Effect of Finishing-Polishing Procedures on Cytotoxicity of Resin-Based Restorative Materials via Real-Time Cell Analysis

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Objective: The aim of this study was to evaluate the effect of finishing and polishing procedures of compomer and bulk-fill composite resins on cytotoxicity against human gingival fibroblasts by xCELLigence analysis. Study Design: Filtek™ Bulk Fill composite and Dyract XP compomer were used. After curing, the specimens were randomly divided into two groups and finishing-polishing procedures were applied to one group; no finishing-polishing procedures were applied to the other group. For the first time in this study, pure gold samples were prepared with the same weight and base area as the test specimens and the wells containing the pure gold samples were determined as the control group. xCELLigence system was used to assess the response of the human gingival fibroblasts after exposure to test specimens. Measurements were recorded for 72 hours after adding specimens. Results: Finishing and polishing procedures caused a significant increase in cell viability of Dyract XP compomer samples at all time periods; the percentage of cell viability reached above 70% after finishing and polishing procedures. However, significant effects were not observed in Filtek™ Bulk Fill composite samples at any time period. Conclusion: Finishing and polishing procedures play an essential role in increasing the biocompatibility of Dyract XP compomer. It is recommended to apply finishing and polishing procedures even though a smooth surface may be obtained in restorations with matrix strips.

Keywords: *Bulk-fill composite, Compomer, Cytotoxicity, xCELLigence, Finishing and polishing procedures, Real-time cell analysis*

INTRODUCTION

Biocompatibility is described as the ability of a material to perform with an appropriate host response when applied as intended. Dental materials are considered biomaterials and they are expected to be non-toxic to living tissues in an organism. [1]

Dental caries is the most common chronic disease of children, [2] and resin-based restorative materials are extensively used in paediatric dentistry due to the increasing demand for tooth-coloured restorative materials, and simple handling properties. [3, 4]

The polymerization of resin-based restorative materials is a chemical reaction between the methacrylate resin monomers resulting in the formation of a highly crosslinked polymer network. [5, 6] It is known that atmospheric oxygen has more tendency to react with free radicals rather than a monomer molecule. This leads to a layer on the surface of resin-based restorative materials, rich in unreacted monomers and defined as oxygen inhibition layer. [7, 8] The degree of polymerization in resin-based restorative materials varies between 55% to 80% and this rate decreases to 35% in the presence of oxygen inhibition layer. [9] However, it has been reported that the degree of conversion further increased to approximately 95% after removal of the oxygen inhibition layer by finishing and polishing techniques. [10]

Unreacted components released from resin-based restorative materials have been considered a reason for mutagenic, genotoxic, cytotoxic, allergic reactions. [11-15] Considering that the resin-based

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restorative materials used in the restoration of cavities are applied to an early age group of pediatric patients, biocompatibility evaluations of resin-based restorative materials become more of an issue.

Several tests such as lactate dehydrogenase (LDH) assay, [16] 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzol-disulfonate (WST-1) assay, [17] 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) assay [18] are used to evaluate the biocompatibility of dental materials. However, these tests are in the format of end-point tests, and continuous monitoring of the cell response is not possible. Therefore, real-time assay systems are designed for dynamic analysis of cell adhesion, proliferation and viability. These systems have promise in a wide range of applications, substantially in toxicity studies. [19-21]

Studies investigating the relation between cytotoxicity and finishing-polishing procedures are limited. [10] To our knowledge, the effect of finishing and polishing procedures on cytotoxicity of resin-based restorative materials has not been evaluated by using a real-time cell analysis.

The present study aimed to evaluate the effect of finishing and polishing procedures on the biocompatibility of bulk-fill composites and compomers that are commonly used in paediatric dentistry, by using real-time cell analysis.

MATERIALS AND METHOD

Specimen preparation

Bulk-fill composite (Filtek ™ Bulk Fill, 3M ESPE GmbH Seefeld, Germany) (n=36) and compomer (Dyract XP Compomer, Dentsply DeTrey Konstanz, Germany) (n=36) in A2 shade were used. The composition of the tested restorative materials is given in Table 1.

