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MUTAGENIC POTENTIAL OF A SELF-ADHESIVE FLOWABLE COMPOSITE

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ABSTRACT

Objective: The aim of this study was to assess the potential mutagenic effects associated to extracts from current self-adhesive flowable resin composite (Vertise Flow,Kerr Corp, Orange, CA, USA) that allows skipping the time-consuming adhesive processes.

Study design: The materials were eluted in dimethyl sulphoxide and the extracts were tested either after 1 day or 7 day incubation period at 370C. Mutagenic effects of the materials were tested on Salmonella typhipmurium strain TA 100 using the standard plate incorporation assay in the absence of S9 fraction from rat liver. The data were statistically analyzed using two-way variance analysis ($p<0.05$).

Results: The dose of the material and incubation as well as the interactions between these factors exhibited varying degrees of influences on the Salmonella typhipmurium colony number. However no mutagenic effect was detected for the self-adhesive restorative material.

Conclusion: It can be concluded that the adhesive restorative material tested in this study has no mutagenic potential.

Keywords: *mutagenicity, AMES test, Salmonella tphymiruim, self-adhesive composite, bulk fill flowables, adhesive resin*

ÖZET

Amaç: Bu çalışmanın amacı, zaman alıcı adeziv prosedürleri kısaltan self-adeziv akışkan rezin kompozit (Vertise Flow,Kerr Corp, Orange, CA, ABD) ekstrelerinin potansiyel mutajenik etkisinin değerlendirilmesidir.

Çalışma dizaynı: Materyal örnekleri dimetil sülfoksit içerisinde bekletilmişler ve 370C' deki inkübasyon sürelerinin 1. ve 7. günlerinde test edilmişlerdir. Bu materyallerin mutajenik etkileri, Salmonella typhipmurium TA 100 suşunda, standart plak korporasyon yöntemi ile S9 fraksiyonu eksikliğinde değerlendirilmiştir. Veriler iki yönlü varyans analizi yardımı ile istatistiksel olarak analiz edilmiştir ($p<0.05$).

Bulgular: Materyal dozu ve inkübasyon süresi ve birbirleri ile ilişkileri Salmonel-la typhipmurium koloni sayıları üzerinde farklı etkiler göstermiştir. Bununla birlikte self-adeziv restoratif materyal için mutajenik etki gözlenmemiştir.

Sonuç: Bu çalışmada test edilen adeziv restoratif materyalin mutajenik etkisi bulunmamıştır.

Anahtar Kelimeler: *mutajenisite, AMES testi, Salmonella tphymiruim, self-adeziv kompozit, bulk fill akışkanlar, adeziv rezin*

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INTRODUCTION

The introduction of adhesion to dentistry around the mid of the last century can be accepted as a big revolution.This revolution and patients attitude due to their esthetic demands has been contributed to the development of new adhesive restoratives. With adhesive technology, the dental clinicians have met with total etching concept and adhesive systems. After the Buonocore attitude about adhesion,various researchers developed new procedures to enhance handling and quality of adhesive restorative materials.The earlier adhesives include three steps in order to achieve bonding to tooth tissue named as the 3-step etch and rinse systems. The aim was later turned into 2-step etch and rinse and the adhesive systems were classified into two general categories such as two step etch and rinse systems that combine primer and bond together applied after etching; and two step self-etch systems, that etch and prime tooth tissue with primer before the application of bond. These materials were accepted as user-friendly and less technique sensitive. With further laboratory efforts 1step self-etch systems were introduced with dental technology that has the possibility to achieve all the steps. These materials are more effective in minimizing technique sensitivity, showed simultaneous demineralization and resin infiltration and reduce postoperative sensitivity however they still requires the polymerization step[1].

Current dental technology goal is combining the benefits of adhesive and composite materials into a self-adhesive restorative. However, it is the biggest question to overcome the hydrophobic–hydrophilic mismatch between restorative material and especially dentin tissue [2].

