Inhibition of Tooth Demineralization by CPP-ACP and NaF: An *In Vitro study* with MicroCT

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1. Introduction

Dental erosion is a common lesion that affects the tooth substance. A high percentage of the young population has dental erosion of varying degree on one or more tooth surfaces. A study from 2014 with participants aged between 16-18 in Norway showed 59% had dental erosions on at least one surface, 44% limited to enamel, while 54% had a combination of affected enamel and dentin.[1] This is a population in the Northern region of Norway.

A study population "Fit Futures" int 2016, showed that: 38% of 16-year-olds, had dental erosions on at least one tooth surface. 18% of these were dental erosion limited to the enamel, while 20% were erosions exposing dentin. A study from 2021 based on 5 year-olds in Bergen in Norway showed 80% to have dental erosions on at least one surface.[2]

It is important to deliver sufficient information about dental erosions for early diagnosis and effective preventive treatment.[3]

1.1 Fluoride

The Norwegian directory of health advice patients in risk of dental erosions to use fluoride products in addition to the daily brushing with fluoride toothpaste. The directory further recommends these additional fluorides to preferably be Stannous fluorides (SnF_2) based on In Situ studies showing the potential of slowing the progression of dental erosions.[4] However, these products are not easily accessible or widely known to the Norwegian public[4] and have a side effect of staining the teeth with discoloration.[5] There is therefore a need of more research done on other agents to aid in preventing and slowing the progression of dental erosions.

The three main effects from fluoride are to strengthen the enamel, help the saliva neutralize low pH and inhibit cariogenic bacterial activity. Strengthening of the dental enamel happens by fluoride substituting the hydroxy-group in hydroxyapatite ($Ca_5(PO_4)_3OH$) creating fluorapatite ($Ca_5(PO_4)_3F$), and reacting with surface calcium ions (Ca) to create calcium-fluoride (CaF_2) on the tooth surface as a protective layer[6]. The critical levels of demineralization of hydroxyapatite are between pH 5.5 – 6, compared to fluorapatite between pH 4.0 – 4.5 [7]. Calcium-fluoride is not easily soluble but will release fluoride-ions when solved, which can again bind to exposed Ca-groups and stop further demineralization or promote remineralization by substituting hydroxy groups in hydroxyapatite.[6]

The root surface is more vulnerable to demineralization by acids due to lower mineralization. Cementum and dentin are both less mineralized than enamel. Thus, demineralization occurs at pH 6.0-6.7 for cementum and dentin. [7, 8]

1.2 A novel agent for preventing demineralization

Casein Phosphopeptide - amorphous calcium phosphate (CPP-ACP) is a complex found and derived from cow's milk. Casein Phosphopeptide (CPP), is a peptide capable of binding calcium by stabilizing amorphous calcium phosphate (ACP) in a solution, thus creating CPP-ACP as a complex. CPP-ACP can release its ACP group and thereby increase saturation of calcium and phosphate in saliva which will help buffer the pH level. CPP can also help the released ACP group to bind with the enamel and dentine which will promote remineralization.[9]

There are many studies using different dentifrices to assess mineralization of enamel and root-caries lesions. The methods used were electric caries monitor, transversal micro-radiography, and micro-computed tomography [10-13]. A study conducted on bovine incisors found CPP-ACP and fluoride (CPP-ACPF) to have a greater effect than CPP-ACP alone on inhibiting enamel demineralization [14]. Another study found no significant difference between CPP-ACP and fluoride gel against enamel demineralization [15]. A third study found CPP-ACPF to be most promising in the release of ions compared to fluoride alone or bioactive glass [16]. A systematic review and meta-analysis done on calcium based preventive agents concluded that CPP-ACP/CPP-ACPF could be considered used as an addition to fluoride, but not yet as a replacement due to lack of clinical trials.[17]

The aim of the study is to compare the ability of CPP-ACP and NaF to reduce tooth demineralization.

2. Materials and Methods

2.1 Specimen preparation

Ten extracted permanent-molars were collected from the University Dental Clinic (Universitetstannklinikken, UTK), and surgical department at the Public Dental Service Competence Centre of Northern Norway in Tromsø (Tannhelsetjenestens kompetansesenter for Nord-Norge, TkNN). They were cleaned and removed of soft tissue, before being stored in a glass jar with 0.1% Chloramine T (Thymin) at 4 ℃. Afterwards, the crown parts were cut into a total of 40 pieces between 3-5mm using a cylinder-diamond bur handpiece (KaVO GENTLE power Lux 25LP 1:5 Contra-Angle Handpiece). The samples were stored in deionized water at 4 ℃ between cutting and scanning.

2.2 Specimen mineralization treatments

The 40 samples were allocated to four treatment-groups as shown in Table 1. Toothpaste containing 1450 ppm sodium fluoride (NaF) as the control group (1). Toothpaste containing 5000 ppm NaF (2). Tooth mousse containing CPP-ACP (3). Tooth mousse with CPP-ACP and 2% NaF (900pm) (4). The samples were coated individually in their given dentifrice for 30 minutes daily for 7 days for a total of 3,5 hours. After each 30-minute treatment, the specimens were rinsed with deionized water (MilliQ), and then stored in individual dram-glass vials with deionized water. The dentifrices and deionized water were renewed every day.