The test specimens were prepared to a height of 4 mm and a diameter of 3 mm in cylindrical plexiglass molds. Bulk-fill composite resin specimen was placed as a single bulk increment of 4 mm, and the compomer specimen was placed as two increments, each with the height of 2 mm. The specimens were covered with Mylar strips and finger-pressed with 1 mm thick glass slides from top and bottom to extrude the excess material. The specimens were then cured by an LED light curing unit with an irradiance of 1000 mW/cm^2. The consistency of the curing light intensity was verified using a radiometer (HILUX, Benlioğlu, Turkey) within each usage.

After curing, the specimens were randomly divided into two groups and finishing-polishing procedures were applied to one half of the group. [Filtek™ Bulk Fill+ (n=18), Dyract XP+ (n=18)]. No finishing or polishing procedures were applied to the other group [Filtek™ Bulk Fill-(n=18), Dyract XP-(n=18)]. The weight of specimens was measured as 0.06 grams by analytical balance. Two samples made from pure gold were prepared with the same weight and base area as test specimens. The wells in which the pure gold samples were applied were accepted as the control group.

The finishing procedure of the specimens was completed with a 12-fluted carbide finishing burs (Meisinger, Neuss, Germany) in a high-speed handpiece. The tungsten carbide burs were changed in every 4 specimens. Afterwards, the specimens were polished with coarse, medium, fine and superfine grit Sof-lex discs (3M ESPE, St. Paul, MN, USA) in a low-speed handpiece for 15 seconds, respectively. Each disc was discarded after each use. After each step of polishing, all specimens were rinsed with water spray for 10 seconds and air-dried for 5 seconds. All specimen preparations, finishing and polishing procedures were carried out by the same investigator to provide standardization. All test specimens were sterilized under UV light for 30 minutes.

Cell culture

Human gingival fibroblast cell lines (HGF-1 (ATCC® CRL-2014™)) were maintained in Dulbecco's modified eagle's medium (DMEM HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS HyClone, Logan, UT, USA) containing Penicillin (100 Units/mL) and Streptomycin (100 µg/mL). The cells were cultured in T75 tissue culture flasks in an incubator (Panasonic Corporation, Tokyo, Japan) with 95% humidity and 5% CO_2 atmosphere at 37°C. The cytotoxicity test was performed using the xCELLigence real-time cell analyzer (RTCA) DP system (ACEA Biosciences, Inc., San Diego, CA, USA) as described previously.[22]

Briefly, xCELLigence real-time cell analyzer (RTCA) DP system uses specially designed disposable 16-well electronic microtiter plates (e-plate). On the bottom of the wells, gold microelectrode rows are embedded, and 4 of them are removed from the centre of each well to allow monitoring of the cells by using an optical microscope. Cellular content changes on these electrodes are detected by the system using the changes in electrical impedance of these sensor electrodes. Electrical impedance changes are converted to a unitless parameter called "Cell Index (CI)" by the xCELLigence software. Increasing the attached cell number of plate surface will also increase the CI. As well as the cell number morphological parameters including cell size, shape and strength of cell adhesion will also affect the changes in CI.

The optimum cell number of human gingival fibroblast cells was determined as 8x10^3 cells/well by proliferation experiments. In each experiment, 2 e-plates with 16 wells were studied simultaneously.

Before seeding human gingival fibroblast cells to the wells of the e-plates, 50 µl of medium was added to each well and a

Table 1: Restorative materials used in the study.

Material type	Material	Composition	Manifacturer
Compomer	Dyract XP compomer	•Urethane dimethacrylate (UDMA) • Carboxylic acid modified dimethacrylate (TCB resin) • Triethyleneglycol dimethacrylate (TEGDMA) • Trimethacrylate resin (TMPTMA) • Dimethacrylate resins • Camphorquinone • Ethyl-4(dimethylamino) benzoate • Butylated hydroxy toluene (BHT) • UV stabilizer • Strontium-alumino-sodium-fluoro-phosphor-silicate glass • Highly dispersed silicon dioxide • Strontium fluoride • Iron oxide pigments and titanium oxide pigments	Dentsply DeTrey Konstanz, Germany
Bulk Fill Composite	Filtek™ Bulk Fill composite	•AUDMA• AFM •UDMA •DDDMA, •YbF3• silan treated ceramic/slica/zirconia • EDMAB • Benzotriazol • Titanium dioxide	3M ESPE GmbH, Seefeld, Germany

