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Evaluation of the Effect of Different Remineralizing Agents and Ozone Application on Artificial Caries

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ABSTRACT
This study aimed to assess the combined and individual effects of different remineralization agents and ozone application on initial caries. Seventy-two premolar teeth were collected. Three different toothpastes (Ipana, ROCS, and Gumgumix) were utilized. The samples were divided into eight groups (n = 8) for experimentation (Distilled water, Ipana+Ozone, Gumgumix+Ozone, ROCS+Ozone, Ipana, Gumgumix, ROCS, Ozone). Microhardness measurements were conducted initially. All samples were then immersed in a demineralization solution for 72 hours to induce initial caries. Then, microhardness measurements were repeated, and pH cycles were carried out for one-week. Finally, microhardness measurements and SEM-EDS analyses were performed. Ipana+Ozone exhibited the highest microhardness value, which was statistically significant compared to other groups except ROCS+Ozone (p < 0.05). Distilled water, Gumgumix+Ozone, and Gumgumix displayed the lowest microhardness values. Ozone application with remineralization agents led to higher precipitation of Ca ions. The demineralized group showed the lowest Ca, P, and F ions. While Ipana+Ozone and ROCS+Ozone had the highest Ca ions, Gumgumix+Ozone had the highest P ions. Within the study’s limitations, it can be concluded that ozone gas positively impacts the remineralization process in initial caries lesions. Additionally, mineral-containing products demonstrated a noteworthy capacity to reverse the demineralization process.

INTRODUCTION
Dental caries is an irreversible microbial disease that causes demineralization of inorganic substances and destruction of organic substances in teeth. Efforts to combat this microbial disease involve developing new strategies to halt the progression and repair initial dental caries without cavity formation (Cury and Tenuta 2014).

Various preventive treatments aim to shift the demineralization process to a remineralization process in early-stage caries. These treatments include changes to patients’ oral hygiene and nutritional habits, fissure sealants, fluoride, chlorhexidine, antimicrobial agent applications, or the combined use of these methods. In recent years, ozone applications have come to the fore as a new treatment model within the scope of preventive approaches or noninvasive approaches in dentistry. Ozone gas, commonly used in the treatment of various medical conditions, has also found application in dentistry due to its potent oxygenation and oxidation properties (Baysan and Lynch 2005; Baysan, Whiley and Lynch 2000; Suh et al. 2019). As a pharmacological approach to treating dental caries, ozone creates a conducive environment for the remineralization cycle by eliminating microorganisms responsible for caries formation and oxidizing all carbohydrates and acids in the carious lesion (Baysan, Whiley and Lynch 2000). The purpose of treating carious lesions with ozone is to reduce causative or contributing risk factors, arrest the caries process, and promote remineralization. Studies show that ozone, acting as a remineralizing agent, enhances the diffusion of salivary ions to the damaged dentin surface, promoting remineralization. In this way, ozone can neutralize acid proteins produced by cariogenic bacteria, which represent the osmotic stimulus responsible for the movement of fluids in the dentinal tubules, leading to hypersensitivity (Floare et al. 2021; Floare et al. 2022).

While fluoride ion remains the gold standard remineralizing agent, concerns about its potential toxicity have prompted increased attention to alternatives. Further, a newly developed mineral gel system, ROCS (R.O.C.S., Moscow, Russia), has been introduced to increase the mineral content around tooth surfaces...
Table 1. Tested materials.

