

Oxidative Alteration in Gingival Fibroblast Cells Induced By Bulk-Fill and Conventional Flowable Composites

Bulk-Fill ve Geleneksel Akıcı Kompozitlerle Uyarılan Dişeti Fibroblast Hücrelerindeki Oksidatif Değişim

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Abstract

Objective: The release of components from dental materials may cause oxidative stress which is crucial factor for tissue damage and cell apoptosis or death. The aim of this study was to evaluate the cytotoxicity of different flowable composites and this materials effect on total antioxidant capacity (TAC) and total oxidant status (TOS) level in human gingival fibroblast cell culture.

Materials and Methods: Gingival fibroblast cells obtained from healthy persons were used for evaluation the cytotoxicity and oxidant status. Six flowable composites used were: two bulk-fill flowable composites (SureFil SDR, X-tra base), a self-adhering flowable composite (Vertise Flow), a highly filled flowable composite (GrandioSO Flow), two conventional flowable composites (Filtek Ultimate, Clearfil Majesty). Specimens in 3 mm diameter, 2 mm height were prepared from each composite (n=6) and were transferred to 24 well plates. Wells without composite material were used as the control group. After 24 h incubation period, cytotoxicity was determined by using the 3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay. Oxidative alterations were assessed using TAC and TOS assay kits. Data were analyzed using the ANOVA and least significant differences post-hoc test.

Results: Cytotoxicity of six materials was significantly different from the control group (p<0.05). Vertise flow was the most cytotoxic material. TAC levels of Vertise flow were significantly different from X-tra base and GrandioSO. TOS levels increased in SureFil SDR and Vertise flow groups but it was not statistically significant difference.

Conclusion: All of the materials used in this study showed cytotoxic effect in human gingival fibroblast cell culture. These materials did not have a significant effect on TOS level. However, TAC level could not prevent the rise of TOS level in Vertise and sureFil SDR.

Öz

Amaç: Dental materyallerden salınan komponentler doku hasarı ve hücre apoptosisi veya ölümünde önemli bir faktör olan oksidatif stres oluşumuna sebep olabilir. Bu çalışmanın amacı; farklı akıcı kompozit rezinlerin sitotoksitesini ve bu materyallerin insan dişeti fibroblast hücre kültüründe total antioksidan kapasite (TAK) ve total oksidan durum (TOD) seviyesine etkisini değerlendirmektir.

Gereç ve Yöntemler: Sağlıklı bireylerden elde edilen diş eti fibroblast hücreleri sitotoksitate ve oksidan durum değerlendirmesi için kullanıldı. Bulk-fill akıcı

kompozit (SureFil SDR, X-tra base), kendiliğinden bağlanan akıcı kompozit (Vertise Flow), yüksek dolduruculu akıcı kompozit (GrandioSO Flow), geleneksel akıcı kompozit (Filtek Ultimate, Clearfil Majesty) olmak üzere altı farklı materyal kullanılarak 3 mm çapında 2 mm yüksekliğinde örnekler hazırlandı (n=6) ve 24'lük doku kültür kabına yerleştirildi. Örnek yerleştirilmeyen kuyucuklar kontrol grubu olarak belirlendi. Yirmi dört saat boyunca inkübe edildikten sonra 3-(4,5 dimetylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) analizi ile materyallerin sitotoksitesi değerlendirildi. Oksidatif değişim TAK ve TOD analiz kitleri ile değerlendirildi. Elde edilen verilerin analizinde ANOVA ve en önemsiz farkla post-hoc testi kullanıldı.

Bulgular: Uygulanan altı materyalin sitotoksitesi kontrol grubu ile anlamlı farklılık gösterdi ($p<0,05$). Vertise flow en sitotoksik materyal olarak tespit edildi. Vertise flow uygulanan grupta TAK seviyesi X-tra base ve GrandioSO'ya göre anlamlı farklılık gösterdi. SureFil SDR ve Vertise flow grubunda TOD seviyesi artış gösterdi fakat bu artış anlamlı değildi.

