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The Effect of Different Remineralizing Agents on Microleakage around Restored Demineralized Enamel: An In vitro Comparative Study

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Aim: This study evaluated the impact of various remineralizing agents on microleakage around restored (with composite resin and glass ionomer restorations) demineralized enamel.
Materials and methods: Two restorative materials were used in this research and five remineralizing agents. Seventy bovine teeth were randomly divided into two groups, 35 of which were restored using resin composite restorations, and the other 35 were restored using glass ionomer restorations. Five teeth in each main group were left untreated and another five teeth in each group were demineralized only without any further treatment. Exposed enamel windows were subjected to five different remineralizing agents; 1: artificial saliva, 2: CCP-ACPF paste, 3: CCP-ACPF varnish, 4: self-assembling peptide; Curodont protect and 5: self-assembling peptide; Curodont repair. All treated groups were then subjected to pH cycling for 30 days. Microleakage test was done using dye penetration method followed by sectioning of the teeth and detection of the leakage score using stereomicroscope.
Results: The results of this study showed significantly increased leakage around glass ionomer restored samples compared to resin composite restored ones.
Conclusion: Demineralized samples have shown the highest score of microleakage while CCP-ACPF varnish have shown the least microleakage score.

Keywords: Remineralization, Microleakage, Self-assembling peptides, CCP ACP fluoride varnish, CCP ACPF paste, Demineralization around restorations.

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Introduction

Regardless of the restorative material used, demineralization around the margins of restorations has been regarded as the principal cause for restoration replacement. Reduced dental restoration durability necessitates several restorative therapies, including the placement of larger and larger restorations and the execution of complex therapeutic procedures[1]. The process of remineralization, which occurs when calcium and phosphate from saliva or other topical sources diffuse into the tooth and, with the aid of fluoride, repairs existing crystal remains rather than producing new ones. This process represents the body's natural repair mechanism for non-cavitated carious lesions. In the oral cavity, demineralization and remineralization take place simultaneously[2]. The balance between demineralization and remineralization governs whether dental caries develop in a progressive, static, or reversible manner[3]. Therefore, any factor that can encourage remineralization in this balance can be used as a weapon in the battle against dental caries disease[4].

The requirement for clinically effective treatments to remineralize early enamel caries lesions is clearly dictated by the principles of minimally invasive dentistry. While the cornerstone of contemporary caries management concepts is fluoride-mediated remineralization. New remineralization techniques, however, have either been commercialized or are currently being developed. They promise to encourage deeper remineralization of lesions, minimize the risks related with using high-fluoride dental care products, and make lifelong caries control easier[5]. These non-fluoride remineralizing systems can be generally divided into techniques for repairing caries lesions by improving fluoride efficacy and biomimetic enamel regenerative technologies[4].

Biomimetic enamel regenerative technologies include the use of self-assembling peptides. The structure of enamel is unique, in that it has no residual cellular components that can aid in the repair process when the enamel is damaged by a cariogenic episode[6]. Due to its low viscosity, monomeric peptide P11-4 can permeate these porosities after application. The peptide undergoes self-assembly to form a viscous fibrous scaffold under the influence of the circumstances seen in a carious environment. The scaffold's anionic peptide P11-4 groups attract calcium ions and can precipitate hydroxyapatite crystals de novo. Ions from tissue fluids are drawn to the nucleator, where they are organised into crystals. Growth of the crystals won't start until the crucial nuclei are stabilised. The scaffold matrix is responsible for this stability. This phenomenon imitates the process in which the enamel matrix proteins self-assemble prior to tooth eruption to direct the precipitation of hydroxyapatite crystals[7].

It is worth noting that the goal to be achieved in remineralizing systems is to reach the synergetic effect of calcium, phosphate with fluoride ions together[8]. Consequently, casein phosphopeptide-amorphous calcium phosphate with fluoride (CPP-ACFP) was considered as an alternative treatment protocol. CPP-ACFP can be applied to a tooth surface as a tooth crème (MI Paste Plus®). It has the same potential as CPP-ACP conjugated with the additional benefits of added fluoride (900 ppm)[9,10]. In the presence of fluoride, the CPP binds greater amounts of calcium and phosphate ions at all pH values. It was suggested that it may continue to function even in highly acidic environments. Also, it had a high potential for deep penetration with no spontaneous precipitation. As a result, CPP-ACFP will remineralize subsurface lesions as well as the prevention of the lesion progression by

forming fluorapatite within the body of the lesion[9,11].

