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Original Article

# Evaluation of the whitening and remineralization effects of a mixture of amorphous calcium phosphate, hydroxyapatite and tetrasodium pyrophosphate on bovine enamel

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# 칼슘인산염, 수산화인회석, 피로인산나트륨 혼합물의 우치 법랑질에서의 미백과 재광화 효과 평가

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**Objectives:** The purpose of the present study was to evaluate the whitening effect, morphological and structural changes, and remineralization of the enamel induced by 3 combined agents: amorphous calcium phosphate (ACP), hydroxyapatite (HA), and tetrasodium pyrophosphate (TSP).
**Methods:** The study was performed on 90 bovine enamel slabs, which were divided into the 6 groups: negative control-distilled water (Group 1); positive control-opalescence F (Group 2); 10% mixed agent (Group 3); 25% mixed agent (Group 4); 50% mixed agent (Group 5); and 100% mixed agent (Group 6). Changes in the shade of the enamel slabs were evaluated using Shade Eye-NCC. Morphological changes were assessed by scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) was used to determine the remineralizing effect of the three agents on enamel slabs.
**Results:** The change in shade of the enamel (ad*) was noted to increase significantly with increase in whitening frequency in all groups. The value of $\Delta n^*$ was significantly greater in all groups except for the negative control group ($P<0.001$). SEM revealed that the control group, Group 5, and Group 6 had similar morphologies. The fluorescence lesion areas in the 4 mixture-treated group were significantly smaller than those in the positive control group ($P<0.001$).
**Conclusions:** These results showed that the mixture of ACP, HA, and TSP was highly effective for bovine enamel whitening and acted by inducing the remineralization of enamel.
**Clinical significance:** We evaluated the applicability of a new mixture containing ACP, HA, and TSP. This mixture would be highly useful in aesthetic dentistry because of its whitening efficiency, which does not compromise the enamel's integrity.

**Key Words:** Amorphous calcium phosphate, Hydroxyapatite, Remineralization, Tetrasodium pyrophosphate, Whitening

## Introduction

The increase in novel disease treatments in an effort to improve quality of life has been accompanied by an increase in aesthetic treatments. In clinical dentistry, tooth whitening is a highly desirable aesthetic treatment, as tooth color is one of the most important factors related to patients' satisfaction with their appearance[1,2]. The growing demand for bleaching as an aesthetic improvement has led to considerable development in bleaching products[3]. Studies using scanning electron microscopy (SEM) have demonstrated that bleaching causes demineralization, degradation, and alters the microhardness and roughness of the enamel surface[4]. Roughness is considered a predisposing factor for bacterial adhesion and stain absorption[5].

Remineralization occurs when calcium and phosphate in the environment along the enamel or dentin crystals, recrystallize on surface crystal remnants. Saliva is the primary source of calcium and phosphate[6]. Saliva does not exert a uniform effect in the mouth and it therefore has distinct localized effects[7]. Several attempts have been made to enhance remineralization.

Amorphous calcium phosphate (ACP) has been shown to be effective in remineralization, and is reported to have an additional whitening effect[8,9]. Hydroxyapatite (HA) is a precipitate generated by a reaction of calcium chloride and disodium phosphate, and increases the concentration of calcium in the oral cavity and remineralization[10]. According to a recent study[11], a bleaching agent containing ACP showed remineralizing and whitening effects. HA, which is the basic element that comprises hard tissues in the human body, is an effective remineralization element[12]. By applying HA to the tooth surface, remineralization can occur, as HA acts as a mineral supplement of calcium and phosphate to the enamel. According to recent studies[13] combination of HA and Hydrogen Peroxide (HP) is effective in tooth whitening; and so HA provides alternatives to oxidizing bleaching agents by its whitening effect. Recently, several studies reported that nano HA repairs early carious lesions as a remineralizing agent[14,15]. Huang S et al.[16] also reported that nano-HA on initial enamel lesions have remineralization potential. Lobene[17] and Scemehorn et al.[18] reported that a toothpaste agent containing tetrasodium pyrophosphate (TSP) had not only an anticalculus effect but also extrinsic stain removal effect. Farrel et al[19]. reported that the bleaching bands were more effective when TSP was added. Attempts to minimize the adverse effects of bleaching treatments by increasing enamel remineralization have been conducted; however, the results are contradictory. In addition, there are few studies that showed a whitening or remineralization effect of a mixture containing ACP, HA, and TSP.

