Cytotoxic Effects of Bulk-Fill Composites on L929 Fibroblast Cells

Efeitos citotóxicos dos compósitos bulk-fill em células de fibroblastos L929

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ABSTRACT

Objective: Unlike traditional composite resins, bulk-fill composite resins could be polymerized as thicker layers. This study aims to contribute to the field by investigating the cytotoxic effects of various bulk-fill composite resins on L929 mouse fibroblast cells in vitro.

Material and Methods: In our study, six bulk fill and one conventional composite resin were used. Composite resin samples (8×4 mm) were prepared in a sterile cabinet by using a glass mod and polymerizing with a led light device (DTE LUX E, Germany). Composite samples (n:3) of which surface area was calculated according to ISO 10993-12: 2012 standards (3 cm²/ml), were kept in media for 24 h and 72 h in 37 ºC incubator, their extracts were filtered in 1:1 and 1:2 proportion and were added on L929 mouse fibroblast cells. Cell viability was examined by the MTT assay and cell death by the LDH test. Cell viability results were evaluated using one-way analysis of variance (ANOVA) test (p<0.05).

Results: When the 1:1 extracts from 4 mm thick bulk-fill composite samples were applied on L929 mouse fibroblast cells, cell viability rates showed significant differences compared to the control group at the end of 24 h and 72 h (except for Estelite Bulk Fill Flow). Although the extracts of the tested composite samples at 1:1 and 1:2 ratio at the end of 72 hours caused a decrease in L929 mouse fibroblast cell viability, the cell viability rate of only PRG-containing bulk fill composite and conventional composite remained below the cell viability ratio (70%) specified in ISO standards. Bulk fill composites did not produce toxic effects (except Beautifill Bulk Restorative) according to the LDH test.

Conclusions: Despite decreasing in general the cell viability, bulk-fill...
INTRODUCTION

Advancements in restorative materials used in dentistry have enabled the use of composite resins in large cavities in posterior teeth [1]. The fact that composite resins are in tooth color makes these materials advantageous in an esthetic sense [2,3]. However, these materials have also disadvantages such as micro leakage and sensitivity occurring due to the polymerization shrinkage [4,5]. Also, as the polymerization depth of conventional composite resins is limited to 2 mm, they are recommended to be used in the layering technique for the restoration of the teeth [6]. The incremental placement of the materials requires longer times in restoration and entails certain risks such as air inflow and contamination between the layers [7]. Furthermore, application of the conventional resins into the deep cavities is more difficult due to the limited depth of cure [8].

In recent years, in order to provide composites that are applicable to the cavity in larger masses and as thicker layers, “bulk-fill” composites have been introduced. As the new-generation bulk-fill composites allow for higher degrees of polymerization than the conventional composites due to their advanced translucent structures, they could be placed into the cavity in larger masses (4-6 mm) [9,10]. In a study evaluating the clinical performance of bulk-fill composite resins in the restoration of cavities in the posterior teeth, it was stated that there was no difference between conventional and bulk-fill composites [11].

Despite the increasing popularity of bulk-fill composite resins, there are concerns about the biocompatibility of these materials. These materials could release monomers in their structures depending on the physical and chemical conditions in oral environment [12]. It is stated in the literature that bisphenol-A glycidyl methacrylate (Bis-GMA), triethylene glycol dimethacrylate (TEGDMA) and urethane dimethacrylate [UDMA] the essential monomers included in the organic matrix of composite resins - create cytotoxic and mutagenic effects on cells [13]. Recently, a nanohybrid ormocer that includes both nanofillers and glass-ceramic fillers has been introduced by the composite industry. Several studies have shown that ormocers release fewer monomer particles and have less cytotoxic effects than the dymethacrylate-based conventional composites. Those studies have carried out the cytotoxicity tests in mouse fibroblasts [14,15].

The aim of this study is to provide a more detailed comparative perspective on the cytotoxicity of bulk-fill composite resins of different contents through an investigation on L929 mouse fibroblast cells using the MTT test in vitro according to ISO 10993-12:2012. The zero hypothesis of the study is that extracts...
from 4 mm samples of bulk-fill composites will not show cytotoxic effects on L929 mouse fibroblast cells.

