Faculty of Dentistry – Mansoura University

MJD

Effect of Incorporation of Bioactive Hydroxyapatite Nano-rods in Self-Etch Adhesive on Microleakage, Nanoleakage Pattern and Elemental analysis of Resin/Dentin Interface

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Abstract:

Objective: To compare the effect of four types of adhesives immediately and after 6 months storage in artificial saliva followed by thermocycling (2000 cycle) on microleakage patterns, the nanoleakage pattern, and elemental analysis of adhesive interface. **Materials and Methods:** A total of 120 extracted sound human permanent molars were selected. And assigned into 4 groups according to adhesive types (one step-self-etch (1S-SE) adhesive, modified self-etch adhesive with hydroxyapatite nanorods (HA-NR), universal adhesive, resin-modified glass ionomer-based adhesive). Each group was further subdivided into two subgroups according to time of testing, (a) immediately evaluated, (b) was subjected to the 6 months storage in artificial saliva followed by thermocycling. For each adhesive group, teeth divided into three groups according to test types: (I) for microleakage (ML) test (n =80), (II) for nanoleakage (NL) test (n =24), and (III) for elemental analysis test (n = 16). For the (ML) test, all collected data were tabulated and statistically analyzed using a statistical package with IBM SPSS. A Chi-squared test was used to compare microleakage scores among different adhesive groups. **Results:** The microleakage was significantly affected by the "type of adhesive material" ($P \leq 0.05$) and the "time". Another Chi-squared test showed that there was no significant difference in microleakage between occlusal and gingival margins among tested groups. Calcium and phosphate levels increased in, the modified self-etch adhesive group. **Conclusion:** Incorporation of bioactive hydroxyapatite nano-rods (HA-NR) into self-etch adhesives decreases microleakage and nanoleakage pattern of resin/dentin interface. It improves Calcium and Phosphate levels at the resin- dentin interface.

Introduction:

Tooth-colored restorations have received plenty of attraction across the world. Evolution in adhesive dentistry performs a dynamic role in conservative dentistry including direct and indirect cosmetic composite restorations.[1] Inability of adhesive systems to completely infiltrate the collagen network with resin monomers, and resin monomers exhibit a concentration gradient as they infiltrate a thick bed of demineralized collagen matrix. Resin-spare zones form within the hybrid layer (HL), which are then filled with water or dentinal fluid.[2] These conditions could accelerate resin elution or offer a water-rich environment for the collagen matrix endogenous collagenolytic enzymes to activate.[3] These water-rich, resin-sparse zones inside the HL, are a major cause for nanoleakage and micropermeability, as well as the consequent degeneration of collagen, which compromise the durability of resin–dentin bonds.[4] Apatite crystallites were used to replace water from intrafibrillar gaps and water-rich, resin-sparse areas of the hybrid layer in the study of remineralization of resin-dentin interfaces.[5] In the current study hydroxyapatite-nanorod fillers were incorporated into the one-step self-etch adhesive to evaluate the marginal adaptation, nanoleakage and elemental analysis of the interface. However, few studies evaluate the mechanical properties of the dentin-resin interface of the hydroxyapatite incorporated adhesive.[6] According to a review of the current scientific literature, there have been no studies that investigated the effectiveness of bioactive hydroxyapatite nano-rods in adhesive systems in terms of microleakage, nanoleakage, and elemental analysis. As a result, this research was a trial to see if the past research gaps might be filled.

Null hypotheses

This study was designed to test the null hypotheses of four different types of adhesives, including one modified adhesive that incorporated with 0.5% bioactive hydroxyapatite nano rods (HAP-NR) immediately after 24h and after 6 months has no effect on the dye penetration at both occlusal and gingival margins, the amount of silver nitrate uptake at the adhesive layer and within the hybrid layer and the elemental analysis at adhesive- dentin interface.

