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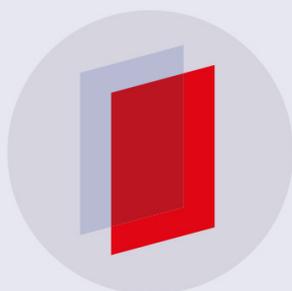
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The effect of theobromine 200 mg/l topical gel exposure duration against surface enamel hardness resistance from 1% citric acid

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Abstract. Theobromine can be used to prevent the demineralization of enamel and can stimulate the growth of new enamels. This study analyzes the effect of theobromine's gel duration exposure on enamel hardness resistance from 1% citric acid. Twenty-eight specimens were divided into three experimental groups; were exposed to theobromine gel 200 mg/l for 16, 48, and 96 minutes; and were then immersed in 1% citric acid. The control group was only immersed in 1% citric acid. Results: A Wilcoxon test showed a significant increase and decrease in enamel microhardness after exposure to theobromine gel and citric acid ($p < 0.05$). A Mann-Whitney test showed a significant increase and decrease in enamel microhardness between different durations of exposure to theobromine gel and immersion in citric acid ($p < 0.05$). The application of theobromine gel 200mg/L increased enamel microhardness but did not contribute to the enamel's hardness resistance after immersion in 1% citric acid. The duration of theobromine gel application affected enamel microhardness and acid resistance.

1. Introduction

Today there is an increased consumption of soft drinks and fruit juices. In Europe, the rate of soft drink and fruit juice consumption exceeds 50% of the total consumption of non-alcoholic beverages [1]. It is well known that acidic foods and beverages are erosive to tooth structure. Tooth erosion is defined as an irreversible loss of enamel and dentin caused by non-bacterial chemical processes. Dental erosion occurs due to the presence of strong acids in the oral environment that derive from acidic foods and beverages such as fruit, fruit juices, carbonated beverages, and sports drinks as well as medicines such as hydrochloric acid tablets, aspirin (acetylsalicylic acid), and vitamin C [2,3]. Enamel is the hardest outer tooth epithelial layer and is largely composed of hydroxyapatite crystals. Enamel is permeable to ions and molecules that can dissolve when in contact with acidic food or beverages. These dissolved ions and molecules can reduce the surface hardness of the tooth [4]. Tooth demineralization occurs when salivary conditions in the mouth are acidic with a pH below 5 [5]. In this condition, the hydrogen ion reacts specifically with phosphate near the surface of the hydroxyapatite (HA) crystals and causes the HA crystals to dissolve. The continuous dissolution of HA can form porosity on the enamel surface, which can lead to pathological conditions [5].

According to Arman Sadeghpour from Tulane University, one of the alkaloids found in chocolate, theobromine, can be used to prevent demineralization of enamel. Theobromine (3,7 dimethylxanthine) is a chemical compound of the alkaloid group in the form of white crystalline powder and only differs from the caffeine molecule by one methyl group (1,3,7 dimethylxanthine). Theobromine is a non-



toxic, natural, and more effective alternative to fluoride in toothpaste. According to Sadeghpour's study, theobromine can stimulate the growth of new enamels. This is because theobromine causes calcium and phosphate from saliva to combine into a crystal unit four times larger than hydroxyapatite [6]. This study is also supported by Tetsuo Nakamoto, who stated that adding theobromine to apatite material can increase the size of crystallite size [7]. Toothpaste with theobromine content has been developed, but gel with theobromine content is not yet available. Gel is clear, semi-solid (colloidal mixture between solid and liquid), translucent, and contains active substances. Gel has several advantages, including its ease of application, its easiness to clean, and its accommodation of faster drug substance release [8]. This study will examine the effect of theobromine topical gel exposure duration for 16 minutes, 48 minutes, and 96 minutes as a remineralization agent that can maintain the hardness of enamel against demineralization with 1% citric acid as a way to improve oral hygiene and protection against tooth damage. This resistance is measured from enamel hardness after demineralization, which previously received theobromine gel exposure. Mineral depositing by the remineralization agent will affect the surface enamel hardness and protect against acid solubility. This study aims to determine the effect of theobromine gel 200 mg/l in 3 different durations, which are 16 minutes, 48 minutes, and 96 minutes, in maintaining tooth enamel hardness against demineralization with 1% citric acid.