Another remineralizing approach that improves fluoride efficacy is the use of fluoride varnishes. Due to the improved release of calcium and inorganic phosphate ions and the bioavailability of CPP-ACP inside the varnish, the combined effects of fluoride and CCP-ACP in a fluoride varnish (MI varnish®) have demonstrated advantageous remineralizing results[12]. Given the relatively neutral conditions under study, the high water solubility of the CPP-ACP complexes may be responsible for the quick release of the ions from MI Varnish®[13].

Maintaining a marginal seal over an extended period of time is crucial for preventing or at least minimising clinical issues including secondary caries and the discoloration of margins caused by microleakage.[14].

Few studies investigated the effect of remineralizing agents on the marginal integrity of demineralized enamel around restorations used routinely in dental practice. In this study microleakage test was performed to evaluate the enamel/restoration interface after being subjected to demineralization, remineralization and pH cycling to test the integrity of this interface after the mentioned challenges.

The null hypothesis of this study is that different available approaches of remineralization will not reduce microleakage of demineralized enamel lesions around margins of two restorative materials that are used routinely in dental practice: resin composite and glass ionomer.

MATERIALS AND METHODS

For the purpose of this study, a total of 70 bovine incisors from the second dentition were used. Teeth with caries, cracks were excluded [15]. After that, a hand scaler was used to remove the blood and soft tissue. The teeth were kept in thymol 0.1% (prepared at the faculty of pharmacy, Ain Shams university, Cairo, Egypt) and distilled water into a closed flask in the freezer (at 4°c) until use, which was no longer than two months to prevent dehydration and bacterial growth[16].

Two restorative materials (resin composite and conventional glass ionomer) and five remineralizing agents (artificial saliva, self-assembling peptide (Curodont repair™), self-assembling peptide (Curodont porotect™), Casein phosphopeptide-amorphous calcium phosphate pastes with fluoride paste (MI paste plus) and Casein phosphopeptide-amorphous calcium phosphate pastes with fluoride Varnish (MI varnish) were used in this study.

A total of fourteen groups (7 nanohybrid resin composite and 7 conventional glass ionomer) with five samples in each group, were used in this study:

Group 1: Baseline (Control) group; not subjected to demineralization or remineralization.

Group 2: Demineralized group; subjected only to demineralization.

Group 3: subjected to demineralization followed by remineralization with artificial saliva and pH cycling for 30 days.

Group 4: subjected to demineralization followed by remineralization with self-assembling peptide (Curodont repair™) and pH cycling for 30 days.

Group 5: subjected to demineralization followed by remineralization with self-assembling peptide (Curodont porotect™) and pH cycling for 30 days.

Group 6: subjected to demineralization followed by remineralization with Casein phosphopeptide-amorphous calcium phosphate pastes with fluoride paste (MI paste plus) and pH cycling for 30 days.

Group 7: subjected to demineralization followed by remineralization with Casein phosphopeptide-amorphous calcium phosphate pastes with fluoride Varnish (MI varnish) and pH cycling for 30 days.

Preparation of the samples:

Using low-speed diamond discs (ContacEZ, Germany), the teeth's roots were cut at the cemento-enamel junction [16]. The cervical part was blocked using resin composite to avoid diffusion of different solutions used into the pulp chamber of the bovine teeth.

Each tooth crown was placed in a mold of rubber base material of a putty consistency (Zhermack Zetaplus putty, Italy) with a one centimeter high ring of polyvinyl chloride (PVC) that set around the tooth segment [17].

The labial surface for each incisor was flattened in a circular motion using three grits of silicone carbide papers (Dongguan Golden Sun Abrasives Co., Ltd., China) 320, 600 and 1200 to remove aprismatic enamel and produce a smooth surface[16]. On the middle third of the labial surface of each tooth, a standardized cylindrical cavity of 1.5 mm in depth X 5 mm in diameter was created using a wheel stone in a high-speed handpiece (air motor NSK, Japan) with water coolant. A graduated periodontal probe (Carl Martin Periodontal Probe, Germany) was used to verify the cavity's depth. In order to prevent tooth desiccation till restoration, the teeth were then kept in distilled water in an incubator at 37°C [18].