Therefore, the objective of this *in vitro* study was to evaluate the applicability of a new mixture containing ACP, HA, and TSP by examining the whitening effect and analyzing the remineralization ability of each compound on early carious enamel. The null hypotheses tested were that (1) inorganic ions of the mixtures increase enamel remineralization, and (2) a new mixture containing ACP, HA, and TSP has a good whitening effect.

## Materials and Methods

### 1. Study material

A mixture of ACP (nanopowder, <150 nm particle size (BET), #693871, Sigma-Aldrich chemical Co., St.Louis, MO, USA), HA (reagent grade, power, synthetic, #289396, Sigma-Aldrich chemical Co., St.Louis, MO, USA), and TSP (≥95%, #P8010, Sigma-Aldrich chemical Co., St.Louis, MO, USA) was used as the study material. A solution containing distilled water (100 ml), ACP (3 g), HA (2 g), and TSP (1 g) was prepared as a 100% mixed solution. The others were diluted to 1/2, 1/4, and 1/10 with distilled water as 50%, 25%, and 10% solutions. Distilled water was used as the negative control and Opalescence F (Ultradent Product. Inc., South Jordan, CA, USA) containing 10% carbamide peroxide was used as the positive control.

### 2. Study overview

Group 1, the negative control group, was treated with distilled water. Group 2, the positive control group, was treated with Opalescence F. Groups 3, 4, 5, and 6 were treated with a solution containing 10% (ACP 0.3%, HA 0.2%, TSP 0.1%), 25% (ACP 0.75%, HA 0.5%, TSP 0.25%), 50% (ACP 1.5%, HA 1%, TSP 0.5%), and 100% (ACP 3%, HA 2%, TSP 1%) of the mixture, respectively (Table 1).

**Table 1.** Study group and characteristics of the treatment materials

| Group | | Contents |
|---|---|---|
| Group 1 (n=15) | - | Negative Control, Distilled Water |
| Group 2 (n=15) | - | Positive Control, 10% Carbamide Peroxide (CP) |
| Group 3 (n=15) | 10% mixed | ACP 0.3%, HA 0.2%, TSP 0.1% |
| Group 4 (n=15) | 25% mixed | ACP 0.75%, HA 0.5%, TSP 0.25% |
| Group 5 (n=15) | 50% mixed | ACP 1.5%, HA 1%, TSP 0.5% |
| Group 6 (n=15) | 100% mixed | ACP 3%, HA 2%, TSP 1% |

ACP: Amorphous calcium phosphate; HA: Hydoxyapatite; TSP: Tetrasodium pyrophosphate.

## 3. Study method

### 3.1. Specimen preparation

The present study was approved by the Institutional Care and Use Committee of the School of Dentistry, K-University, in Daegu. For this study, we selected 300 Sound bovine teeth that were cleaned and stored in 0.1% thymol solution under refrigeration for no more than a week. Three hundred cylindrical enamel specimens, 3 mm long and 8 mm in diameter were prepared using bovine central teeth. The specimens were embedded in self-cured acrylic resin at right angles to the long axis of the acrylic cylinder. The enamel surface was ground with an Automatic Polisher (Metaserv® 250 Grinder-Polisher, Buehler, USA) under cool water sequentially, using 240, 400, 600, and 800 grit polishing sand paper.

### 3.2. Specimen discoloration

A cola beverage (Coca Cola, pH 2.26±0.20), currently on the market, was stirred for over 6 hours to remove the carbon dioxide gas and then the specimens were immersed in the cola for 24 hours. Specimens were subsequently rinsed with triple distilled water. Discoloration was confirmed with the naked eye after the rinsing step. After measuring the initial color of 150 discolored specimens, 15 specimens were distributed into each group according to their brightness (L*).

### 3.3. Handling of mixture

Of the 150 specimens, the 90 specimens near the average brightness value were divided into 6 groups with 15 specimens per group. All test mixtures used in this study were prepared the morning of the day of use. One test-cycle was defined as follows: the specimen was treated with 20 ml of the test-mixture for 10 minutes except for Group 2 (the positive control), and was then dipped into artificial saliva for 170 minutes. The artificial saliva consisted of 2.2 g/L gastric mucin, 0.381 g/L NaCl, 0.231 g/L $CaCl_2$, 0.738 g/L $KH_2PO_4$, 1.114 g/L KCl, 0.02% sodium azide, and trace of NaOH to pH 7.0[20]. Five liters of artificial saliva were freshly prepared on a daily basis.

