Spectrophotometer analysis of bulk-fill composites on various beverages: An in-vitro study

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Abstract

Aim: This in-vitro Study evaluated the Color Stability of Universal Shade (IvB) Bulk-fill Composite resin in Different Beverages in different time interval.

Materials and Methods: 60 cylindrical samples were prepared in metal ring mould (10 mm internal diameter and 4 mm height). Specimens of the Bulk-fill composite resin were randomly divided into 6 groups and stored in artificial saliva for 7 days. Specimens were immersed in Distilled Water, Coca Cola, Coffee, Tea, Orange Juice, Beer solution 100sec/per day and kept in artificial saliva between each cycle. The samples were stored in incubator at 37 degree Celcius, 100 percent relative humidity. Measurement of color changes of each sample were carried out at baseline, 24 hours (1 day), and 168 (7 days) after immersion in staining solutions, with a reflectance spectrophotometer under the D65 (daylight) illuminant over a white background using CIE L* a* b* parameters and the readings were recorded.

Results: When a day-wise comparison of color stability in the Bulk fill resin composite, the following was observed on day 1, the spectrophotometer reading in bulk fill composite were maximum with coca cola and least in beer. On day 7, the spectrophotometer reading in bulk fill composite were maximum with coca cola and least in beer.

Conclusion: Coca cola exhibited significantly more staining susceptibility than that of Coffee, Tea, Beer, Orange Juice ($P < 0.001$). The intensity of discoloration is proportional to the absorption potential of coca cola solution; the greater the coca cola absorption the stronger discoloration was observed.

Keywords: Bulk fill composites, Spectrophotometer.

Introduction

Ideal aesthetics vary between culture, generations, gender, and the dentist. View of aesthetics must not be the only determinant of the final result. The term aesthetics was coined by Alexander Baumgarten, in 1735, it drives from the German asthetisch or French esthetique, both derived from the greek word "esthetic-sensitive-sentient", and "to perceive-feel-sense". Color stability of composite resin is an important property influencing its clinical longevity, which continues as a challenge inherent to material.[1] Color changes can occur due to various etiologic factors; extrinsic discoloration can occur due to staining in the superficial layer of resin composite, water absorption, surface roughness, smoking and diet.[2,3] The Physical and mechanical properties of dental composites are directly influenced by the degree of conversion achieved during polymerization.[7] Lower degree of conversion provides composites with an inferior mechanical properties and greater discoloration and degradation which results in restorations with poor wear resistance and poor color stability.[12] The discoloration of resin-based restorative materials may be the result of several extrinsic & intrinsic factors.

In oral conditions, composite resins are exposed to different dietary beverages such as coffee which might result in absorption and adsorption of colorants in coffee into the resin surface[5] and consequently undesirable color change. Therefore, the objective of this study was to evaluate the acidic effect of various beverages on the color stability of bulk fill composite.

The present in-vitro study was conducted to evaluate the color stability of relatively new class of resin composite (Bulk fill composite) in commonly ingested acidic beverages.

Materials and Mathods

Sixty specimen of universal shade (IVB) bulk fill resin Tetric-N-ceram composite material (Ivoclar Vivadent). 60 cylindrical samples were prepared in the metal ring mould (10 mm internal diameter and 4 mm height). Composite were filled in each specimen according to manufacturer's instructions and Polyester strip was placed on a glass slab and Teflon matrix. After filling a mould to excess, the material surface was covered with another polyester strip and glass slide and compressed with a device (500gm) for 20sec. To further compress the material and removal of the excessive material. All the specimens were light cured using LED curing light (Blue phase C8, Ivoclar Vivadent, Astria), in accordance to manufacturer instruction. The polymerisation of specimens was carried at 4 quadrants on each top and bottom side against the strip and glass plate and then for another similar amount for irridation but without the glass plates. The curing tip was positioned to perpendicular to specimens surface. The power output density used was 1000mW/cm.[2]

Then all specimens were finished with a series of different grid abrasive finishing disc (Shofu Super Snap

Dental India Private Limited, New Delhi, India) in a sequence of decreasing abrasiveness with intermittent movement, under constant water cooling. Polishing procedure was kept to minimum time 10s for each step to avoid microcrack formation.