<table>
<thead>
<tr>
<th>Brand</th>
<th>Ingredient</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ipana Pro-Expert</td>
<td>Aqua, Hydrated Silica, Sorbitol, Sodium Lauryl Sulfate, Cellulose Gum, Aroma, Sodium Fluoride, Sodium Carboxymethyl Cellulose, Zincibar Officinalce Extract,</td>
<td>The Procter &amp; Gamble Company, Ohio, USA</td>
</tr>
<tr>
<td>Gumgumix Toothpaste</td>
<td>Calcium Carbonate, Glycerin, Aqua, Di Calcium Phosphate, Honey, Sorbitol, Glycyrrhiza Glabra Root, Extract, Aroma, Xanthan Gum, Calcium Carboxymethyl Cellulose, Zingiber Officinalce Extract, Potassium Sorbate, Menthol, Sodium Benzonate</td>
<td>Beka Pharmaceuticals, Istanbul, Turkey</td>
</tr>
<tr>
<td>ROCS Mineral Gel</td>
<td>Aqua, Glycerin, Xylitol, Hydroxyethylcellulose, Calcium Glycerophosphate, Polysorbate-20, Aroma, Methylparaben, Magnesium Chloride, Hydroxypropyl Guar</td>
<td>R.O.C.S., Moscow, Russia</td>
</tr>
</tbody>
</table>

and in the oral cavity. This system reportedly creates a bioavailable source of calcium, phosphate, and magnesium and can be used to treat early caries and erosive lesions. The remineralizing effect of this agent on white spot lesions and tooth sensitivity has also been reported (Damar, Çelikel Ad and Pınar Erdem 2023).

Ginger, one of the antibacterial extracts, is capable of remineralizing initial caries lesions and preventing macromolecules from free radical and oxidative stress (Zick et al. 2008).

Enamel is the most complex and hardest tissue in the human body, with a content of 96% hydroxyapatite, 4% water, and organic material. It is damaged throughout life by being constantly exposed to factors in the oral environment (Kreulen et al. 2010; Lacruz et al. 2017). Lesions in enamel, which cannot renew or reshape itself, are irreversible and are repaired with an artificial material. For this reason, early diagnosis and treatment of initial caries are of great importance in minimally invasive dentistry (Elsayad 2011).

This study aims to assess both the individual and combined impacts of various remineralization agents and ozone application on initial caries. The null hypothesis posits that the application of ozone will have a positive effect on the remineralization process of the tested materials.

Materials and methods

A total of 72 premolar teeth extracted for orthodontic reasons were collected for the study. Inclusion criteria of teeth for the study:

1. No caries or abrasions on the buccal surface,
2. Containing sufficient thickness of enamel on the buccal surface,
3. No discoloration,
4. Teeth without hypoplasia and cracks.

Soft tissue residues on the tooth surfaces were cleaned with handpieces. The teeth were kept in a refrigerator at + 4°C in 0.01% thymol solution until use. Enamel sections were created from the buccal surfaces of the samples with the help of a diamond separator. The obtained buccal tooth sections were placed on acrylic blocks with their buccal surfaces facing upwards. The middle third of the samples were abraded with 600-grit silicon carbide sandpaper and standard flat surfaces were obtained. The samples were washed with distilled water and kept in distilled water until the experiments were performed.

Initial microhardness measurements were carried out using the Shimadzu HMV-II Vicker’s hardness device (HMV-II, Shimadzu Corporation, Kyoto, Japan), by applying a diamond pyramid tip with a load of 300 g for 10 seconds, from 3 different points on the flat surfaces of the samples, and the average was recorded.

The materials tested are presented in Table 1. The samples were divided into 8 groups (n = 8) to carry out the experiments (Table 2):

Standard remineralization and demineralization solutions were prepared using previously published protocols. The remineralization solution (artificial saliva) was composed of 3.8 mg/L Mg²⁺ (MgCl₂·6 H₂O), 84.36 mg/L PO₄³⁻ (K₂HPO₄/KH₂PO₄) 50 mg/L Ca²⁺ (calcium lactate), 0.05 mg/L fluoride, 625 mg/L KCl, 400 mg/L carboxymethylcellulose, and 2000 mg/L methyl-4-hydroxybenzoate, with the pH adjusted to 7.2 using 1 M KOH. The demineralizing solution (DS) served as an acid challenge simulating the acids generated in the dental plaque with intake of cariogenic meal. The DS was composed of 314.2 mg/L Ca (NO₃)₂·4 H₂O, 174.18 mg/L KH₂PO₄, 1225 mg/L CH₃COOH with the pH adjusted to 4.5 using 1 M KOH.