In this way, 2 cycles were performed on every group except for Group 2 daily. The specimens were stored in distilled water at 4°C under 100% relative humidity, except during the dipping procedures. These procedures were repeated for 6 days.

The Group 2 specimens were coated with Opalescence F, a bleaching solution, for 3 hours and the same procedure was applied except for the preparation time. After each preparation procedure, all specimens were rinsed twice with running water for 30 seconds each and then kept under 100% relative humidity conditions except for the dipping procedure.

### 3.4. Measurement of the shade change

To measure the shade change according to the mixture treatment, the shade of the enamel was measured before and after dipping the specimen in the mixture solution using Shade Eye-NCC (150131, SHOFU Co., Japan). Repeated measurements were then performed three times under dry conditions. The tip of the shade measurer was consistently applied above the 0.5-1.0 mm sample's surface at right angles to the surface. Three sites were chosen for the measurements. The L*, a*, and b* values were then obtained. The magnitude of the total color difference is represented by ⊿E* calculated by the following equation: $\Delta E^* = \{(L^*)^2+(a^*)^2+(b^*)^2\}^{1/2}$.

### 3.5. Morphological examination

Specimens (n=7) were sputter-coated with gold to a 180~200 Å thickness using gold ion deposition equipment (IB-3, Eiko Co., Japan) in a vacuum. Then, scanning electron microscopy (SEM, S-4200, Hitachi, Co., Japan) with an energy dispersive X-ray spectrophotometer (EDS) was used to examine the surface morphological changes at 2000x magnification at an acceleration voltage of 15 kV.

### 3.6. Analysis of remineralization

The remaining specimens (n=8) were cross-sectioned longitudinally using a low-speed diamond saw (Isomet; Buehler Ltd., Lake Bluff, IL, USA), and the sectioned enamel surface was polished with 100-grit silicon carbide paper and decending grades of 6-, 3-, and 1-μm diamond paste (Buehler Ltd., Lake Bluff, IL, USA), as described by Lee et al.[21]. The fluorescence images were examined using a confocal laser scanning microscope (CLSM, LSM 510, Carl Zeiss, Germany) under a fluorescein-5-isothiocyanate (FITC) field at an excitation wavelength of 488 nm and an emission wavelength of 515 nm. According to the method reported by Paris et al.[22]. 3 points were selected from the enamel surface to the bottom of the decalcification area. The decalcified depth was measured and the mean was calculated using a laser scanning microscope (LSM) image browser (Carl Zeiss, Germany).

## 4. Statistical analysis

To compare the color change and fluorescence depth according to the pre/post-treatment, one-way ANOVA tests were conducted, with Tukey's multiple comparison tests for post hoc analysis. Repeated measures ANOVA was used to ex-

**Table 2.** Shade change (⊿E*) of the specimen according to the whitening times

| Group | After 1 day* | After 2 days* | After 3 days* | After 4 days* | After 5 days* | After 6 days* |
|---|---|---|---|---|---|---|
| Group 1† | 6.93±2.49[a] | 6.13±2.41[a] | 8.38±3.28[a] | 7.77±1.70[a] | 8.37±1.53[a] | 9.72±1.35[a] |
| Group 2† | 30.4±7.55[b] | 39.92±8.53[b] | 47.45±8.14[b] | 51.21±7.12[b] | 51.07±6.24[b] | 54.72±7.14[b] |
| Group 3† | 21.2±4.11[c] | 26.37±4.55[c] | 31.52±4.38[c] | 34.37±3.71[c] | 34.42±1.86[c] | 37.30±1.73[c] |
| Group 4† | 25.24±3.61[bc] | 33.09±5.39[bc] | 35.75±5.81[c] | 36.18±6.17[c] | 38.58±5.54[c] | 31.94±1.92[c] |
| Group 5† | 26.65±2.89[b] | 34.22±3.80[b] | 38.78±2.87[c] | 41.09±2.94[c] | 41.32±3.04[c] | 42.92±0.92[c] |
| Group 6† | 28.90±3.63[b] | 34.75±4.86[b] | 37.46±3.81[c] | 41.21±2.54[c] | 42.00±3.87[bc] | 45.72±4.02[c] |

Values are reported as the Mean±S.D.
*Significantly different among the groups at each time point by one-way ANOVA.
†Significantly different among the experimental times by repeated measures ANOVA.
a,b,cThe same letters indicate no significant different among the groups according to a Tukey's multiple comparison at each whitening time.