Table 1. Demonstration of 4 different products and their contents

Group/Brand	Active agent	Name
1. Colgate®	1450ppm NaF	Sensitive Pro-Relief™
2. Colgate®	5000ppm NaF	Duraphat® 5mg/g
3. Recaldent™	CPP-ACP	GC Tooth Mousse®
4. Recaldent™	CPP-ACPF (900ppm NaF)	GC MI Paste Plus®

2.3 Demineralization procedure

An acid buffer containing 0.244g $CaCl_2$, 0.303g NaH_2PO_4, and 2.86 ml glacial acetic acid (100%) was adjusted to pH 4.4 with KOH (1M). Each sample was placed in 10ml of the acid-buffer solution at 37°C. Samples were removed, rinsed in deionized water (MilliQ) and scanned after 24h, 74h, and 120h in the solution. The acid-buffer was replaced every 24h hours.

2.4 MicroCT-scanning

SkyScan 1272 MicroCT (Bruker-MicroCT, Kontich, Belgium) was used to scan the samples. The 01-mm aluminum filter was selected for the scanning, and flat-field calibration and exposure modification was performed before each scan. 10 Samples were mounted per cycle on a carousel controlled by the SkyScan software. Each scan had the following settings: 80kV source voltage, 125uA source current, 5um image pixel size, 0.4 degrees rotation step, frame averaging of 6, random movement of 15 and a 180 degrees rotation. The chronological scanning cycles are illustrated in Figure 1.

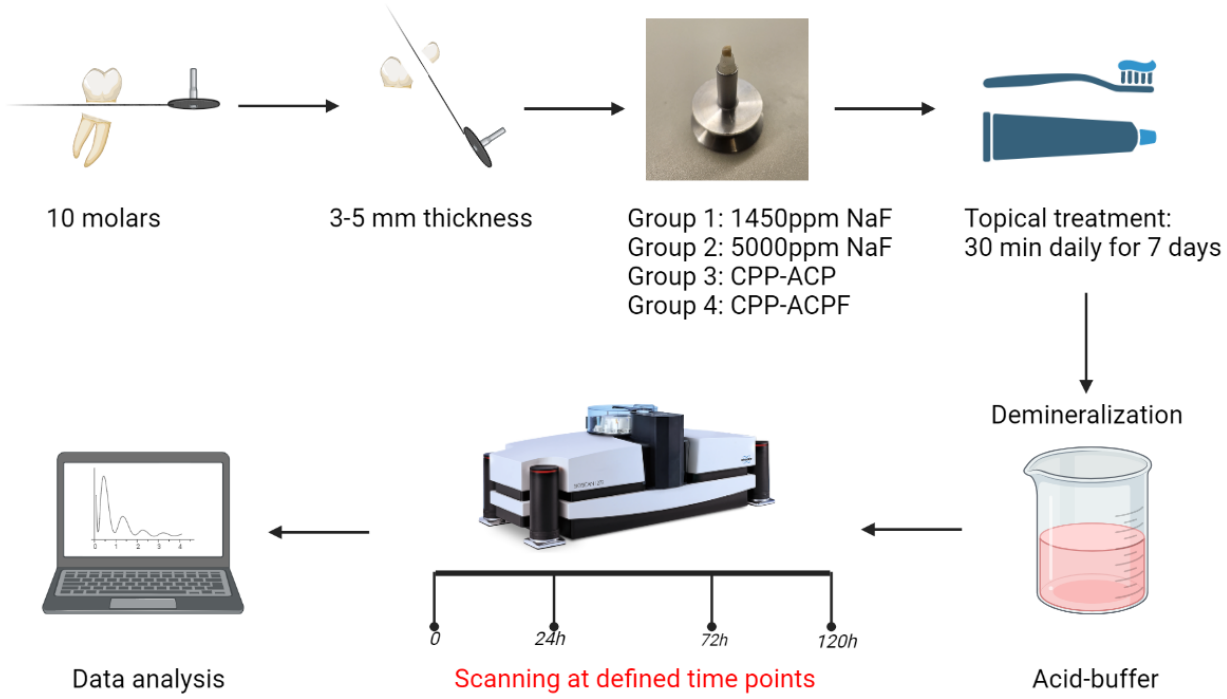


Figure 1. Flow chart of study design

2.5 Data analysis

After the scans were done, the images from each scanning were reconstructed using NRecon V1.7.2 software (Bruker-MicroCT, Kontich, Belgium). The same parameters were used for every sample in every group in each cycle: Output format 8-bit BMP, Dynamic range 0.000-0.100 (greyscale contrast), Smoothing 0, Beam hardening correction 80%, Ring artifacts reduction 3, Misalignment compensation -1,5. The images were then analyzed using CTAn 1.20.3.0 software (Bruker-MicroCT, Kontich, Belgium) to generate the values of the volume of each sample in all cycles. First, a thresholding was done to segment the foreground from background using a logarithmic scale from the image dataset. The first threshold was set between 30-255 to include all hard tissue, then "despeckling" was done to remove all pixels not attached to the largest object from the binary image. The Region of interest (ROI) was set with "Shrink wrap" of 2D space, then the ROI was converted to an image by "Bitwise operations", then the image was "Reloaded", then saved again in a separate folder as the Volume of interest (VOI) by "Save bitmaps". Now since the datasets were analyzed for two parts a second thresholding was done. For the combined analysis of total enamel and dentin, the lower grey threshold was set to 45, and the upper grey threshold was set to 255. For the analysis of enamel alone, the lower grey threshold was set to 140, and the upper grey threshold was set to 255. Finally, the 3D analysis was done to calculate the basic 3D parameters of the binary image whereby Object volume (mm^3) was of interest for further statistical analysis.