In the initial stage of the study, artificial carious lesions were created by keeping all samples in demineralization solution for 72 hours (37°C). The solution was renewed daily. A shaking incubator was used to perform

Table 2. Groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Materials</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>Distilled water (control group)</td>
</tr>
<tr>
<td>Group 2</td>
<td>Ipana toothpaste + Ozone gas</td>
</tr>
<tr>
<td>Group 3</td>
<td>Gumgumix toothpaste + Ozone gas</td>
</tr>
<tr>
<td>Group 4</td>
<td>ROCS mineral gel + Ozone gas</td>
</tr>
<tr>
<td>Group 5</td>
<td>Ipana toothpaste</td>
</tr>
<tr>
<td>Group 6</td>
<td>Gumgumix toothpaste</td>
</tr>
<tr>
<td>Group 7</td>
<td>ROCS mineral gel</td>
</tr>
<tr>
<td>Group 8</td>
<td>Ozone gas</td>
</tr>
</tbody>
</table>
the experiments. After demineralization, microhardness measurements were repeated and recorded.

Ipana toothpaste containing NaF, Gumgumix tooth cream containing ginger-honey (herbal) and ROCS mineral gel were applied by electric toothbrush (Oral-B iO, The Procter & Gamble Company, Ohio, USA) for 30 seconds three times a day. HealOzone (Curozone, KaVo, Biberach, Germany) ozone generating system which delivers ozone gas at a concentration of 2.100 ppm ±10% was used and silicone application cups were used to prevent air contact with the teeth during ozone application, and ozone application was carried out for 20 seconds. Group 1, the control group, was kept in distilled water while the experiments were carried out and was not subjected to any treatment. Daily eating habits were tried to be imitated by performing 18 hours of remineralization and 6 hours of demineralization per day for 7 days in total.

After the pH cycle, microhardness measurements were repeated and 2 samples from each group were reserved for Scanning Electron Microscopy (SEM) analysis. SEM analysis was performed with EVO LS10 (Carl Zeiss, Jena, Germany) at x100, x500, x1000, x5000, and x10000 magnifications. Energy dispersive X-ray spectrometer (EDS) analysis was performed at x5000 magnification, and the presence of Calcium (Ca), Phosphate (P), and Fluoride (F) ions was observed (Figure 1).

The obtained data were analyzed with the SPSS 22 software. As a result of the normality analysis of the data obtained in this study, the ANOVA and/or T test were preferred for the comparisons between groups for the normally distributed variables. In addition, the non-parametric Kruskal-Wallis H test and Mann-Whitney U test were preferred for the non-normally distributed variables.

Results

Microhardness results

Average microhardness values are given in Table 3. Initial and post-deminerolization microhardness values were not statistically significant between the groups (p > 0.05).

After the pH cycle, Group 2 (Ipana toothpaste + Ozone gas) showed the highest microhardness value, and this value was statistically significant when compared to other groups except Group 4 (ROCS mineral gel + Ozone) (p < 0.05). Group 1 (distilled water), Group 3 (Gumgumix toothpaste + Ozone), and Group 6 (Gumgumix toothpaste) showed the lowest microhardness values.

EDS results

Average EDS analysis results are presented in Table 4. Ozone application with remineralization agents resulted in higher precipitation of Ca ions. The lowest Ca, P, and

![Figure 1. Flow chart.](image-url)
Table 3. Microhardness values (mean (standard deviation)).

