Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Original Research

Cytotoxic effects of new-generation bulk-fill composites on human dental pulp stem cells

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Received October 28, 2017; **Accepted** February 15, 2018; **Published** February 28, 2018

Doi: http://dx.doi.org/10.14715/cmb/2018.64.3.11

Abstract: This study was performed to evaluate possible DNA damage in cells of human origin exposed to dental composites *in vitro* using a cytotoxic assay. Five bulk-fill composites were filled in molds and irradiated for 20 s. DPSCs were inoculated into 24-well plates. After the insert membrane was inserted and composites were added and the experiment was continued for 24/72 hours. In order to investigate the effects of the materials on DPSCs; its effect on apoptosis-regulating Bcl-2 gene, Human Beta-Defensins (HBDs 1-2) gene, Interleukin 6, 8, 10 expression level was examined. Also in order to check the cellular viability and stress factors; MTT assay, Total Antioxidant and Oxidant Status kits were used. At both irradiation times, all composites significantly affected analyses parameters used in primary DNA damage assessment or induced significant formation of cellular death. Cytotoxicity was detected in TE<SS<FBF<XB<VBF groups at 24 hour, and after 72 hour this sequence has changed.

Key words: Cytotoxicity; Apoptosis; Human beta defensins; Stem cells.

Introduction

A new resin based dental composite material class, the bulk-fill composites, has been introduced in the last few years. They provide convenience to speed up the restoration process by enabling up to 4- or 6-mm thick increments to be cured in one step, so it can help clinicians save time (1). It is preferred for these materials to have no side effect and to be biocompatible on the oral tissues (2-6). *In vitro* cytotoxic tests, genotoxic tests and gene suppression in the cell layers can be applied to assess the biological effects of restorative materials (7). The reliability of these tests are supported by different tests and techniques in order to (8-10) avoid misleading results when compared to the results developing under *in vivo* conditions.

Dental composites have micro monomers and these monomers in the organic matrix of restorative materials causes gene mutation in dental pulp stem cells and degradations in the DNA strand. These residual monomers cause degeneration in DPSC cells and an increase in Bcl-2 gene expression regulating apoptosis (11-14).

The asset of apoptotic cells increases the release of Human Beta Defensins (HBDs). They depress the proinflammatory cytokines in the relevant tissue, activate the mast cells and provide the degranulation of the cells (15). By now, 4 human beta defensins have been isolated (hBD-1,-2,-3, and -4) (16, 17). It is shown that odontoblastic cells similar to epithelial cells (cells inside the dentinal tubules of the teeth) structurally contain HBD-1 and HBD-2. Stimulation of odontoblast-like cells and microbial lipopolysaccharides with recombinant HBD-2 causes changes in the proinflammatory and anti-inflammatory cytokines (18-20). As a result of the HBD-2 and HBD-1 increase; dental pulp increases the release of inflammatory cytokines and causes the tooth to lose its viability.

In the studies, the defense is expected to be at high levels in endodontic infections, decayed teeth, in the cases having infection in the apical of teeth and in the teeth with deep restoration. However as a result of our researches, the effect of HBDs on gene expression in dental pulp tissues has not been clarified in the literature (21, 22).

As a result of the literature review; the studies investigating the cytotoxic effects of restorative materials have been reached, however, no study investigating the effects of these materials on Bcl-2 gene regulating apoptosis, HBDs-1 and HBDs-2 gene expression, and IL-6, IL-8 and IL-10 cytokines via MTT, TAS and TOS analyses has been found. 9 different biological parameters were assessed using the Human Dental Pulp stem cells in this study for the first time.

Materials and Methods

Specimen preparation

For bulk fill composites (Table 1), 4x6 mm standard molds were used. After placing the restorative material inside the molds, strip bands were attached on the lower and upper surfaces of the molds and they were pressed with the glass slide in order to develop a smooth surface and then, they were polymerized for 20/40 seconds via LED light device (Elipar Freelight II, 3M-ESPE, St.Paul,MN,USA) according to the instructions of the producing company. After the samples were hardened, their edges and surfaces were polished by using the polishing disks (Sof-Lex™ Diamond Polishing System;

Table 1. Materials used in this study.

	Abbreviation	Material Name	Manufacturer	Material Type	Matrix type	Filler Content	Filler content %
Group 1	SS	Surefil SDR Flow	Dentsply Caulk / ABD	Bulk Fill Flowable Composite	Dimethacrylate Resin, BPADMA TEGDMA, UDMA BHT	Silicate glass, Silicate oxide Hybrid glass fiber	80
Group 2	XB	X-tra Base Flowable	Voco Cuxhaven GERMANY	Bulk Fill Flowable Composite	BisGMA, BISEMA, UDMA and Procrylat	Zirconia, Silica Particles, IteriumTrifluoride,	75
Group 3	VBF	Venus Bulk Flow	Voco Cuxhaven GERMANY	Bulk Fill Flowable Composite	UDMA, EPBADMA	Ba-Al-F Silicate glass, YbF_3, and SiO_2	65
Group 4	FBF	Filtek Bulk Flow	3M / ESPE - USA	Bulk Fill Flowable Composite	UDMA, BisGMA, BISEMA, ProAcrylate Resins TEGDMA	YBF3 fillers, Zirconium Silica Particles	64.5
Group 5	TEF	Tetric-Evo Flow Bulk Fill	Ivoclar Vivadent Austria	Bulk Fill Flowable Composite	Dimethacrylate Resin	Glass particles, Theprepolymer, Itanium trifluoride, Mixed oxides	76

3M ESPE, St. Paul, MN, USA).