Materials and Methods:

Four different types of adhesives including the "modified-bioactive" adhesive have been evaluated; 1S-SE adhesive (Opti Bond™ All-In-One) (Kerr, Orange, CA, USA PVPA Italia, s.r. l) non-modified (NM),1S-SE adhesive (Opti Bond™, All-In-One) with (HA-NR) Modified (M), methacryloyloxydecyl dihydrogeh phosphate (MDP) containing adhesive (Scotchbond universal adhesive) (3M-ESPE, MN, St Paul, USA), Resin Modified Glass Ionomer bonding agent (RMGI) with Riva conditioner (Riva Bond LC, SDI, Australia), as well as a nanohybrid resin composite restorative material (Filtek Z250XT, 3M-ESPE, St. Paul, MN, USA), hydroxyapatite-nanorod (HAP-NR) fillers are used in this in vitro study and artificial saliva which was prepared in Faculty of Pharmacy, Mansoura University. The artificial saliva is composed of Metyle-p hydroxybenzoate, KCL Sodium C carboxymethyl, cellulose, calcium phosphate, $MgCl_2.6H_2o$, $CaCl_2.2H_2o$, K_2po_4, kH_2po_4, and pH adjusted to 6.75with KOH.

In this laboratory study, a total of 120 freshly human permanent molars were utilized. The selected molars were assigned randomly into four groups according to adhesive types into four different groups:

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Group 1; receive one-step self-etch adhesive (Opti Bond™ All-In-One) (NM) (non-modified),

Group 2; receive "modified-bioactive" adhesive (Opti Bond™, All-In-One containing hydroxyapatite nanorods) Modified (M)

Group 3; receive MDP containing adhesive (Scotchbond universal) (U)

Group 4; receive resin-modified glass ionomer-based adhesive (RMGI) (Riva Bond). (R)

The groups according to time of testing then subdivided into two subgroups; subgroup (a) was immediately after 24h evaluated, while subgroup (b) was subjected to the 6 months storage. For each adhesive group, teeth were divided into three groups according to test types: (I) for microleakage test (n=80), (II) for nanoleakage test (n =24), and (III) for elemental analysis test (n = 16).

1. Specimen's preparation for microleakage testing

For the microleakage test (n=80), half of the restored teeth (n=40) were stored in artificial saliva for 6 months and then subjected to thermocycling (2000cycle) (5°c-55°c) for an aging period. Box-shaped class V cavity was prepared on the buccal surface of each tooth with dimensions 3x3mm, a depth of 2mm. The prepared cavities were restored with the same type of restorative material a nanohybrid resin composite (Filtek, Z250 XT, 3M-ESPE, St. Paul, MN, USA) using different types of adhesives, according to the corresponding group. All the adhesive systems and resin composite restorations were applied and cured with a light-emitting diode (LED) light-curing unit (Eli par ^{TM} Deep Cure-S LED Curing Light). The intensity of the LED unit was monitored via a radiometer (Demeter LC, Kerr, Germany) 1300mW/cm^2 with a wavelength between 350-520nm according to the manufacturer's instructions.

To measure microleakage, the restored teeth were immersed in 2% methylene blue dye for 24 hours at room temperature and rinsed for 60 seconds with distilled water. The specimens were sectioned buccolingually through the restoration with a precision diamond saw (Isomet 4000 saw, Buehler, USA) under profuse water cooling to maintain an equal plane. An Optical Stereomicroscope was used to examine microleakage in the occlusal and gingival margins for only one half from each specimen at 30x magnification (Nikon eclipse MA 100). Along the restoration margins, the degree of microleakage was measured using the scoring criteria.[7]

Microleakage was scored according to the following criteria

- 0 = no leakage
- 1 = leakage one third into the restoration.
- 2 = leakage two thirds into the restoration.
- 3 = leakage to the deepest part of the restoration.

2. Specimens Preparation for nanoleakage evaluation by scanning electron microscope (SEM)

For the nanoleakage test, 6 additional teeth from each group were used (n =24). Half of the restored teeth (n=12) were stored in artificial saliva for 6 months and then subjected to thermocycling for an aging period. The samples were then encased in an acrylic resin block and the occlusal enamel and superficial dentin were removed for each tooth, exposing the mid dentin area (Figure 1). Then all bonding agent was applied on cut surfaces according to the manufacturer's instructions and covered with a nano-hybrid composite restorative material. The restorative material was incrementally built-up (3mm-thickness) to form a crown segment (Figure 1). To prepare the specimen for NL evaluation, the restored teeth were vertically sectioned with a precision diamond saw (IsoMet 4000 saw, Buehler Ltd., Lake Bluff, USA) under running water cooling to obtain two adhesive-dentin halves. All surfaces received two coats of nail varnish applied 1mm from the bonding interface.

