QUANTITATIVE EVALUATION OF THE POWER OF AMELOGENIN AND CHITOSAN IN NATURAL REPAIR OF DENTIN DEFECTS USING ENERGY-DISPERSIVE ANALYTICAL X-RAY ELEMENT ANALYSIS

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ABSTRACT

Objectives: Evaluate the potential of amelogenin and chitosan to repair eroded dentin at different times by means of a chemical element analysis.

Methodology: Twelve human lower first molars were collected. Radicular part was removed, while coronal part was sectioned mesiodistally. Two dentin slabs were obtained from each molar. Samples were immersed in Coca Cola beverage (Coca-Cola® Co., Egypt) for 25 hours. Samples in group I were stored in artificial saliva, while in group II they were treated with agarose hydrogel. Agarose hydrogel was loaded with chitosan in group III and with Emdogain in group IV. Hydrogels were applied to samples for 5 hours. This procedure was repeated daily for 15 days. Dentin mineral contents (Ca & P) were tested using energy dispersive analytical X-ray.

Results: The baseline mean values of Ca & P in sound dentin samples were (23.08±2.23) & (7.91±3.09), while were significantly decreased into (6.88±2.30) & (4.04±1.28) after immersion in Coca Cola. On comparing Ca & P contents of different groups after 5 days treatment, ANOVA showed no significant differences in both elements, while after 10 days a highly statistically significant differences were recorded in all groups (p=0.000). After 15 days, a highly significant differences in Ca content were recorded (p=0.000), while no significant differences were recorded in P content (p=0.167). Emdogain group represented the best results.

Conclusion: Emdogain exhibits promising remineralizing effect on erosive dentin, as it has the highest mean difference when compared to chitosan and control groups.

KEYWORDS: Amelogenin- Chitosan- Erosive dentin- Remineralization.

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INTRODUCTION

Dental erosion is the process by which tooth hard tissue is lost through chemical disintegration in the absence of oral microorganisms.[1,2] Acids and certain chemicals can erode the surface of teeth and dental restorations, resulting in structural damage.[1,3,4] When tooth substance loss is mostly caused by dental erosion, the condition is known as erosive tooth wear and typically manifests as a lesion with a smooth, melted appearance of dentin and enamel.[2,5] Even though there are less research on erosive tooth wear in adults than in children and adolescents, adult prevalence ranges widely from 2% to 100%, with an increasing frequency as people age.[6-10]

Dentin hypersensitivity, dental discomfort, poor dental aesthetics, and/or compromised oral function are possible effects of dental erosion.[11] Since intrinsic gastrointestinal and extrinsic dietary acids are the etiological factors of dental erosion, some self-care measures were developed to reduce dentition's exposure to these acids. To manage dental erosion and its clinical ramifications, these measures are taken in addition to the preventive and restorative strategies recommended by dental professionals.[11-14] Specifically, acidic beverages such juices, iced tea, sparkling water, energy drinks, soft drinks, and sports drinks may provide a risk of tooth erosion. [15,16]

The major structural protein in dentin is a highly crosslinked type I collagen and around 10% of organic dentin.[17] It is also composed of non-collagenous proteins and proteoglycan molecules which form the backbone of collagen network incorporating hydroxyapatite crystals within.[17] Tubule diameter increases and peri-tubular dentin dissolves initially as a result of the erosive process.[18] This may result in the organic matrix being exposed and the intertubular dentin becoming demineralized, leaving a rough and porous surface.[18,19] Apart from the breakdown of minerals, there is also enzymatic degradation of collagen.[20,21]

