Effect of Green Tea, Stevia Extract Solutions, and Fluoride-Based Mouthwash on Remineralization of Incipient Enamel Lesion: An In Vitro Study

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ABSTRACT:

Background: Remineralization of early enamel lesions is considered one of the approaches of minimal invasive dentistry. **Aim:** This in vitro study was conducted to evaluate the remineralizing effect of green tea, stevia extract, and fluoride-based mouthwashes on incipient enamel lesions using a laser fluorescence device (Diagnodent). **Materials and Methods**: Sixty human extracted premolars were randomly distributed into six groups of 10 teeth according to the remineralizing agent used. Group I: 0.5% stevia aqueous solution; Group II: 5% stevia aqueous solution; Group III: 0.5% green tea aqueous solution; Group IV: 5% green tea aqueous solution; Group V: Fluoride mouthwash (positive control group) and Group VI: artificial saliva (negative control group). Laser fluorescence testing (Diagnodent) was used for baseline assessment, after 48 hours, demineralization, and after 1 week of the remineralization phase. **Results:** After 7 days of remineralization, the negative control group showed the highest laser fluorescence mean value while all experimental and positive control groups showed the lowest laser fluorescence mean values. 5% stevia and 0.05 % Green tea mouthwashes showed an insignificant difference between baseline and remineralization. **Conclusions**: Stevia and green tea mouthwashes had a positive impact on incipient enamel remineralization.

Keywords: Enamel remineralization, stevia extract, green tea extract, fluoride, Incipient enamel lesions.

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INTRODUCTION:

Dental caries is an infectious disease affecting many populations worldwide. Dental caries is a dynamic reversible process of demineralization and remineralization of the tooth inducted by acid-producing bacteria in the oral cavity. Non-cavitated carious lesions could be arrested and reversed with proper plaque control and remineralization therapy. (1)

Mechanical tooth brushing in conjunction with the use of mouthwash as a daily oral hygiene routine is effective in reducing dental caries incidence.

Minimally invasive dentistry dictates the need for remineralization of early enamel lesions. Novel approaches had been launched to remineralize initial enamel lesions such as toothpastes, mouthwashes, sealants, and gels. Topical use of remineralizing agents had been reported to provide ions that favored the subsurface mineral gain achieving a reorganization of the enamel prismatic structure. (2, 3)

Fluoride-mediated remineralization is the cornerstone for caries prevention as well as for incipient lesion remineralization. Fluoride has an inhibitory effect on *Streptococcus mutans*, an acid-producing bacteria that cause demineralization of tooth structure. (4) Furthermore, fluoride enhances calcium and phosphate ion precipitation on the enamel surface and replaces the –OH group in the hydroxyapatite (HAp)-forming fluorapatite crystals (FAP) which have high resistance to dissolution in an acidic environment. (5)

However, these fluorapatite crystals limit further incorporation of the calcium and phosphorus ions affecting the subsequent mineralization process of the subsurface enamel lesions. (6) Additionally, fluoride fails to guide the formation of well-ordered and oriented mineral crystals on the enamel surface. (7)

Nowadays, prime attention has been directed toward the use of natural products-based mouthwashes as they have minimal side effects and are alcohol and/or sugar-free. (8)

Green tea contains minerals mainly potassium, phosphorus, calcium, and magnesium. Green tea is characterized by its high fluoride content (over 1500 ppm) as well as its high polyphenols (catechins) content like epigallocatechin gallate (EGCG). Green tea can act as an enamel matrix metalloproteinases (MMPs) inhibitor. The enamel remineralization efficiency of green tea had been reported by in vitro and in vivo studies. (9, 10, 11)

Stevia is a natural herbal non-caloric sweetener extracted from Stevia rebaudiana. Stevia contains tannins, xanthines (theobromine and caffeine), and flavonoids. Stevia has two main glycosides; Stevioside and Rebaudioside A. (12) Stevia has an antibacterial effect against streptococcus mutans. (13) Nevertheless, its enamel remineralizing effect is still controversial. Some studies reported its demineralization potential. (14) However, other studies proved that stevia increases salivary flow rate, salivary pH as well as salivary buffering capacity. (15, 16)

The current in vitro study was conducted to compare and evaluate the remineralizing effect of green tea, stevia extract, and fluoride-based mouthwashes on incipient enamel lesions using a laser fluorescence device (Diagnodent).