2.6 Statistical analysis

The Object volume (mm^3) from the output files were used to calculate total mineral change (TMC), and enamel mineral change (EMC) for each scanning point as percentages that were then used in the statistical analysis. TMC and EMC were calculated as means and standard deviations. All values were analyzed by One-way Analysis of variance (ANOVA) using Bonferroni comparison, with TMC or EMC as the response variable and treatment groups as factor variable. All statistical calculations and analysis were performed with STATA for windows (STATA Ver. 16.1, Copyright 1985-2019 StataCorp LLC, 4905 Lakeway Drive, Texas 77845 USA). The significance level was set to alfa = 0.005

2.7 Summary of methods

In this study, microcomputed tomography (MicroCT) was utilized to determine the inhibiting effect of CPP-ACP and NaF on demineralization of human teeth. We selected three different commercially available dentifrices to compare with a toothpaste containing the most common concentration of sodium fluoride used in Norway (1450 ppm).

3. Results

3.1 Enamel percentage

A one-way ANOVA was conducted to determine if the percentage of enamel was different between the groups. The ANOVA showed no statistically significant difference between groups ($F(3,36) = 1.18$ $p = 0.3293$).

3.2 Total mineral change (TMC)

Three one-way ANOVA's were conducted to determine if the TMC was different between the groups after 24h, 72h, and 120h in the acid-buffer solution. The total of 40 pieces were classified into four groups: 1450ppm NaF (n = 10), 5000ppm NaF (n = 10), CPP-ACP (n = 10) and CPP-ACPF (n = 10).

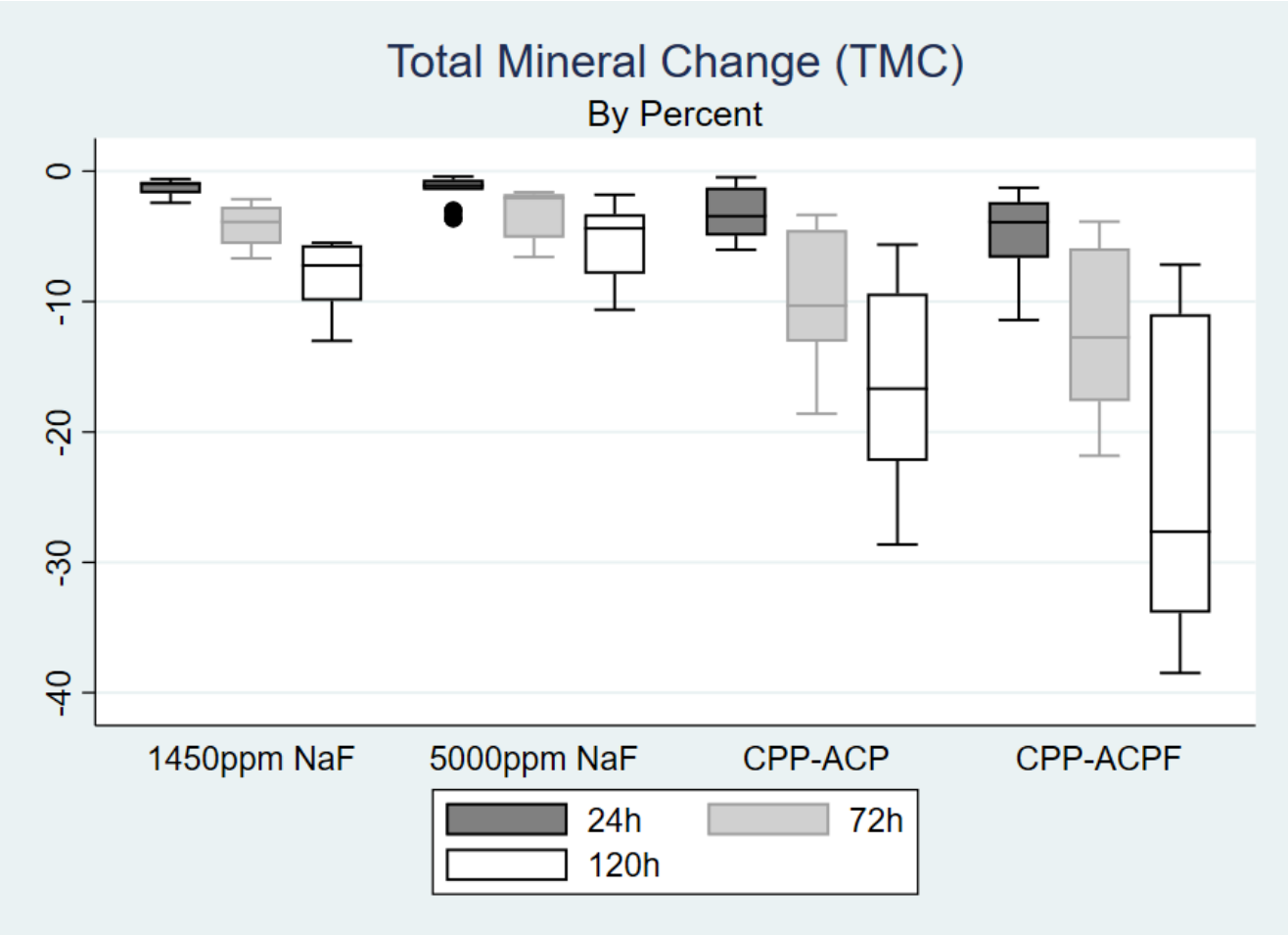


Figure 2. Combined boxplot with medians, interquartile ranges (box), minimum and maximum borders and outliers (dots), illustrating total TMC after 24 hours (dark grey), 72 hours (light grey) and 120 hours (white) acid exposure.