MATERIAL AND METHODS

Preparation of the Samples

In the study, GrandioSO x-tra (Voco, Cuxhaven, Germany), Tetric N Ceram Bulk-Fill (Ivoclar Vivadent, Lihtenštáytn), Estelite Bulk-Fill flow (Tokuyama, Tokyo, Japan), Filtek Bulk-Fill Posterior Restorative (3M ESPE, USA), Admira Fusion x-tra (Voco, Cuxhaven, Germany), Beautifil Bulk Restorative (Shofu, Japan) and Filtek Z250 (3M ESPE, USA) composite materials were used (Table I). 8x4 mm samples of composites were prepared by using a glass mod in a sterile cabinet and placed in sterile tubes. The composites were polymerized for 20 s using a DTE LUX E (Germany, 1200 mW/cm², tip diameter 8 mm) led device.

Cylinder-shaped samples of composite having a 3 cm²/ml surface area, which is calculated according to ISO 10993-12: 2012 standards [16], were incubated in 2 ml serum-free Dulbecco’s modified eagle medium (DMEM) (HyClone Laboratories, Inc., Logan, UT, USA) (control group in serum-free medium) for 24 and 72 h, at 37 oC, in an incubator with 5% CO2. The tubes were covered with pieces of aluminum foil to prevent the composite samples immersed into the serum-free DMEM medium from being exposed to light. The extracts of composite samples were filtered after 24 h and 72 h periods, diluted with DMEM medium (1:1 and 1:2) and cytotoxicity experiments were conducted.

Cell Culture

The L929 fibroblast cell line stored at −196 °C was let thaw in a water bath at 37 °C and centrifuged. The cells were kept in DMEM, which is supplemented with 10% fetal bovine serum (PAA Laboratories, Linz, Austria), at 37 °C and 5% CO₂ in a humidified incubator. Once the cells reached the optimal density (1×105 cells/ml), the cell suspension was prepared according to the descriptions in ISO 10993-5: 2009 [17] by calculating the cell number of the desired density for a 96-well cell culture plate using DMEM medium including 10% FBS and 1% antibiotic.

Table I - Bulk fill resin composites and their components

| Material | Type | Composition | Filler content (w/w) | Lot Number |
|----------|------|-------------|----------------------|------------|
| GrandioSO x-tra (Voco, Cuxhaven, Germany) | Bulk fill | Bis-GMA, UDMA, TEGDMA | Barium glass; ytterbium trifluoride; mixed oxide; catalysts, stabilizers, and pigments | 77-79  W93364 |
| Tetric N Ceram Bulk Fill (Ivoclar Vivadent, Lihtenštáytn) | Bulk fill | Dimethylacrylate | Barium glass, ytterbium trifluoride, mixed oxide, additives, catalysts, stabilizers, and pigments | 75-77  6643739 |
| Estelite Bulk Fill flow (Tokuyama, Tokyo, Japan) | Bulk fill | Bis-GMA, Bio-MePP, TEGDMA, UDMA | Spherical filler; Composite Filler (200 nm spherical Gd2O3-ZrO2) | 70  W114 |
| Filtek Bulk Fill Posterior Restorative (3M ESPE St. Paul, USA) | Bulk fill | UDMA, UDMA, EGDMA, EDMAB | Ytterbium trifluoride; ceramic/filler/zirconia | 76.5  100372 |
| Admira Fusion x-tra (Voco, Cuxhaven, Germany) | Bulk fill | - | Inorganic fillers (Organically modified ceramics) | 84  1704051 |
| Beautifil Bulk Restorative (Shofu, Japan) | Bulk fill | Bis-GMA, Bio-MePP, TEGDMA | S-PRE filter based on fluoroborosilicate glass | 87  1815383 |
| Filtek Z250 (3M ESPE St. Paul, USA) | Conventional (Microhybrid) | Bis-GMA, UDMA, Bio-MePP, TEGDMA, Bio-EMA | Silica filler 20 nm ve 4-11 nm zirkonyum | 78.5  N717544 |

*BisGMA: Bisfenol diglisidilmetakrilat, BisEMA: bisfenol-etilmetakrilat, UDMA: üre탄 dimetakrilat, PEGDMA: polietylenglikol dimetakrilat, TEGDMA: trietilenglikol dimetakrilat; Bis-MePP: 2,2-bis (4-metacryloxydiphenyl) propane.
Then the cell suspension was allocated into the 96-well cell culture plate [100 µl/well] and incubated for 24 h in a 5% CO2 incubator. After the incubation, DMEM was removed and the media remaining of the two different dilutions, in which the composites were immersed, were similarly allocated into wells (100 µl/well) and the materials were incubated for another 24 h in a 5% CO2 incubator. Finally, the MTT assay was performed.