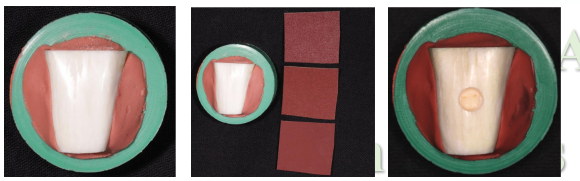

Figure 1:* (a) *Bovine incisor embedded in acrylic ring block with enamel facing upward,* (b): *Smoothened labial surface using three grits silicon of carbide papers (i):320, (ii): 600 and (iii): 1200* (c): *Cylindrical cavity preparation with dimensions 1.5depth x 5mm diameter.

Cavities were restored randomly with either conventional glass ionomer or nanohybrid resin composite following the manufacturer's instructions for each material. Regarding resin composite selective etching of enamel margins was done for 30 seconds followed by rinsing for 30 seconds and drying of the cavities followed by application of the universal adhesive and light curing for 20 seconds. The application of resin composite was done in oblique layers and finally finishing and polishing. Regarding glass ionomer application, conditioning of the cavities was done for 10 seconds using dentine conditioner followed by rinsing. Application of the glass ionomer was done after activation of the glass ionomer capsule and trituration for 10 seconds followed by application of a nano-varnish. After 24 hours glass ionomer was finished and polished.

Demarcation of the enamel window: A graph paper with dimensions 10mm x 10mm were adhered to a double face adhesive and placed on the middle third of the labial surface of all the bovine incisors to form a window. Except on the double face adhesive all around the crown surface was coated with acid resistant nail varnish (Amanda, Milano) in three layers leaving a two mm space around the tooth/restoration interface to differentiate the central window from adjacent area for a total exposed surface of approximately 10 mm2 that was subjected to the demineralizing solution and remineralizing agents. Once the paint dried the double face adhesive was removed and samples were ready with an exposed, central isolated window of the restoration surrounded by enamel [19].

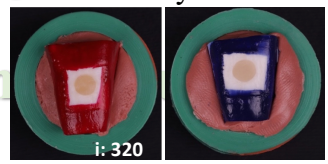

Figure 2: Demarcated enamel window surrounding: (a)resin composite and (b): glass ionomer restorations.

Preparation of the demineralization solution and Artificial enamel caries formation:

The demineralizing solution was prepared using the chemicals listed in ***table (1)*** at pH 4.4 [20]. A total 70 sample were subjected to demineralizing solution for producing artificial enamel carious lesions of ICDAS II

score[21]. The remaining 10 samples (5 nanohybrid resin composite and 5 glass ionomer restored bovine samples) were not subjected to demineralization and used as baseline (control) samples. Artificial enamel lesion was created in the exposed window of the enamel surface. Each sample was immersed separately in 20 mL of an unstirred demineralizing for 96 hours at 37°C in an incubator and pH 4.4 that was checked daily using pH meter (Adwa, AD110, Hungary)[22]. The solution was replaced every 24 hours during the entire period of the demineralization of each specimen[23]. The specimens were taken out after four days, rinsed in deionized water for a minute, and then dried using absorbent paper before being placed in deionized water for storage until use. As verified using this protocol produced a lesion depth about 200–300 µm [24].

Table (1): List of materials used, products name and their composition, manufacturer and patch number.