The first one-way ANOVA of TMC after 24h showed a statistically significant difference between groups ($F(3,36) = 6.84$, $p = 0.0009$). A Bonferroni comparison revealed that TMC was statistically significantly negative in the CPP-ACPF group compared to the 1450ppm NaF group (-3.59776 %, $p = 0.002$), the 5000ppm NaF group (-3.39224 %, $p = 0.004$), and CPP-ACP group (-1.5362 %, $p =0.617$).

The second one-way ANOVA of TMC after 72h showed a statistically significant difference between groups ($F(3,36) = 10.37$, $p = 0.0000$). A Bonferroni comparison revealed that TMC was statistically significantly negative in the CPP-ACP group compared to the 5000ppm NaF group (-6.57739 %, $p = 0.011$), the CPP-ACPF group compared to the 5000ppm NaF group (-9.42931 %, $p = 0.000$), and 1450ppm NaF group (-8.26078 %, $p =0.001$).

The third one-way ANOVA of the TMC after 120h showed a statistically significant difference between groups ($F(3,36) = 12.78$, $p = 0.0001$). A Bonferroni comparison revealed that TMC was statistically significantly negative in the CPP-ACPF group compared to the 1450ppm NaF group (-15.922 %, $p = 0.000$), the 5000ppm NaF group (-18.4116 %, $P = 0.000$), and the CPP-ACP group compared to the 5000ppm NaF group (-11.0794 % $p = 0.012$).

3.3 Enamel mineral change (EMC)

Three one-way ANOVA's were conducted to determine if the EMC was different between the groups after 24h, 72h, and 120h in the acid-buffer solution. The total of 40 pieces were classified into four groups: 1450ppm NaF (n = 10), 5000ppm NaF (n = 10), CPP-ACP (n = 10) and CPP-ACPF (n = 10).

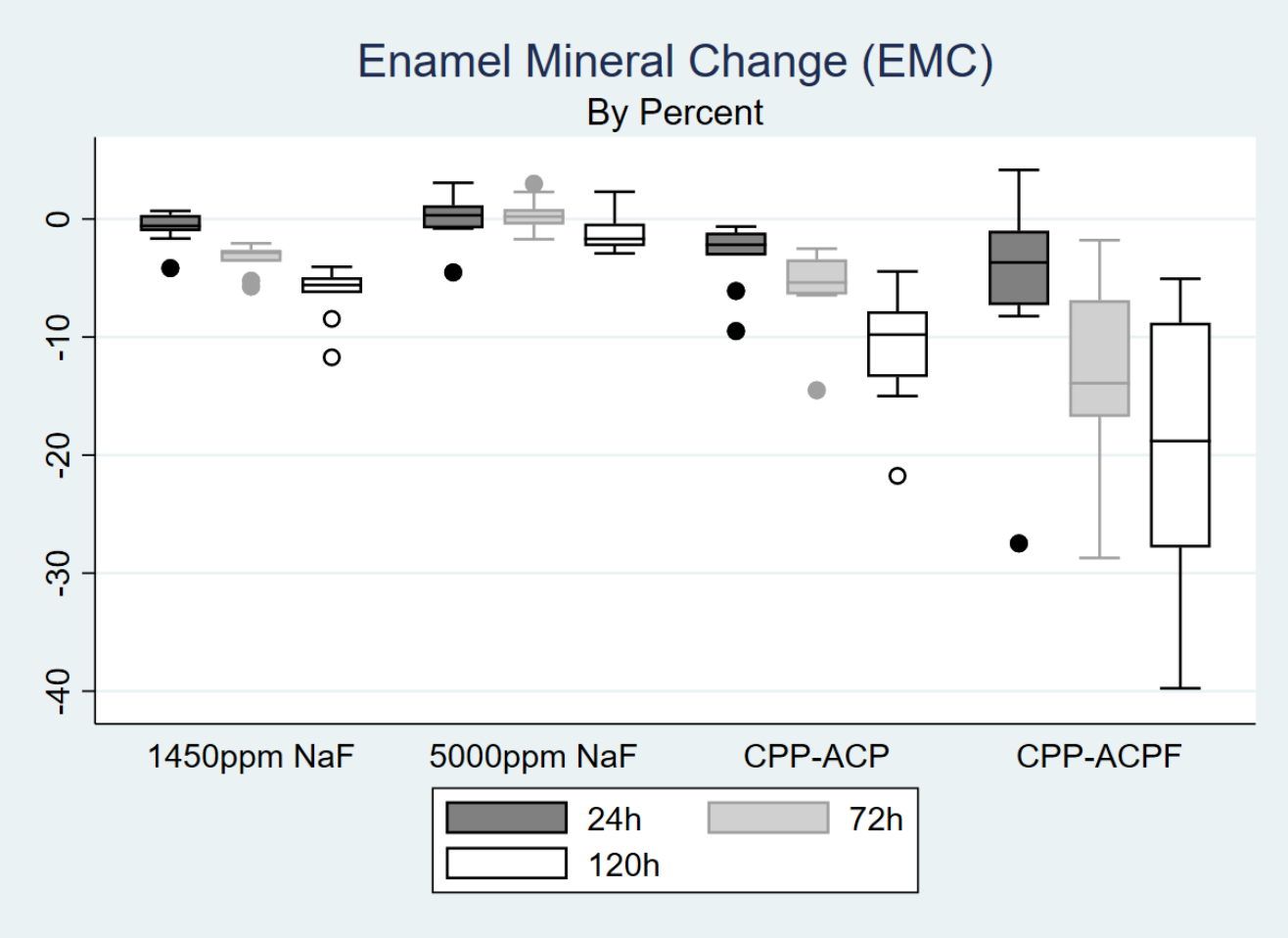


Figure 3. Combined boxplot with medians, interquartile ranges (box), minimum and maximum borders and outliers (dots), illustrating total EMC after 24 hours (dark grey), 72 hours (light grey) and 120 hours (white) acid exposure.