Sonuç: Çalışmada kullanılan tüm materyaller sitotoksik etki göstermiştir. Bu materyaller TOD seviyesi üzerinde önemli etki oluşturmamıştır. Ancak TAK seviyesi, Vertise ve SureFill SDR grubunda TOD seviyesindeki yükselmeyi engelleyememiştir.

Introduction

Flowable composites were introduced in 1990s and they have been designed to provide improved adaptation and polymerization stress relief. The low viscosity of these materials allows them to shape itself to fit in the difficult access cavity areas (1,2). Flowable composites are suitable for small class III or class V restorations, enamel defects, margin repairs or as cavity liners (3,4). However, there are some problems associated with their poor mechanical properties, lower filler content, polymerization shrinkage and weak adhesion (1,3). Manufacturers have recently introduced new generation flowable composites, so called higher filler loading flowable resin composites, bulk-fill flowable composites and self-adhering flowable composites for elimination of negative effects and improving clinical requirements (3,5). Higher filler loading flowable resin composites for posterior restorations have better wear resistance compared with some resin composites (6). Bulk-fill composite resins specifically designed for placement in single layers of 4 to 5 mm and have lower polymerization shrinkage and stress values when compared with conventional flowable composite resins (7,8). Self-adhesive flowable composites includes acidic monomer like glycerol phosphate dimethacrylate (GPDM) and can be bonded to tooth structures without using adhesive systems (9).

The biocompatibility has gained considerable interest during recent decades and is the important factor for evaluation of materials clinical success, as well as the physical properties (10). Chemical composition of composite material, degradation of material, degree of monomer conversion, surface treatment and conditions within the oral cavity may cause to release substances into the oral

environment (11,12). Inadequate polymerization can cause to release methyl methacrylate (MMA), hydroxyethyl methacrylate (HEMA), bisphenol A diglycidyl dimethacrylate (Bis-GMA), triethyleneglycol dimethacrylate (TEGDMA), and urethane dimethacrylate (UDMA) from the resin matrix (13,14). These monomers are associated with cytotoxicity and oxidative stress in tissue or cell and influence the signal transduction pathways and complex regulatory cellular networks. Moreover, this monomer deplete the amount of glutathione and cause to increase the formation of reactive oxygen species (ROS) (14,15). As a result of aerobic metabolism, ROS are occurred in cells. Low concentrations of ROS are compatible with normal physiological functions, whereas high concentrations of ROS are considered to be harmful to cells, leading to oxidative stress (16). Antioxidant systems neutralize these reactive molecules and protect cells from potential cytotoxic effects. But when the balance between oxidants and antioxidants is disrupted, oxidative stress occurs (15,17).

The objective of this study was to evaluate *in vitro* cytotoxicity of different flowable composites materials and their effects on total antioxidant capacity (TAC) and total oxidant status (TOS) levels in human gingival fibroblast (HGF) cell culture.

Materials and Methods

This study was granted ethical approval by the Ethical Committee of the Atatürk University Faculty of Dentistry (22.04.2016/23) and conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from all patients. The cytotoxic effects of flowable composites on HGF cell culture, were evaluated using the direct contact test method and 3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide (MTT) assay. Oxidative alterations were determined by measuring of TAC and TOS level.

Preparation of Samples

Six different flowable composite materials were used in the study. The materials used and their contents are demonstrated in Table 1. Samples in a disc shape, 3 mm diameter and 2 mm height, were prepared from each restorative material. Six samples in disc shape using a teflon mold were prepared from each restorative material. The materials placed in the mold were covered with transparent tape (mylar strip) and left to harden between two glasses. Resin containing materials was polymerized using a visible blue LED light device (Elipar Freelight II, 3M-ESPE, St. Paul, MN, USA) at a wavelength of 450 nm according to manufacturer instructions. The edges and surfaces of samples were straightened using polishing discs (Sof-Lex; 3M ESPE, St. Paul, MN, USA). Samples were transferred to well plates, Wells without flowable composite material were determined as the control group.

Preparation of the Cell Culture

Cultured HGF cells were used in this study. Gingival tissue samples were obtained from young healthy donors tissue overlying impacted third molars by the informed written consent of the patients (aged 18-25). The gingival tissue pieces immediately were placed in 2 cc Dulbeccos Modified Eagles Medium/F12 (DMEM: Gibco BRL, New York, USA). The samples were washed by normal saline and then by DMEM medium for 2-3 min. Gingival tissue was cut into approximately 1x1 mm pieces in size and placed in 50 cc falkon for washing small parts and blood cells residues.