background measurement of the device was performed to obtain a reference value. In the meantime, the cells were treated with 0.05% trypsin/EDTA. Flasks were left in a 37°C incubator for 1–5 min. After detached cells were observed under a microscope, 5 mL of media was added to the flask to stop trypsinization. The cell resuspension was centrifuged at 400 × g for 5 min. The pellet was resuspended with 10 mL of fresh medium and counting of the cells was performed using a hemocytometer.

Cytotoxicity test

Following the counting of the cells, 150 µl of cell suspension that contained 8×10^3 cells were seeded to the wells of the e-plate. Proliferation, attachment and spreading of the cells were monitored by the xCELLigence system. Approximately 24 h after seeding, while cells were in the log growth phase, wells of e-plates were replaced with 150 µl of the fresh medium and gold, bulk-fill composite and compomer specimens were added to the wells. Cell index was normalized at the point of cell medium replacement. Data was taken every 15 minutes to confirm the proliferation percentages of the cells. Measurements were recorded at 24, 48 and 72 hours after adding specimens. The experiment was repeated three times independently.

Statistical analysis

Shapiro Wilk's test was used to determine the compatibility of the data for normal distribution. "Two-way repeated measures ANOVA (One Factor Repetition)" test was used for repeated measurements. The analyzes were performed with R Studio (RStudio: Integrated Development for R. RStudio, Inc., Boston, MA). The confidence interval was set to 95% and $p<0.05$ were considered to be statistically significant. The results were reported as Mean values ± Standard Deviation.

While calculating the cell viability percentage, the viability of the control group in each period time was accepted as 100% viable and the following formula was used:

$$\text{Viability rate \%} = \text{Normalized CI}_{\text{sample}} / \text{Normalized CI}_{\text{control group}} \times 100$$

RESULTS

Dynamic monitoring of recorded measurement is given in Figure 1. Normalized CI values of all groups related to the time intervals are given in Table 2 and Figure 2.

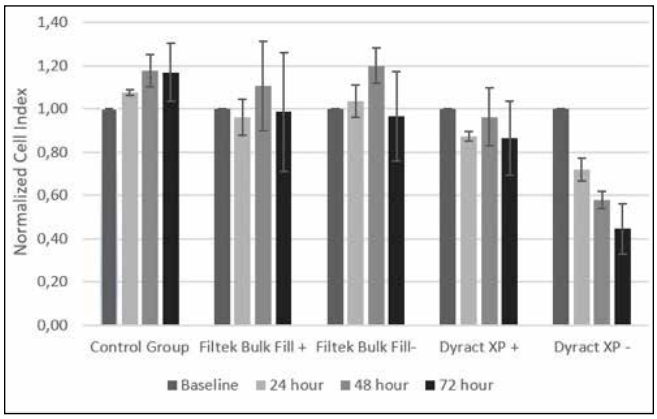

Figure 2: Evaluation of cytotoxic effect for all groups.