Following the nail polish had dried, the specimens were immersed in an aqueous solution of 50wt % ammoniacal silver nitrate (PH = 9.5) for 24 hours at 37°C in complete darkness, then rinsed with distilled water for 5min. before spending 8 hours in a photo-developing solution (Kodak GBX fixer and replenishers, Rochester, NY, USA) under fluorescent light to allow reduction of the diamine silver ions to metallic silver grains within voids along with the bonded interface.

The nails varnish was removed using a periodontal scalar after a 5-minute wash in distilled water. To remove the surface layer of silver, specimens were wet polished using waterproof silicon carbide papers with grits of 800-, 1200-, 2000-, and 4000-grits. Submerging the specimens in an ultrasonic bath for 10 minutes removed the debris (digital ultrasonic cleaner, Codyson, china) and air drying. Specimens were attached on aluminum stubs with carbon adhesive tape and graphite paint (Ted Pella, Inc., Moorestown, NJ, USA). All specimens were gold-sputtered (SPI Module - Sputter Carbon / Gold Coater, EDEN instruments, Japan) and viewed in the backscattered mode under a high-resolution scan electron microscope (SEM) with an accelerating voltage of 20kv, the working distance of 7-13mm, and magnifications of x500, x1000, and x2000. (SEM Quanta FEG 250 with field emission gun, FEI Company-Netherlands).[8]

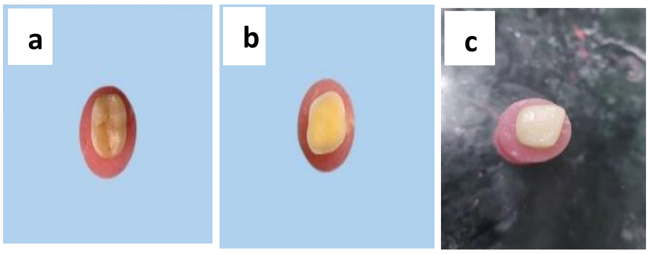

Figure 1: a; embedded of the specimens in the acrylic resin block,
b; preparation of flat dentin surface,
c; crown segment of composite restoration

3. Specimen's preparation for elemental analysis of resin-dentin interface

Elemental analysis of resin-dentin interface was assessed for 4 teeth from each group (n=16). The resin/dentin interface was subjected to energy dispersive x-ray (EDX) analysis with EDX software attached to a field emission scanning electron microscopy (JSM-6510LV, JEOL, Japan) at an accelerating voltage of 20kv.

Results:

1. Microleakage test results

Regarding adhesive types, all the tested groups showed highly significant differences among each other p= 0.01 (Table 1). The Chi-squared test revealed no statistically significant difference in microleakage between occlusal and gingival margins among the tested groups (Table 2). Concerning the effect of time, there was a significant difference of microleakage between the immediate subgroup and delayed subgroup where the highest number of specimens that had microleakage for all groups was found in case of delayed subgroups while the least one was recorded in immediate subgroups (Table 3). According to the microleakage test result, the modified adhesive group showed the least microleakage in both immediate and delayed subgroups, while the RMGI adhesive was the highest microleakage. The scoring of microleakage evaluation is shown in Figure 2**.**

2. Nanoleakage evaluation

The HL created by (RMGI adhesive) showed the most severe silver uptake as silver deposits within the adhesive layer for both immediate and delayed groups. Conversely, the silver uptake was spot mode and it was lower in modified adhesive in both immediate and delayed groups.

While in the non-modified adhesive immediate group, a thin continuous layer of silver nitrate uptake along with resin dentin interface and reticuler pattren extending toward dentin. There were spots of silver grains deposit at the interface and reticuler pattrens as terminal branches that extended vertically from the HL surface to the adhesive layer in the non-modified adhesive delayed group. In universal MDP containing adhesive, a thin reticular pattern of discontinuous islands of silver deposits at the base of the HL is presented. However, after storage and thermocycling, there was water-trees structures at HL and extended toward the dentin at the base of the HL and exhibiting substantial increase in size and density.