Product name	Material	Manufacturer	Composition	Patch number
Prime and bond ® universal	Universal adhesive	Dentsply Sirona (Germany)	-Phosphoric acid modified acrylate resin -Bi and multifunctional acrylate	LOT: 2009000399
Meta-Etchant	Phosphoric acid etchant	META ®BIOMED	-Phosphoric acid 37% - Water -Xanthan gum	LOT: 1510000795
Ceram. x® Sphere TEC™ one universal	Nanohybrid Resin Composite	Dentsply Sirona (Germany)	**-Resin matrix:** polysiloxane combined with poly-urethane-methacrylate as well as bis-EMA and TEGDMA. **-Filler system:** The filler load ranges from 77-79 weight-% total (59-61% by volume)	LOT: 1910000725
Conditioner	Ketac™ Conditioner	3M™ Ketac™ (Germany)	-Water (70-80%by weight) -Polyacrylic acid (20-30%by weight)	LOT: 1905027
GC Fuji IX GP Fast	Glass ionomer	GC Corp., Tokyo, Japan	**Powder**: Fluro - alumino silicate glass – Polyacrylic acid powder **Liquid**: Polyacrylic acid – Polybasic carboxylic acid	LOT: 1905082
Equia coat	Varnish	GC Corp., Tokyo, Japan	-Methyl methacrylate (40-50%)	LOT: 1812061
GC MI Paste Plus ®	Casein phosphopeptide -amorphous calcium phosphate pastes with fluoride **(CCP-ACPF)**	GC Corp., Tokyo, Japan	-Sodium fluoride 0.20%W/W -900 ppm Fluoride	LOT: 201202D
MI Varnish ®	**CPP-ACP Fluoride Varnish**	GC Corp., Japan	-1-8% Sodium fluoride -1-5% CPP-ACP	LOT: 1912101
Curodont™ Repair	Self-assembling peptide	Credentis AG, Switzerland),	-P11-4 peptide (Not Applicable)	LOT: 1012435
Curodont™ Protect	Self-assembling peptide	Credentis AG, Switzerland),	-P11-4 peptide -Sodium monofluorophosphate (900 ppm F)	LOT: 18-477.1
Artificial saliva			-Methyl-p-hydroxybenzoate (2gm/L) -Sodium Carboxymethyl Cellulose (10gm/L) -Potassium chloride (0.625gm/L) -$MgCl_2 . 6H_2O$ (0.059gm/L) -$CaCl_2 . 2 H_2O$(0.166gm/L) -Dipotassium hydrogen phosphate "K_2HPO_4" (0.804gm/L) -Potassium dihydrogen phosphate "KH_2PO_4"(0.326gm/L) -Potassium hydroxide -PH 7.2	
Demineralizing solution			-Calcium Chloride (0.244 gm/L) - Sodium dihydrogen phosphate (0.343 gm/L) -Acetic acid (3.13 mL) -Sodium hydroxide -PH: 4.4	

Application of remineralizing agents

-Artificial saliva: Samples in this group were treated with artificial saliva which was prepared using the chemicals listed in ***table (1)*** at pH 7.2.

-Professionally applied self-assembling peptide (Curodont Repair™) application: First the enamel window was exposed to 3% sodium hypochlorite for 20 seconds followed by thorough rinsing with water for 20 seconds, this was followed by 37% phosphoric acid etching for 20 seconds followed by 20 seconds rinsing with water[25]. The self-assembling peptide (Curodont repair™) was provided in glass vials of powdered form. It was reconstituted with 50 µl of distilled water right before use. Using a microbrush, the mixed solution was then applied to the enamel window, where it was left for 5 minutes to allow for diffusion and self-assembly[26]. Samples were then immersed in an artificial saliva solution[27].

Professionally applied casein phosphopeptide amorphous calcium phosphate with fluoride varnish (MI

varnish®) application:. A microbrush was used to apply the varnish in a thin, uniform layer, and it was left undisturbed on the teeth for two minutes. Following this application, each sample was placed into a separate container with 10 ml of artificial saliva[12].

-Patient applied self-assembling peptide (Curodont protect™) application:. Size of a pea from curodont protect™ gel was distributed with a swab on the lesion and kept undisturbed for two minutes. Removal of the cream was done using through rinsing under deionized water. This was done twice weekly for the 30 days treatment period [28].

Patient applied casein phosphopeptide amorphous calcium phosphate with fluoride paste (MI Paste Plus®) application: A pea size of the MI Paste Plus® was distributed with a swab on the lesion and kept undisturbed for three minutes according to manufacturer's instructions [29]. Removal of the paste was done through rinsing under deionized water. This was done for the whole treatment period 30 days/ three times daily.

pH cycling: The pH-cycling methodology replicates a high caries risk environment in vivo[30]. All treatment regimens employed artificial saliva as the remineralization medium and an acetic acid demineralizing solution as the acidic challenge medium.Samples were stored in an incubator with temperature adjusted to 37°C degrees [31]. pH cycling sequence was as follows; Day one is all-day storage in artificial saliva; subsequent days' treatments are as follows: one hour three times of acidic challenge followed by immersion of the test samples in artificial saliva at 37°C [31] and this was repeated for 30 days [32]. Regarding the artificial saliva group, test samples were only subjected to pH cycling without any treatment for the whole 30 days.

After the pH cycling period, samples in all groups were rinsed with and stored in deionized water before being tested. Then measurements were recorded for each sample.