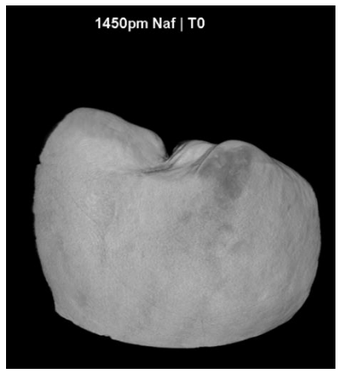
The first one-way ANOVA of EMC after 24h showed no statistically significant difference between groups ($F(3,36) = 2.03$, $p = 0.1276$).

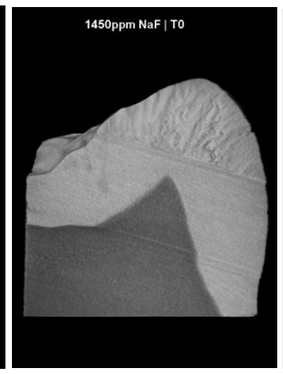
The second one-way ANOVA of the EMC after 72h showed a statistically significant difference between groups ($F(3,36) = 15.79$, $p = 0.0000$). A Bonferroni comparison revealed that EMC was statistically significantly negative in the CPP-ACP group compared to the 5000ppm NaF group (-6.1272 %, $p = 0.023$), the CPP-ACPF group compared to the 1450ppm NaF group (-9.55674 %, $p = 0.000$), the CPP-ACP group (-7.10074 %, $p = 0.006$), and the 5000ppm NaF group (-13.2279%, $p = 0.000$).

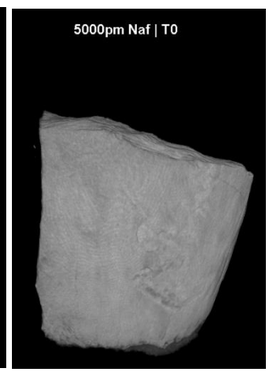
The third one-way ANOVA of the EMC after 120h showed a statistically significant difference between groups ($F(3,36) = 15.88$, $p = 0.0000$). A Bonferroni comparison revealed that EMC was statistically significantly negative in the CPP-ACP group compared to the 5000ppm NaF group (-9.71226 %, $p = 0.009$), the CPP-ACPF group compared to the 1450ppm NaF group (-13.692 %, $p = 0.000$), the 5000ppm NaF group (-18.8122 %, $p = 0.000$), and CPP-ACP group (-9.0999 %, $p = 0.017$).

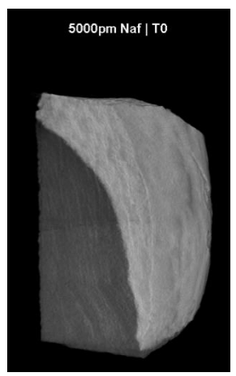
1450ppm NaF	5000ppm NaF	CPP-ACP	CPP-ACPF