2. Materials and Methods

This study is a laboratory experimental study. The samples used were enamel samples of human premolars that were divided into 3 test groups (initial, after theobromine 200 mg/l gel exposure, and after demineralization with citric acid solution) with exposure times of 16 minutes, 48 minutes, and 96 minutes. One group ($n = 7$) was only treated with immersion in 1% citric acid solution and acted as a descriptive comparison. After theobromine gel exposure, the three test groups were tested for their hardness. Subsequently, the test group was immersed in a 1% citric acid solution with pH 4 for 150 seconds to determine whether the enamel could maintain its hardness. Measurement of surface enamel hardness using the Knoop Microhardness Tester Shimadzu Japan was performed before treatment, after being exposed to theobromine gel, and after immersion in 1% citric acid.

The duration of theobromine exposure for 16 minutes, 48 minutes, and 96 minutes was an assumption of fluoride topical gel usage for 1 month, 3 months, and 6 months. According to Gunawan, 6 months is chosen as the longest time limit because the fluoridation effect occurred after fluoride consumption for 6 months. The length of time in a single exposure, which is 4 minutes per week, is a simulation of topical gel application time in individuals with low levels of caries, which is 1-2 minutes each year [5]. One month and 3 months were chosen to compare whether theobromine takes the same or less time than fluoride in improving and maintaining enamel surface hardness against acids. Data analysis was done using SPSS program version 17. After conducting a Wilcoxon test, a Friedman test was done to determine the significance of increase and decrease of enamel surface hardness values in each test group. Then, a Kruskal-Wallis test was done to determine the significance of the increase and decrease of enamel surface hardness among the test groups followed by Post Hoc analysis using the Mann-Whitney test.

3. Results and Discussion

3.1 Results

In this study, an enamel hardness test was performed after theobromine gel 200 mg/l exposure with three different time durations, which are 1 month (16 minutes), 3 months (48 minutes), and 6 months (96 minutes) followed by immersion in 1% citric acid solution. The mean values of hardness were obtained using the Knoop Microhardness Tester; these values can be seen in Table 1. The mean value of the enamel hardness values on theobromine 200 mg/l gel exposure with durations of 1 month (16

minutes), 3 months (48 minutes), and 6 months (96 minutes) followed by immersion in 1% citric acid solution are described.

Table 1. Mean Value of Micro Hardness Enamel (KHN) Before and After Exposure to Theobromine Gel and Acid Immersion

Hardness value after treatment	Exposed Group		
	1 months (16')	3 months (48')	6 months (96')
Initial Hardness	402 ± 15.39	412.43 ± 2.93	413.71 ± 4.31
Final Hardness after <i>Theobromine Gel</i> 200 mg/l Exposure	425 ± 5.51	448.86 ± 11.39	465.43 ± 8.65
Final Hardness after Citric Acid 1% Demineralization	280.29 ± 10.48	302.14 ± 8.66	333.14 ± 8.27

Table 1 shows the average difference in surface enamel hardness values before and after exposure to theobromine gel 200 mg/l with exposure times of 16 minutes, 48 minutes, and 96 minutes followed by immersion in 1% citric acid. The result of the exposure was an increase in the mean value of enamel hardness from 402 ± 15.39 KHN to 425 ± 5.51 KHN (5.70%) in theobromine gel exposure for 16 minutes; an increase in the mean value of enamel hardness from 412, 43 ± 2.93 KHN to 448.86 ± 11.39 KHN (8.79%) in theobromine gel exposure for 48 minutes; and an increase in the mean value of enamel hardness from 413.71 ± 4.31 KHN to 465.43 ± 8.65 KHN (12.55%) in theobromine gel exposure for 96 minutes. After exposure to the gel, the tooth was immersed in 1% citric acid solution for 150 seconds. As a result, as shown in Table 1 and Figure 1, there was a decrease in the mean value of enamel hardness in the 16-minute group from 425 ± 5.508 KHN to 280.29 ± 10.484 KHN (34.09%). In the 48-minute group, the value of hardness decreased from 448.86 ± 11.393 KHN to 302.14 ± 8.668 KHN (32.68%), and in group of 96 minutes the value of enamel hardness decreased from 465.43 ± 8.658 KHN to 333.14 ± 8.275 KHN (28.44%).