The application of salts and ions, including hydroxyapatite, calcium phosphate, fluoride, and oxalates, and proteins that encourage remineralization are some of the various therapeutic modalities that include dentin blocking method.[22] Because dentin lesions lack remaining seed mineral crystals and have a heterogeneous structure with a high organic content, the classical ion-based crystallization paradigm is ineffective for dentin remineralization.[23] Recently, multiple strategies of biomimetic remineralization have been reported. It has been shown that these methods work well for restoring dentin tissue microstructures. It illustrates various strategies for addressing this problem by backfilling dentin collagen with liquid-like amorphous calcium phosphate nano-precursor particles, which mimic the natural mineralization process. This bottom-up remineralization strategy is thought to be a workable way to remineralize and plug patent dentinal tubules because it is not reliant on seed crystallites.[24] Several medical specialties, including dentistry have highlighted the application of chitosan extracts.[25-27] The biopolymer chitosan is produced when chitin is deacetylated and is found naturally in the cell walls of fungi, yeasts, insects, and primarily the shells of crustaceans.[28] This material exhibits great potential as it is non-toxic, biocompatible, biodegradable, bio-adhesive, and promotes teeth remineralization by means of calcium and phosphate deposition. Because of its amino groups, the chitosan molecule chemically permits substitution processes and creates cross-links with dentin collagen.[28-32] The chitosan amine group (NH3+) is drawn to the collagen carboxyl group (COO−) through electrostatic interaction, which gives it its adhesiveness.[28] Through this process, the organic matrix becomes more chemically and physically stable, resists degradation, and is less susceptible to the activity of metalloproteinases.[33,34] Chitosan's application in restorative dentistry is particularly intriguing because of all these factors taken together, especially when it comes to minimally invasive cavities.[35,36]

Emdogain® is a contemporary commercially available product. It has garnered substantial attention in recent years as it comprising enamel matrix proteins (90% amelogenin). Enamel matrix proteins' biomineralization activity has a promising role in tissue regeneration.[37] Energy dispersive spectroscopy is an analytical method that makes it possible to identify the components contained in the sample under study. It works well with a wide range of solid samples, including biological tissues, metals, and ceramics.[38]

The current in vitro study's objective was to evaluate the potential of amelogenin and chitosan to repair artificially eroded dentin at different times by means of a chemical element analysis. The study's null hypothesis stated that these materials' potential to remineralize would not vary over time or between them.

MATERIALS & METHODS

Ethical approval

This study had the Scientific Research Ethics Committee's approval from Faculty of Dentistry, Suez Canal University (approval number:712/2023/30-10-2023).

Samples preparation

A total of twelve human lower first molars were collected for use in the current study.[39] All molars were extracted from patients for therapeutic purposes. The study omitted any teeth with cracks, fractures, white spot lesions, fillings, hypoplastic diseases, or obvious cavities.[40] Any soft tissue that was still present was manually scaled, then molars were immersed in 1% chloramine-T solution for 72 hours for disinfection.[41] After examination, molars were kept in distilled water with 0.1% thymol in a refrigerator at 4°C to prevent the growth of bacteria or fungi until the study start (they were utilized within 1 month of their extraction).[40,42]

Every molar had its radicular part removed and its coronal part sectioned mesiodistally by a diamond coated disc (Buehler, IL, USA) under water coolant. Two dentin slabs of 3 mm thickness each were obtained from each molar by using hard tissue microtome (Yushuoda Hard Tissue Microtome, Liaoning, China). A total of twenty-four dentin samples were obtained and mounted on resin blocks, and 600 grit silicon carbide sheets were used to polish the samples. Dentin mineral contents (Ca & P) were examined utilizing the environmental scanning electron microscope (JOEL JSM 6360 Scanning Electron Microscope) and energy dispersive analytical X-ray (EDX).

Beverage exposure

Dentin samples were then coated with waterproof nail varnish leaving a workable window exposed of approximately 3 × 3 mm at the center by using sticky tape.[43] All samples were immersed in Coca Cola beverage (Coca-Cola® Co., Egypt) (10 samples/250 ml) for a total of 25 hours (Coca Cola was replaced every 5 hours). Following Coca exposure, samples were rinsed with distilled water, dried, and subjected to EDX analysis to evaluate Ca & P contents.[44] According to the experimental hydrogels, dentin samples (n=24) were divided randomly into 4 groups as follows (n = 6): group I: received no treatment (control); group II: treated with agarose hydrogel; group III: treated with agarose hydrogel+ chitosan; group IV: treated with agarose hydrogel+ Emdogain.