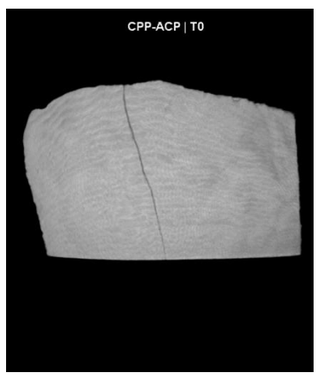
T0

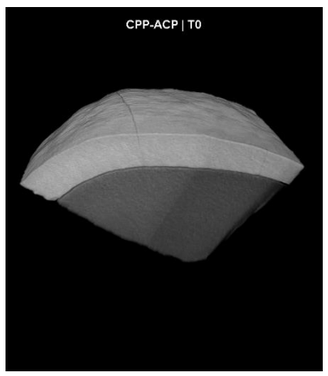


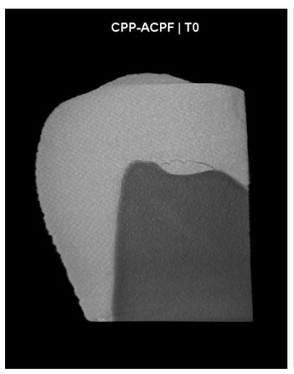


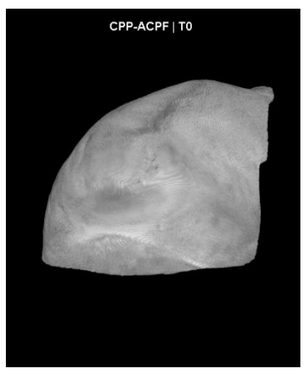




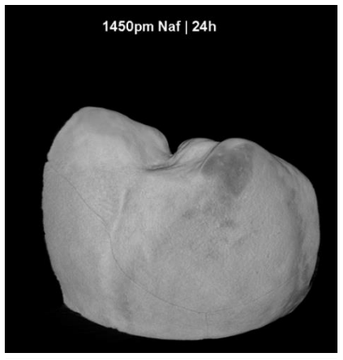


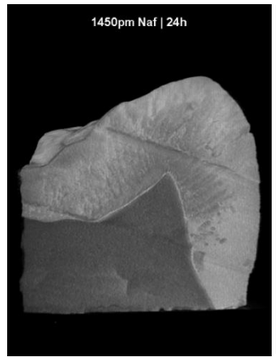


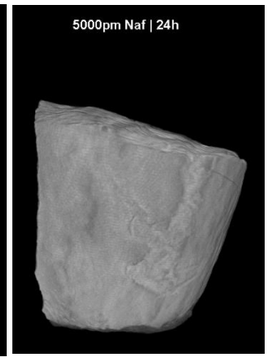


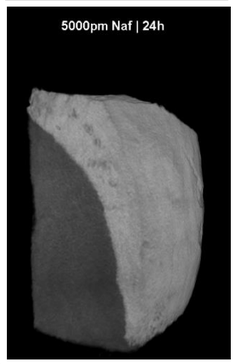


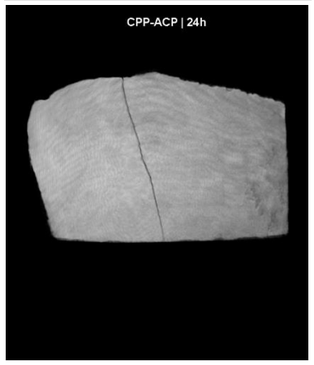
24h

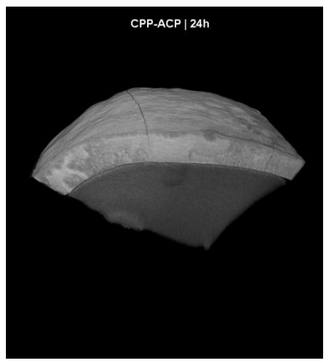


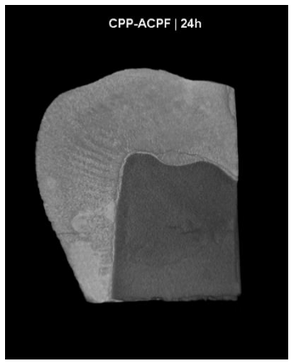


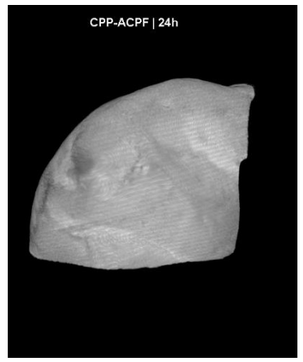




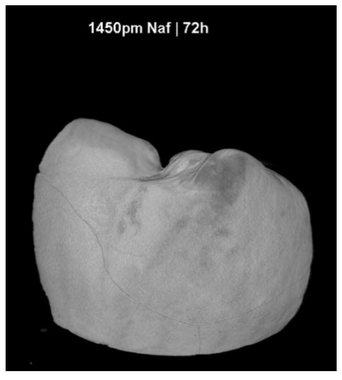


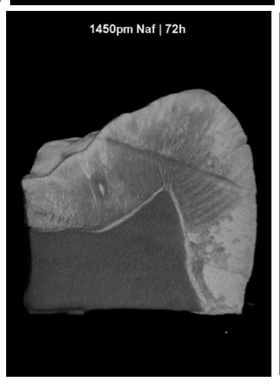


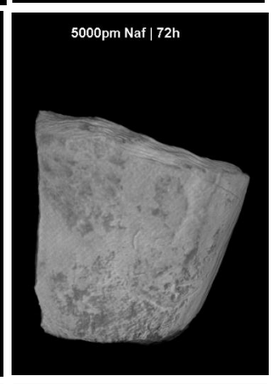


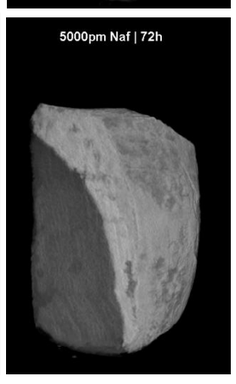


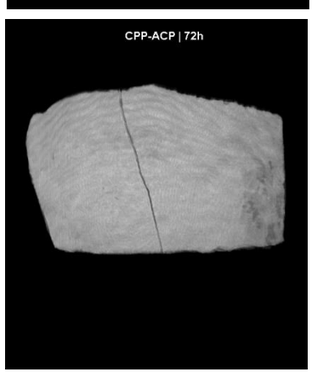
72h

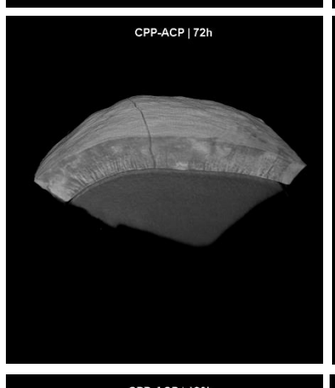


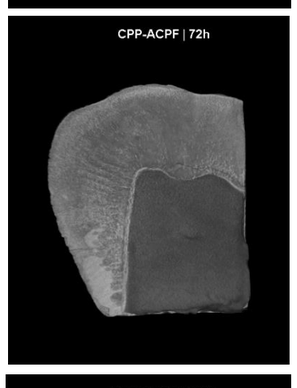


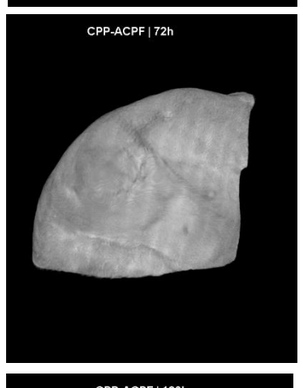












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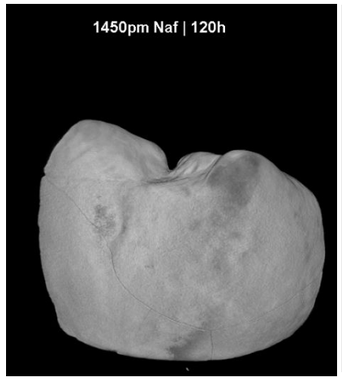