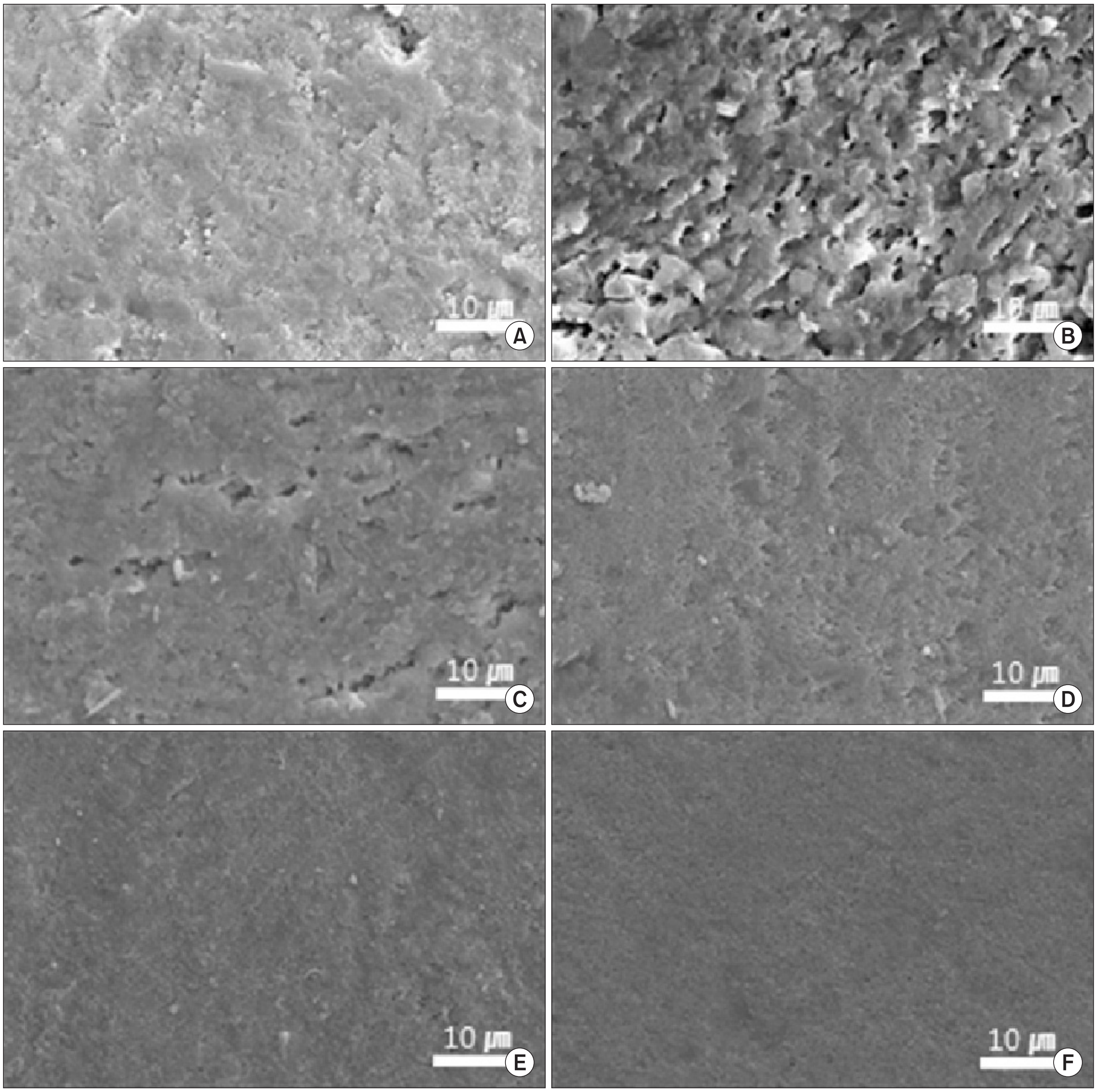


**Fig. 1.** Morphology of the superficial enamel surface after 6 days of treatment in each group (A: Group 1, B: Group 2, C: Group 3, D: Group 4, E: Group 5, F: Group 6) (×2,000).

amine the color difference according to the number of treatments. All statistical analyses were carried out using SPSS 18.0 for Windows (SPSS Inc., Chicago, Il, USA). A *P*-value of 0.05 was considered to be significant.