Overall, all adhesives presented higher levels of silver uptake after storage and thermocycling but the least one was modified adhesive and the highest was RMGI adhesive.

3. Elemental analysis of resin-dentin interface using energy dispersive x-ray (EDX) mapping

The EDX elemental analysis of the tested groups is shown in Tables 4, 5, 6 and 7. The major elements are represented by weight percent. The EDX elemental analysis revealed that the Ca and P levels at adhesive - dentin interface of all groups except RMGI adhesive group were increased after 6 months storage and thermocycling while Ca/p ratio decreased after storage in all groups.

The highest level of Ca and P was found in the modified adhesive in the immediate and delayed subgroup followed by universal, non-modified adhesives and the least was in RMGI adhesive. The highest Ca/p ratio was in the modified adhesive delayed subgroup (Table 5) and the least was in RMGI adhesive group (Table7).

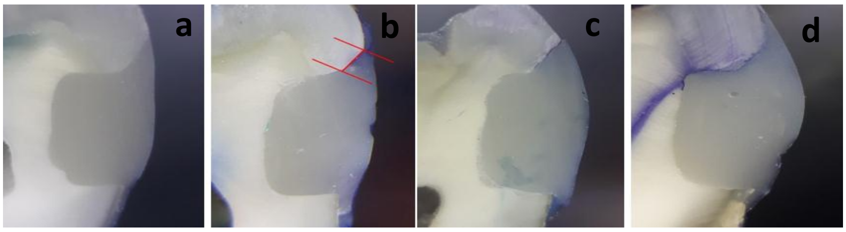

Figure 2: Scoring of microleakage evaluation. a) Score 0, b) Score 1, c) Score2 , d) Score 3.

Table 1: Microleakage scores for total and according to the adhesive groups

		Groups			P-value
		Occlusal number	**Gingival number**	**Total number**	
Scores	**No leakage**	34	32	66	0.686
	Leakage one third into the restoration	35	34	69	
	Leakage two thirds into the restoration	7	6	13	
	Leakage to the deepest part of the restoration	4	8	12	
Total		**80**	**80**	**160**	

Abbreviation: NM, Non-modified adhesive; M, Modified adhesive; U, Universal adhesive; R, Riva Bond LC adhesive. *Superscript indicate a significant difference between groups (P<0.05) using Chi-square test

Table 2: Microleakage scores for total and according to the occlusal and gingival margins

		Groups			P-value
		Occlusal number	**Gingival number**	**Total number**	
Scores	**No leakage**	34	32	66	0.686
	Leakage one third into the restoration	35	34	69	
	Leakage two thirds into the restoration	7	6	13	
	Leakage to the deepest part of the restoration	4	8	12	
Total		**80**	**80**	**160**	

Table 3: Microleakage scores for total and according to the immediate and delay groups

		Groups			P-value
		I (N)	D (N)	Total (N)	
Scores	**No leakage**	48	18	66	0.000*
	Leakage one third into the restoration	20	49	69	
	Leakage two thirds into the restoration	4	9	13	
	Leakage to the deepest part of the restoration	8	4	12	
Total		**80**	**80**	**160**	

Superscript indicate a significant difference between groups (P<0.05) using Chi-square test

Table 4: Shows mineral content at adhesive/ dentin interface in non-modified group

Elements	Immediate	Delayed
	Wt.%	**Wt.%**
Ca	13.46	15.28
P	4.63	8.85
Si	37	0.69
Sb	0	4.64
Ca/p	2.90	1.72

Abbreviation: Ca, calcium; P, phosphate; Si, silicon; Sb, antimony; Ca/p, calcium phosphate ratio

Table 5: Shows mineral content at adhesive/ dentin interface in modified adhesive group

Elements	Immediate	Delayed
	Wt.%	**Wt.%**
Ca	20.27	25.95
P	8.01	13.19
Si	2.14	0.26
Mg	0.52	0
Ca/p	2.53	1.96

Abbreviation: Ca, calcium; P, phosphate; Si, silicon; Mg, magnesium; Ca/p, calcium phosphate ratio

Table 6: Shows mineral content at adhesive/ dentin interface in universal group

Elements	Immediate	Delayed
	Wt.%	**Wt.%**
Ca	16.71	24.12
P	7.38	13.51
Si	3. 70	0.38
Sb	5.98	1.68
Ca/p	2.26	1.78