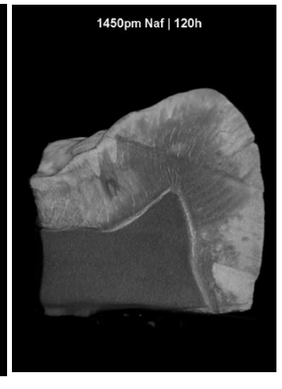

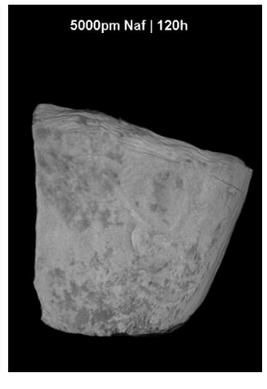

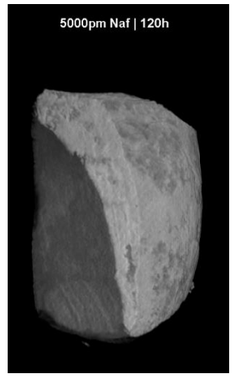

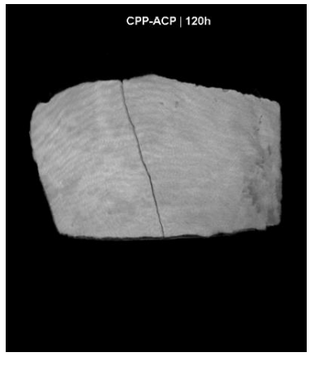

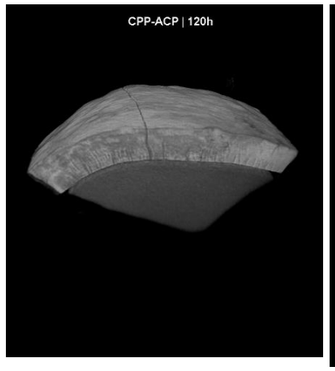

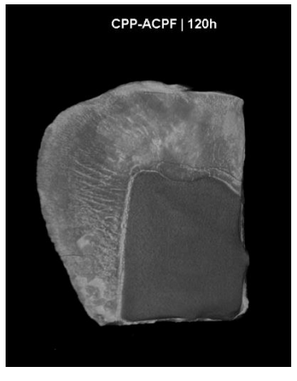

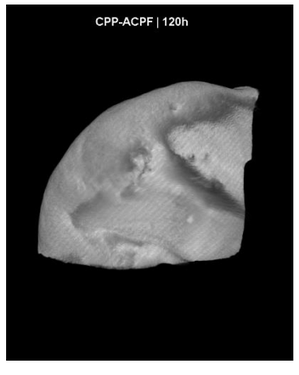

Figure 4. MicroCT-images of a sample from group 1 (1450ppm NaF) at each defined time point to visualize the loss of mineral.

1450ppm NaF timepoint	Mean (%)	Standard error (%)
TMC 24h	-1.224451	0.1959463
TMC 72h	-4.233937	0.5408439
TMC 120h	-7.981103	0.8232095
EMC 24h	-0.716829	0.4436187
EMC 72h	-3.343905	0.3786553
EMC 120h	-6.188537	0.7216392

Figure 2. Mean and standard error of TMC and EMC from group 1 (1450ppm NaF) at each defined time point.

5000ppm NaF timepoint	Mean (%)	Standard error (%)
TMC 24h	-1.429968	0.3329618
TMC 72h	-3.065408	0.5996093
TMC 120h	-5.491483	0.9864305
EMC 24h	-0.0148442	0.6212577
EMC 72h	0.3272873	0.4594231
EMC 120h	-1.068353	0.5734524

Figure 3. Mean and standard error of TMC and EMC from group 2 (5000ppm NaF) at each defined time point.

CPP-ACP timepoint	Mean (%)	Standard error (%)
TMC 24h	-3.286009	0.6306333
TMC 72h	-9.642803	1.589433
TMC 120h	-16.57092	2.400823
EMC 24h	-3.040768	.8689213
EMC 72h	-5.799908	1.065814
EMC 120h	-10.78062	1.578841

Figure 4. Mean and standard error of TMC and EMC from group 3 (CPP-ACP) at each defined time point.

CPP-ACPF timepoint	Mean (%)	Standard error (%)
TMC 24h	-4.822213	1.06637
TMC 72h	-12.49472	2.122786
TMC 120h	-23.9031	3.838706
EMC 24h	-5.422466	2.748497
EMC 72h	-12.90065	2.529916
EMC 120h	-19.88052	3.567709

Figure 5. Mean and standard error of TMC and EMC from group 4 (CPP-ACPF) at each defined time point.

5. Discussion

The 40 different pieces all varied in size and composition of enamel and dentin, As *Figure 4* illustrates, some pieces consisted of less than 20% enamel, while others consisted of above 80% enamel. Therefore, the values used to assess the effect of the individual agents were relative; expressed as the mean percent change in enamel for each group, instead of the mean absolute change. This means that if any specific group had significantly more percentage enamel as opposed to dentin than another group, any positive results might be attributed to this rather than the given mineralization agent. However, the results from the analysis of enamel percentage reveals that the enamel to dentin ratio does not significantly differ between the groups. Although not significant, the 1450ppm NaF group showed a higher mean enamel percentage and less variation than the other groups. The 5000ppm NaF group showed the highest variation, and the CPP-ACP and CPP-ACPF groups were almost identical.