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>Post demineralization</th>
<th>Post remineralization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>327.30 (39.07)</td>
<td>108.51 (1.70)</td>
<td>92.14 (13.50)</td>
</tr>
<tr>
<td>Ipana toothpaste + Ozone gas</td>
<td>297.48 (23.19)</td>
<td>105.51 (4.25)</td>
<td>187.28 (25.28)</td>
</tr>
<tr>
<td>Gumgumix toothpaste + Ozone gas</td>
<td>302.80 (20.30)</td>
<td>103.23 (10.28)</td>
<td>84.90 (12.24)</td>
</tr>
<tr>
<td>ROCS mineral gel + Ozone gas</td>
<td>326.37 (23.76)</td>
<td>101.80 (11.40)</td>
<td>164.75 (16.44)</td>
</tr>
<tr>
<td>Ipana toothpaste</td>
<td>303.44 (28.60)</td>
<td>104.65 (6.50)</td>
<td>114.55 (15.37)</td>
</tr>
<tr>
<td>Gumgumix toothpaste</td>
<td>306.95 (24.01)</td>
<td>109.94 (3.14)</td>
<td>79.93 (15.86)</td>
</tr>
<tr>
<td>ROCS mineral gel</td>
<td>340.72 (22.40)</td>
<td>110.08 (2.77)</td>
<td>131.84 (17.14)</td>
</tr>
<tr>
<td>Ozone gas</td>
<td>318.21 (27.52)</td>
<td>115.23 (2.41)</td>
<td>113.93 (16.16)</td>
</tr>
</tbody>
</table>

*Different lowercase letters indicate statistically significant differences in the row.
Different uppercase letters indicate statistically significant differences in the column.
Significance level was established as p < 0.05.

Table 4. EDS Analysis (x5000 magnification) Results.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Ca</th>
<th>P</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>66.79</td>
<td>31.79</td>
<td>1.25</td>
</tr>
<tr>
<td>Ipana toothpaste + Ozone gas</td>
<td>67.10</td>
<td>33.46</td>
<td>1.48</td>
</tr>
<tr>
<td>Gumgumix toothpaste + Ozone gas</td>
<td>64.58</td>
<td>34.03</td>
<td>1.38</td>
</tr>
<tr>
<td>ROCS mineral gel + Ozone gas</td>
<td>67.73</td>
<td>33.12</td>
<td>1.83</td>
</tr>
<tr>
<td>Ipana toothpaste</td>
<td>65.06</td>
<td>31.87</td>
<td>1.24</td>
</tr>
<tr>
<td>Gumgumix toothpaste</td>
<td>63.5</td>
<td>32.97</td>
<td>1.33</td>
</tr>
<tr>
<td>ROCS mineral gel</td>
<td>64.52</td>
<td>33.91</td>
<td>1.56</td>
</tr>
<tr>
<td>Ozone gas</td>
<td>65.31</td>
<td>32.29</td>
<td>1.41</td>
</tr>
<tr>
<td>Demineralized</td>
<td>51.13</td>
<td>31.15</td>
<td>1.23</td>
</tr>
</tbody>
</table>

F ions were observed in the demineralized group. While the highest Ca ions were observed in Group 2 (Ipana toothpaste + Ozone) and Group 4 (ROCS mineral gel + Ozone), the highest P ions were observed in Group 3 (Gumgumix toothpaste + Ozone gas).

**SEM results**

In the distilled water group, the demineralization process led to the loss of the inorganic structure on the enamel surface, resulting in a rough, irregular, porous structure. Crystals belonging to the prisms dissolved on the irregular enamel surface, causing small cavity formations. Enlargements in the interprismatic areas and occasional dissolutions on the outer walls of the prism sheaths were observed (Figure 2).

In Group 2 (Ipana toothpaste + Ozone gas), gaps and irregularities in the prismatic and interprismatic enamel areas were significantly reduced compared to other groups. Homogeneous calcified sediments were observed in a wide area (Figure 3).

In Group 3 (Gumgumix toothpaste + Ozone gas), reductions in gaps within the interprismatic areas were noted, with a concentration of non-homogeneous calcified sediments in these areas (Figure 4).