Total sixty specimens was randomly divided into six groups comprised of 1 control group & 5 experimental groups.

Staining Protocol

Group 1: Experimental Group - Tea Solution (n=10)

Specimens were stored in 37°c tea for preparation of tea solution. 3 gm of tea was prepared in 50ml boiling water and added to 50ml of boiled milk the tea was freshly prepared daily prior to each test period.

Group 2: Experimental Group - Coca Cola (n=10)

Specimens were stored in 37°c cola carbonated soft drink. The lids of the container tightly closed to prevent escape of carbonic gas in order to maintain an acceptable label of carbonic gas a new bottle used everyday. Samples were immersed in 100ml of coca cola solution.

Group 3: Experimental Group - Coffee Solution (n=10)

Specimens were stored in 37°c coffee for preparation of coffee solution. 1.5gm of coffee was prepared in 50ml boiling water and added to 50ml of boiled milk. The coffee was freshly prepared daily prior to each test period.

Group 4: Control Group - Distilled Water (n=10)

Specimens were stored 100ml distilled water and the solution was changed daily. Distilled water was used as a control and to investigate intrinsic color changes in the restorative material.

Group 5: Experimental Group - Orange Juice (n=10)

Specimens were stored in 37°c orange juice. The lids of the container tightly closed to prevent change in pH of orange juice in order to maintain an acceptable label of pH a new bottle used everyday. Samples were immersed in 100ml of orange juice.

Group 6: Experimental Group - Beer (n=10)

Specimens were stored in 37°c BEER. The lids of the container tightly closed to prevent escape of carbonic gas in order to maintain an acceptable label of carbonic gas a new bottle used everyday. Samples were immersed in 100ml of beer solution.

Preparation of Artificial Saliva

The artificial saliva used in this study was of the following composition: Sodium chloride (NaCl) 0.400 g; Potassium chloride (KCl) 0.400 g; Calcium chloride monohydrate (CaCl2H2O) 0.795 g; Sodium dihydrogen phosphate (NaH2PO4) 0.69 g; Disodium sulphide hydrate (Na2S x 9H2O) 0.005 g; Urea 1.0 g; Distilled water 1000 ml. The pH was then adjusted to 6.9 with Sodium hydroxide (NaOH) or Hydrochloric acid (HCl).

The pH Measurements

Six beverages were used in this study: Coca-Cola, coffee, Tea, orange juice and beer. The pH of each beverage was determined using a pH meter (HI 221; Hanna Insruments Inc., Woonsocket, RI, USA). Ten pH readings of the freshly prepared drinks were obtained in order to get a mean pH measurement for each beverage.

Staining Process

Ten specimens of each group were placed in a beaker with the closed air-tight lid, having 100 ml of the respective experimental staining solution, ensuring that both surfaces of the specimens were exposed. The duration of immersion in the respective solutions for each sample group was 100 seconds per day. Then the specimens were rinsed thoroughly with 500 ml distilled water and stored in artificial saliva in a glass container with the closed air-tight lid, till the next immersion into their respective experimental staining solutions. All specimens were stored in light proof container at 37°c with 100% relative humidity in an incubator. After 7days of immersion in the artificial saliva solution, the specimens rinse with distilled water & blotted dry with absorbent paper before the final measurement. The same operator measured Pre-immersion, 1-day Post immersion & 7-day Post immersion values for each group, to minimize the operator error in the study. The mean value for each group and variables were calculated where color change (ΔE) represents the initial and final measurement. The readings were recorded at Pre-immersion, 1-day Post immersion & 7-day Post immersion.

Testing of Samples
Color Assessment

The color of composite resins can be determined by varying methods, including visual assessment and instrumental measurement by a colorimeter or spectrophotometer.