Preparation of experimental hydrogels

Calcium chloride ($CaCl_2$) agarose hydrogel was prepared by dissolving 0.5g agarose powder (Genetic Analysis Grade, Fisher Bio-Reagents, UK) into 100 ml of 0.13M (1.91g) of $CaCl_2$ solution. The latter was prepared by dissolving $CaCl_2.2H_2O$ (Analytical Reagent Grade, Fisher Chemical, UK) in deionized water (Sigma-Aldrich, St. Louis, MO, USA). On the other hand, Na_2HPO_4 agarose

hydrogel containing 500 ppm fluoride was prepared by dissolving 0.5g agarose powder into 100 ml of 0.26M (4.63g) of Na_2HPO_4 solution containing 500 ppm (0.3g) fluoride. The latter was prepared by dissolving $Na_2HPO_4.2H_2O$ (EMSURE-Merck KGaA, Germany) and NaF (DHARMA, USA) in deionized water.[45] Both mixtures were left to soak for 30 minutes then heated at 150ºC till completely dissolved and left at room temperature till gelation and then stored in the refrigerator till using.[46]

In case of "chitosan hydrogel", Calcium chloride (Ca Cl_2) hydrogel was prepared by dissolving 0.13M (1.91g) of $CaCl_2$ into 1% (v/v) acetic acid (Laboratory Reagent Grade, Fisher chemical, UK). One gram of chitosan (ACROS ORGANICS. New Jersy, USA. Geel, Belgium.) was added, stirred and heated at 150ºC till completely dissolved. Finally, 0.5g agarose was added to the previous solution.[47] On the other hand, in case of "Emdogain hydrogel" (Straumann, Basel, Switzerland.), 4ml of the previously prepared Ca Cl_2 hydrogel was added to 0.2ml of Emdogain (30mg/ml) to achieve a final concentration of 1.5mg/ml Emdogain + $CaCl_2$ agarose hydrogel.[46]

Application of hydrogels

At the time of application, the prepared hydrogels were preheated to 55ºC in water bath. A layer of 1mm thickness of Calcium chloride (Ca Cl_2) hydrogel was first applied on the surfaces of dentin samples utilizing a plastic syringe, and allowed it to gel for around two hours. A second layer of Na_2HPO_4 hydrogel was then added carefully. The combined hydrogels were kept on the dentin surfaces for 5 hours, rinsed in distilled water (Sigma-Aldrich, St. Louis, MO, USA), and then stored in artificial saliva which was prepared by using [Na3PO4 (3.90mM), KCl (17.98mM), NaCl (4.29mM), MgCl2 (0.08mM)] (Sigma-Aldrich, St. Louis, MO, USA), CaCl2 (1.10mM) (Analytical Reagent Grade, Fisher Chemical, UK), [NaHCO3 (3.27mM), H2SO4 (0.50mM)].[46,48] Every day for thirty days, this process was repeated. The control

group's samples were kept for 30 days in artificial saliva which was replenished daily.[49] Finally, all dentin samples were examined under EDX for evaluation of weight % of Ca & P contents after 5, 10, and 15 days.

Data analysis

For statistical analysis, IBM SPSS Statistics Version 20 for Windows was used. The mean and standard deviation (SD) of quantitative data were reported. When a one-way analysis of variance (ANOVA) revealed significance, Tukey's test was run afterward. P less than 0.05 was regarded as significant.[43]

RESULTS

The baseline means and standard deviation of the weight % of the Ca & P elements in sound dentin samples were calculated and represented as (23.08±2.23) & (7.91±3.09). After immersion of samples in Coca Cola beverage for 25 hours, these values were significantly decreased into (6.88±2.30) & (4.04±1.28) respectively. Table & figure1 shows mean and standard deviation of weight % of Ca of groups I, II, III, and IV after 5 days of treatment, respectively, as (22.99±1.49), (25.92±4.96), (24.84±2.46), and (21.83±3.11). It also shows mean and standard deviation of weight % of P of groups I, II, III, and IV respectively, as (11.58±2.31), (9.96±2.39), (10.83±2.37), and (11.79±1.19). On comparing Ca & P contents of different groups after 5 days of treatment, ANOVA showed no statistically significant differences in both elements. The mean values of (Ca) & (P) contents were (P=0.391 & 0.619) respectively.