Before the materials prepared were placed in 96-well plates; they were sterilized with pressurized vapor in the autoclave at 121°C at 1 atm pressure for 15 minutes. This process was repeated for all the materials used in the study.

Preparation of Cell Culture

Preparation of Human Dental Pulp stem cells (DPSCs)

DPSCs (Fig. 1) were provided by ATCC company. Procedures were performed according to this source (24), but shortly, the cells coming in cryofalcons were dissolved in series at normal room temperature and centrifuged for 5 minutes in a 1200 rpm +4 degrees centrifuge (Bachmann, Germany) by adding 2 cc DMEM medium (Dulbecco's Modified Eagle's Medium, Gibco, USA) . The settled cells were mixed in the new medium (DMEM/f12, 1/10 FBS, 1% antibiotic (penicillin - streptomycin - amphotericin B)) and inoculated in a 25 cm^2 flask. When the cells covered 80% of the flask, the passage process was performed by adding 0.4 cc Trypsin /EDTA. The cells were taken in a sterile tube and centrifuged for 5 minutes at 1200 rpm. After the supernatant liquid of the cells forming a pellet was removed, new medium was added and the incubation process was completed by inoculating 250 µl and 10^5 cells in every well of a 24-well cell plate and then this was kept in an incubator containing 5% CO_2 at 37 degrees.

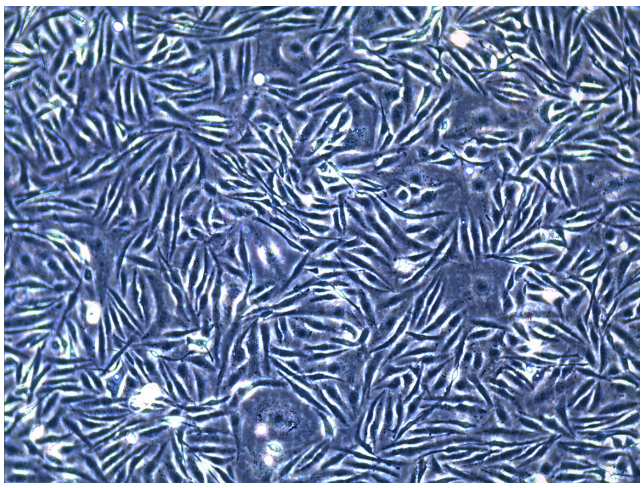

Figure 1. Dental pulpa stem cells with a 10x magnification.

Preparation of insert membrane well plates

It is aimed to enable the transition of the components that were dissolved from the restorative materials or were not polymerized due to the membrane and thus to have them contact with the cells on the medium bottom**.** When the tubule diameter of dentin at the enamel dentin border was only 1 µm, the dentinal tubule diameters reached to 3-4 µm on the pulp side. Based on this information, inserts with a pore thickness of 3 µm were used in the present study (Fig. 2).

The cells in the form of logarithmic reproduction that were active and had a surface covered at the rate of 90-95% were included in the experiment. The samples

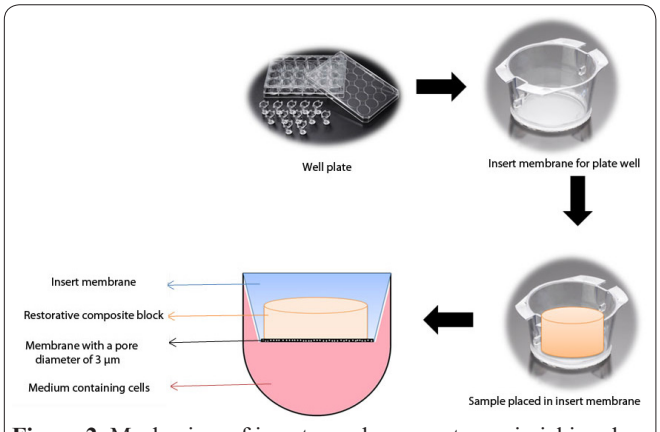

Figure 2. Mechanism of insert membrane system mimicking dentin tubules.

sterilized under UV light for 2 hours were individually placed with the help of a sterile forceps in a sterile cabinet in such a way not to contact directly with cells after they were loaded to insert membranes placed into 24-well plates. The cells were left for incubation in the incubator of 5% CO2 at 37 ºC for 24 and 72 hours.

Determination of BCl-2, HBD-1, HBD-2, IL-6, IL-8, and IL-10 expression

Samples were processed blindly for the detection of mRNA transcripts of HBD-1 and HBD -2. The total cellular RNA was isolated from each sample.

RNA isolation

Medium of 24-well plates was taken and 0.1 cc Trypsin/EDTA was added to every well. After they were incubated in the incubator for 5 minutes, they were centrifuged at 1200 rpm for 5 minutes. The cells forming a pellet were processed for RNA isolation and cDNA synthesis.