MATERIALS & METHODS:

Study Design

Sixty human-extracted premolars were randomly selected and used in this study. The teeth were equally randomly distributed into six groups of 10 teeth according to the remineralizing agent used. Group I: 0.5% stevia aqueous solution; Group II: 5% stevia aqueous solution; Group III: 0.5% green tea aqueous solution; Group IV: 5% green tea aqueous solution; Group V: Fluoride mouthwash (positive control group) and Group VI: artificial saliva (negative control group). Laser fluorescence testing (Diagnodent) was used for baseline assessment, after 48 hours, demineralization, and after 1 week of the remineralization phase.

Materials

a) A commercially available green tea brand; (Lipton, Unilever Mashreq Co.; Egypt)

b) Stevia extracts powder (The Agriculture Research Center, Giza, Egypt)

c) Amine Fluoride 0.125 gm mouthwash (Ezafluor mouthwash, Multipharma Co. Egypt)

Research Ethical Approval

The study was approved by the Research Ethics Committee (REC) of the Dentistry Faculty, Al-Ahram Canadian University, Egypt. Research number (IRB00012891‡29).

Sample size Calculation

The sample size was calculated depending on a previous study as a reference. [17] According to this study, the accepted sample size was 12 per group, when the response within each subject group was normally distributed with a standard deviation of 10, the true mean difference was 12, total sample size increased to 15 to compensate for 20% drop out when the power was 80 % & type I error probability was used 0.05. PS Power 3.1.6. for sample size calculation.

Samples Preparation

Sixty freshly sound extracted premolars for orthodontic reasons were collected, cleaned, and disinfected with 5.25% sodium hypochlorite solution for 60 minutes then stored in artificial saliva at room temperature. [18] Decoronation of all teeth was done by sectioning the roots 2 mm cervical to the cemento-enamel junction using a diamond saw with water coolant (Isomet®5000 Linear Precision Saw; Buehler Ltd., Lake Bluff, USA) [19] Residual tissues and debris were removed by scraping the crowns with a hand scaler and washing under running tap water; then polishing with fluoride-free pumice paste was done. A colored varnish coating was applied on all teeth surfaces leaving a window of 2 mm X 2 mm on the middle third of the buccal and lingual surfaces [20, 21]

Each tooth was sectioned into two halves in a mesio-distal direction to obtain buccal and lingual halves. Plastic molds of 3 mm in

height and 20 mm in length were poured with cold cure acrylic resin (Acrostone, Egypt) to prepare the blocks. Each half (Buccal and Lingual) was fixed on the custom-made acrylic block using superglue to get ready for surface treatment [20] Then a waterproof permanent marker was used to numerically code all blocks' bases. After grouping, all samples were kept in a separate glass container of 10 ml of artificial saliva at 37°C (D180-P air jacket CO2 incubator, Egypt).

Incipient Enamel Lesions Preparation

All samples were immersed in a demineralizing solution (10 mL for each specimen) for 48 hours. The demineralizing solution was composed of 2.2 mM calcium chloride, 2.2 mM potassium dihydrogen phosphate, 0.05 M acetic acid, and 1 M potassium hydroxide (KOH) to maintain a pH of 4.4. [20] After Rinsing the samples with distilled water, they were stored in artificial saliva to simulate the oral cavity conditions. The demineralizing solution was renewed every 12 hours to prevent depletion of the solution.