In Group 4 (ROCS mineral gel + Ozone gas), calcified precipitations were observed in some of the small cavity formations on the irregular enamel surface. Spaces in the interprismatic areas were reduced, but calcified sediments were not homogeneous (Figure 5).

**Figure 2.** SEM images of the distilled water group, magnification at (a): x100 (b): x500 (c): x1000 (d): x10000.

**Figure 3.** SEM images of the Ipana + Ozone group, magnification at (a): x100 (b): x500 (c): x1000 (d): x10000.
In Group 5 (Ipana toothpaste), calcified precipitates were observed in interprismatic areas and dissolution areas on the outer walls of the prism sheaths. Gaps decreased in interprismatic areas (Figure 6).

In Group 6 (Gumgumix toothpaste), despite surface irregularities, small cracks, and porous structures on the enamel surface, calcified sediments were observed in the pits, albeit dispersed and non-homogeneous (Figure 7).

In Group 7 (ROCS mineral gel), small cavity formations due to dissolution occurred in interprismatic areas on the irregular enamel surface. Non-homogeneous calcified precipitates were observed in the dissolution areas on the tooth surface (Figure 8).

In Group 8 (Ozone gas), the enamel surface exhibited an irregular appearance with occasional flattening and widening in interprismatic areas (Figure 9).

In the demineralized group, small-sized cracks on the surface, a rough surface, expansions in interprismatic areas, and occasional dissolution on the outer walls of the prism sheaths were observed (Figure 10).

**Discussion**

Dental caries is the most common disease worldwide, dependent on pH. For enamel tissue, mineral loss begins below pH 5.5, initiating the demineralization process (Abou Neel et al. 2016).

White spot lesions (initial caries) represent a stage where caries progression can be detected and halted. Understanding the histological structure of these lesions emphasizes the importance of employing minimally invasive techniques for the most appropriate treatment (Guerrieri et al. 2012). Numerous methods have been proposed for the treatment of these lesions, including lasers, ozone therapy, herbal-based products, bioactive agents, and calcium-phosphate-containing remineralization agents (Scheifele, Studen-Pavlovich and Markovic 2002).

Ozone therapy has recently gained widespread use in dentistry, addressing various areas such as prophylaxis and caries prevention, remineralization of pit and fissure caries, remineralization of root and flat surface caries, tooth whitening, and reducing tooth sensitivity. It has been demonstrated to prevent caries formation and support remineralization by eliminating bacteria in the carious lesion (Almaz and Sönmez 2015).

Ozone exerts its effects by attacking glycoproteins, glycolipids, and other amino acids, disrupting enzymatic control systems within cells. Consequently, the cell membrane's permeability increases, leading to membrane expansion and ultimately halting cell viability. The oxidizing effect of ozone is hypothesized to remove proteins from demineralized areas, facilitating increased diffusion of calcium and phosphate ions and, thereby, promoting the remineralization of lesions (Baysan and Lynch 2007).

Likewise, the potent oxidizing properties of ozone may influence proteins in demineralized enamel, leading to their removal. This, in turn, facilitates lesion remineralization by enhancing the diffusion of remineralizing agents. Due to these positive properties, ozone is frequently researched for its use in dentistry (Baysan and Lynch 2004).

In studies, it has been reported that explaining the fluoride effect on the remineralization of the enamel surface is challenging. Calcium and phosphate ions are
Figure 6. SEM images of the Ipana group, magnification at (a): x100 (b): x500 (c): x1000 (d): x10000.

Figure 7. SEM images of the Gumgumix group, magnification at (a): x100 (b): x500 (c): x1000 (d): x10000.

Figure 8. SEM images of the ROCS group, magnification at (a): x100 (b): x500 (c): x1000 (d): x10000.

Figure 9. SEM images of the Ozone group, magnification at (a): x100 (b): x500 (c): x1000 (d): x10000.