Isolation was performed by using the Qiagen RNA isolation kit. 1cc Qiazole solution was added to the cells in the form of a pellet that were settled in the tube and they were kept at room temperature for 5 minutes. Then, 200µl chloroform was added, agitated for 15 seconds, and kept at room temperature for 2-3 minutes. The samples were centrifuged at 12.000 g for 15 minutes at the temperature of 4 degrees. The colorless fluid at the top was transferred to another tube and it was vortexed by adding 1:1 ethanol alcohol. 700µl of the sample was taken and placed in a collection tube and after it was closed, it was centrifuged at room temperature at 8.000 g for 15 seconds. This stage was repeated once more. 700µl RW1 solution was added into the RNeasy column. The cap of the column was closed and it was centrifuged at 8.000 g for 15 seconds. Then, 500 µl RPE was centrifuged at RNeasy column at 8.000 g for 15 seconds. Then, 500 µl RPE was added to the RNeasy column and centrifuged at 8.000 g for 2 minutes. After this stage, a new 1.5 cc tube was placed in the RNeasy column, 30-50µl RNasefree water was added and its cap was closed, it was centrifuged at 8.000 g for 1 minute.

cDNA synthesis

For the cDNA synthesis; 2µl from the genomic DNA wipeout buffer 7x solution and RNA 1µg and RNasefree water were prepared to have a total volume of 14µl and after they were kept at 42 degrees for 2 minutes, they were again placed in the ice. Then, a total of 20 µl including reverse transcription master mix 1 µl, Quantrscript RT buffer 5x 4 µl, RT primer mix 1 µl and RNA 14 µl were mixed and placed in the RT-PCR device. Heat was given at 42 degrees for 15 minutes and at 95 degrees for 3 minutes and then it was kept up to -20 degrees.

MTT, Oxidant and Antioxidant Analyzes

MTT, TOS, and TAS experiments were carried out in order to determine the cell viability and the level of stress factors. Cell fluid was taken from the samples after their interaction with cells for 24 and 72 hours and it was kept at -20 ℃ to determine TAS and TOS levels. MTT solution was mixed as 10% with new cell medium and added into 24-well plate in order to determine viability of cells.

MTT Assay

When the DPSCs covered 90-95% of the plates, the materials were placed on the membranes and they were left for interaction with the cells for 24 and 72 hours. In order to determine the cytotoxicity ratio of the materials, MTT test was applied at the end of 24-hour and 72-hour incubation period in the incubator containing 5% CO_2 at 37 °C. At the end of the period, MTT solution (3-(4.5- dimethylthiazol -2-yl)-2.5- diphenyltetrazolium bromide) of 10% was added and incubated in the incubator containing 5% CO_2 at 37 °C for 4 hours and afterwards, by adding DMSO (Dimethylsulfoxide)(Sigma, USA), the absorbance value was read at 550 nm wavelength in (optical density) a spectrophotometer device (µQuant, BadFriedrichshall, Biotek) and the living cell count was obtained. The experiment was repeated for 3 times.

$$\text{Viability \% ratio} = \frac{sample\ absorbance\ value}{control\ group\ absorbance\ value} \times 100$$

Total oxidant status (TOS)

In total oxidant status (TOS) assay, the assessment is done by measuring spectrophotometrically the density of the color related to the amount of oxidants in the sample. In the present study, TOS (Total Oxidant Status) kits manufactured by RealAssayDiagnostics® (Turkey) company were used.

The components in the kit were Reactive 1 Solution, Reactive 2 Solution, Standard 1 solution, and Standard 2 Solution. In order to determine the TOS level; 500 µl Reactive 1 solution was added to the wells in which 75 µl plasma sample was present and after reading the initial absorbance value at 530 nm, 25 µl Reactive 2 solution was added in the same well and second absorbance was read at 530 nm at the end of the waiting period of 10 minutes at room temperature. Standard 2 solution in the kit was used for Standard 2. By using the absorbance values obtained and the following formula, TOS levels were determined in mmol Trolox Equiv./L.

$$\text{TOS} = \frac{\Delta example}{\Delta ST2} \times 20$$

Δ ST2 (Δstandard 2 = ST2 second reading - ST2 first reading), Δ Sample (ΔSample= Sample second reading-Sample first reading)

Principle of the Total Antioxidant Status (TAS) Measurement

In TAS assay; antioxidant capacity was determined by inhibiting formation of the 2-2'-azinobis (3-ethylbenzothiazoline 6-sulfonate= ABTS+) radical cation. In the assay process, Real AssayDiagnostics® (Turkey) commercial kit was used.

The components of the kit were Reactive 1 Solution, Reactive 2 Solution, Standard 1 solution, and Standard 2 Solution. In order to determine the TAS level; 500 µl Reactive 1 solution was added in the wells containing 30 µl sample and first absorbance was read at 660 nm. Then, 75 µl Reactive 2 was added to the same wells and allowed to wait at room temperature for 10 minutes. At the end of the waiting period, second absorbance value was read at 660 nm. While distilled water was used for Standard 1, Standard 2 solution in the kit was used for Standard 2. The absorbance values obtained were placed

Table 2. MTT analysis results of DPSCs after 24 hours.

24 hours	Percentage of MTT analysis results					
	Control (ACTB gene)	XB	SS	FBF	VBF	TE
	0,173	0,073	0,164	0,09	0,066	0,185
	0,164	0,066	0,172	0,051	0,068	0,183
	0,168	0,0695	0,168	0,0705	0,067	0,184
Percentage %	100	41,246	99,703	41,839	39,762	109,19

Table 3. TAS analysis results of DPSCs after 24 hours.

24 hour	TAS analysis results					
	Control	XB	SS	FBF	VBF	TE
	5,84	1,74	6,15	2,25	3,67	6,21
	5,57	1,54	6	2,18	3,54	6,43
	5,49	1,68	5,94	2	3,16	6,125
avg.	5,64	1,65	6,03	2,14	3,45	6,25

according to the following formula and TAS levels were determined in mmol Trolox Equiv./L.

$$TAS = \frac{(\Delta ST1 - \Delta example)}{(\Delta ST1 - \Delta ST2)}$$

Δ ST1 (Δstandard 1 = ST1 second reading - ST1 first reading), Δ ST2 (Δstandard 2 = ST2 second reading - ST2 first reading), Δ Sample (ΔSample= Sample second reading- Sample first reading)

Detection of apoptosis by Annexin-V binding

The AnnexinV-FITC apoptosis detection kit (Boivision) was used to identify connecting to Annexin V, which had a stable affinity for phosphatidylserine, in order to analyze apoptosis (47).