Remineralizing agents Preparation

Preparation of 0.5% and 5% Stevia Aqueous Extracts

Powder of 0.5 g and 5 g weight were mixed with 100 ml of boiled distilled water and poured into a sterile glass flask for 5 minutes. Extracts filtration was done through a muslin cloth for coarse residue then filtered through Whatman No. 1 filter paper and finally kept in an airtight container. [22]

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Enamel Surface Treatment with Remineralizing Mouthwashes:

All experimental groups (I, II, III, IV, V) were immersed in 20 ml of the corresponding mouthwash (0.5% stevia, 5% stevia, 0.5% green tea, 5% green tea, fluoride mouthwash respectively) for 5 min at 37°C 3 times per day for 7 days. At the end of the week, samples were rinsed carefully with distilled water. All samples were dried with clean absorbent then returned to the incubator and stored in 20 ml of artificial saliva. Samples of the negative control group were stored in the artificial saliva during the whole experimental time and not subjected to any type of treatment. Artificial saliva was changed daily to avoid the risk of the artificial saliva's saturation which may interfere with the treatment process. [23] Artificial saliva was prepared by mixing 500 ml distilled water, 20 g potassium chloride, 0.843 g sodium chloride, 0.051 g magnesium chloride, carboxymethyl cellulose, 20 ml tricalcium phosphate, and 0.05 M sodium hydroxide to maintain a pH of 6.8 [24]

Laser Fluorescence Testing

DIAGNOdent pen (KaVo, Bilberach/Riss, Germany) was used at baseline, after Initial enamel lesions creation, and after 7 days of remineralizing phase. Laser fluorescence testing was done three times for each specimen and the mean value was calculated and recorded. The concept of using a diode laser light source and a fiber optic cable depends on transmitting this light to a handheld probe. This light is absorbed inducing infrared fluorescence by organic and inorganic materials. Reflected fluorescence is collected at the probe tip, displaying a digital number between 0 and 99. Based on the previous study by Lussi et al; (1999), a Scoring system according to the manufacturers' instructions is used. Score 0-4: no caries, score 5-10: enamel caries limited to the outer half of the enamel thickness, score 11-20: enamel caries limited to the inner half of the enamel thickness without obvious spread in the dentin and score ≥ 21: caries spread in the dentin. [25]

Statistical Analysis

Statistical analysis was performed with SPSS 16 ® (Statistical Package for Scientific Studies), Graph pad prism and windows. Exploration of the given data was performed using the Shapiro-Wilk test and Kolmogorov-Smirnov test for normality which revealed that data originated from nonparametric data. Accordingly, a comparison between different intervals was performed using Friedman`s test, while a comparison between different groups was performed using the Kruskal-Wallis test.

RESULTS:

Comparison of Enamel Fluorescence treated with Green Tea, Stevia Extract Solutions, and Fluoride-Based Mouthwashes at baseline, after 48 hrs of demineralization, and after remineralization.

Comparison between different groups as shown in **table (1)** and **figure (1)** revealed significant differences at all intervals as $P<0.05$, multiple comparisons were also performed and revealed that:

In all groups except artificial saliva baseline was significantly lower than demineralization while there was insignificant difference between baseline and remineralization, while in artificial saliva baseline was significantly lower than demineralization while there was insignificant difference between demineralization and remineralization

Effect of material

Comparison between different groups revealed significant difference at all intervals as $P<0.05$, multiple comparisons was also performed and revealed that:

- At baseline: there was insignificant difference between all groups as $P=0.06$.
- After 48 hrs. of demineralization: there was insignificant difference between all groups as $P=0.058$.

- After remineralization: there was a significant difference between all groups as P=0.0001*, as artificial saliva (11.75 ± 1.25) was significantly the highest while there was insignificant difference between all other groups.

Table (1): Mean and standard deviation of all groups at baseline, after 48 hrs. of demineralization and after remineralization.

	Baseline		Demin. 48hrs		Remineralization		P value (Friedman`s test)
	M	SD	M	SD	M	SD	
0.5% Stevia	7.76 aA	3.24	10.27 bA	3.16	6.15aA	3.51	0.03*
5% stevia	7.27 aA	2.09	11.33 bA	3.46	7.78 aA	4.58	0.003*
0.05 % Green tea	7.38 aA	1.56	10.07 bA	3.20	7.07 aA	6.13	0.01*
5% Green Tea	7.15 aA	1.21	10.51 bA	5.42	7.28 aA	6.49	0.04*
Artificial Saliva	8.69 aA	1.97	13.13 bA	1.45	11.75 bB	1.25	0.0001*
5% Fluoride (Positive Control)	8.57 aA	1.45	12.15 bA	3.83	8.82 aA	5.24	0.01*
P value	0.06		0.058		0.0001*		