In the present in-vitro study, a reflectance spectrophotometer were used to measure color change for each specimen.

This system uses two D65 (daylight) light sources illuminated at 45° and reflected to the detector screen at 0°. CIE L*a* b* parameters and equations were used.

Spectrophotometer Set-Up:

A customized positioning jig made from self-cure acrylic was fabricated to obtain consistent and standardized orientation of specimen for color measurement. All specimens were placed within the circle on the neutral grey background. The indices on the jig allow consistent repositioning of the sensor headpiece of the spectrophotometer over the jig. The black jig also eliminates the influence of external light during color evaluation. Prior to any measurement, the spectrophotometer were calibrated to white and green ceramic tiles supplied by the manufacturer.

Measurement of color changes of each sample were carried out at baseline, 24 hours (1 day), and 168 (7 days) after immersion in staining solutions, with a reflectance spectrophotometer under the D65 (daylight) illuminant over a white background using CIE L* a* b* parameters and the readings were recorded.

{L* represents value (ranges from (100) white to (0) black), a* refers to chromacity in red and blue axis (+a red/ -a green) and b* refers to chromacity in yellow and blue axis (+b yellow/ -b blue)}.

In the present in-vitro study, a reflectance spectrophotometer were used to measure color change for each specimen. This system uses two D65 (daylight) light sources illuminated at 45° and reflected to the detector screen at 0°. CIE L*a* b* parameters and equations were used. The color difference in each sample between two immersions was calculated by Commission Internationale de l'eclairge (CIE) L*a*b* formula

$$\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$$

Results

Descriptive statistics of color changed of bulk-fill composite resin material was analyzed and expressed in terms of *mean and standard deviation*. The intra group (pre and post immersion values) comparison of the mean value of color change of the specimens was done by *Paired T test*, table no.1. The inter-group comparison was done by *One way ANOVA test* followed by *Tukey's Post-hoc Analysis* test. Data obtained was compiled on a MS Office Excel Sheet (v 2010, Microsoft Redmond Campus, Redmond, Washington, United States). Data was subjected to statistical analysis using Statistical package for social sciences (SPSS v 21.0, IBM).

When a day-wise comparison of Color Stability in the Bulk fill resin Composite, the following was observed on day 1, the spectrophotometer reading in bulk fill composite were maximum with COCA COLA and least with BEER. (Table No-2) On day 7, the spectrophotometer reading in bulk fill composite were maximum with COCA COLA and least with BEER. (Table 3.), (Fig. 1)
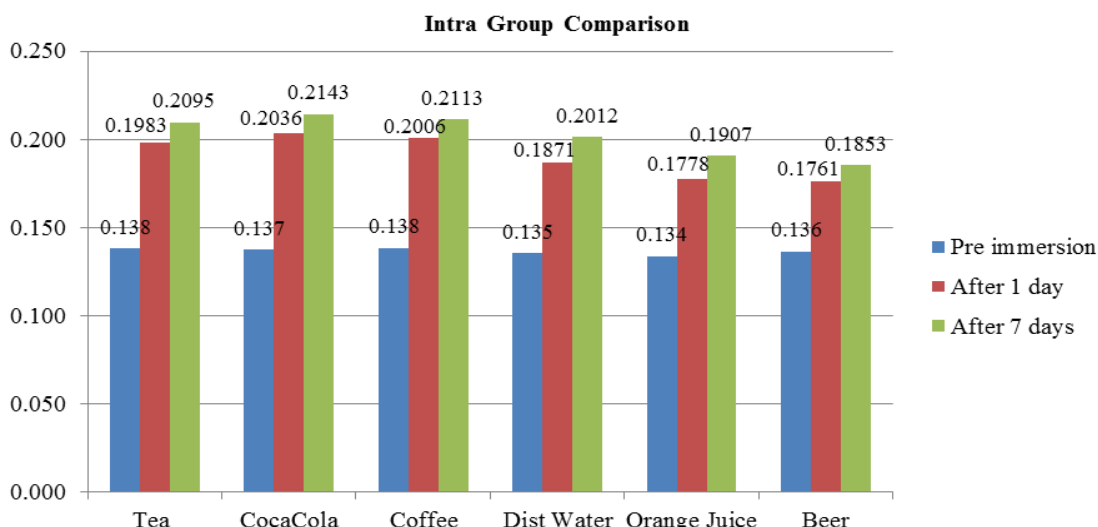