# Results

## 1. Analysis of the shade change

Table 2 listed the quantitative analysis results of the shade change ($\Delta E^*$).

The shade change ($\Delta E^*$) revealed statistically significant increases with an increasing treatment time of solution treatments in all groups by 1.3 to 1.8 times ($P<0.001$). A comparison of the degree of tooth whitening according to the number of treatments of mixed solution revealed a statistically significant difference in the shade change ($\Delta E^*$) in all groups ($P<0.001$) (Table 2). To quantitatively analyze the enamel shade change, the positive control (Group 2) was designated at 100%, and whitening effect was observed in 67% of Group 3, 77% of Group 4, 83% of Group 5, and 85% of Group 6.

## 2. SEM observations

Fig. 1 showed SEM images after 6 days of the mixture treatment. Compared to the untreated group 1, the Opalescence® 10% treated positive control (Group 2) showed a prominent typical pattern of demineralization through the dissolution of the organic/inorganic enamel element. In Groups 3, 4, 5, and 6 treated with ACP, HA, and TSP, enamel remineralization was confirmed by the enamel rod recovery. In particular, the relatively high mixture concentrations in Groups 5 and 6 produced a higher level of enamel remineralization than Groups 3 and 4, but there was no significant difference between Groups 5 and 6 (Fig. 1).

## 3. Observation of CLSM

Fig. 2 showed the loss of inorganic substance and remineralization in the sectioned specimen after 6 days of treatment. The right side of the central band is the air space and the left is the enamel. In the 10% CP-treated positive control (Group 2), enamel mineral loss was observed to a depth of 55-60 μm. Groups 3, 4, 5, and 6, which had been treated with the mixed