The samples were seeded in the well plate (Corning, New York, USA) then 2 cc DMEM/F12 medium with 10% heat-inactivated fetal calf serum (FCS), 100 U/mL penicillin, 100 µg/mL streptomycin and 1% amphotericin B (Gibco BRL, New York, USA) added and incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO₂ (Incubator ESCO, SINGAPORE) and the old medium was replaced with fresh medium twice a week. The morphology of primary HGF resulted in spindle shaped cells (Figure 1). When the plate obtain 70-80% confluency (the cell number was 1x10⁶ cells/

Table 1. Flowable composites used in this study

Material name	Material type	Content	Manufacturer/lot no
SureFill SDR Flow	Bulk- fill flowable composites	UDMA with hybrid glass filler. Barium and strontium, alumino-fluoro-silicate glasses	DENTSPLY, milford, USA 1312000155
X-tra base	Bulk-fill flowable composites	Bis-EMA, Aliphatic dimethacrylate	VOCO, GmbH, Cuxhaven, GERMANY 1422161
Vertise™ flow	Self-adhering flowable composites	GPDM, HEMA, methacrylate co-monomers	KERR, USA 2894473
Clearfil majesty flow	Highly filled, microhybrid flowable composites	TEGDMA, hydrophobic aromatic dimethacrylate, Silanated barium glass filler Silanated colloidal silica dl-Camphorquinone Accelerators Pigments	KURARAY, Medical Inc, Okayama, JAPAN BM0004
Grandio SO flow	Highly filled flowable composites	HEDMA, Bis-GMA, TEGDMA, Bis-EMA	VOCO, GmbH, Cuxhaven, GERMANY 1331164
Filtek™ Ultimate	Nanohybrid flowable composites	Bis-GMA, TEGDMA, Silane treated ceramic, Ytterbium fluoride	3M ESPE, St. Paul, MN, USA N629174

UDMA: Urethane dimethacrylate, Bis-EMA: Ethoxylated Bisphenol A dimethacrylate, Bis-GMA: Bisphenol A diglycidyl dimethacrylate, GPDM: Glycerol phosphate dimethacrylate, HEMA: Hydroxyethyl methacrylate, TEGDMA: Triethyleneglycol dimethacrylate

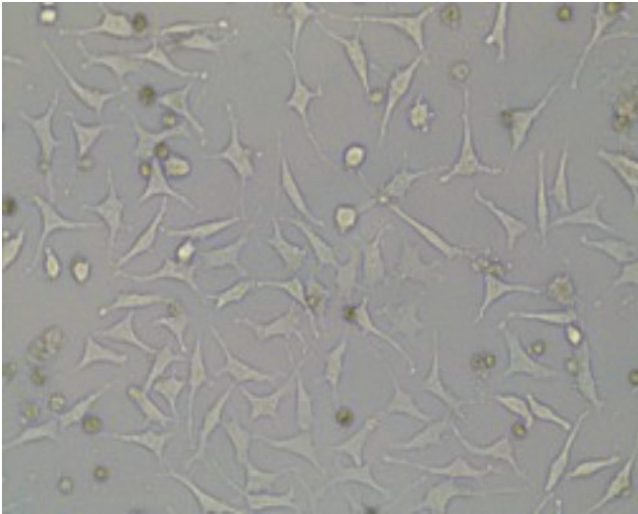


Figure 1. The morphology of cultured cells, growing from the tissue origin (100 μ diameter).

mL), the primary HGFs were trypsinated. HGFs were used in the third passages.

MTT Assay

After 24 h material-cells interaction periods, MTT assay was carried out with a commercially available kit (Sigma, USA). MTT reagent (10 μ L) was added into the cell culture. The plate was incubated in CO₂ incubator at 37 °C for 4 h, in this period NAD(P)H oxidoreductases reduced a purple formazan intracellularly and then 100 μ L of crystalline solvent solution was added to each well. The intensity of the formazan was measured at 570 nm with a Biotek Spectrophotometer (μ Quant, Biotek, Winooski, USA).