Friedman's statistic test was performed to determine the significance of surface enamel hardness change in each test group: 16 minutes, 48 minutes, and 96 minutes. The results of Friedman's statistic test showed a significantly different change in surface enamel hardness value ($p = 0.05$) in each test group. The Wilcoxon statistical test was used in each test group to determine the difference between the mean initial hardness value, the mean value of hardness after gel exposure, and the mean value of hardness after demineralization. The results of this test show that in groups of 16 minutes, 48 minutes, and 96 minutes, there was a significant difference between the mean value ($p < 0.05$) after gel exposure and after demineralization. The Kruskal-Wallis statistical test was performed to determine the mean values of enamel hardness between the three treatment groups (baseline, after gel exposure, and after demineralization). The obtained mean value of initial enamel hardness was over p ($p = 0.05$), which means there is no significant difference in the mean value of initial hardness. The mean significance value of enamel hardness after gel exposure was less than p ($p = 0.05$), which means there is a significant difference in the mean value of enamel hardness between groups after theobromine gel exposure. The mean significance value of enamel hardness after demineralization was less than p ($p = 0.05$), which means there was a significant difference in the mean value of enamel hardness between groups after immersion in 1% citric acid solution.

Post Hoc analysis was performed as a continuation of the Kruskal-Wallis test and showed significant differences in hardness values after gel exposure and after demineralization. Post-hoc analysis with Mann-Whitney test in the group after gel exposure showed a significant increase in the mean value of enamel hardness ($p < 0.05$) in the three durations of gel exposure: between 16 minutes and 48 minutes duration, between 48 minutes and 96 minutes duration, and between 16 minutes and 96 minutes duration. A significant difference in the mean value of enamel hardness was also seen in all three test groups after demineralization: between the 16 minutes and 48 minutes, between the 48 minutes and 96 minutes, and between the 16 minutes and 96 minutes. In this study, as shown in Table

1.2, treatment was performed in the comparison group in which the enamel hardness value of seven dental specimens were measured and then immersed in 1% citric acid solution without gel exposure. The value of enamel hardness decreased from 386.71 ± 15.82 KHN to 257.29 ± 14.55 KHN (33.46%). While in the theobromine gel exposure group for 16 minutes, there was a decrease in the value of enamel hardness from 402 ± 15.39 KHN to 280.29 ± 10.48 KHN (30.32%); in the 48-minute theobromine gel exposure group, there was a decrease in the hardness value from 412.43 ± 2.93 KHN to 302.14 ± 8.66 KHN (26.76%); and in the 96-minute theobromine gel exposure group, the value of enamel hardness decreased from 413.71 ± 4.3 KHN to 333.14 ± 8.27 KHN (19.46%). The decrease in value was seen from the hardness value of enamel after immersion in an acidic solution to the initial hardness value before exposure to theobromine gel for 16 minutes, 48 minutes, and 96 minutes.

3.2 Discussion

Based on the results in this study, there was a significant increase in enamel hardness in theobromine gel exposure of 200 mg/l for 16 minutes, 48 minutes, and 96 minutes. This increase was considered significantly different based on a Friedman's statistic test, which was followed by a Wilcoxon test ($p < 0.05$). From the results, it can be seen that theobromine gel 200mg/l can be quite effective as a remineralization agent at exposure for 16 minutes, 48 minutes, and 96 minutes. Increased hardness of this enamel, according to Nakamoto, relates to theobromine properties that can increase the size of apatite crystals that have been tested by the x-ray diffractometry test in which an increase in apatite crystal size is observed. Moreover, there was less release of Ca, P, and Mg ions in the theobromine group compared to the control group. This was consistent with a study by the Patent Cooperation Treaty (PCT), which showed significant increases in tooth enamel values in the group given exposure to theobromine paste (an increase of 27.5%) compared to the group given exposure to NaF paste (an increase of 8.5%) and the control group (increase of 9.4%) [9].