The latest developments in self-adhesive restorative materials are promoted as materials require neither etching nor a bonding agent[3]. Special phosphate dimethacrylate monomers, like glycerol phosphate dimethacrylate (GPDM), allows chemical interaction of the phosphonate groups with calcium ions of tooth tissue can be accepted as a way to reproduce self-adhesive restorative material [4]. Firstly introduced self-adhesive flowable composites were introduced mainly for cavity sealing and restoration. They include monomers mediating adhesion with tooth tissue thus they do not require any adhesive pretreatment[5]. These materials did not improve the bond strength to enamel when compared to etch-and-rinse adhesives[6] however they have similar shear bond strength with self-etch and etch and rinse adhesive systems on superficial and deep dentin[4]. Vertise Flow (VF; Kerr, Orange, CA, USA)was a self-adhesive flowable composite that includes the monomer glycerol phosphate dimethacrylate (GPDM) designed to bond to tooth tissue without a separate adhesive and etching step according to these contributions [2].

The continuous advancements in dental technology have raised questions about the biological safety of new materials and techniques. There are several investigations on the biocompatibility of dental materials generally focused on the characterization of cytotoxic effects in vitro [7].However, until recently the efforts to obtain information on the key events leading to cell damage have been scarce. One of the well-known major consequences of dental monomers on living tissue is their induction of DNA mutations, which, if not repaired, could lead to birth defects or malignant transformation of the

tissue as indicated by the induction of genotoxic effects. The *Salmonella typhimurium*/ microsome assay (Salmonella test; Ames test) is a widely accepted short-term bacterial assay for identifying substances that can produce genetic damage that leads to gene mutations. Also its low cost, simplicity, and speed make the Ames test an important and widespread part of biological examinations of dental materials and of standardization protocols[8]. The objective of this study was to determine the mutagenic potential of this self-adhesive flowable composite Vertise Flow related to its monomer ingredients.

MATERIALS AND METHODS

Chemicals, positive mutagens and tester strains

D-glucose, d-biotin, crystal violet, and sodium chloride were purchased from Sigma Chemicals (Sigma Aldrich, Deisenhofen, Germany), ampicillin trihydrate and dimethyl sulphoxide (DMSO) was from Fluca (Sigma Aldrich, Deisenhofen, Germany), Oxoid agar, Oxoid nutrient broth no. 2 from Oxoid Ltd. (Oxoid Ltd. , Hampshire, England), and citric acid monohydrate,sodium ammonium phosphate, sodium hydrogen phosphate were obtained from Merck (Merck, Darmstadt, Germany). The positive mutagens sodium azide (NaN3) was purchased from Sigma Chemicals (Sigma Chemicals, Deisenhofen, Germany), and Daunomicina was purchased from Deva Holding (Deva Holding, Istanbul, Turkey). Sodium azide was used on S. typhimurium TA 100 and Daunomicina was used on S. typhimurium strains in the absence of a metabolically active microsomal fraction from rat liver (S9). S. typhimurium TA 100 was kindly provided by Dr. Bruce N Ames (University of California, Berkeley, CA, USA).

Preparation of test substances

Five disc shaped specimens (Table 1) were prepared by placing Vertise Flow into teflon molds according to the manufacturers' instructions in laminar flow (Bioair, Siziano, Italy) to obtain sterile conditions . The dimensions of the discs were 5 mm in diameter and 2 mm and applied as bulk fill; than the surfaces were covered with transparent strip to prevent the formation of air-inhibited surface layer and light cured with LED (Elipar Free Light, 3 M ESPE, AG, Germany, 1007 mW/ cm^2) 40 s.