Total Antioxidant Capacity and Total Oxidant Status Assay

TAC assay kit was used to determine antioxidant levels of samples by inhibiting formation of a free radical, 2,20-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) compound (Rel Assay Diagnostics, Gaziantep, Turkey) on HGF cell cultures for 24 h. The assay was calibrated with Trolox equivalent as a stable antioxidant. After cells were incubated for 24 h, the medium was removed from the well plate and new medium was placed. Standard solutions were added to wells, according to kit protocol, reagent 1 solution was added to each well and first spectrophotometric reading was carried out at 660 nm. Reagent 2 solution was added to each well and the well plate was incubated at room temperature for 10 min. Second spectrophotometric

reading was obtained at 660 nm. In TAC assay kits, ascorbic acid was used as an organic antioxidant compound for a determined positive control.

The TOS assay was carried out with a commercially available kit (Rel Assay Diagnostics, Gaziantep, Turkey) on HGF cell cultures for 24 h. According to assay protocol, complexes formed with the ferric ion are oxidized to ferrous ion by oxidants presented in the sample. In the acidic environment ferrous ions form a chromogen-colored pigment. The pigment density measured spectrophotometrically is related to the total amount of oxidant molecules in the sample. Hydrogen peroxide (H₂O₂) was used for assay calibration. After 24 h cell incubation periods, medium was removed from the plate and new medium was placed. Standard solutions in kit were added to each well. According to assay protocol reagent 1 solution was added to each well. First spectrophotometric reading was carried out at 530 nm. After the first reading, reagent 2 solution was added to each well and then incubated for 10 min at room temperature. Second spectrophotometric reading was done at 530 nm. In TOS assays, H₂O₂, ROS was used as a positive control.

Statistical Analysis

Analysis of variance (ANOVA) was used to analyze the effects of the six flowable composite resins on MTT, TAC and TOS levels. Significant main effects were analyzed post-hoc using LSD multi-comparison test. All statistical analyses were performed via SPSS 20 (SPSS Inc., Chicago, IL, USA) using a confidence interval of 95%.

Results

The cytotoxicity of flowable composites was measured by using MTT assay and expressed as a percentage of the control groups (Figure 2). Cell viability in all experimental groups was significantly decreased compared with the control group ($p < 0.05$). Vertise flow exhibited significant cytotoxicity on cultured HGF and caused cytotoxicity at levels of 41%. Filtek ultimate and X-tra base showed lower cytotoxicity at levels of 18.5 and 21.7%. Vertise flow had a statistically significant difference compared with X-tra base and Filtek ultimate ($p = 0.034$, $p = 0.015$).

Table 2 presents the level of TAC and TOS in HGF cell culture after application of dental flowable composites. TAC level of Vertise was significantly different from X-tra base and GrandioSO ($p = 0.034$,

$p=0.049$). TAC level of Filtek Ultimate was significantly different from X-tra base and GrandioSO ($p=0.025$, $p=0.036$). TOS level increased in SureFil SDR and Vertise groups but it was not statistically significant difference. The lowest TOS level was seen in Filtek Ultimate. Table 3 demonstrates the differences (p value) between groups in terms of MTT, TAC and TOS.

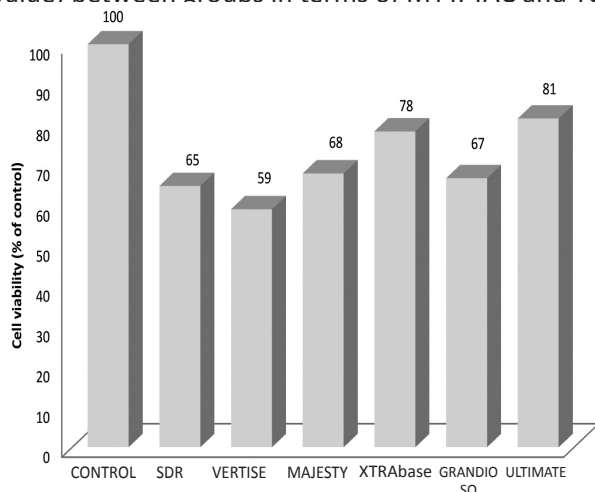


Figure 2. Cytotoxicity of flowable composites were measured by using MTT assay and expressed as a percentage of the control groups. Low cell viability means high cytotoxicity
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Discussion

Cell culture assay has gained interest in recent years and are assumed to be appropriate methods to evaluate the biocompatibility of the restorative dental materials, since they are standardized, easy to apply, repeatable, cost-effective and take less time (18,19). In the present study biocompatibility of six different flowable composites on HGF were investigated with the MTT assay and TAC and TOS biological parameters assay in cell culture.