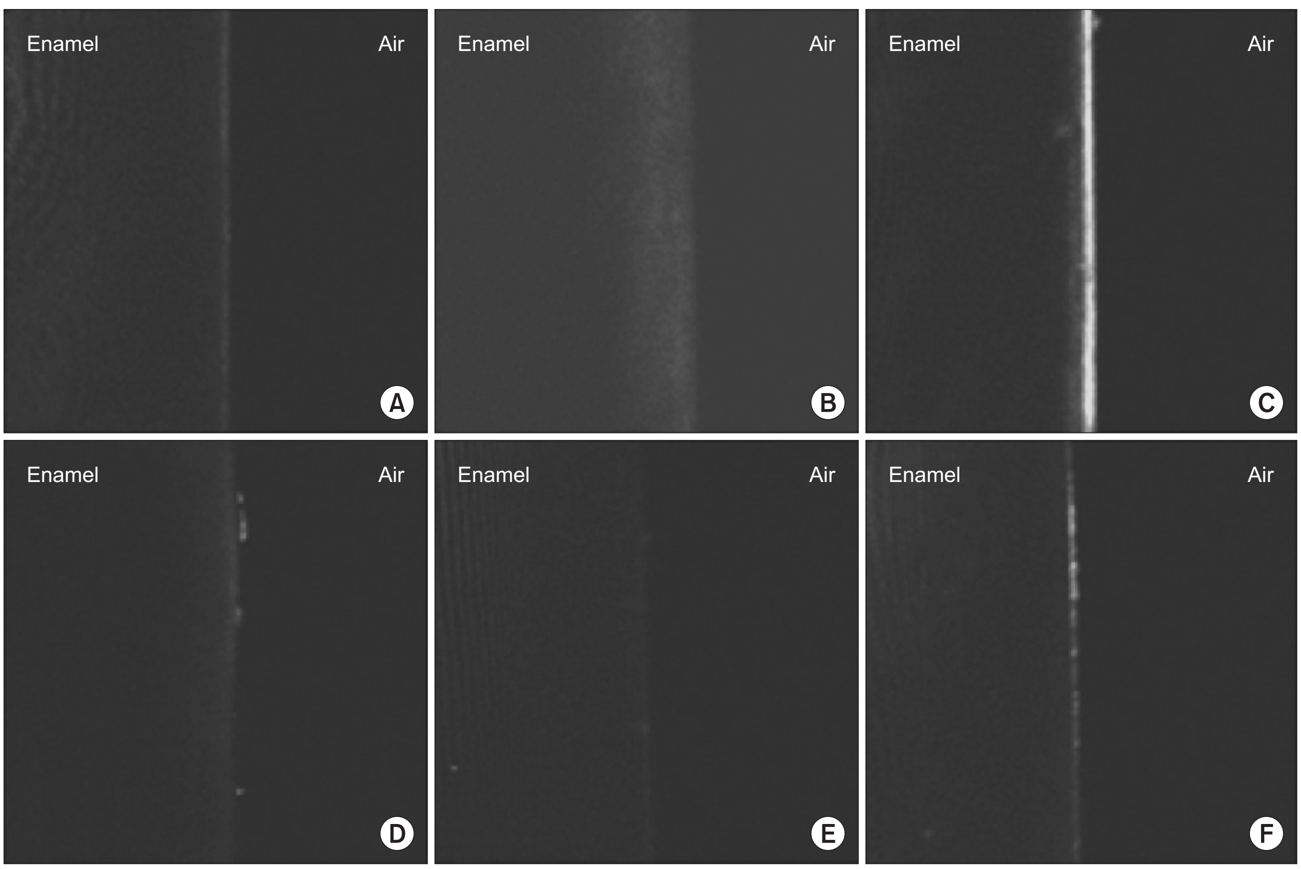


**Fig. 2.** Fluorescent lesions after whitening in each group at 6 days as determined by CLSM; note the demineralized lesion in the center, emits a bright color (A: Group 1, B: Group 2, C: Group 3, D: Group 4, E: Group 5, F: Group 6).

**Table 3.** Fluorescence lesion depth at 6 days by CLSM

| Group | Fluorescence lesion depth (μm) | *P* value* |
|---|---|---|
| Group 1 (n=8) | 6.15±0.88[a] | <0.001 |
| Group 2 (n=8) | 64.45±7.48[b] | |
| Group 3 (n=8) | 11.13±1.01[a] | |
| Group 4 (n=8) | 8.49±1.34[a] | |
| Group 5 (n=8) | 7.03±0.88[a] | |
| Group 6 (n=8) | 4.98±0.51[a] | |

Values are Mean±S.D.
*Significantly different among groups by repeated measures ANOVA procedure.
[a,b]The same letters indicate no significant difference between the groups according to a Tukey's multiple comparison.

solution, showed a pattern of mineral loss similar to that of the untreated group (Group 1). In our study, weak or missing fluorescence indicated an area of remineralization. The mean fluorescence depth was measured to evaluate the quantitative remineralization (Table 3). Less mineral loss was observed in the mixed solution-treated group than the group treated with Opalescence® 10% (Table 3, Fig. 2). In particular, the mineral loss recovered slightly in Group 6.

## Discussion

This study provided the first empirical evidence that a mixture of ACP, HA, and TSP causes whitening and remineralization effect while retaining the enamel's integrity. The mixture solution was applied to the tooth surface and the enamel remineralization effect was revealed by mineral supplements to enamel deficient in calcium and phosphate. The remineralization promotes the whitening effect by affecting the light transmission of the tooth surface and increasing the surface reflection.

Bleaching agents, mainly oxidizers, act on the organic structure of the dental hard tissues, slowly degrading them to chemical by-products, such as carbonates, which are lighter in color, and carbon dioxide[23]. Generally, unstable peroxides convert to unstable free radicals. These free radicals can oxidize or reduce other molecules[24]. According to Mor et al.[25], at-home bleaching agents can roughen the tooth surface and composite restoration, increasing bacterial adhesion. The rough surface of the restoration can also cause patient discomfort and accelerate the plaque and food debris buildup by increasing surface energy, which can cause secondary caries and gingivitis. Therefore, ongoing work focuses on reducing this kind of side effect with an increase in aesthetic treatment.

First of all, we selected the soft drink (Coca cola) for the discoloration and demineralization process to confirm the whitening effect and remineralization effects of mixtures. Several researches[26] used cola, wine, coffee, and tea as staining solutions in enamel, and composites for intrinsic and extrinsic discoloration. Bayindir et al.[27] reported the highest delta E values of prosthodontic materials at the 24 hour immersion period, similar to the results of Ergun et al.[28]. Cola quantitatively determines the stain development process within 24 h, so it has advantages to other staining solution. We selected Cola for the staining model to access the effect of whitening on extrinsic discolored teeth by enhanced the remineralization activity.