Preparing the raw data files for statistical analysis was done in NRecon and CTAn. First, NRecon processed the raw TIFF-files into BMP-files with the settings expressed in the methods, then the BMP files were imported to CTAn. Further, a thresholding was done in CTAn to specify what part of the scan was to be used for calculating 3D parameters. After thresholding, a “despeckle” was done to remove any pixel not attached to the largest object. Despeckling was done for every sample after every scan and the program would remove different amounts of pixels every time. But in the analysis where enamel was segregated from dentin prior to despeckling, the command ither wrongfully removed more pixels from the baseline scan than the following scan or, removed to few pixels from the scan following the baseline scan. Both scenarios would result in the program making it seem like an individual sample had gained mineral after acid exposure. This happened to a few of the samples; some automatically marked as outliers by STATA.

The inhibiting effect of demineralization from the different mineralizing agents appear to be both time and tissue dependent. The TMC was significant between groups at all times of acid exposure, however, with increasing levels of significance the more acid exposure. The EMC on the other hand, was not significantly different between groups after 24h of acid exposure, but after 72h and 120h. This indicates that the inhibiting effect may have been stronger on the enamel then the dentin. An explanation may be in the structure difference between enamel and dentin, particularly in the way fluoride easily binds to calcium ions on enamel surface or substitutes the hydroxy group.

A study on bovine teeth showed a dose response between inhibition of demineralization and the fluoride content in a mineralizing agent[14]. The same study also found a concentration of 9000ppm NaF to have the lowest mean mineral loss than all other groups, and CPP-ACPF more effective than CPP-ACP. However, the dentin tubules in bovine teeth are of a wider diameter and higher number than in human teeth. This results in the radiodensity of coronal bovine dentin to be lower than human

teeth, in contrast to bovine enamel that is of a higher radiodensity than human enamel[18]. Bovine dentin may therefore have a larger capacity of absorbing fluorides than human teeth, resulting in different outcomes in studies using human teeth; all things being equal.

CPP-ACP used as a prevention against dental erosions seems to be more realistic than it being a form of treatment. Because of the time-limited inhibition effect of CPP-ACP, it might useful as an addition to fluoride only if taken often enough. The most important measure to treat dental erosions is finding and limiting the cause. Dental erosions are ither endogen or exogen, or a combination of both. Bulemia and gastro esophageal reflux are conditions that can cause dental erosions due to the strong stomach-acid. However, the greatest factor in the rise of dental erosions as a public health concern in scandinavia, is the increased consume of soft drinks and sports drinks.[19]

The treatment recommendations from the Norwegian government (by Helsedirektoratet.) for dental erosions are to use extra fluoride products like mouth wash, fluoride tablets and toothpaste containing CaCO3, KNO3 or arginine[4]. The recommendation also states stannous fluorides as the preferred fluoride addition based on limited evidence. However, toothpastes containing stannous fluorides are not available to the Norwegian public making it impossible to follow this recommendation. Moreover, stannous fluorides can cause staining of teeth.[5]

A study on the remineralizing potential of CPP-ACP with and without fluoride explained the possible differences in effects between the two. The study was done on human enamel by creating initial caries like lesions and storing them in artificial saliva. While CPP-ACP alone contributed calcium and phosphate ions promoting remineralization, the fluoride ion in CPP-ACPF could disrupt remineralization by interacting with the ACP-group and rendering calcium, phosphate and fluoride less available. Furthermore, the adverse effect of adding fluoride to CPP-ACP seems not to be an issue In Situ; were the interaction between saliva and dental pellicle creates an environment were CPP-ACP and fluoride has a synergistic effect.[20]

It is however more interesting to see how effective CPP-ACP is without fluoride to be able to properly compare it to the toothpaste commonly used. If CPP-ACP had faired statistically worse than 1450ppm NaF, and CPP-ACPF had fared as well or better, one could easily attribute the effect to fluoride rather than CPP-ACP itself.

CPP-ACP does not have any known side effects and exists in virtually all products containing milk, is a safe to ingest (unless the consumer is intolerant to milk protein) and generally tastes milder than conventional toothpastes.[21] It is therefore a safe alternative to fluoride and does not pose any of the risks associated with fluoride.

CPP-ACP may have additional beneficial effects against dental erosions that are not assessed in this study. In vivo, CPP-ACP may help buffer saliva against acids by fortifying the natural Phosphate buffer-system with calcium and phosphate ions, but also saturating dental biofilm.[22] A systematic

review on randomized control trials also noted the possible limitations affecting CPP-ACPF In vitro, compared to In vivo and In Situ studies.[23]

The study design allowed investigation of the inhibiting effect of the different agents by introducing acid after topical application of these. This is an inherent limitation as to investigate and compare the different agents remineralizing abilities. This would be interesting as to assess potential for treatment of dental erosions and could be done by topical application also after a set time of acid exposure.

6. Conclusion

The goal of the study is to provide knowledge that will assist clinicians in selecting appropriate agents as prophylaxis against dental erosions. In this regard, this study suggests that CPP-ACP might have similar outcomes compared to fluoride, at a certain concentration, as an addition to patients with - or in risk of dental erosions. However, the results from this study alone is not sufficient to recommend CPP-ACP to be taken as an addition to fluoride as prevention against dental erosions. Thus, well-designed clinical trials on CPP-ACP compared to fluorides assessed by systematic reviews and meta-analysis are needed.

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