Figure 10. SEM images of the demineralized group, magnification at (a): x100 (b): x500 (c): x1000 (d): x10000.
needed to create fluorapatite crystals during fluoride application. Therefore, calcium, phosphate, and fluoride ions play a crucial role in the remineralization-demineralization process (Zhang et al. 2011). In laboratory studies (Kilik and Gurbuz 2021; Vitiello et al. 2022), these ions are mostly investigated by SEM-EDS analysis to evaluate the capacity of remineralization agents. In this study, ginger-honey (herbal) based toothpaste, a remineralization gel, fluoride-containing toothpaste, and ozone gas were investigated. The Ca/P ratio was evaluated with EDS analysis.

According to the findings of this study, the combination of Ipana toothpaste and ozone gas resulted in higher microhardness values after treatment. Notably, since the other tested materials do not contain fluoride ions, these results suggest a positive interaction between ozone gas and fluoride ions. It is well-established that certain elements can integrate into calcium salts, rendering them more resistant to acids, with fluoride being the most significant among these elements (Joiner et al. 2009). Hence, many dentists often opt to administer topical fluoride in the treatment of white spot lesions.

The combination of ROCS mineral gel and ozone gas demonstrated higher Ca ion levels in EDS analysis, and the microhardness values were also satisfactorily high. However, these results did not exhibit a significant difference compared to Group 2, which was treated with Ipana toothpaste and ozone gas. Similar to the results of this study, Akgün et al. (Akgün et al. 2021) showed in their in vitro studies that ROCS mineral gel can fill the gaps between enamel prisms with calcium, phosphate, and magnesium ions. The manufacturer recommended that this product must be used for at least 2 weeks. In this in vitro study, it was observed that microhardness values and Ca/P ratio were still high in Group 4 treated with ROCS mineral gel and ozone gas in the pH cycle that lasted only 1 week.

Bilgin et al. (Gocmen et al. 2016) reported that a mixture of ginger and honey had a remineralizing effect on initial enamel lesions. Korkut et al. (2017) showed that Gumgumix toothpaste remineralized initial caries better than the control group containing 1450 ppm fluoride. On the contrary, in this study, it was observed that Ipana toothpaste with 1450 ppm fluoride content had a higher remineralization capacity than Gumgumix toothpaste. Additionally, the combined application of Ipana and ozone showed the highest microhardness values.

Supporting the microhardness results of this study, SEM evaluation of the group treated with Ipana toothpaste and ozone gas revealed significantly fewer gaps and irregularities in the prismatic and interprismatic enamel areas compared to other groups. Homogeneous calcified sediments were observed extensively in this group. Similarly, in Group 4 treated with ROCS mineral gel and ozone gas, there was a reduction in gaps and irregularities in the prismatic and interprismatic enamel areas, though these calcified precipitates were not homogeneous. Conversely, in Group 3 treated with Gumgumix toothpaste and ozone gas, the decrease in gaps was concentrated only in the interprismatic area.

Amaechi et al. reported that demineralization at 37°C significantly increased initial enamel lesions compared to demineralization at 20 °C. In our study, after the demineralization process, average microhardness values decreased to 105 ± 4 VHN (Amaechi, Higham and Edgar 1998). Consequently, none of the tested samples were remineralized to the initial values. Additionally, 72 samples were collected to evaluate the remineralization process of the tested materials, and this number is not sufficient to draw any definitive conclusions. Further studies with larger sample sizes are needed to follow long-term results.

**Conclusion**

Based on the findings of this study, it is concluded that ozone gas has a positive impact on the remineralization process in initial carious lesions, supporting the acceptance of the null hypothesis. Additionally, ROCS mineral gel demonstrates the ability to remineralize initial caries, and the fluoride present in Ipana toothpaste is identified as a significant contributor to the remineralization process.

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**Disclosure statement**

No potential conflict of interest was reported by the author(s).

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Data availability statement

The data that support the findings of this study are available from the corresponding author, ÖE, upon request.

Ethical approval

Ethical approval was received for the study from Marmara University Dentistry Ethics Committee (Grant Number: 2022–56).

References