In addition, table & figure 2 displays weight % of Ca for groups I, II, III, and IV, along with its mean and standard deviation after 10 days of treatment respectively as (6.90±2.83), (18.41±2.76), (16.03±4.26), and (24.03±4.76). It also shows mean and standard deviation of weight % of P of groups I, II, III, and IV respectively, as (5.11±1.01), (9.69±1.57), (7.94±1.71), and (14.35±2.16).

On comparing Ca content of different groups after 10 days of treatment, ANOVA showed highly statistically significant differences in Ca content of all groups (p=0.000). The mean Ca value of group IV was significantly higher than that of groups I, II, III (P= 0.000, 0.008, 0.046) respectively. Furthermore, mean Ca value of groups II and III was significantly higher than that of group I (p=0.005, 0.022) respectively.

On comparing P content of different groups after 10 days of treatment, ANOVA showed highly significant differences in P content of all groups (p=0.000). The mean P value of Group IV was significantly higher than that of groups I, II, III

(p=0.000, 0.009, 0.001) respectively. Furthermore, mean P value of groups III and II was significantly higher than that of group I (p=0.010, 0.026) respectively.

Furthermore, table & figure 3 shows mean and standard deviation of weight % of Ca of groups I, II, III, and IV after 15 days of treatment, respectively as (14.51±2.89), (15.59±2.35), (15.51±1.92), and (24.38±0.96). It also shows mean and standard deviation of weight % of P of groups I, II, III, and IV respectively, as (7.52±1.14), (6.85±3.39), (9.48±0.73), and (10.68±3.39). On comparing Ca content of different groups after 15 days of

TABLE (1) Distribution of mean and standard deviation weight % of Ca and P in all groups (after 5 days of treatment):

Steps Mineral contents	5 days treatment				
	Group I	Group II	Group III	Group IV	P- value
(Ca) Mean ± S.D	22.99±1.49	25.92±4.96	24.84±2.46	21.83±3.11	0.391
(P) Mean ± S.D	11.58±2.31	9.96±2.39	10.83±2.37	11.79±1.19	0.619

TABLE (2) Distribution of mean and standard deviation weight % of Ca and P in all groups (after 10 days of treatment):

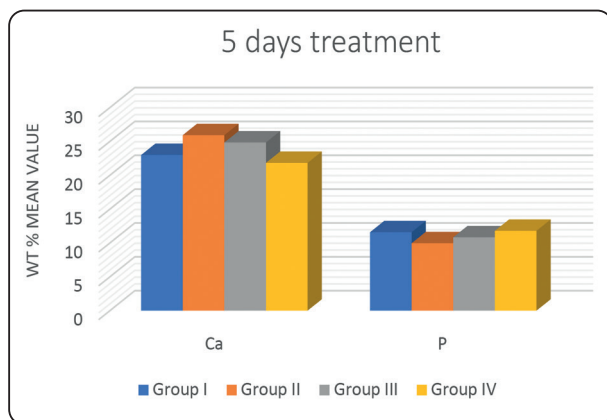| Steps Mineral contents	10 days treatment				
	Group I	Group II	Group III	Group IV	P- value
(Ca) Mean ± S.D	6.90±2.83	18.41±2.76	16.03±4.26	24.03±4.76	0.000**
(P) Mean ± S.D	5.11±1.01	9.69±1.57	7.94±1.71	14.35±2.16	0.000**