Cytotoxicity Test

MTT ([3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide], Sigma, USA) was combined with PBS, homogenized and an MTT solution with a final concentration of 5 mg/ml became ready for the cell viability test. The secreted media in 96-well cell culture plate that were incubated for 24 h was removed after incubation, then 100 µl/well DMEM medium and 13µl/well MTT solution were filled in clusters and incubated for another 30 min at room temperature. Absorbance levels of the samples were read at 450 nm and 650 nm reference wavelength in microplate spectrophotometer (BIO-TEK µQuant, BIO-TEK Instruments, Inc, USA). All experiments were triplicated. Cytotoxicity percent was calculated as: (absorbance of sample – absorbance of control sample) / (absorbance of high control sample – absorbance of control sample) × 100.

Statistical Analysis

Statistical analysis of the data was performed using the SPSS 22.0 program (SPSS Inc., Chicago, IL, USA). Cell viability rates belonging to the 1:1 and 1:2 diluted extracts of the composite samples obtained at the end of 24 h and 72 h periods were compared by using one-way analysis of variance (ANOVA) and Tukey multiple comparison tests (p<0.05).

RESULTS

Bulk fill composite resins tested, only Filtek Bulk Fill Posterior and Beautifil Bulk Restorative extracts (1:1) at the end of 24 h showed significant differences in cell viability compared to the control group (p <0.05). The cell viability values of the extracts (1:2) of these composite resins (except Beautifil Bulk Restorative) did not differ significantly compared to the control group (p>0.05), (Table II).

At the end of 24 h, the fluid bulk-fill composite extract showed the highest cell viability among the composite sample extracts, while the pre-reacted glass-ionomer (PRG) containing bulk-fill composite (Beautifil Bulk Restorative) provided the lowest rate. Although the organically modified ceramic-
based (ormocer) bulk-fill composite (Admira Fusion x-tra) exhibited a higher rate of cell viability than PRG-based composite and Filtek Bulk Fill Posterior composite, it did not show a statistically significant difference compared to the other bulk-fill composites.

At the end of 72 h, both 1:1 and 1:2 extracts of the samples showed significant results in terms of cell viability, in comparison to the control group (p<0.05), except for Estelite Bulk Fill Flow. Beautiful Bulk Restorative composite exhibited the lowest rate of cell viability (p<0.05), (Table III). When the extracts of bulk-fill composites at the end of 24 and 72 were diluted by 1:2 the cell viability increased (Table II and III). At the end of 72 h, Beautiful Bulk Restorative and conventional composite (Filtek Z250) remained below the ISO cell viability standard (70%) at 1:1 (Table III and Figure 1) and 1:2 dilution (Table III).

![Figure 1 - Cytotoxicity (MTT test) results of the extracts (1:1) of composites in L929 cells after 24 and 72 h incubation. The results (mean) of three independent experiments are shown as % of the untreated control.](image)

When the LDH test results of the extracts of composites (1:1 ratio after 72 h) are examined; The increase in the LDH activity of the Beautiful Bulk Restorative and conventional composite (Filtek Z250) groups was statistically significant compared to the control group (p<0.05). The use of Beautiful Bulk Restorative composite induced 45.9% cell death in L929 cells according to LDH release assay which indicated the breakdown (necrosis) of the cell membrane (Figure 2).

**Table II - Cell viability percentage of the extracts of the composites at the end of 24 h according to MTT test**

| Material          | Cell absorbance value (t1) | Cell absorbance value (t2) | Cell Viability (%) t1 | Cell Viability (%) t2 |
|-------------------|----------------------------|----------------------------|----------------------|----------------------|
| GrandioSO x-tra   | 1.03±0.05a                 | 1.04±0.06a                 | 92.8                 | 93.6                 |
| Tetric N-Ceram    | 1.02±0.06a                 | 1.08±0.06a                 | 91.9                 | 107.3                |
| Estelite Bulk Fill Flow | 1.12±0.13a             | 1.12±0.29a                 | 98.9                 | 109.0                |
| Estelite Bulk Fill Posterior | 0.80±0.09b         | 0.97±0.11b                 | 72.1                 | 87.4                 |
| Admira Fusion x-tra | 1.09±0.04a                | 1.02±0.08a                 | 92.2                 | 100.9                |
| Beautiful Bulk Restorative | 0.14±0.02c           | 0.52±0.07b                 | 72.6                 | 46.8                 |
| Filtek Z250       | 0.14±0.03a                 | 0.18±0.03a                 | 100.9                | 100                  |
| Control           | 1.11±0.03a                 | 1.10±0.06a                 | 100                  | 100                  |

* Different letters indicate statistical difference between the groups. p<0.05.