Means with the same superscript letters (small per raw / capital per column) were insignificantly different as P>0.05

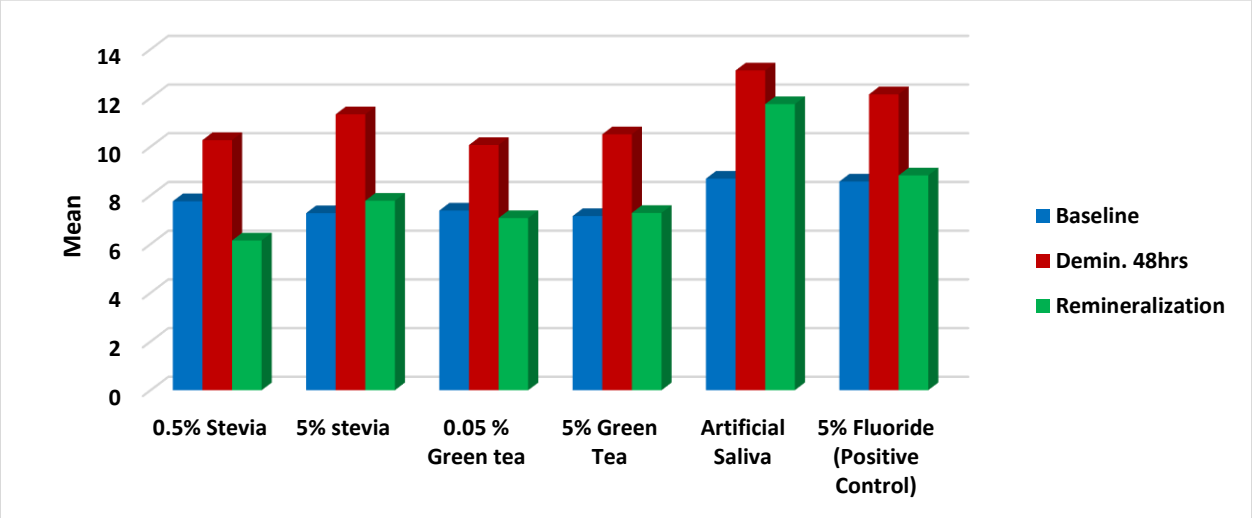

Figure (1): *bar chart showing Mean of all groups at baseline, after 48 hrs. of demineralization and after remineralization.*

DISCUSSION:

Dental caries is one of the global health issues in both industrialized and developing communities. [26] It is a multifactorial dynamic disease that causes a cycle of demineralization and remineralization of dental hard tissues. [27]

Following caries prevention protocols resulted in a significant decline in dental caries levels in high caries-risk groups with low socio-economic status in developed countries [28]. However, there are still great challenges in dental caries prevention and treatment.

Fluoride can prevent dental caries when used in different forms [29] Even though the beneficial effects of fluoride on dental health are well-established, it is very crucial to regulate the amount of fluoride intake. Excessive ingestion of fluoride may cause toxic and harmful effects. Oral hygiene products are the major source of fluoride toxicity. According to fluoride poisoning data collected by the American Association of Poison Control (AAPC), toothpaste ingestion remains the main source of toxicity followed by fluoride-containing mouthwashes and supplements. [30] This gave rise to investigating natural products for their remineralizing effect as they are safer with no side effects.

Therefore, this in vitro study was conducted to evaluate the remineralizing effect of different concentrations of an aqueous solution of green tea, stevia, and fluoride on artificially demineralized enamel using laser fluorescence, DIAGNOdent pen.

Fluoride promotes the formation of appetite and enhances the remineralization of teeth. [19] When the concentration of fluoride ion is low (under 100 mg/L), fluorapatite is formed, which improves the acidity resistance of enamel. When the concentration of fluoride ion is higher, it combines with calcium ion to form calcium fluoride, promoting remineralization. [31]

DIAGNOdent pen is a useful and reproducible method that could be used to quantify WSLs and test the effectiveness of preventive treatment. [32]