Abbreviation: Ca, calcium; P, phosphate; Si, silicon; Sb, antimony; Ca/p, calcium phosphate ratio

Table 7: Shows mineral content at adhesive/dentin interface in RMGI adhesive group

Elements	Immediate	Delayed
	Wt.%	**Wt.%**
Ca	19.34	7.43
P	7.55	4.79
F	0.13	0.15
Sb	7.54	3.24
Al	5.60	1.70
Ca/p	2.56	1.55

Abbreviation: Ca, calcium; P, phosphate; F, fluoride; Sb, antimony; Al, aluminum

Discussion:

The use of nanofillers has caused a shift in the restorative dentistry paradigm in recent years. The mechanical and adhesive properties of the material have been significantly altered by the inclusion of organic and inorganic fillers.[9] Researchers have selected a biomimetic mineralization strategy based on the replication of biological mineralization using nanotechnology.[10] Hydroxyapatite crystals play a crucial part in the mineralization processes within tooth dentin, and it is artificially incorporated to stimulate dentin and enamel remineralization.[11]

Nanofillers have been proposed in studies studying the modification of adhesive systems to reduce susceptibility to degradation by reducing hydrophilicity, minimizing enzymatic collagen degradation, and lowering stress contraction relative to a reduction in a polymeric matrix.[12] This partly provides support to the findings of the current study, as non-modified specimens showed higher microleakage compared to modified specimens. Furthermore, the presence of phosphate and calcium at the resin–dentin interface validated a complex interaction observed within dentin for bioactive hydroxyapatite nanorods as shown by EDX analysis. As a result, it is hypothesized that (HAP-NR) in the form of nanorods achieves a stable release, resulting in improved dentin resin interaction and possible remineralization.[13]

The least microleakage in the modified group and the highest level in the Riva group. Both immediate and delayed subgroups of modified adhesive showed significant differences with non-modified adhesive. This might be due to the non-modified self-etch adhesive used in the current study containing hydroxyl ethyl methacrylate (HEMA), water, acetone, and ethanol, which function as a semipermeable membrane, causing the adhesive to absorb more water, influencing marginal adaption.[14] In contrast, the highest performance of modified adhesive can be explained by the action of hydroxyapatite, which reduces water uptake and improves the adhesive mechanical properties.[15] In addition, the RMGI adhesive group pretreated with PAA has a significant difference from other groups. According to the current study, they revealed the highest level of microleakage compared to all other tested groups. This can be related to a hydric imbalance of the GIC materials and the presence of a great amount of HEMA which might contribute to higher retention and increase permeability to water.[16] Also the RMGI adhesives employed in this investigation contain the hydrophilic monomer 2-hydroxyethyl methacrylate (HEMA). Water solubility and adhesive material sorption are essential variables in the restoration of long-term stability.[17] On the other hand, water can reduce the mechanical properties of adhesive agents and weaken the connections between them and the tooth surface.[18]

However, Neelakantan et al.[19] disagree with these findings and found a lower amount of nanoleakage for glass ionomer-based bonding agents compared with resin-based dentin adhesives. They attributed this due to the presence of discrepancies in the depth of resin infiltration and dentin demineralization or the presence of water that has not evaporated during polymerization. The results of this study approve the results of Sano et al.[20] and De Munck et al.[21] However, the modified adhesive group showed a spot mode of silver nitrate uptake, and all the other groups showed reticular nanoleakage patterns. The amount of silver nitrate uptake in HLs created by RMGI based adhesive (water-based adhesive) is considerably higher than the amount of silver nitrate uptake in HLs created by other adhesive groups, which confirm the outcome of the microleakage test and elemental analysis in the current study although statistical analysis was not performed..

Conclusion:

Within the limitations and based on the outcome of the present study, the incorporation of 0.5% of bioactive hydroxyapatite nanorods in self-etch adhesive can result in:

1. Decrease in the dye penetration at both occlusal and gingival margins in immediate and delayed time.

2. The amount of silver nitrate uptake decreases also in both tested periods compared to a non-modified adhesive system.

3. The elemental analysis revealed an increase in the percentage of calcium and phosphate ions in both testing times at the adhesive/ dentin interface.

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