Pulp and gingival fibroblasts are highly exposed to resin monomers after releasing from composite fillings to the oral cavity and this materials cause morphological changes and inflammatory reaction in cell (11). Therefore, primary cultures of human pulp and gingival fibroblasts were selected as optimal for biocompatibility testing of dental materials. Several approaches are possible to determine the cytotoxicity of material like LDH release, MTT formation, XTT formation, neutral red uptake, kenacid blue binding, acid phosphatase activity, sulforhodamine B binding and resazurin binding (20). But, MTT and NR tests are more sensitive and more reliable in the evaluation of a material's toxic properties (21,22). According to the ISO 10993-5 specification, direct tests, indirect tests

Table 2. Mean and standart deviation of MTT, total antioxidant capacity and total oxidant status in human gingival fibroblast cell culture after 24 h application of dental flowable composites

Groups	Sample size (n)	MTT Mean \pm SD (count of cell/ μm^2)	TAC Mean \pm SD (mmol trolox Equiv./L)	TOS Mean \pm SD (mmol H_2O_2 Equiv./L)
Control	6	0.251 \pm 0.03 ^a	0.89 \pm 0.01 ^{a,b}	3.64 \pm 0.96 ^a
Surefil SDR flow	6	0.162 \pm 0.04 ^{b,c}	0.89 \pm 0.01 ^{a,b}	4.03 \pm 0.64 ^a
Vertise flow	6	0.148 \pm 0.04 ^c	0.90 \pm 0.01 ^a	3.91 \pm 1.28 ^a
Clearfil majesty flow	6	0.170 \pm 0.03 ^{b,c}	0.89 \pm 0.01 ^{a,b}	3.37 \pm 1.06 ^a
X-tra base	6	0.196 \pm 0.04 ^b	0.88 \pm 0.01 ^b	3.60 \pm 1.44 ^a
Grandio SO flow	6	0.167 \pm 0.01 ^{b,c}	0.89 \pm 0.01 ^b	3.64 \pm 0.67 ^a
Filtek ultimate	6	0.204 \pm 0.03 ^b	0.90 \pm 0.00 ^a	2.90 \pm 0.82 ^a

In the same column, the groups identified by different superscript lowercase are statistically different ($p<0.05$)

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, TAC: Total antioxidant capacity, TOS: Total oxidant status, SD: Standard deviation

Table 3. P-value between groups in terms of MTT, total antioxidant capacity and total oxidant status

	Control		Surefil SDR flow			Vertise flow			Clearfil majesty flow			X-tra base			Grandio SO flow			Filtek ultimate				
	MTT	TAC	TOS	MTT	TAC	TOS	MTT	TAC	TOS	MTT	TAC	TOS	MTT	TAC	TOS	MTT	TAC	TOS	MTT	TAC	TOS	
Control	x	x	x																			
Surefil SDR flow	0.000	0.736	0.519	x	x																	
Vertise flow	0.000	0.300	0.659	0.514	0.481	0.838	x	x														
Clearfil majesty flow	0.001	0.680	0.644	0.724	0.454	0.271	0.317	0.151	0.368	x	x											
X-tra base	0.018	0.257	0.941	0.131	0.145	0.472	0.034	0.034	0.606	0.242	0.468	0.698	x	x								
Grandio SO flow	0.01	0.331	0.989	0.827	0.193	0.510	0.385	0.049	0.649	0.892	0.572	0.654	0.193	0.870	0.952	x	x					
Filtek ultimate	0.041	0.240	0.218	0.064	0.398	0.65	0.015	0.887	0.098	0.129	0.116	0.437	0.718	0.025	0.246	0.100	0.036	0.223	x	x		