To quantitatively analyze the enamel shade change, the positive control (Group 2) was designated at 100%, and whitening effect was observed in 67% to 85% at Group 3 to 6. Naked eye observation indicated that the mixed solution treated groups showed a similar effect to that of Group 2 (the positive control). Based on the results, we concluded that the mixtures of ACP/HA/TCP have concentration-dependent synergistic effects.

In the case of discolored dentin, bleaching agents placed on the enamel do not readily reach the stain. Better results are expected if the stain is on the enamel surface or the enamel is defective and porous. Changes in tooth structure due to extrinsic factors have been widely investigated though SEM. The method requires proper specimen preparation and examination conditions: these procedures change the natural condition and/or part of the specimen structure. We have previously confirmed the validity evaluation of SEM in our earlier study[21]. Microhardness is usually used for quantitative measurement, but SEM of TEM is usually used in imaging analysis. SEM is usually used to measure the histomorphologic changes of the enamel surface and to confirm the demineralization or remineralization[29,30]. In this study, SEM revealed the typical demineralization effect in Group 2. On the other hand, remineralization could be easily observed in Groups 3, 4, 5, and 6. To confirm the result, when observed under confocal scanning microscopy, the depth of mineral loss in Group 2 was 60 μm. In contrast, no mineral loss was observed in the other groups, which were treated with the mixture solution, i.e., similar to Group 1 (distilled water). These findings suggested that the inorganic ions in the mixtures of ACP/HA/TCP recover enamel surface morphology by inducing the remineralization effect of enamel, and are thus effective in bovine enamel whitening.

We used the ratio of 3%, 2%, 1% for the ACP/HA/TSP. ACP is used in paste as 10% CPP-ACP, and is incorporated in various commercial products such as GC Tooth-Mousse or MI paste (GC, IL). Several reports[16] showed that 1%-10% HA has similar effects to 10% CPP-ACP. And commercial dentifrice products commonly contain 3.3% TSP (i.e. Crest Tartar Control). For synergistic effects of mixtures, we combined the 3 components and then used 1/3$^{rd}$ the amount of total gram.

This study could not explain which was actually the most effective, because we did not test each ingredient separately for the remineralization effect. Nevertheless, this study has important clinical implications in choice of a new bleaching system containing ACP, HA, and TSP with good whitening effect. According to the present and earlier results, we confirmed the hypotheses that inorganic ions of a new mixture containing ACP, HA, and TSP increases enamel remineralization and has a good whitening effect.

Our study had some limitations. First, a cola beverage on the market was used to discolor the teeth and the mixed solution was applied. This procedure differs from the mechanism of endogenous and extrinsic discoloration, and a range of whitening effects could be observed naturally. Second, the qualitative and quantitative analysis of the chemical composition of the specimen and the degree of inorganic ion deposition could not be compared after treatment with the mixed solution. Third, we did not test the whitening effect using additionally treated mixtures in the representative bleaching systems. Further studies are necessary to address these limitations; to simulate the oral cavity environment by inducing possible exogenous pigmentation; to check the concentration of the inorganic ions in the teeth when treated the mixures; *in vitro* study to discoloring the teeth using stable method like as stookey method; and *in vivo* safety assessment when using the mixture solution.

## Conclusions

We examined the tooth whitening effect using *in vitro* models prior to performing clinical trials. The results revealed that the inorganic ions of the mixture of ACP, HA, and TCP had an effect on the remineralization of bovine enamel without adversely affecting the whitening efficacy as follows.

1. The degree of tooth whitening according to the number of treatments of mixed solution revealed a statistically significant difference in the shade change ($\Delta E^*$) in all groups ($P<0.001$).

2. SEM revealed the typical demineralization effect in Group 2. On the other hand, remineralization could be easily observed in Groups 3, 4, 5, and 6.

3. Under confocal scanning microscopy, the depth of mineral loss in Group 2 was 60 μm. In contrast, no mineral loss was observed in the other groups, which were treated with the mixture solution, i.e., similar to Group 1 (distilled water).

Future clinical study is required to examine the optimal concentration of the mixture solution and confirm its whitening ability.

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