Fig. 1: Intra Group comparing of Color Stability showing in decreasing order Group 2 followed by Group 3 and least by Group 6.

Table 1: Inter Group Comparison of color Stability At Pre Immersion

	Groups	N	Mean	Std. Deviation	Std. Error	Minimum	Maximum
Pre immersion	Group 1	10	.138060	.0075316	.0023817	.1319	.1554
	Group 2	10	.137313	.0028395	.0008979	.1332	.1421
	Group 3	10	.138229	.0025974	.0008214	.1319	.1407
	Group 4	10	.135469	.0052179	.0016501	.1301	.1442
	Group 5	10	.133619	.0042613	.0013475	.1301	.1450
	Group 6	10	.136398	.0042137	.0013325	.1303	.1452
	Total	60	.136515	.0048128	.0006213	.1301	.1554

* = statistically significant difference (p<0.05)
** = statistically highly significant difference (p<0.01)
 # = non significant difference (p>0.05)… for all tables

There was a statistically non significant difference seen for the values between the Groups (p>0.05)

On Comparing Mean and Std. Deviation value in table no.1, Group (0.138 ± 0.007) showed maximum Color changes followed by Group 3 (0.138 ± 0.002) and least in Group 5 (0.133 ± 0.004)

Table 2: Inter Group Comparison Of Color Stability At 1 Day Post Immersion

	Groups	N	Mean	Std. Deviation	Std. Error	Minimum	Maximum
After 1 day	Group 1	10	.198333	.0132621	.0041938	.1728	.2103
	Group 2	10	.203638	.0103148	.0032618	.1908	.2205
	Group 3	10	.200572	.0017717	.0005603	.1964	.2029
	Group 4	10	.187054	.0056322	.0017811	.1802	.1940
	Group 5	10	.177750	.0067379	.0021307	.1711	.1945
	Group 6	10	.176131	.0029388	.0009293	.1720	.1806
	Total	60	.190580	.0133425	.0017225	.1711	.2205

There was a statistically highly significant difference seen for the values between the Groups (p<0.01) with highest mean for Coca cola followed by Coffee & least for Beer.

On Comparing Mean and Std. Deviation value in table no.2, Group 2 (0.203 ± 0.010) showed maximum Color changes followed by Group 3 (0.200 ± 0.001) and least in Group 6 (0.176 ± 0.002)

Table 3: Inter Group Comparison Of Color Stability At 7 Days Post Immersion

	Groups	N	Mean	Std. Deviation	Std. Error	Minimum	Maximum
After 7 days	Group 1	10	.209492	.0026861	.0008494	.2058	.2127
	Group 2	10	.214271	.0084777	.0026809	.2019	.2322
	Group 3	10	.211314	.0014231	.0004500	.2082	.2127
	Group 4	10	.201170	.0074049	.0023416	.1902	.2101
	Group 5	10	.190713	.0070315	.0022236	.1860	.2091
	Group 6	10	.185273	.0027941	.0008836	.1824	.1911
	Total	60	.202039	.0121795	.0015724	.1824	.2322

There was a statistically highly significant difference seen for the values between the Groups (p<0.01) with highest mean for Coca cola followed by Coffee & least for Beer.