Microleakage examination test: Each sample was covered with two layers of nail polish (except for 1 mm around the tooth-restoration interface then vertically downward immersed in a solution of 2% methylene blue dye (SD Fine-Chem limited, Mumbai, India) for 24 hours at 37 °C temperature incubator. Samples were then removed from the dye solution and thoroughly rinsed with water. Under water spray, samples were sectioned buccolingually using a low speed diamond saw (Top Dent, Edenta Golden, Swiss). After being cleaned under running water, the specimens were dried using tissue paper. Dye penetration was assessed with a camera connected to a stereomicroscope (Nikon SMZ 745T,China), and the images were analyzed with a dedicated software (RI viewer for Windows).

The mean scores were determined using the following criterion [33]:

0- no dye penetration;
1- dye penetration in enamel;
2- dye penetration beyond the dentin-enamel junction, without reaching the axial wall;
3- dye penetration in the axial wall.

The evaluation and quantification of the marginal dye microleakage was based on the ISO/TS 11405 guidance [34].

Statistical analysis:

Mann-Whitney U test was used for analyzing the effect of restorative material on the microleakage score. Regarding the effect of different remineralizing agents, Kruskal-Wallis test was used, followed by a Dunn's post hoc test with Bonferroni correction, and for repeated measurements, a Friedman's test followed by a Nemenyi post hoc test. At $p<0.05$, the significance level was established. R statistical analysis software

4.1.3 was used to conduct the statistical analysis.

RESULTS

Mann-Whitney U test showed that glass ionomer (3.00±0.00) had a significantly higher microleakage score than composite (2.54±0.61) ($p<0.001$).

Kruskal-Wallis test showed that regarding the demineralized and curodont repair™ groups, both nanohybrid resin composite and conventional glass ionomer had the same mean microleakage score (3.00±0.00). Regarding the baseline, curodont protect™, MI varnish® and MI paste plus® groups, conventional glass ionomer had a higher microleakage value than nanohybrid resin composite, yet the difference was not statistically significant. While regarding artificial saliva group, conventional glass ionomer had a significantly higher value than nanohybrid resin composite ***(figure 3)***.

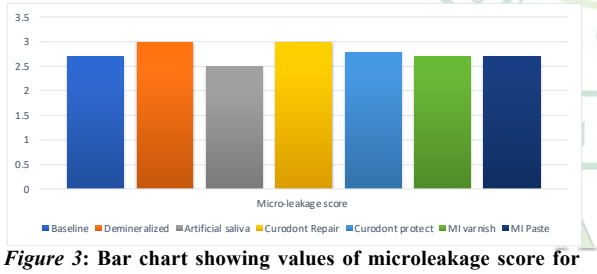

***Figure 3*: Bar chart showing values of microleakage score for tested remineralizing agents.**

DISCUSSION

The idea of remineralization and demineralization of tooth surfaces served as inspiration for the prevention of caries around the restoration margin. Fluoride is one of the best remineralizing agents for preventing caries. Nevertheless, some concerns have been expressed about fluorosis and excess fluoride intake[35]. In recent years, alterative materials for fluoride have been introduced. These non-fluoride remineralizing systems can be categorized into: remineralizing methods that increase the effectiveness of fluoride to treat caries lesions and biomimetic enamel regenerative technologies[4]. While fluoride operates primarily on the superficial mineral layer of the caries lesion, biomimetic treatments attempt to mineralize the deeper caries lesion leading to further mineralization[35].

The comparison between different available approaches of remineralization to test their ability to remineralize demineralized enamel lesions around margins of two restorative materials that are used routinely in dental practice: resin composite and glass ionomer was to be beneficial. Additionally, two strategies of remineralizing agent application were tested in our study, the professionally applied ones that are applied at the dental office by the dentist and home care products that are applied as a part of the patient routine hygiene measures to mimic the clinical conditions.

Because bovine enamel and human enamel react similarly to acidic challenges and remineralization settings, bovine enamel is still employed as a substitute for testing demineralization and remineralization of enamel[36]. In addition it was found that lesions in bovine enamel formed faster in comparison to human enamel however, with no remarkable difference in their mineral distribution characteristics[37]. Additionally, artificial enamel lesions are more consistently reproducible than natural lesions and thus provide a reliable experimental model[38]. All these reasons in addition to the large surface area of bovine incisors justified its use in this study. Enamel surface was polished to the remove the aprismatic layer which is less permeable to treatment agents and acidic solutions due to its higher mineral content compared to the enamel subsurface[39].