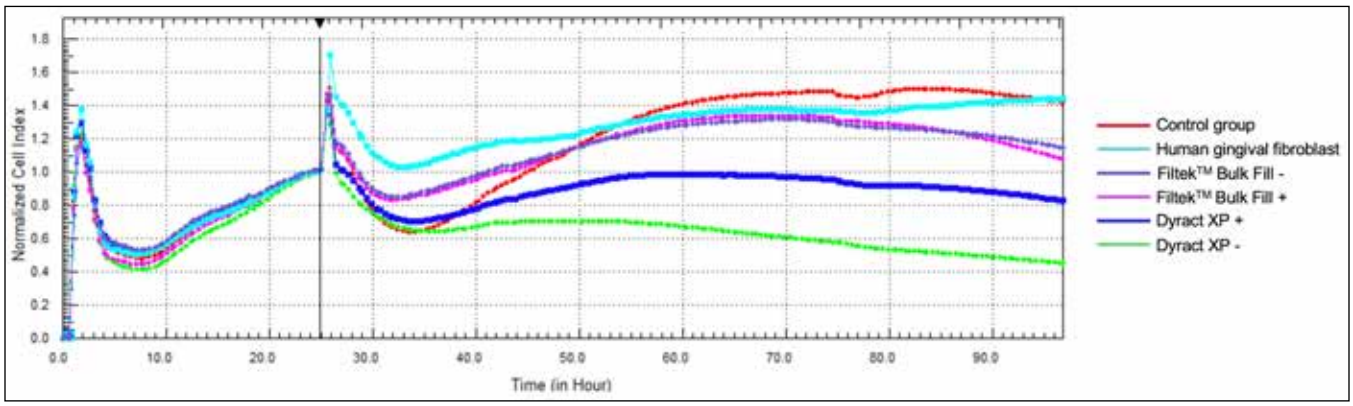

Figure 1: Dynamic monitoring of all groups in cultured human gingival fibroblast cells. Normalization time is marked with a vertical line.

Table 2: Means and standard deviations of normalized CI values for all groups.

Time	Control Group	Dyract XP -	Dyract XP +	Filtek™ Bulk Fill -	Filtek™ Bulk Fill +
Normalized time	1.00±0.00^{aA}	1.00±0.00^{aA}	1.00±0.00^{aA}	1.00±0.00^{aA}	1.00±0.00^{acA}
24 hour	1.0756±0.0141^{bcA}	0.7191±0.0520^{bcB}	0.8743±0.0211^{bcCE}	1.0368±0.0744^{acAF}	0.9610±0.0829^{acDEF}
48 hour	1.1777±0.0745^{bcAC}	0.5782±0.0399^{bB}	0.9620±0.1331^{acA}	1.1992±0.0809^{bcDC}	1.1053±0.2071^{aAC}
72 hour	1.1685±0.1346^{acA}	0.4453±0.1149^{cB}	0.8635±0.1724^{acA}	0.9667±0.2061^{aA}	0.9853±0.2726^{bcA}

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

While normalized CI values of Dyract XP- were significantly lower than the control group at all time periods (p≤0,001), normalized CI values of Dyract XP+ exhibited a significant decrease compared to the control group only at the 24 hour interval (p = 0.001). Dyract XP+ exhibited significantly higher normalized CI values than Dyract XP- at all time periods.

There was no significant difference between the normalized CI values of Filtek™ Bulk Fill- and the control group. Normalized CI values of Filtek™ Bulk Fill+ were significantly lower than the control group only at the 24 hour interval (p <0.05). Finishing and polishing procedures did not cause a significant difference between the groups of Filtek™ Bulk Fill- and Filtek™ Bulk Fill+ at any time period.

Dyract XP- exhibited significantly lower normalized CI values than Filtek™ Bulk Fill- at all time periods. However, there were no significant differences between the normalized CI values of the Dyract XP + and Filtek™ Bulk Fill+ groups at any time period.

The percentages of cell viability for the control and experimental groups at 24, 48 and 72 hours intervals are shown in Table 3. The cell viability was below 70% at 24, 48 and 72 hours in Dyract XP-; however it was above 70% in Dyract XP+, Filtek™ Bulk Fill+ and Filtek™ Bulk Fill- at all time periods.

Table 3: Percentages of cell viabilitiy.

Time	Control Group	Dyract XP -	Dyract XP+	Filtek™ Bulk Fill -	Filtek™ Bulk Fill +
Normalized time	100%	100%	100%	100%	100%
24 hour	100%	**66,85%**	81,28%	96,39%	89,34%
48 hour	100%	**49,09%**	81,68%	101,82%	93,85%
72 hour	100%	**38,10%**	73,89%	82,72%	84,32%

DISCUSSION

In the present study, real-time cell analysis demonstrated that finishing and polishing procedures have an important role in the biocompatibility of restorative materials. Recent studies revealed that resin monomers such as TEGDMA, UDMA, HEMA, Bis-GMA are able to increase the reactive oxygen species and oxidative stress, which is responsible for cell death. [23, 24] The monomers also have been found to be associated with DNA strand breaks, caspase activation, delay in cell cycle and inhibition in cell proliferation. [25] Therefore, it is crucial to obtain a biocompatible polymer structure after polymerization in resin-based restorative materials.