Statistical analysis

IBM SPSS Statistics 22 (IBM SPSS, Turkey) program was used for the statistical analyses to assess the results obtained in the study. In order to analyze the data, OneWay ANOVA(One Way Analysis of Variance) method was used and the significance values were compared with the control group. The significance values between the groups were assessed at the levels of $p<0.05$ and $p<0.001$ and a significant difference was found between the groups. We used power analyzed for stablieshed sample size (12 well for each sample). Indipendent value in our experiment are brand of material, time of materyal exposure, Bcl-2, hBD-1, hBD-2, IL-6, IL-8, and IL-10, TAS, TOS and Annexin V.

Results

MTT Analysis Results of the materials on DPSC cells after 24 hours

While cell viability was almost 100% in SS, cell regeneration of 9% was observed in TE group. In XB, FBF, and VBF groups; cell degeneration of approximately 60% was observed. Reproductive capability of mesenchymal cells (DPSC) is very rapid. They can completely cover an empty medium in 3 days. SS and TE maintained cell survival within 24 hours only compared to the control group and a cellular degeneration was observed to a large extent. After 24 hours, the cells lost their proliferation and division abilities in XB, FBF and VBF. Almost all the mesenchymal cells, playing an important role in the formation of dentin bridge, maintain their viability in TE group and SS group after 24 hours (Table 2, Fig. 3).

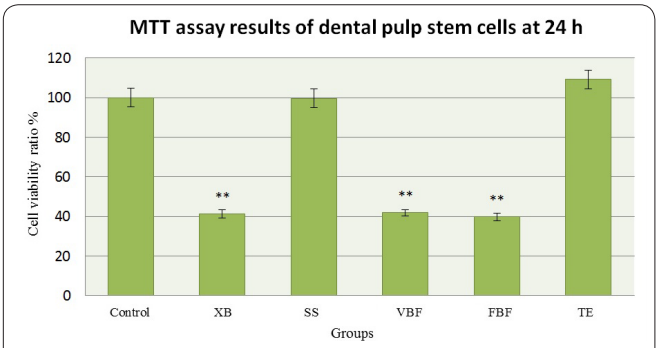

Figure 3. Viability rate in cells exposed 24h to different dental materials. Each value is expressed as mean ± standard deviation (n = 12). Values followed by different small letters differ significantly at * $p<0.05$ and ** $p<0.001$.

TAS and TOS Analysis Results of the materials on DPSC cells after 24 hours

When the total antioxidant values were examined; a decrease was observed in the antioxidant level in the other groups except for SS and TE. Due to the increase in cell degeneration, antioxidant level in XB, FBF and VBF reduced by approximately 60% compared to the control group. (Table 3, Fig. 4) It was observed that TOS values were lower in SS and TE groups than the control group(Table 4, Fig. 5).

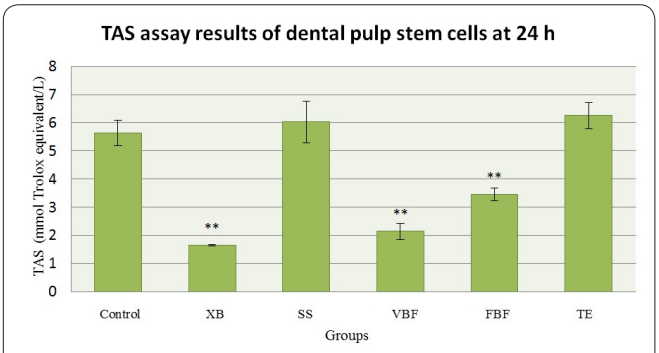

Figure 4. TAS levels of different dental material on cells for 24h. Each value is expressed as mean ± standard deviation (n=3). Values followed by different small letters differ significantly at * $p<0.05$ and ** $p<0.001$.

Table 4. TOS analysis results of DPSCs after 24 hours.

24 hour	TOS analysis results					
	Control	XB	SS	FBF	VBF	TE
	2,6	6,37	1,95	4,46	6,12	1,84
	2,47	6,48	1,78	4,44	5,64	1,66
	2,38	6,15	1,8	4,21	5,8	1,45
avg.	2,48	6,34	1,84	4,37	5,86	1,65

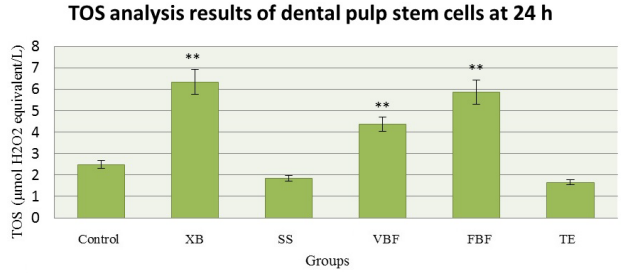

Figure 5. TOS levels of different dental material on cells for 24h. Each value is expressed as mean ± standard deviation (n=3). Values followed by different small letters differ significantly at * $p<0.05$ and ** $p<0.001$.