Fig. (1) Bar chart showing mean values of (Ca) & (P) content weight %) after 5 days of treatment between different groups.
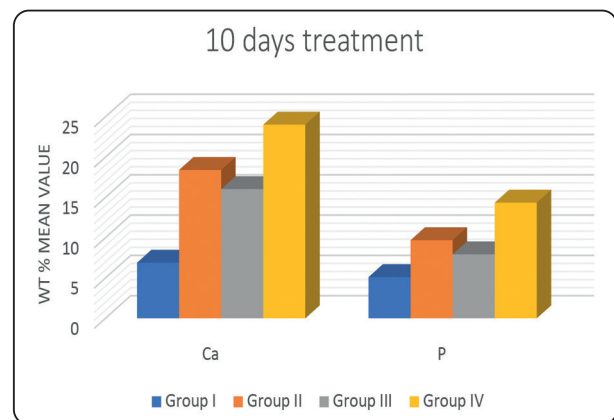

Fig. (2): Bar chart showing mean values of (Ca) & (P) content (weight %) after 10 days of treatment between different groups.

TABLE (3) Distribution of mean and standard deviation weight % of Ca and P in all groups (after 15 days of treatment):

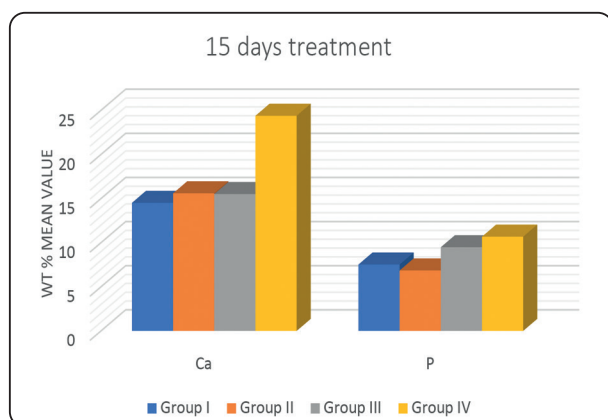| Mineral contents	Steps	15 days treatment				
		Group I	Group II	Group III	Group IV	P- value
(Ca) Mean ±S.D		14.51±2.89	15.59±2.35	15.51±1.92	24.38±0.96	0.000**
(P) Mean ±S.D		7.52±1.14	6.85±3.39	9.48±0.73	10.68±3.39	0.167

Fig. (3) Bar chart showing mean values of (Ca) & (P) content (weight %) after 15 days of treatment between different groups.

treatment, ANOVA showed highly statistically significant differences in Ca content of all groups (p=0.000). The mean Ca value of group IV was significantly higher than that of groups I, II, III (P= 0.000, 0.000, 0.000) respectively. On comparing P content of different groups, ANOVA showed no statistically significant differences in P content of all groups (p=0.167).