**Table III - Cell viability percentage of the extracts of the composites at the end of 72 h according to MTT test**

| Material          | Cell absorbance value (t1) | Cell absorbance value (t2) | Cell Viability (%) t1 | Cell Viability (%) t2 |
|-------------------|----------------------------|----------------------------|----------------------|----------------------|
| GrandioSO x-tra   | 1.16±0.09a                 | 1.17±0.12a                 | 72.1                 | 78.0                 |
| Tetric N-Ceram    | 1.26±0.03a                 | 1.22±0.08a                 | 78.3                 | 81.3                 |
| Estelite Bulk Fill Flow | 1.50±0.07bc             | 1.52±0.12b                 | 93.1                 | 101.3                |
| Estelite Bulk Fill Posterior | 1.19±0.08a            | 1.25±0.14a                 | 73.9                 | 83.3                 |
| Admira Fusion x-tra | 1.32±0.10ab              | 1.12±0.14a                 | 81.2                 | 74.6                 |
| Beautiful Bulk Restorative | 0.18±0.01e          | 0.43±0.10d                 | 11.2                 | 28.7                 |
| Filtek Z250       | 0.71±0.08d                 | 0.91±0.16c                 | 44.1                 | 60.7                 |
| Control           | 1.16±0.13c                 | 1.50±0.19b                 | 100                  | 100                  |

* Different letters indicate statistical difference between the groups. p<0.05.

**DISCUSSION**

Bulk-fill composite resins are commonly preferred by dentists for the restoration of...
teeth as they could be applied in thick layers. However, due to the failure of led light to reach a sufficient depth during the polymerization of these materials, the insufficiently polymerized monomer particles may remain free in their structure. It has been reported that the biocompatibility of composite resins is correlated with the amount and structure of the organic substances released [18] and monomers released from the resin matrix because of the insufficient polymerization may produce cytotoxic results over time [19,20]. In our in vitro study, we tried to examine the cytotoxic effects occurring on L929 mouse fibroblast cells depending on the use of bulk-fill resins of different contents in 4 mm thickness.

ISO 10993-12: 2012 proposed several cell culture testing models to evaluate the cytotoxicity of dental materials [16]. These are direct contact (direct method), indirect contact with a barrier (indirect method), and the extract method in which the extracts from biomaterials are added onto the cells. In the ISO 10993-5: 2009 standard, it was stated that the tested materials may have toxic potential if the cell vitality is below 70% after MTT test [17]. Lim et al. [21] compared those in vitro test models use to evaluate the cytotoxicity of composite resins and suggested the extract test due to its higher sensitivity if a single test model is planned to be used in the studies.

L929 mouse fibroblast cell lines are the most widely used cells to evaluate the in vitro cytotoxicity of dental materials [22,23]. Among the major advantages of mouse fibroblast cell lines it is stated that they are practical to use, contain one single type of cell and provide more accurate cytotoxic responses [23]. Therefore, in our study L929 mouse fibroblast cell line was preferred.

It is stated that the extent to which the polymerization of the restorative composite resins is accomplished, has an impact on the toxicity [24], and the oxygen inhibition layer formed on the surface of the composites after polymerization increases the monomer release [25]. In their study evaluating the cytotoxicity of the composites, Couchman et al. [26] suggested that the curing time decreased cytotoxicity by increasing the degree of polymerization. In the literature, it is also reported that there is no correlation between the oxygen inhibition layer formed during the polymerization of the samples by covering them with a glass and the amount of monomer release [27]. In our study, composite materials were covered with 1 mm glass coverslip and polymerized for 20 seconds with high intensity (DTE LUX E, Germany, 1200 mW/cm²) led light device.

In a study on the toxicity of bulk-fill composites carried out on mouse fibroblast cells, Toh et al. [28] reported that extracts obtained from 4 mm samples showed more cytotoxicity than 2 mm samples. In a similar study on the toxicity of fluid and paste bulk-fill composite resins conducted on L929 mouse fibroblasts by Demirel et al. [29] it was reported that at the end of 72 h composite extracts caused a statistically significant decrease in cell viability level, which is in line with the former. In a study on human pulp cells, by examining the toxic effect of bulk-fill composite samples in terms of whether it change at the layers of different polymerization depths [0-2, 2-4 and 4-6 mm] Lee et al. [30] stated that as the irradiation depth increased the more toxicity occurred, and the highest cytotoxicity was observed in the layer of 4-6 mm depth. However, Nascimento et al. [31] reported that bulk-fill resins exhibited low level and/or no cytotoxicity on L929 cells, except for Opus, which showed more moderate cytotoxicity, as pointed out in the MTT assay index.

