($p<0.01$). In lesion volume (ΔQ) in comparison between baseline and after treatment there is statistical significance for 1450 ppm F, alternating Ca/P and saliva (607.44 ± 676.72; 555.8 ± 431.7; 1.81± 2.74) ($p<0.01$) compared to Tris-HCL which showed no statistical significance. The area of the lesion showed decrease in the lesion in groups 1450 ppm F, alternate soaking with calcium and phosphate and artificial saliva (Diff ± SE) (58106±311.28; 483.63±245.16; 9.56±24.80) more then Tris-HCL. The value of ΔF, ΔQ and Area in 1450 ppm F and alternate soaking with Ca/P produced significant higher remineralisation ($p<0.01$) than the Tris-HCL and saliva groups.

Conclusion: The novel alternate soaking treatment in this model resulted in significantly greater remineralisation than saliva alone.

Appendix 3: Certificate for best oral presentation at the Leeds Dental Institute Research Day (June 2024).



School of Dentistry
FACULTY OF MEDICINE AND HEALTH

UNIVERSITY OF LEEDS

POSTGRADUATE RESEARCH DAY
26 JUNE 2024

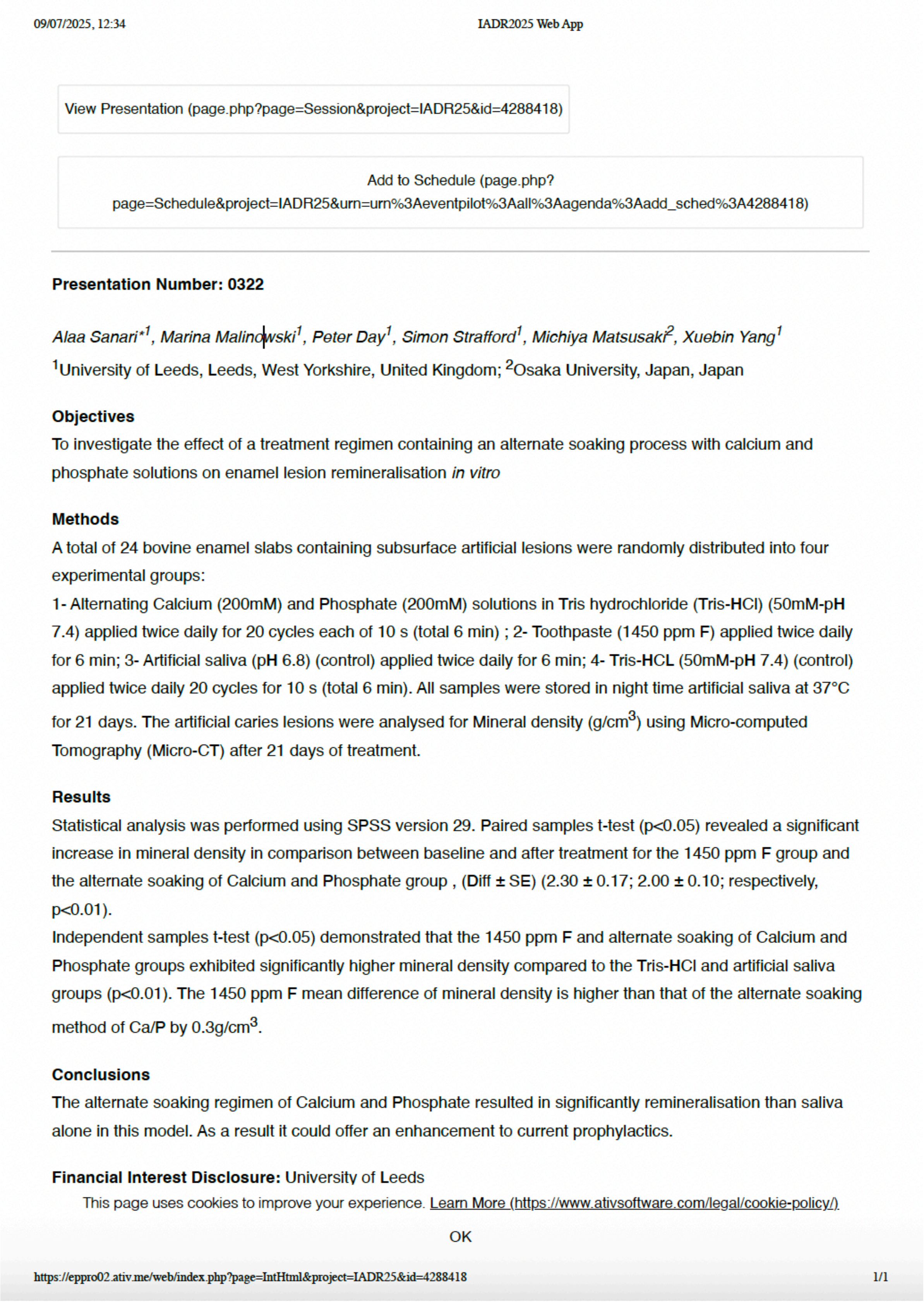
.........Alaa Sanari.........