The energy dispersive X-ray analysis (Figure 4) and the scanning electron microscopy images (Figure 5) revealed mineral deposits on the surfaces of dentin samples when each experimental group was compared with the control group.
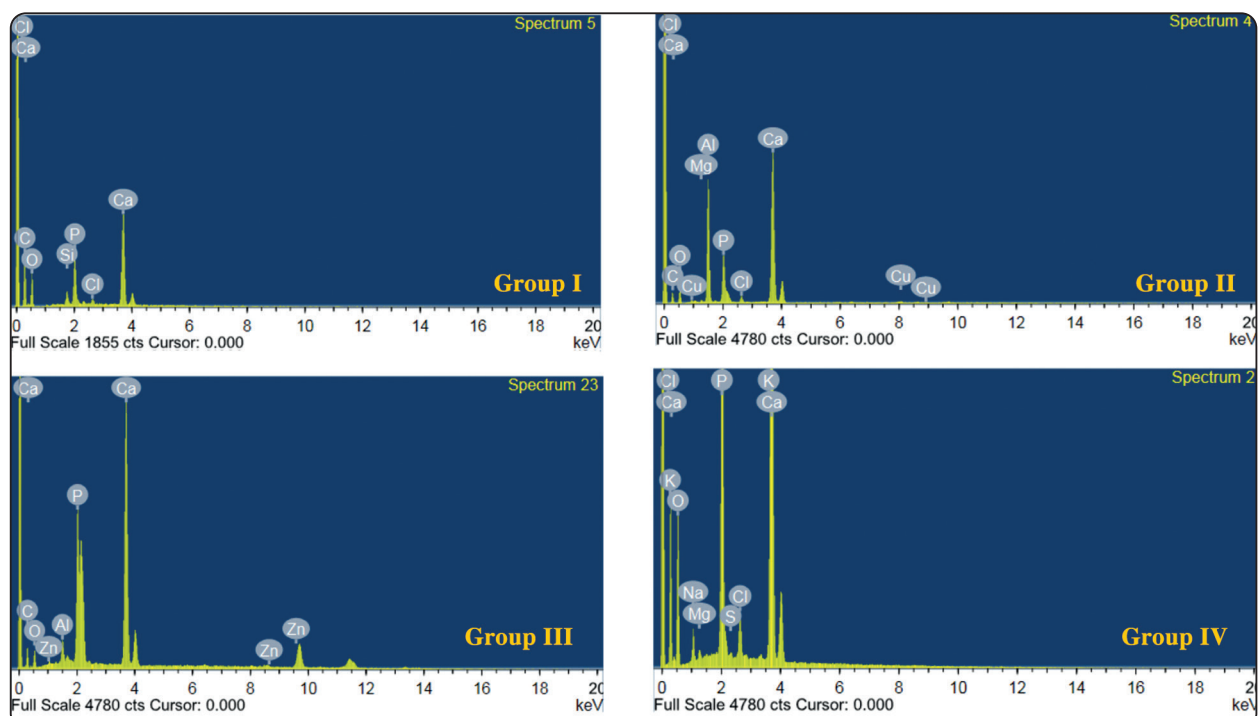

Fig. (4) Energy dispersive X-ray spectroscopy elemental analysis of dentin samples in a distinct group following remineralization (15 days treatment)
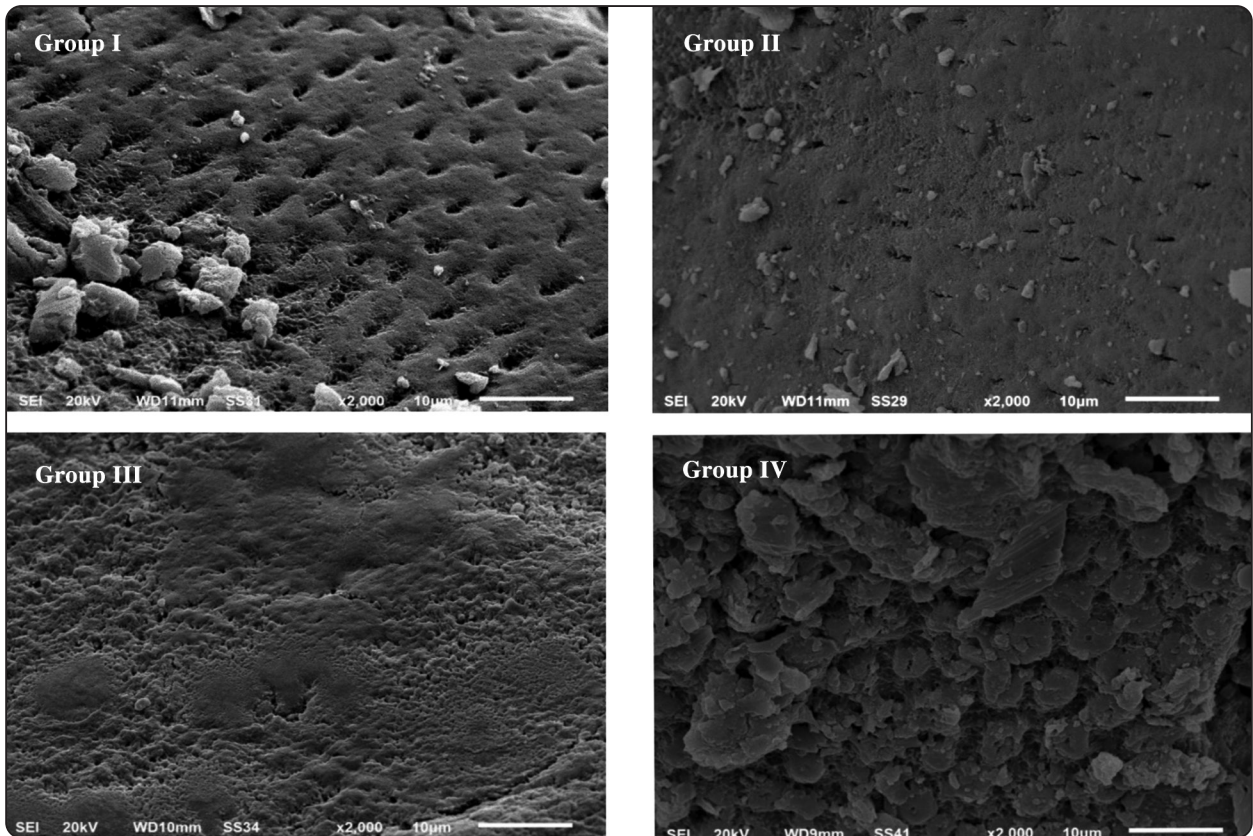
Fig. (5) Scanning electron microscopy structural characterization of dentin samples in various groups following remineralization (15 days treatment)