Table 1: Chemical composition and application procedure of Vertise Flow

Material	Manufacturer	Composition	Application procedure
Vertise Flow	Kerr, Orange, CA, USA	Resin: GPDMA, HEMA, Bis-GMA, catalysts Fillers: prepolymers, silanated Ba-glass, SiO2, YF3	Dispense a thin layer (<0.5 mm) on a forcefully dried surface; use a provided applicator with a brushing motion for 15–20 s; light cure for 20 s; syringe additional material in increments of less than 2 mm and light cure each increment for 20 s.
HEMA hydroxyethyl methacrylate, Bis-GMA bisphenol glycidyl dimethacrylate, GPDMA glycerolprosphoric acid dimethacrylate, SiO2 silicium oxide, YF3 ytterbium tri-fluoride			

Extract Preparation

The specimens were eluted in 10 mL dimethyl sulphoxide (DMSO) and the extracts were tested after an incubation period of 24 h at 37^0C and 168 h at 37^0C in a fully humidified air atmosphere incubator containing 5% CO2 (n=15). The ratio of sample surface area to the volume of the culture medium was adjusted to approximately $3cm^2$/mL as recommended by ISO.

Cytotoxicity testing

Prior to mutagenicity testing, cytotoxic amounts of the adhesive material extracts were evaluated. The rationale behind this test was to determine whether the test concentrations of the materials would have any cytotoxic effect 0.1 ml of a diluted of an overnight bacterial culture (TA 100 strain) was added to 2.5 ml top agar along with different concentrations of the tested chemicals. The top agar was poured onto nutrient agar plates and assessment of cytotoxicity was performed after 24 h incubation at 37^0C[9].

Mutagenicity tests

Mutagenicity tests were conducted by the standard plate incorporation test as previously described by Maron and Ames[10]. Two test strains of *Salmonella typhimurium* and TA 100 were used to detect frame-shift and base-pair mutation, respectively. Dimethyl sulfoxide samples of 25, 50, 75, 100 μL were plated into minimal agar plates with 2.5 ml of top agar previously supplemented with 0.05 mM histidine-biotin solution had been previously added. Overnight culture of TA 100 (0.1 ml) and the contents were mixed and poured on agar plates. Oxoid nutrient broth no. 2 was used for overnight culture. For plate incorporation assays, 0.1 ml of bacterial tester strain, and different concentrations of test extracts from different adhesive materials were added separately to 2.5 ml of molten top agar. For this assay, Daunomicina and Sodium azide (N_aN_3) (known mutagens) without S9 were used as positive control of the TA 100 strains, respectively in the absence of S9 fraction. The negative control was DMSO that we used as solvent. Three plates were conducted for each dose group and each experiment repeated independently two or three times. The strains were checked routinely for ampicillin resistance, ultraviolet-light sensitivity, crystal-violet sensitivity, histidine requirement and spontaneous reversion rate. After 72 h of incubation, revertant colonies were counted. The mutagenicity was expressed as the number of revertants per plate.

Statistical analysis

The data were analyzed by one way ANOVA. Doses higher than the mean of the control group and consequent mutagenic condition were defined as ‘‘mutagenic’’, whereas an increase in dose approaching to, but not reaching a two-fold increase was defined as ‘‘weak mutagenic’’.

RESULTS

For a substance to be considered mutagenic in the Ames Test, the number of revertant colonies per plate containing the test material must be at least two fold higher than the revertant colony number of the vehicle control or there must be an increase in the revertant colony number in a dose dependent manner in plates containing test substance in compare to the vehicle control plates. As a sequel of cytotoxicity tests, it was found that none of the test doses of the materials exhibited cytotoxic effects, even for increased doses. The number of revertants in the positive control group was significantly increased in comparison with the negative control (solvent) which

verified the conducted assay.The mutagenic effects of the materials had correlations with applied dose, and the incubation procedure as well as the combination of these parameters for some materials. Considering the bacterial colonization on the test plates of the materials with different doses and incubation periods it was seen that neither the incubation period nor the applied doses have significant effect on Vertise Flow's mutagenicity ($p > 0.05$) (Table 2).

Table 2 : Mutagenicity of Vertise Flow

Applied Doses (mL)	Incubation Period	
	24 h	168 h
25	123± 2.8	150± 12
50	120± 3.2	143± 8.3
75	134± 1	138± 7
100	137± 4	138± 7
Vehicle Control	140± 1.5	146± 6
Positive Control	800± 24 [a]	

[a] The correlation between dose, and incubation period is significant in comparison with the control group ($p<0.05$)
*DMSO was used as the vehicle control.
*NaN3 was used as positive control.