On Comparing Mean and Std. Deviation value in table no.2, Group 2 (0.214 ± 0.008) showed maximum Color changes followed by Group 3 (0.211 ± 0.001) and least in Group 6 (0.176 ± 0.002)

Table 4: Pair Wise Comparison Using Tukey's Post Hoc Tests

Dependent Variable	(I) time	(J) time	Mean Difference (I-J)	Std. Error	Sig.	95% CI	
						Lower Bound	Upper Bound
grp 1	Pre	1D	-.0602728*	.0039985	.000**	-.070187	-.050359
	Pre	7D	-.0714318*	.0039985	.000**	-.081346	-.061518
	1D	Pre	.0602728*	.0039985	.000**	.050359	.070187
	1D	7D	-.0111590*	.0039985	.025*	-.021073	-.001245
grp 2	Pre	1D	-.0663253*	.0035245	.000**	-.075064	-.057587
	Pre	7D	-.0769583*	.0035245	.000**	-.085697	-.068220
	1D	Pre	.0663253*	.0035245	.000**	.057587	.075064
	1D	7D	-.0106330*	.0035245	.015*	-.019372	-.001894
grp 3	Pre	1D	-.0623430*	.0008911	.000**	-.064552	-.060134
	Pre	7D	-.0730850*	.0008911	.000**	-.075294	-.070876
	1D	Pre	.0623430*	.0008911	.000**	.060134	.064552
	1D	7D	-.0107420*	.0008911	.000**	-.012951	-.008533
grp 4	Pre	1D	-.0515852*	.0027542	.000**	-.058414	-.044757
	Pre	7D	-.0657012*	.0027542	.000**	-.072530	-.058873
	1D	Pre	.0515852*	.0027542	.000**	.044757	.058414
	1D	7D	-.0141160*	.0027542	.000**	-.020945	-.007287
grp 5	Pre	1D	-.0441310*	.0027447	.000**	-.050936	-.037326
	Pre	7D	-.0570940*	.0027447	.000**	-.063899	-.050289
	1D	Pre	.0441310*	.0027447	.000**	.037326	.050936
	1D	7D	-.0129630*	.0027447	.000**	-.019768	-.006158
grp 6	Pre	1D	-.0397330*	.0015099	.000**	-.043477	-.035989
	Pre	7D	-.0488750*	.0015099	.000**	-.052619	-.045131
	1D	Pre	.0397330*	.0015099	.000**	.035989	.043477
	1D	7D	-.0091420*	.0015099	.000**	-.012886	-.005398

There was a statistically highly significant / significant difference seen for the values between all pairs of Groups (p<0.01, 0.05) between all time pairs

Discussion

A crucial property of esthetic restorative materials is their long term color stability. Although improvements in esthetic restorative materials have been achieved during recent years, discoloration represents a significant problem for direct tooth colored restorations. Various studies reported the overtime color change of light cured composite resins due to extrinsic or intrinsic discoloration. Changes in color can be the result of intrinsic discoloration due to physio-chemical reactions in the deep portions of the restoration or the result of extrinsic discoloration due to accumulation of plaque and stains. Changes in color depend on several factors, such as the staining agent, the surface roughness, contact time with or immersion in coloring environments and the type of composite resin used. Previous studies on color stability have shown that beverages such as coffee, tea, red wine and cola can cause staining of composite resins to varying degrees. The structure of the resin matrix and characteristic of the filler particles directly affect the susceptibility, may be explained by the nature of the resin matrix and also may be correlated with the dimension of the filler particles. The affinity of the resin matrix for stains is modulated by its conversion rate and its chemical characteristics, water sorption rate being particularly important.[12]

With the improvement of restorative materials and the demand for aesthetic restorations, the composite resin has become the material of choice for anterior teeth restorations. But, despite the improvement of its physical and chemical properties, color stability is still a limitation on the longevity of the restorations. There are in the literature a large number researchers reporting that the resin materials are susceptible to staining after immersion in solutions such as coffee, tea, and other beverages. However, when referring to the association, immersion in solutions/ brushing, few studies have been carried out to demonstrate the changes in physical properties of composites.[7]

Discoloration of teeth is classified as extrinsic, intrinsic or internalized, which may detract from esthetic appearance.[9] In visible light-cured composite resin system, camphorquinone is generally used as the photoinitiator. However, if curing is inadequate, unconverted camphorquinone will cause a yellowish discoloration. Further, other components of the photoinitiator system — namely tertiary aromatic or aliphatic amines which act as so-called synergists or accelerators, they also tend to cause yellow or brown discoloration under the influence of light or heat.