Gene expression (IL-6, IL-8, IL-10, BCL-2, HBD-1, HBD-2, and ACTB) Analysis Results of the Materials on DPSC cells after 24 hours

When examining the analysis results, it was observed that IL-6 increased 29 times in XB group, 25 times in VBF and 18 times in FBF compared to the control group. Similarly, when the results of IL-8 gene expression were examined, it was observed that there was an increase in these groups compared to the control group. If the cellular damage on DPSC cells is high, damage on the neuron and gingivo-fibroblast cells on the lower layer is also high. While the inflammatory factors was high in VBF, FBF, and XB; IL-10 level that would suppress the inflammatory status was low in these groups. Cell degeneration ratios were higher in VBF, FBF, and XB groups.

The IL-10 value increased 11 times in SS group and 28 times in TE group. It was observed that the smaller the BCL-2 gene expression value, the greater the cell degeneration. When the results of BCL-2 gene expression are observed; the cells were suppressed 5 times in XD, 19 times in VBF and 24 times in FBF and they were protected 13 times in SS and 36 times in TE. An increase of 36 times was observed in TE group compared to the control group. When the results of BCL-2 gene expression were examined, regeneration was observed in the cells in TE and SS groups. When examining HBD-1 gene expression results; high value of HBD-1 gene expression showed high cellular death in the materials. While this value was low in TE and SS groups; HBD-1 value was high in FBF, VBF and SS groups. An increase of approximately 58 times was observed in FBF group compared to the control group. Also it was observed that after 24 hours, HBD-2 gene was suppressed and not expressed (Table 5, Fig. 6).

MTT Analysis Results of the materials on DPSC cells after 72 hours

A decrease of 4% was observed in cell viability in SS group after 72 hours. In TE group, cell regeneration of 24% was observed in the cells. Cell regeneration was observed at the rate of 11% in XB, 7% in FBF and approximately 8% in VBF group. Although regeneration was observed in DPSC in XB, FBF, and VBF groups after 72 hours; cell viability ratio in these groups was 50% lower than the control group. It was observed that the cells lost their proliferation and division abilities after 72 hours except for SS and TE groups. In such case, it is not possible to mention the biocompatibility of XB, FBF, and VBF groups. (Table 6, Fig. 7)

TAS Analysis Results of the materials on DPSC cells after 72 hours

When the total antioxidant values were examined; a decrease was observed in the antioxidant level in the

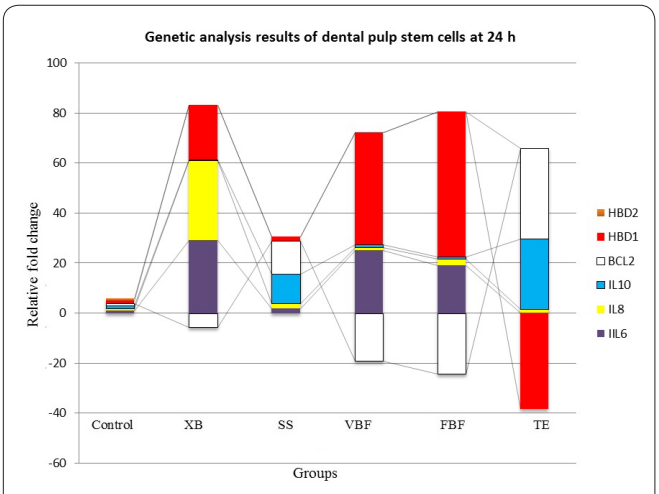

Figure 6. Gene expression level analysis of Bcl-2, hBD-1, hBD-2, IL-6, IL-8, and IL-10 in compare to housekeeping ACTB gene and control groups.24h exposure time of Dental Pulpa Stem Cells to dental materials (n=3).

Table 5. DPSCs, percentage values of gene expression analysis results after24 hours.

	Control	XB	SS	VBF	FBF	TE
IL-6	1	29,23	1,76	25,03	18,99	0,28
IL-8	1	31,93	2,16	1,34	2,6	1,21
IL-10	1	0,23	11,76	1,03	0,99	28,28
BCL-2	1	-5,65	13,24	-19	-24,31	36,24
HBD-1	1	21,95	1,76	45,03	58	-38,28
HBD-2	1	ND	ND	ND	ND	ND

Table 6. MTT analysis results of DPSCs after 72 hours.

72 hour	Percentage of MTT analysis results					
	Control (ACTB gene)	XB	SS	FBF	VBF	TE
	0,253	0,101	0,269	0,098	0,093	0,212
	0,132	0,1	0,098	0,087	0,091	0,287
	0,1925	0,1005	0,1835	0,0925	0,092	0,2495
Percentage %	100	52,20779	95,32468	48,05195	47,79221	129,6104

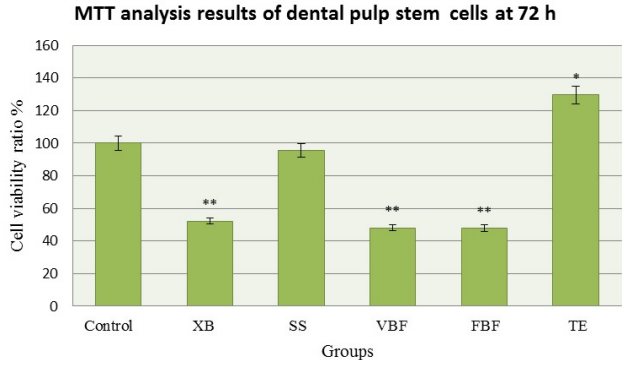

Figure 7. Viability rate in cells exposed 72h to different dental materials. Each value is expressed as mean±standard deviation (n=12). Values followed by different small letters differ significantly at * p<0.05 and ** p<0.001.

groups other than SS and TE. Antioxidant level had a slight increase in TE and SS groups compared to the control group. Due to the increase in the number of apoptotic cells, antioxidant level reduced by about 60 in XB, FBF and VBF % compared to the control group. It is observed that the cells were more vulnerable in XB group than the other groups (Table 3, Graph 2). While the TAS value was 5.32 in the control group, it was 3.67 in VBF group and 3.15 in FBF group. This value was 2.68 in XB group. While TE group had the highest antioxidant value, XB group had the lowest antioxidant value.) (Table 7, Fig. 8).