A clinical scoring system known as the International Caries Detection and Assessment System (ICDAS) enables the identification and evaluation of caries

activity. ICDAS was created to be used for epidemiological, clinical research, and practise purposes[40]. In addition to allowing for the detection of dental caries at the non-cavitated level based on visual evaluation of the tooth surface, ICDAS has been demonstrated to have content validity and correlational validity with histological lesion depth[41]. With a demineralization solution of ten Cate and Duijsters, demineralized enamel lesions with ICDAS score II (white lesion that is seen without air-drying and placed anywhere between the inner ½ of enamel and the outer ⅓ of dentin) were produced[42]. With a lesion depth of 200–300 µm and no surface erosion[30], this technique results in subsurface enamel demineralization[24],[20].

Maintaining a marginal seal over an extended period of time is crucial for preventing or at least minimising clinical issues including secondary caries and the discoloration of margins caused by microleakage.[14]. In this study microleakage test was performed to evaluate the enamel/restoration interface after being subjected to demineralization, remineralization and pH cycling to test the integrity of this interface after the mentioned challenges.

There are various methods for identifying microleakage. These include scanning electron microscopy, neutron activation analysis, fluid filtration, and the use of dyes, chemicals, and radioactive tracers[43]. The dye leakage method was employed in this investigation because it is a simple, economical, quick and efficient way that does not demand the use of sophisticated laboratory equipment.Methylene blue (2 %) was used in this study because of its low cost, ease of application and low molecular weight of the dye, which is smaller than bacteria. Microleakage studies are usually done qualitatively[18]. It is reported that microleakage tests may be reliable test to predict in vivo performance[44]. The findings of an in vitro microleakage research should be viewed as a theoretical maximum degree of leakage, which may be higher than what is anticipated in vivo, according to Bajabaa et al[45].

Despite the evolution of different types of GIC, it was found that none of these materials have a complete marginal sealing, which is considered as a major reason for microleakage[46]. In the present study, conventional glass ionomer (Fuji IX) showed statistically significant higher microleakage compared to resin composite. Dimensional changes in materials during setting alter the material's structure and may have an impact on adhesion, considerably influencing the development of the marginal gap and subsequent microleakage[47]. Due to the limited time of the study, the setting contraction of glass ionomer may have not been compensated by the subsequent water sorption which is responsible for closing the marginal gaps at glass ionomer-tooth interface[48]. Mali et al. reported a similar result with conventional glass ionomer having greater microleakage in comparison to resin glass ionomer and composite[46]. They claimed that the presence of tubular fluid in dentin controls the dehydration of Fuji IX in order to explain the findings of their investigation. The properties of glass ionomer cement may have been altered in in-vitro conditions and in the absence of dentinal fluid, which could have contributed to the elevated leakage values in the current investigation[46].

Regarding the resin composite group, demineralized samples have shown the highest score of microleakage although there was no significant difference between different groups. This may be due to the fact that demineralized enamel is more porous and has less mineral content than sound enamel[49].

High microleakage value of Curodont repair™ samples could be attributed to

pretreatment of enamel margins with 37% phosphoric acid etchant as a step of the self-assembling peptide application which removes the pseudo-intact surface hypermineralized layer and exposes the hypomineralized porous body of the lesion. This result is in accordance with ***Moosavi et al.,*** who tested the effect of surface treatments of demineralized enamel on microleakage and have shown that demineralized group (without any subsequent treatment) showed more microleakage at the enamel- adhesive interface compared to sound teeth [49].

MI varnish® have shown more microleakage resistance although it was not significantly different from other groups. This may be due to the precipitation of calcium fluoride from CCP-ACP fluoride varnish treated demineralized enamel that may have a great inhibitory effect on microleakage[49]. Lower microleakage scores of both MI paste® and artificial saliva treated demineralized enamel samples may be due to the perseverance of the pseudo-intact hypermineralized surface layer that partially impeded the diffusion of methylene blue dye compared to other groups.

Conclusions

Within the limitations of this study, the following could be concluded:

1-Microleakage at enamel/restoration interface is aggravated by demineralization and pH cycling regardless the type of the restorative material used.

2-Conventional glass ionomer has shown extensive microleakage at enamel restoration interface regardless of the remineralizing agent used.

3-MI varnish may improve the marginal seal at enamel/restoration interface.

4- Use of 37% phosphoric acid as a step self-assembling peptide Curodont repair ™ application may impair the marginal interface.

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