Previous studies concerning color stability have shown that beverages (such as coffee, tea, red wine, and cola) and mouthrinses have varying degrees of staining effect on auto- and light-cured composite resin restorative materials. The staining potential of these drinks and solutions vary according to their composition and properties.[9]

In the present in-vitro study the resin composites used were bulk-fill which are sculptable resin composite. Unlike low-viscosity bulk-fill composites, which must be covered by a 2-mm occlusal capping layer using another methacrylate-based hybrid composite suitable for posterior teeth, with this bulk-fill composites it is possible to completely restore the whole cavity with one material.

Tetric N-Ceram is a light-cured, hybrid composite for direct restorations in posterior teeth, and may also be used for Class V restorations, extended fissure sealing in molars and premolars and for reconstructive build-up. Tetric N-Ceram is available in 3 shades; IVA for slightly reddish teeth, IVB for slightly yellowish teeth, and IVW white for fillings in deciduous teeth and very light teeth. Tetric N-Ceram bulk-fill can be applied in "bulk" increments of up to 4 mm without any adverse effect on the material's polymerization behavior or mechanical properties. It can be cured with conventional LED curing lights with a light intensity ≥ 500 mWcm2in 20 seconds and using a light source such as Bluephase G2 light cure unit (Ivoclar Vivadent, Schaan, Liechtenstein) with a light intensity ≥ 1000 mWcm2in just 10 seconds. Tetric N-Ceram bulk-fill achieves this via incorporating advanced composite-filler technology, a pre-polymer shrinkage stress reliever, the photo initiator Ivocerin® (polymerisation booster), and a light sensitivity filter.

The choice of a single color tone may be a limitation of this study; however, in accordance with previous studies, only the A3 Vita shade was selected for each type of the composites tested in this study. To assess color change of dental materials, visually and/or specific instruments have been proposed. The methodology used in the present study was in accordance with the previous researches that used spectrophotometry and the CIE L*a*b* coordinate system, which is a widely used tool for dental purposes. Various studies reported the advantages of using the CIE L*a*b* coordinate system, such as its repeatability, sensitivity, and objectivity. This technique is well suited for the determination of small color variations (ΔE).

This study was designed to simulate the frequent and long term consumption of an individual drinking a carbonated beverage Coca Cola, Tea, coffee, fruit juice (orange) and beer on the Color Stabilty on Tetric N-Ceram bulk-fill resin composites. These beverages were selected to simulate an individual's habit of consumption of an acidic food, sour fruits, and drinks.[6]

During consumption, food or drink contacts teeth or restoration surfaces for only a short time before it is washed away by saliva. However, in previous studies, substrates usually had contact with acidic food or drink for a prolonged period of time and the situation did not account for the role of saliva. Therefore, in the present study, the restorative materials were immersed in these drinks for 2 min a day, rinsed with distilled water and then were stored in artificial saliva at 37°C for the rest of the day to simulate the washing effect of saliva and human's body temperature.

As the specimens were not exposed to any mechanical forces so the changes observed in color would be attributed

to the chemical reaction between the beverages and materials and to the difference in composition of the material. The color results obtained in the present study indicate that pH of the solutions has a critical influence on the restorative materials. Coombes reported that a low pH of acidic food and drink leads to erosive wear in materials.[14] The acids in these drinks promoted the release of unreacted monomers by penetrating into the resin matrix, thereby resulting in color changes values.