In the Kruskal-Wallis statistic test showing significant difference, continued Post Hoc analysis of Mann-Whitney test and the results showed significant differences in the mean of the inter-group treatment of the theobromine gel group at 16 minutes, 48 minutes, and 96 minutes. At the exposure time of 16 minutes, the mean value of enamel hardness increased to 425 ± 5.51 KHN (an increase of 5.7%); at the exposure time of 48 minutes, the mean value of enamel hardness increased to 448.86 ± 11.39 KHN (an increase of 8.79%); and at the exposure time of 96 minutes, the mean value of enamel hardness increased to 465.43 ± 8.65 KHN (an increase of 12.55%). The best results were obtained in the 96-minute treatment group. This is due to the longer exposure of the enamel surface to theobromine, which is a tooth remineralization agent. The results obtained are in accordance with those obtained by Muljadi, which suggested that the longer the fluoride (which is considered the same as theobromine as the remineralization agent) is in contact with the enamel, the higher the increase in enamel hardness [10]. In this study, the longer the theobromine gel comes into contact with enamel, the more likely it is that the resulting apatite crystals will increase the hardness of the enamel.

The demineralization process in this study was simulated using 1% citric acid solution with pH 4. Citric acid is an acid that is widely contained in soft drinks and has a high demineralization potential [11]. A study conducted by Lussi *et al.* and Attin *et al.* showed that citric acid acts as a chelator agent that can bind calcium on the surface of an enamel, which indicates the potential demineralization of acidic beverages in everyday life [11,12]. Michele *et al.* in his study showed that immersion of citric acid (in vitro) pH 4 for 150 seconds was equal to 40 minutes of enamel exposure with orange juice (in situ), and there was a significant decrease in surface hardness value after immersion in an acidic solution [13]. This is also supported by Frank Lippert, who performed a demineralization cycle by immersing the sample using a citric acid solution (pH 4) for 150 seconds, which resulted in a significant decrease in surface hardness value [14]. According to Olsson C.E.G [15], the decrease in hardness occurs because the enamel reacts with acidic (H^+) ions and dissolves the hydroxyapatite into calcium and phosphate ions [5].

After exposure to theobromine gel 200 mg/l at 3 different times, the whole group was immersed in a 1% citric acid solution with a pH of 4. Friedman and Wilcoxon test results showed a significant

decrease in the value of enamel hardness in all test groups seen against initial hardness values and hardness values after theobromine gel exposure. The decrease in the initial hardness value of enamel after acid immersion in the 16-minute gel exposure group was 30.32%. In the 48-minute gel exposure group, decreased hardness of enamel after acid immersion was 26.76% against initial hardness values. A 19.46% decrease in initial enamel hardness occurred in the 96-minute gel exposure group after immersion in acid. This decline in the value of enamel hardness was judged to be statistically significant. There was a significant decrease in the value of hardness between the initial hardness value and the hardness value after acid immersion in all test groups. This showed that theobromine gel exposure at 16 minutes, 48 minutes, and 96 minutes was not sufficient to maintain enamel resistance to 1% citric acid. This significant decrease in the value of hardness was due to the 1% citric acid (pH 4), which had considerable erosive properties and caused demineralization. This is in accordance with previous research, which found that citric acid is strong and can cause faster erosion, especially at a low pH. This acid is also twice as destructive to enamel as hydrochloric acid or nitric acid because of its great affinity for calcium [16]. Citric acid is commonly used in soft drinks and is very erosive.