DISCUSSION

Since the question whether biomaterials have adverse effects on the body is of major concern. Incomplete polymerization of dental resin composites and resin-based bonding agents under clinical conditions result in unreacted resin monomers that may be released from the resin matrix into the aqueous environment of oral cavity. An immediate question is whether the released monomers can reach sufficient concentration to induce a significant cellular effect.There have been several evidences showing genotoxicity of resin monomers. Direct interaction between nucleotides and resin monomers, production of DNA damaging intermediates, or inhibition of DNA repair systems might be responsible for the mutagenicity of resin monomers [11]. Released monomers can reach sufficient concentration to induce a significant cellular effect. It has been estimated that the concentrations of some monomers released from the dentinal adhesives can be in the millimolar range after diffusion through the dentin layer. For instance, HEMA leaching from dentin adhesives may reach concentrations as high as 1.5–8 mmol/l [12]. Therefore, the concentration of dental monomers in the pulp may be in the millimolar range, high enough to be considered as potentially harmful for pulp cells. Ames test used in this study has been recommended as the mutagenesis screening test for chemicals and environmental samples because of its extensive database and good correlation with carcinogenicity. This assay which was specifically developed to detect chemically induced mutagenesis developed by Bruce Ames[13] is generally preferred as an initial screen to determine the mutagenic potential of new chemical technology as the most rapid, simple, sensitive and economical screening method. Ames test has also a good correlation with carcinogenicity[14] thus this method generally used to detect the possible mutagenic and genotoxic effects of dental materials. An Ames test with *Salmonella typhimurium* revealed that a monomer ingredients of resin composites glycidyl methacrylate (GMA) could cause mutagenicity through base-pair substitution and frame shift mutation in the genetic code[15]. None of the article was assessed the mutagenic effects of Vertise Flow, only in one literature it was mentioned that Vertise Flow may cause cellular damage in gingival

and pulp fibroblasts in vitro[16]. This material has been generally used for cavity lining and restorative treatments applied directly to tooth tissues without any adhesive procedure [5]. It is already seriously mentioned in the literature that chemical activity of adhesive restorative materials is important for the restored tooth prognosis [17]. Like all adhesive materials, Vertise Flow may release components with possible harmful effects to dental structures especially pulp tissue and cause a wide spectrum of pulpa dentinal reactions. Thus biological safety and monomer release of this self-adhesive material remains in close contact with living dental tissue over a long period of time are very important. Vertise Flow includes GPDMA, HEMA and BisGMA as monomer component. In the literature no assay was evaluated the biocompatibility of GPDMA, however it was observed that HEMA and Bis-GMA could induce the enhancement of DNA migration in human lymphocytes[18] and they have genotoxic effects at chromosome level in V79 cells[19]. These monomers' genotoxic effect was also showed in the in vitro Mammalian Cell Gene Mutation Test (HPRT Test) in CHO (Chinese hamster ovary) cells[20]. Nevertheless, hydroxylated metabolites of Bis-GMA monomer were not found to be mutagenic in L929 cells or *Salmonella typhimurium* [21]. However we could not observed any mutagenic effect of Vertise Flow including HEMA and BisGMA in our study. The Ames Salmonella/microsome test generally detects 83% of the carcinogens as mutagenic with completed protocol. This ratio pointed out that Ames' test is not able to state all carcinogens[10]. Mutagenicity tests may exhibit false-positive results, andit is not possible to draw a conclusive statement based solely on a single study[22]. In addition to this the mutagenic potential of Vertis Flow was tested in the absence of S9 fraction.Within this limitation this material did not lead to mutagenicity, but also a possible mutagenic effect could be detected in the presence of S9. Thus this test must be repeated in the presence of S9.

CONCLUSIONS

In the light of the results of this study, Vertise Flow can be considered safe in terms of mutagenicity within these parameters. However all clinicians must consider the possible mutagenic potential of all dental restorative materials in their clinical practice.

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