Coca-Cola has a pH of 2.89 which is the lowest among the beverages tested in the present study. Coca-Cola is a carbonate beverage containing carbonic acid and phosphoric acid which promotes dissolution of the resin matrix, thus promoting the dislodgement and leaching out of filler particles and color changes of restorative materials.[10]

Some beverages though acidic appear to be less erosive than others while their pH is nearly similar. This might be possibly related to the type of acid used in the beverage formulation. Orange juice is composed of citric acid while Coca-Cola is composed of phosphoric and carbonic acid. Phosphoric acid softens the resin materials more readily than citric and carbonic acid.[13]

Tetric N-Ceram samples immersed in coffee, showed no significant change in micro hardness with control. Although the pH of coffee is nearly 7, coffee is composed of water, and the effect of water uptake can degrade polymer materials.59When polymer materials absorb water, there is hydrolysis of the coupling agents and loss of chemical bond between filler particles and the resin matrix. Filler particles dislodge from the outer surface of the material increasing the color changes.

With respect to the samples immersed in beer showed significant difference in color with the control in Tetric N-ceram group (P = 0.68). This could be attributed to the ethanol concentration in beer (8% volume) combined with pH levels of 4.3 and susceptibility of the resin matrix of Tetric N Ceram to degradation. It has been reported that exposure to ethanol degrade the cross-linked matrix in the dental composite, and leads to the hydrolysis of the filler-matrix interfaces eventually leading to a decrease in mechanical and chemical properties. It may be speculated that softening of the resin matrix would favor dislodgment of filler particles from the matrix, thereby allowing for the formation of a roughened surface and color changes which was observed in this study.

In comparison to Tetric N-Ceram was found to be less affected by low pH of beverages. Tetric N-Ceram contain Bis-EMA and a reduced amount of TEGDMA, which promote better resistance to the action of chemical substances.[14]

According to the Spectrophotometer results of this study, material tested became changes in color after immersion in the beverages.

A study done by Ardu et al in which Wine proved to have the highest staining potential followed by coffee, tea, orange juice, and cola, which had the lowest staining potential. The highest color change measured against a white background was observed for Durafill (Heraeus

Kulzer) in wine (_E = 62.3), while the least staining was found for Enamel HFO (Micerium) in cola (_E = 3.5). The highest color change measured against a black background was observed for EsthetX (Dentsply) in wine (_E = 46.0), while the least staining was observed for Enamel HFO in cola (_E = 2.5).[8]

Another study done by Park et al which revealed coffee induced a significant color change (ΔE^*: 3.1~5.6) in most specimens but the other Solutions (distilled water and green tea) induced only a slight color change. Overall, coffee caused unacceptable color changes to the resin nanocomposites.[6]

Same study conducted by Malekipour et al in which Tea and coffee produced the most discoloration, whereas, water exhibited the least color change after immersion for 14 days in Z100 ($P < 0.05$). After one day of immersion, coffee caused the lowest discoloration in the test composite, compared to tea, cola, distilled water, and lemonade ($P < 0.05$) and concluded that Staining solutions and immersion time are significant factors that affect color stability of composite resins.[15]

In our study Coca-Cola produced greater color changes in the restorative material as compared to other beverages where as study done by P Sarveshwar Reddy et al showed more surface roughness by coke beverage and coffee has showen more color changes with respect to other groups. So, the association of a low pH and the presence of a strong inorganic acid leading to an aggressive attack on the surface of restorative materials could possibly explain to an increase in the color changes seen.

Conclusion

On the basis of the results of this study, it can be concluded that:

All the beverages used in this study changes in color in the material tested.

Coca-Cola causes more color changes than other beverages in the material tested. The total changes of color after 1, and 7 days of composite immersion in coca cola were higher than the clinically acceptable threshold, and they are comparable to the color changes observed with coffee, tea, orange juice and beer, which are known as strong staining agents. The intensity of discoloration is proportional to the absorption potential of coca cola solution; the greater the coca cola absorption the stronger discoloration was observed. Within the limitation of this study long term use of these method should be followed up in clinical studies. However, more in-vitro studies need to be conducted to co-relate the result of this study.

Conflict of Interest: None.

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