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, TAC: Total antioxidant capacity, TOS: Total oxidant status

and extract tests are used for material and cellular contact methods in tests for *in vitro* cytotoxicity. In previous studies demonstrated a good association between direct and indirect contact tests, while the least sensitivity was seen in the extract tests (23,24). Based on previously performed studies direct material cellular contact method and MTT assay were used in this present study. The *in vitro* antioxidant/oxidant capacity of materials was determined by measuring TAC and TOS levels with commercially available kits. The major advantage of this test is to measure the antioxidant capacity of all antioxidants and all oxidant in a biological sample.

The main finding obtained from this study is that the restorative materials showed different degrees of cytotoxicity. The rank order of cytotoxicity for materials; Filtek Ultimate<X-tra base<Clearfil Majesty Flow<Grandio SO flow<SureFill SDR Flow<Vertise. This is not surprising; substances released from dental materials cause toxicity in cell culture (13). Self-adhering flowable composite material Vertise flow showed higher cytotoxicity. This might be due to the presence of an acidic monomer in compound. Two main components of this material are an acidic functional monomer (GPDM) and a functional monomer (HEMA). GPDM etches dentine and enamel, and improves wettability(25).Tadin et al.(11)reported serious toxic effects of Vertise flowable composites on pulpal fibroblast and to cause increasing apoptotic cells. X-tra base resin formulated with ethoxylated bisphenol A dimetacrylate (Bis-EMA) showed cell viability at level 78%. In a toxicity study by Barbosa et al., (26) UDMA and Bis-EMA showed lesser cell death than HEMA. Bis-GMA and TEGDMA are proven cytotoxic agent. However cytotoxicity caused by Bis-GMA is higher than that caused by

the other monomers. Lower concentrations of Bis-GMA cause necrosis of HGF while higher ones cause cell apoptosis (27,28).

Imbalance between antioxidant defence system and free radicals cause oxidative stress. In the current study, alterations in the oxidant and anti-oxidant level of HGF cells were determined after exposure to flowable composite materials. Experimental studies support that, resin monomer effect redox balance and enhance ROS production and there are evidences about materials toxicity related with ROS production (16,29). Vertise and Filtek Ultimate had significant effect on TAC. SDR was slightly but not significantly induced level of TAC. Although increased TAC level, TOS level increasing was not prevented in Vertise and SDR. The decrease in cell survival was also observed by application of these two flowable materials. Increased TOS level and cytotoxicity were closely associated for SDR and Vertise flowable composites. Because oxidative stress is a common final mechanism of cell death (30). Schweikl et al. (31) reported dental monomers exposed cells indicated oxidative stress as a result of increased ROS and this oxidative stress acts as a signal for pathways activation which control cell death and viability through the redox sensitive activation of antioxidant proteins. Gallorini et al. (32) observed in their study that the resin monomer HEMA differentially caused oxidative stress in RAW264.7 mouse macrophages. In a study performed by Chang et al. (33) showed that Bis-GMA induced the expression of hemoxygenase-1 which is an oxidative responsive gene and stimulate ROS production, apoptosis and cell death in pulp cell culture.

Conclusion

The result of this study provides a better understanding toward the protective effect of antioxidant capacity against formation of oxidative stress induced by flowable resin composite. The limitation of this study included testing only total levels of antioxidant and oxidant status. Evaluation of the different antioxidant enzyme such as superoxide dismutase, catalase, glutathione is recommended for further studies.

Ethics

Ethics Committee Approval: This study was granted ethical approval by the the Ethical Committee of the Atatürk University Faculty of Dentistry Atatürk (date/approval number: 22.04.2016/23).

Informed Consent: Informed consent was obtained from all patients.

Peer-review: External and internal peer-reviewed.

Authorship Contributions

Concept: N.Ç., M.İ.Y., Design: A.T., M.İ.Y., Data Collection or Processing: A.T., Analysis or Interpretation: A.T., Literature Search: M.İ.Y., N.Ç., Writing: N.Ç., M.İ.Y.

Conflict of Interest: No conflict of interest was declared by the authors.

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