TOS Analysis Results of the materials on Human dental pulp stem cells after 72 hours

TOS level was found to be 2.15 in the control group. The highest TOS value was obtained in FBF group (7.09) and the lowest TOS value was obtained in SS group (2.02). Antioxidant values increased 3 times in VBF, FBF, and XB compared to the control group. This 3-times increase showed that there was excessive stress in the cells. Oxidant level in TE and SS were lower than the other groups. Cells were exposed to stress less in TE and SS groups compared to the other groups. As a result of the present study, an increase was observed in the apoptosis ratio in XB, FBF and VBF groups except for SS and TE materials. (Table 8, Fig. 9)

Gene expression (IL-6, IL-8, IL-10, BCL-2, HBD-1, HBD-2, and ACTB) Analysis Results of the Materials in Human dental pulp stem cells after 72 hours

When the genetic results were assessed; it was found that while HBD-1, IL-6, IL-8, IL-10 and BCL-2 genes were expressed, HBD-2 gene expression cannot be taken. After 72 hours; suppression was observed in SS and TE groups in terms of IL-6 and IL-8 inflammatory factors. While there was an increase in IL-6 and IL-8 values after 24 hours; these values were suppressed after 72 hours. When the results of IL-10 gene expression were examined; the increase in TE and SS groups

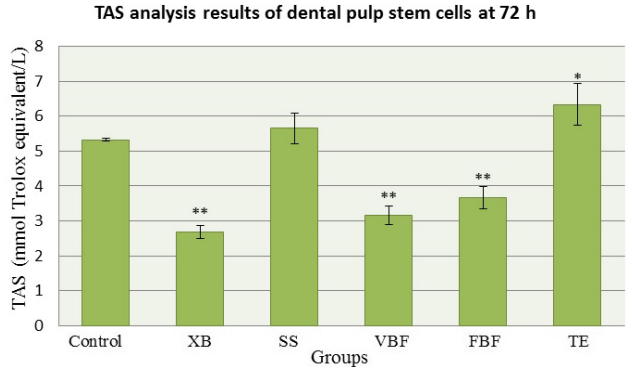

Figure 8. TAS levels of different dental material on cells for 72h. Each value is expressed as mean±standard deviation (n=3). Values followed by different small letters differ significantly at * p<0.05 and ** p<0.001.

Table 7. TAS analysis results of DPSCs after 72 hours.

72 hour	TAS analysis results					
	Control	XB	SS	FBF	VBF	TE
	5,4	2,81	5,74	2,98	3,71	6,5
	5,33	2,65	5,53	3,357	3,69	6,32
	5,24	2,59	5,66	3,11	3,59	6,21
avg.	5,32	2,68	5,65	3,15	3,67	6,34

Table 8. TOS analysis results of DPSCs after 72 hours.

72 hour	TOS analysis results					
	KONTROL	XB	SS	FBF	VBF	TE
	2,2	6,52	1,89	6,95	6,8	2,4
	2,1	6,31	2,2	7,22	6,73	2,2
	2,16	6,2	2	7,09	6,62	2,25
avg.	2,15	6,34	2,02	7,09	6,71	2,28

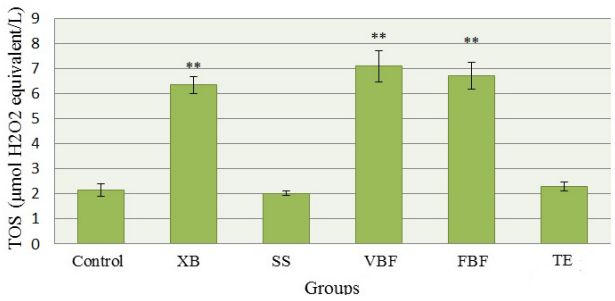

Figure 9. TOS levels of different dental materyal on cells for 72h. Each value is expressed as mean ± standard deviation (n=3). Values followed by different small letters differ significantly at * $p<0.05$ and ** $p<0.001$.

compared to the control group showed that IL-6 and IL-8 gene expression results were compatible with IL-10 results.

The IL-10 value increased 16 times in SS group and 28 times in TE group. In other words, the cells in these groups protected themselves more. It was observed that the smaller the BCL-2 gene expression value, the greater the degree of cell degeneration. When the results of BCL-2 gene expression were examined; the increase was 4.25 times in XB group, 19 times in SS group, 22 times in VBF group and 1.34 times in FBF group. An increase of 39 times was observed in TE group compared to the control group. When the results of BCL-2 gene expression were examined, regeneration was observed in the cells in TE, SS, and VBF groups. Even though a cell regeneration was observed in VBF; cell viability ratio was low in VBF group. When examining the HBD-1 gene expression results; high value of HBD-1 gene expression showed high cellular death in the materials. When examining the results of HBD-1 gene expression; this value was lower in TE and SS groups and HBD-1 value was high in FBF, VBF and SS groups. An increase of approximately 15 times was observed in other groups compared to the control group. However, it was observed that HBD-2 gene was suppressed and not expressed after 72 hours (Table 9, Fig. 10).