DISCUSSION

Erosion is a surface-softening lesion that is prone to wear and cannot be remineralized with traditional treatments.[50] Remineralization is the net increase in the tooth's minerals content which serves to replenish the minerals lost during demineralization. The traditional approach is using solutions containing phosphate and calcium ions at varying fluoride concentrations. Dentin remineralization is a clinically relevant procedure for treating dental erosion, caries, and dentin hypersensitivity. It's crucial to comprehend the interactions between dentin components and biomaterials in order to choose and create compounds that include materials that interact more directly with dentin components through ionic and chemical affinity.[51]

Dentin tissue recovery is a complicated process because it requires the reconstruction of two distinct phases: inorganic apatite and organic type I collagen, which are connected in a certain spatial relationship. This suggests that the complete recovery of demineralized dentin requires more than just remineralization; rather, the collagen matrix's structure must be restored and the two phases must be connected in a certain way.[51]

The scientific discipline of biomimetics employs biomimicry, which mimics nature's process of material synthesis. In restorative dentistry, its primary objective is to create a hard tissue bond that will enable the hard tissues to regain their full function. This makes the whole crown into a unit that offers almost normal function with biologic and cosmetic results by allowing functional stresses to travel through the tooth.[52]

Numerous in vitro studies have demonstrated the ability of cell-free biomimetic mineralization

techniques to regenerate tissue microstructures resembling dentin or enamel. These included bioactive glass, nano-HAP and proline, amelogenin, chitosan, and gelatin.[53,54]According to reports, these techniques may be able to self-heal teeth imperfections, making them effective for treating little tooth defects but ineffective for treating larger ons.[45]

In the current study, agarose which is considered as a biomimetic mineralization system was chosen due to its potential effect for repairing exposed dentin. Chitosan was added to the agarose to function as a reservoir for calcium and phosphate ions, while Emodogain was added to mimic the biomineralization process that organic matrix proteins trigger in the formation of tooth enamel. The application of the hydrogels was lasted for 5 hours/daily which corresponds with the least amount of human sleep time, since people could apply the hydrogels in their mouths for an entire night when used clinically.[46,55-57]

Scanning electron microscope in conjunction with EDX was used to examine dentin surfaces throughout the study steps. The concurrent EDX analysis was carried out to provide accurate quantitative analysis and distribution for weight % of (Ca) and (P) elemental chemical composition of the investigated dentinal surfaces. Following the findings analysis, the null hypothesis was rejected because the remineralizing ability of the amelogenin and chitosan used in this study was influenced by time and materials.