Has been awarded the prize for
Verbal Presentation

Dr Thuy Do ....
Director of Postgraduate Research Studies

Professor Gail Douglas ....Gail VA Douglas..........
Director of Research and Innovation

Appendix 4: Abstract and Certificate of attendance and Oral Presentation at IADR Conference (June 2025).



09/07/2025, 12:34 IADR2025 Web App

Presentation Number: 0322

Alaa Sanari[1], Marina Malinowski[1], Peter Day[1], Simon Strafford[1], Michiya Matsusaki[2], Xuebin Yang[1]*

[1]University of Leeds, Leeds, West Yorkshire, United Kingdom; [2]Osaka University, Japan, Japan

Objectives

To investigate the effect of a treatment regimen containing an alternate soaking process with calcium and phosphate solutions on enamel lesion remineralisation *in vitro*

Methods

A total of 24 bovine enamel slabs containing subsurface artificial lesions were randomly distributed into four experimental groups:

1- Alternating Calcium (200mM) and Phosphate (200mM) solutions in Tris hydrochloride (Tris-HCl) (50mM-pH 7.4) applied twice daily for 20 cycles each of 10 s (total 6 min) ; 2- Toothpaste (1450 ppm F) applied twice daily for 6 min; 3- Artificial saliva (pH 6.8) (control) applied twice daily for 6 min; 4- Tris-HCL (50mM-pH 7.4) (control) applied twice daily 20 cycles for 10 s (total 6 min). All samples were stored in night time artificial saliva at 37°C for 21 days. The artificial caries lesions were analysed for Mineral density (g/cm^3) using Micro-computed Tomography (Micro-CT) after 21 days of treatment.

Results

Statistical analysis was performed using SPSS version 29. Paired samples t-test ($p<0.05$) revealed a significant increase in mineral density in comparison between baseline and after treatment for the 1450 ppm F group and the alternate soaking of Calcium and Phosphate group , (Diff ± SE) (2.30 ± 0.17; 2.00 ± 0.10; respectively, $p<0.01$).

Independent samples t-test ($p<0.05$) demonstrated that the 1450 ppm F and alternate soaking of Calcium and Phosphate groups exhibited significantly higher mineral density compared to the Tris-HCl and artificial saliva groups ($p<0.01$). The 1450 ppm F mean difference of mineral density is higher than that of the alternate soaking method of Ca/P by $0.3 g/cm^3$.

Conclusions

The alternate soaking regimen of Calcium and Phosphate resulted in significantly remineralisation than saliva alone in this model. As a result it could offer an enhancement to current prophylactics.

Financial Interest Disclosure: University of Leeds

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FOR DENTAL, ORAL, AND
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VERIFICATION OF ATTENDANCE AND PRESENTATION

The International Association for Dental, Oral, and Craniofacial Research verifies that:

Alaa Sanari

attended the 2025 IADR/PER General Session & Exhibition in Barcelona, Spain, June 25-28, 2025, and presented the research listed on the following page:

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Activity Transcript

IADR2025

Issued to **Alaa Sanari** on Jun 26, 2025

Date	Title	Type
Thu, Jun 26 11:00am - 12:15pm (Europe/Madrid)	**The Effect of Alternate Soaking Method on Enamel Lesion Remineralisation in Vitro**	Poster Session

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