Discussion

For all the materials that are used in restorative dentistry and can be polymerized, the risk of creating a biological reaction due to insufficient polymerization is specified (23). Even though a complete polymerization in the composite resins seems theoretically possible, it is known that methacrylate monomers in the composite materials contain double bond at the rate of 25-50% and they remain without being reactive. Thus, it is reported that these monomers that are not completely polymerized pass through dentinal tubules and reach to pulp and thus they may cause biological reactions (23-30). It is very important to know the cytotoxic properties of the restorative materials that are frequently used in dentistry. For this purpose, in the present study, it was aimed to identify the toxic materials and to give information to the literature in this regard.

The cell population that takes place in the (perivascular) bed surrounding the veins of dental pulp and is called as "dental pulp stem cells (DPSC)" is considered to be the mesenchymal stem cells (MSC). These cells are multipotent cells that can show high proliferation, can be cloned and have high plasticity capability (31-33).

Marigo et al., (34) assessed the cytotoxic effects of Surefil SDR bulk-fill flowable composite resin and three conventional flowable materials (Venus Diamond Flow, Filtex Supreme XTE Flowable and Enamel plus HRi Flow) on DPSC cells via MTT analysis. They analyzed the correlation between the results obtained and the conversion grade. Marigo et al., observed a slight cytotoxic effect in all the test groups. In the present study; it was found while the cell viability ratio was 99.7% after 24 hours, a cell degeneration of 6% was observed in DPSC cells after 72 hours. Besides the MTT analysis parameter; different parameters such as the gene expressions, inflammatory cytokine values, TAS and TOS were analyzed. The results of these parameters were explained in the results section in detail.

Franz et al.(35), assessed the cytotoxic effect of packable and non-packable dental composites on L-929 mice fibroblasts after 72 hours. In their study, they spe-

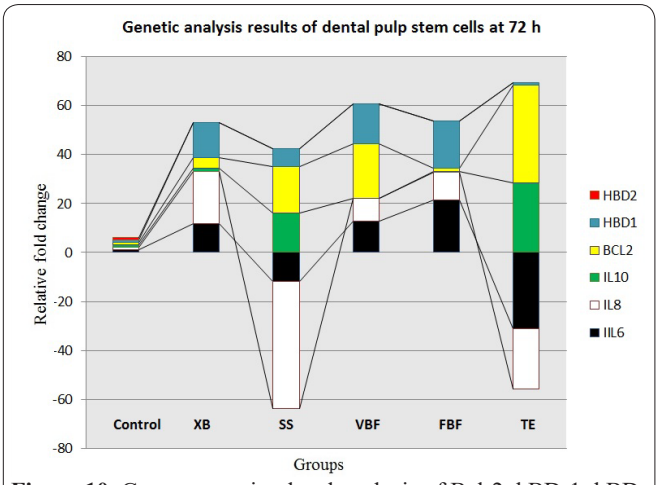

Figure 10. Gene expression level analysis of Bcl-2, hBD-1, hBD-2, IL-6, IL-8, and IL-10 in compare to housekeeping ACTB gene and control groups.72h exposure time of Dental Pulpa Stem Cells to dental materials (n=3).

Table 9. DPSCs, percentage values of gene expression analysis results after 72 hours.

	Control	XB	SS	VBF	FBF	TE
IL-6	1	11,96	-11,77	12,93	21,43	-31,12
IL-8	1	21,2	-52	9,17	11,4	-24,53
IL-10	1	1,2	16	0,17	0,4	28,53
BCL-2	1	4,25	19,03	22,11	1,34	39,8
HBD-1	1	14,51	7,32	16,25	19,14	1,13
HBD-2	1	ND	ND	ND	ND	ND

cified that the composite materials showed a toxic effect within the first 24 hours of application and they observed an increase in the cytotoxic effect over time. Similarly, in the present study, it was found that while toxic effect was observed in XB, FBF, and VBF in the first 24 hours, SS and TE groups were biocompatible. Cell regeneration was 11% in XB, 7% in FBF, 8% in VBF and 20% in TE group after 72 hours, cell degeneration was 4% in SS group. Similarly, in the present study, a toxic effect was observed in the first 24 hours in the groups, and, this toxic effect decreased in all the groups except for SS group after 72 hours. After 72 hours, there was a cell increase of approximately 12% in the other groups except for SS group.

In a restorative treatment, there is a dentin barrier between the material and the pulp tissue that prevents the transition of the monomers into the pulp. In the dentin barrier test, dentin is placed between the test material and the target cells as a diffusion or adsorption barrier (36). In the present study, it was tried to imitate the physiological structure of the teeth by using the insert membrane system.

In their study, Dommisch et al., (18) proved that human beta defensins (hBD-1, -2) played a role in host defense of the human dental pulp via reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemical analysis. In their study, Dommisch et al., also specified that human β-defensins played a significant role in the development of odontoblasts that are responsible for the dentin production. Additionally, they specified that human β-defensins are an agent responsible for local host defense and minor gene expression was observed in HBD-2 in the dental pulp cells of the undecayed teeth and noninfected teeth. In the present study; it was observed that HBD-2 gene was not expressed after 24 hours and 72 hours in DPSC cells. While cytotoxic effects of DPSC cells were assessed, the studies having an experiment period lasting for more than 72 hours can provide more detailed information about the gene expression.

In the study conducted by Trichaiyapon et al., (37) to compare the cytotoxic effects of flowable composite and mineral trioxide aggregate (MTA) materials on human periodontal ligament cells (PDLC), they found that newly prepared MTA and flowable composite resin materials which can release fluoride were more toxic and other flowable composites were less toxic. In contrast to the results of the study by Trichaiyapon et al.,, it was determined in the present study that VBF and FBF, being flowable composite resins, had high toxicity.