The Kruskal-Wallis statistical test followed by Post Hoc analysis with a Mann-Whitney test showed a significantly different change in hardness value ($p < 0.05$) in all groups exposed to theobromine and then immersed in 1% citric acid. From percentage calculations, the enamel sample group that had received 16 minutes of theobromine gel had decreased hardness by 30.32% after demineralization with 1% citric acid compared to the initial hardness value. While the enamel test group with 48 minutes and 96 minutes of gel exposure experienced a decrease in hardness of 26.76% and 19.46% respectively compared to initial hardness values. When compared with descriptive enamel specimens directly immersed in 1% citric acid solution without exposure to theobromine gel (Table 2), the percentage of enamel group degradation decreased with theobromine gel exposure; this percentage was still lower than the percentage decrease in enamel hardness directly immersed in citric acid 1%, which was 33.46%. The percentage value showed that 96 minutes of theobromine gel exposure was more effective in reducing the percentage decrease in enamel hardness against acid immersion compared with theobromine gel exposure for 16 minutes and 48 minutes. However, the third duration of exposure is not effective enough to restore the initial value of enamel hardness.

The effect of theobromine 200 mg/l gel in increasing the initial hardness value of enamel and maintaining enamel hardness after demineralization is allegedly supported by the ability of theobromine ability to increase the crystal size of apatite enamel. According to previous study, this is likely due to interstitial reactions by theobromine ions to apatite because theobromine ion size is smaller than the diameter of microtunnel enamel. The substitution of other ions in apatite crystals will cause a change in the physical properties of the apatite. The crystal structure will become denser due to the enlargement of apatite, which will lead to the emergence of forces between the larger atoms. The greater the attraction force between atoms, the greater the force required to separate the bonded atoms. This suggests that theobromine may make the enamel more resistant to the acid because it requires a stronger acid to have a greater force to react with (draw) one of the ions of the apatite crystals and cause solubility and degradation of enamel hardness [17]. But the possibility of this explanation needs to be further investigated. This result also conforms to Nakamoto's assertion that the increasing crystal sizes of enamel causes the enamel surface to become harder and more resistant to the acidic erosion properties [7]. The addition of minerals that play an important role in tooth remineralization such as calcium and phosphate in theobromine gel will also optimize the remineralization effect in enamel, but this needs further investigation. In this study, theobromine gel consisted of a pure theobromine powder plus carboxymethyl cellulose (CMC), which acts as a thickener and has no additional minerals for tooth remineralization. Based on research conducted by the Patent Cooperation Treaty (PCT), efforts are being conducted to enhance and maintain enamel hardness by introducing theobromine elements (at least calcium and phosphate) to the gel [8].

The results of this study indicate that the longer the exposure of enamel to theobromine gel, the greater the ability to maintain enamel hardness against citric acid 1%. These results were confirmed by Indah [18], who stated that more acid-resistant enamels were longer-lasting enamels exposed to

theobromine. In this study, a SEM (Scanning Electron Microscope) examination was performed to show the comparison of enamel surface damage levels among untreated groups, the group receiving only acid immersion, the group that was given cocoa solution for 5 minutes and then immersed in acid, and the group that was given cocoa solution for 10 minutes and then immersed in acid. The group that was given cocoa solution for 10 minutes showed less damage. In other words, the duration of theobromine exposure time affects the decrease in enamel surface damage in the demineralization process, which also affects the increase of theobromine's ability in maintaining the value of enamel hardness from solubility [18]. Based on the above description, it was shown that theobromine gel 200 mg / L exposure can significantly increase the hardness of enamel at exposure times of 16 minutes, 48 minutes, and 96 minutes. Increased enamel hardness is related to theobromine properties that can increase apatite crystal size, which causes apatite crystals to become more resistant to acids. However, in its application, it is necessary to add additional minerals such as calcium and phosphate to theobromine gel so it can enhance its effects of enamel endurance and solubility.

4. Conclusion

Gel with theobromine 200 mg/l content can increase surface enamel hardness with duration exposures of 16 minutes, 48 minutes, and 96 minutes. However, after immersion of 1% citric acid (pH 4) for 150 seconds, surface enamel hardness with theobromine gel exposure for 16 minutes, 48 minutes, and 96 minutes decreased hardness. The enamel surface with 96 minutes of theobromine exposure showed the lowest decrease in hardness compared to 48 minutes and 16 minutes. Further studies of the apatite crystal enlargement mechanisms caused by theobromine on human's HA tooth and the theobromine gel effect on enamel hardness with control groups using artificial saliva or additional calcium and phosphorus minerals should be conducted.

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