All enamel specimens (experimental, positive, and negative control groups) were subjected to the demineralization phase for 48 hours. The demineralizing solution was used in this study of pH 4.4 to simulate the pH drop that happens in the oral cavity as it can cause enamel dissolution and demineralization. [19] Artificial saliva was used as a storage medium for all experimental and negative control teeth to simulate the remineralizing capacity of human saliva [33], as it contains inorganic electrolytes (calcium, phosphorus, and fluoride) that are important elements in the remineralization process. [34]

All specimens were subjected to laser fluorescence assessment using a DIAGNOdent pen at baseline, after demineralization and after remineralization with different concentrations of stevia and green tea, fluoride mouthwash (positive control) as well as the specimens stored in the artificial saliva without any treatment (negative control). Regarding laser

fluorescence readings after demineralization for 48 hours, there was an increase in the reading indicating demineralization and loss of minerals in all groups. These results confirmed the demineralizing effect of the demineralizing solution used in our study. [19]

Laser fluorescence results showed a statistically significant difference between the demineralized enamel and the treated enamel with green tea, stevia, and fluoride which were capable of remineralizing enamel tissues. The remineralizing ability of green tea could be attributed to the action of tea on the collagen network which stabilized the collagen and maintained it in an expanded state thus the interfibrillar spaces are kept open for remineralization. [35] Additionally, green tea is rich in F ions ranging from 3.2 mg kg/1 to 400 mg kg/1 by weight in dry samples. [36, 37, 38] Furthermore, the high pH value of green tea which is about 6.3 may be the reason for being a strong remineralizing agent. [39]

Several studies were in agreement with the result of the present study as they reported the remineralizing effect of green tea using different methods of assessment. [36, 37,38] Babu et al., 2017, revealed that green tea and sodium fluoride showed an increase in enamel fluorescence indicating the remineralization of demineralized enamel samples. Green tea has a higher amount of remineralization ability as compared to black tea. They referred to this result to the high catechin fraction which is responsible for its antibacterial properties. [36] Bozorgi et al., 2018, stated that microhardness values increased significantly after treating the teeth with green tea as compared to the microhardness values following demineralization. [40] Several studies concluded that green tea extract could reduce teeth erosion. [41] Green tea varnish significantly reduced white spot lesions around orthodontic brackets. [42]

The stevia leaves contain compounds such as diterpenoid steviol-glycosides, sesquiterpenes, bis-nor-diterpene, sterols, and flavonoids which have many systemic therapeutic properties. [43,44] The 5% stevia group showed better remineralization compared to the values of the 0.5% stevia group. This was in contrast with Kishta-Derani et al who concluded that 5% stevia recorded the greatest microhardness loss. [44] Demirez et al evaluated the Ca, P, and Ca/P ratio of teeth using the EDX analysis, the stevia group showed better results than the distilled water [45]. It was postulated that stevia has the capability to reduce dental caries by reducing the amount of acid produced in the mouth as a result of food decomposition by the microorganisms, decreasing the cell surface hydrophobicity, inducing cell aggregation, inhibiting the production of extracellular polysaccharide, and finally by preventing bacteria from adhering to the enamel. [44] Nutrients in Stevia include water (80-85%), protein, fiber, monosaccharides, lipids, essential oils, vitamin C, b-carotene, vitamin B2, and vitamin B1. In addition, it contains antioxidant compounds like apigenin, quercetin, isoquercitrin, luteolin, miocene, kaempferol, chlorogenic acid, and caffeic acid. It also includes minerals such as

cobalt, magnesium, iron, potassium, and phosphorus. [47]

The amine fluoride mouthwash-treated group showed less remineralization potential when compared to the green tea and stevia groups. This could be attributed to the in vitro study environment that lacks the oral soft tissues which serve as reservoirs for fluoride. [48] In an in vivo environment, fluoride may be retained on a large surface area of soft tissue as the tongue, which may increase the availability of the active agents and impact remineralization in a different way than what happened in this study. [14]

CONCLUSION:

Under the limitations of the current in vitro study, it was concluded that green tea and Stevia mouthwashes had a positive impact on incipient enamel remineralization.

RECOMMENDATIONS:

Further studies are recommended to be conducted to evaluate and compare the enamel remineralization effect of the Stevia, green tea, and fluoride-containing mouthwashes In Vivo for longer follow-up periods to explain the extract concentration effect.

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