In their study, Ramezani et al., (38) investigated the effect of dental restorative materials on TAS level in saliva. They found that composite restorations caused a significant increase in the TAS level compared to undecayed teeth and amalgam restorations. In the present study it was observed that antioxidant level increased in TE and SS groups compared to the control group; whereas, TAS level decreased in VBF, FBF and XB since cellular death increased.

In the study it is reported that Ethoxylated bisphenol A dimethacrylate (bisEMA), which is the basic monomer of composite materials, from the polymerized composite materials Venus® bulk fill, Surefil® SDR™ flow, Filtek™ Bulk Fill and Sonic Fill™. Additionally, they betrayed that the molecular weight of BİSEMA varied between 452 and 892 g/mol in the composite materials. They pointed out that high ratio of BisEMA in the composite material affected the toxicology of the resin composites (39). Similarly, when examining the material contents of XB, FBF, and VBF which were used in the present study and are toxic materials, it was observed that BisEMA was present (Table 1). Higher toxicity in FBF compared to XB group made us think that ethoxy group of BisEMA was higher in FBF.

In their study, Longo et al. (40), assessed the cytotoxicity and cytokine expression of silorane and methacrylate-based composite resins. They proved that cytotoxicity was not observed in silorane-based composites within 6 hours and 12 hours and toxic symptoms were observed after 24 hours. In the present study, it was observed that there were toxic symptoms in XB, FBF, and VBF after 24 hours. It was determined that a slight cell regeneration was present in the materials after 72 hours; however, despite the cell regeneration, cell viability values were lower in XB, VBF and FBF than the control group. A cell increase of approximately 12% was observed in XB, FBF, VBF, and TE groups after 72 hours.

In their study, Ilday et al., (41), investigated the effect of composites containing silorane on gingival crevicular fluid by assessing IL-6, IL-8, and tumor necrosis factor alpha (TNF-α) parameters. As a result of the study, they stated that composites containing epoxy resin had negative effects on oral environment and gingival tissue. In the present study, the cytotoxic effects of new-generation bulk-fill flowable composites containing silorane on human dental pulp cells were investigated by assessing IL-6, IL-8, and IL-10 cytokines. And similar to the study by Ilday et al., the result of the present study revealed that the composites containing epoxy resin caused toxic effect in dental tissues.

Different techniques and methods are used by different researchers to investigate the cytotoxic effect of the materials. In their study, Moharamzadeh et al. (45), investigated the effect of resin monomers on human gingival fibroblasts and keratinocytes by using the sandwich enzyme-linked immunosorbent assay (ELISA) experiment. They observed that dental resin monomers were toxic, however they did not express the IL-1 gene of the cells used. On the other hand, in the present study, IL-6, IL-8 and IL-10 parameters were examined when assessing the cytotoxic effect of the materials in human dental pulp cells and it was observed that these cytokines were expressed. As mentioned above, the use of different tests and techniques in the assessment of cytotoxic studies provides detailed information for the researchers.

In their study, Fleming et al., (42)examined the cytotoxic effect of Filtek™ Z250, Admira and X-tra fil are observed via direct contact method by using murine undifferentiated pulp cell line (OD-21). In a 14-day period, they determined a decrease in the viabilities of murine undifferentiated pulp cells. In the present study, DPSC cells were used instead of murine pulp cells. Instead of the direct contact of the cells with restorative materials, the dentinal tubules were simulated and the insert membrane technique was used. In this technique, membranes with pores of 3 micrometers were used and thus, direct contact of the restorative materials with the cells was

prevented. Thus, in the present *in vitro* study, a system that simulated dentin and allowed the diffusion of the test materials such as dentin was used. When the results obtained were examined; as in the study by Fleming et al. it was found that while cell degeneration was observed in some groups after 72 hours, cell regeneration was seen in other groups. When TAS and TOS values were examined; lower TOS values indicated that the biocompatibility of SS and TE groups was better. When TOS results were compared with MTT results and TAS results; it was observed that the data were correct. Under normal conditions, TAS and TOS values are compatible with each other. However in the present study; TAS level increased in TE and SS insomuch that TOS results were found to be lower even than the control group. This is an expected process in the treatment of teeth restored with composites. Thus, the low TOS level causes less stress formation in DPSC cells and the successful completion of the dentin bridge. When the long-term TAS and TOS analysis results of restorative materials were examined; the increase in TAS antioxidant level showed that the cells were protected more. The antioxidant values of VBF and FBF groups were lower than the control group. Cell defense was not sufficient in VBF and FBF groups. Based on the MTT results, the low cell viability values in VBF and FBF groups supported this result. When examining the values obtained; it was found that the analysis results of TAS, TOS, and MTT were compatible with each other. It was observed that cell proliferation was provided in TE group and the cells in VBF, FBF, SS, and XB groups were not proliferated, and the cells were degenerated.

The investigation on biocompatibilities of resin-based dental materials, which have gained widespread and effective use in the practice of dentistry, has still been ongoing. The studies on biocompatibilities of such materials have also brought about the innovative approaches to reduce cytotoxicity. When all the filling materials were compared with the control group after 24 and 72 hours in terms of the cell proliferation percentages; it was observed that the cell proliferation was significantly high in TE group; whereas, the cell proliferation was lower in SS, XB, FBF, and VBF compared to the control group.

Conflict of Interest

Authors Hakan Kamalak, Aliye Kamalak, Ali Taghizadehghalehjoughi, declares that they have no conflict of interest.

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