In our study, at the end of 72 h, the cell
viability rate of the Estelite Bulk Fill Flow composite used as 4 mm layers did not cause a statistically significant difference compared to the control group. However, ormocer-based (Admira Fusion x-tra), PRG-containing bulk-fill composite (Beautifil Bulk Restorative) and the other bulk-fill composites (GrandioSO x-tra, Tetric N-Ceram Bulk Fill, Filtek Bulk Fill Posterior) caused a significant reduction in the cell viability. As the bulk-fill composites diminished the viability of L929 mouse fibroblast cells, the null hypothesis of the study was rejected.

Legrand et al. [32] the release of LDH culture medium as a result of damage to the cell membrane indicates cell death. Increased LDH activity is associated with an increase in dead cell numbers and a decrease in glucose consumption. In our study, Beautiful Bulk Restorative and conventional composite (Filtek Z250) showed more LDH activity than the control group with extracts in the ratio of 1:1 after 72 h. Our results were in accordance with Legrand.

Schubert et al. [33] found that Admira Fusion had significantly less cytotoxic effect on mouse L929 cells and human gingival fibroblasts than Filtek Supreme XTE and GrandioSO. The absence of certain classic resin monomers in Admira Fusion apparently allowed for lower cytotoxicity and better biocompatibility, compared to resin-based dental restoratives, which is of great importance for the clinical practice. In our study, bulk-fill composite (Admira Fusion x-tra) containing ormocer caused a significant decrease in cell viability at the end of 72 h compared to the control group, even if it did not at the end of 24 h.

It is stated that both the resin content of the composites and the degree of monomer conversion play a determining role in cytotoxicity levels [34]. Although bulk-fill composites have many advantages, there could remain some unpolymerized monomers at a depth of 4 mm. Those monomers, Bis-GMA, TEGDMA and UDMA, released from the structure of composites have been proven to be cytotoxic in many studies [35,36]. The toxicity grading of these monomers has been reported to be as Bis-GMA > UDMA > TEGDMA [36]. In our study, Estelite Bulk Fill Flow, which has similar monomers (Bis-GMA, Bis-MPEPP, TEGDMA, UDMA), showed the highest cell viability, while Beautifil Bulk Restorative showed the lowest one.

In this study, Beautifil Bulk Restorative was observed to cause a significant reduction in cell viability in both 24 and 72 h of experimentation periods. This product contains PRG filler in its resin matrix, unlike the other tested bulk-composites. The fluoro-aluminosilicate glass is pre-reacted with polyacid by forming a glass-ionomer matrix structure and blended with resin. Resin-based restorative materials containing PRG filler have been reported to provide higher fluoride release than compomers due to their glass ionomer hydrogel matrices [37]. In line with the results of this study, in a previous study, Toh et al. [28] found that Beautifil Bulk Restorative was to be more cytotoxic than all other tested bulk-fill composites. They suggested that the cytotoxic effects of Beautifil Bulk Restorative might be caused by the release of fluoride and other ions, such as PRG fillers including aluminum, boron, sodium, silicon, strontium, and zinc.

In the previous literature on the toxic effects of conventional composites on L929 mouse fibroblasts, it was stated that the decrease in cell viability after the first 24 h was not significant, while it was observed to reach more significant levels after 72 h [33,38]. The data obtained in our study shows that composites keep releasing cytotoxic materials after the first 24 h following polymerization.
Furthermore, the reduction in cell viability turned out to be higher in the 72 h extracts.

The findings of this in vitro study, which aimed to examine the cytotoxic effects of bulk-fill composites, are limited to the data collected on a single type of cell line and a two types of cytotoxicity test applied. Then, our study provides only a general and elementary-level evaluation about the cytotoxicity of bulk-fill composites. The tests models applied onto different cell lines or cells that are sourced from humans’ oral environment could give different responses in terms of cytotoxicity.

**CONCLUSION**

At the end of 72 h, the majority of bulk-fill composites decreased the cell viability but they did not cause unacceptable cytotoxic effects to L929 mouse fibroblasts, except PRG-containing bulk fill composite (Beautifil Bulk Restorative), which was cytotoxic.

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**Conflict of interest**

The authors have no proprietary, financial, or other personal interest of any nature or kind in any product, service, and/or company that is presented in this article.

**Regulatory Statement**

This study was conducted in accordance with all the provisions of the local human subjects oversight committee guidelines and policies of: World Medical Association Declaration of Helsinki.

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