Sound dentin samples were first examined under EDX showing Ca & P mean values of (23.08 ± 2.23) & (7.91 ± 3.09) respectively. After Coca Cola beverage, these mean values were significantly decreased into (6.88 ± 2.30) & (4.04 ± 1.28) representing the effectiveness of Coca Cola in causing demineralization of dentin samples. These results came in agreement with the study of Han et al., 2017 denoting the removal of the inorganic components from the surface of dentin exposing the organic matrix (mainly type I collagen).[45]

Regarding findings of different comparisons between the different experimental groups after 5 days of treatment, the current results revealed that such period of material application was not enough to explore a significant effect of any of the tested materials which were all similar to the results obtained in the control samples.

However, concerning comparison of the results after 10 days of treatment, the EDX analysis showed that (Ca) & (P) values in group IV treated with agarose hydrogel + Emdogain were significantly higher than those in groups I, II & III (control group, agarose hydrogel, agarose hydrogel+ chitosan) respectively. These results could be attributed to the presence of type I collagen matrix in group I which only exists on the demineralized dentin surface and was not able to promote the mineral crystal deposition due to the absence of NCPs and/or lack of seed mineral crystals, as well as, the absence of fluoride ions.[45,47] In contrast to group II where the agarose hydrogel is the most versatile growth media for crystals, as agarose hydrogel loaded with calcium and phosphate ions acts as a template to induce nucleation and growth.[58,59] In addition to group III where chitosan acts as a scaffold for the formation of stable calcium phosphate-based layer onto the surface of demineralized dentin by increasing the cross-linking between the dentin collagen fibers and agarose molecules. Chitosan physically adsorbs onto saliva, preventing acid erosion of the hydroxyapatite surface in addition to the cross-linking that occurs between chitosan and saliva.[60,61]

Furthermore, after 15 days of treatment, the current comparison between the experimental groups showed that (Ca) value in group IV was significantly higher than those in groups I, II & III. These results were attributed to the aggregation of amelogenin with calcium and phosphate ions followed by development of oriented apatite crystals.[55] The presence of amelogenin in Emdogain can guide the formation of highly ordered apatite crystals, besides it has a role with the presence of fluoride in promoting the oriented bundle formation

of needle-like fluoridated HAP forming an enamel-like layer.[61]

LIMITATIONS

1. Study limitations include the in vitro nature of the experimental methodology and the artificial duration of beverage consumption. With the in vitro design, the tooth is exposed to the beverage for a predetermined amount of time without taking into account factors like salivary clearance, remineralization capability, movement inside the mouth during swallowing, or rate of beverage consumption. These restrictions probably make all beverages' observed degradation potential worse.

2. There is a significant degree of variety amongst related studies because there is no standard technique for these kinds of investigations.

3. Given the length of time required for dentin remineralization, it may be difficult to repeat the procedure in an in vivo setting. Furthermore, the exposed scaffold would be rapidly destroyed before full crystal regrowth by diet texture, oral temperature, and pH.

4. Due to the necessity of gathering human teeth, it proved impossible to obtain the necessary quantity, which necessitated the sectioning of teeth as a means of compensation.

CONCLUSION

• The following conclusions could be made given the constraints of this study:

• The Emdogain group has the biggest mean difference when compared to chitosan and control groups, suggesting that it has a promising remineralizing impact on erosive dentin.

• Emdogain and chitosan groups have the greatest surface coverage, respectively.

• The calcium and phosphate ions may also be an effective way to treat dentin erosion over time.

RECOMMENDATIONS

1. Longer-term research is needed to confirm the beneficial effects of these biomimetic remineralizing materials.

2. More research on different kinds of biomimetic materials ought to be conducted along these lines.

DECLARATION STATEMENT

• No conflict of interest is disclosed by the authors.

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