Various *in vitro* test models have been recommended by the International Standards Organization (ISO) 10993 series for evaluating the cytotoxicity of dental materials. Those series include direct contact tests, indirect contact tests and extract tests. [26] In this study, the direct contact test model was used to mimic Class 2 and Class 5 restorations located close to gingiva in clinical conditions. Cell viability assays were performed on fibroblasts as they are the predominant cell type in gingival connective tissue. It was reported that human gingival fibroblasts reacted more sensitively to resin-based composites compared to mouse fibroblasts. [27] Therefore, human gingival fibroblasts were preferred in simulating the intraoral environment and to get more informative results in the present study.

Real-time cell analysis systems allow label-free and non-invasive assessment of cellular changes. The impedance-based technology allows the measurement of CI values through many time points and creates a line graph that reflects the biological status of the cells. Continuous quantitative readout of the viability of the cells helps to obtain more realistic results compared to single end-point values of conventional cytotoxicity tests. [21, 28, 29] It is therefore preferable to evaluate the biocompatibility of the materials by using the xCELLigence system.

Pure gold samples were used as the control group to prevent the cell death that may occur due to the immersion of the test specimens into the cell culture, which could adversely affect the study result. Since in many studies, it has been reported that pure gold is a stable and non-toxic substance, [30-34] samples made from pure gold were prepared with the same surface area and weight in order to have the mechanically same effect as the test specimens. To date, no other study using pure gold as a control group has been reported. Therefore this is the first study evaluating the cytotoxic effects of dental materials using this protocol.

In this study, normalized CI values of Dyract XP+ were significantly higher than Dyract XP- at all time periods. This finding indicates that although the formation of the oxygen inhibition layer was reduced by using Mylar Strips and glass, unreacted monomers remained on the surface of test specimens and unreacted monomers were removed by the finishing and polishing procedures. Finishing and polishing procedures did not cause a significant difference in the cell viability of Filtek™ Bulk Fill composite groups. The reasons could be that the Filtek™ Bulk-fill composite has a higher conversion rate, sufficient polymerization depth and contains a high molecular weight monomer network. [35-39] The biocompatibility of Filtek™ Bulk Fill composite has been evaluated in various studies on different cells and using different test methods. [35, 40, 41] However, we could not find any previous study investigating the effects of finishing and polishing procedures on the biocompatibility of Filtek™ Bulk Fill composite, which makes it difficult to compare to the results of our study. There are different monomers in the structure of the restorative materials used in our study. The fact that finishing and polishing procedures caused different effects on the biocompatibility of the Dyract XP and Filtek™ Bulk Fill could be explained by the differences in monomer contents in materials. The normalized CI values of Dyract XP- were lower than Filtek™ Bulk Fill- at all time periods. However, Dyract XP+ exhibited similar normalized CI values with Filtek™ Bulk Fill+ at all time intervals. Therefore, it could be possible to interpret that the disadvantages of the Dyract XP compomer due to cytotoxicity can be avoided with finishing and polishing procedures.

Time is also a significant factor, which may have an influence on cytotoxicity. Some studies stated that acute release of monomers occurs in the first 24 hours. [42, 43] On the contrary, some recent research reported that monomer elution is not completed in 24 hours and some monomers continue to leach out long term. [43-45] In the present study, the highest decrease in cell viability was observed in the first 24 hours for both Dyract XP- and Dyract XP+. However, the cell viability continued to decrease and significantly lower cell viability was observed at 48 and 72 hours in Dyract XP-. This indicates that the monomer release was not completed in the first 24 hours. On the other hand, in Dyract XP+, no toxic effects that

could significantly reduce cell viability were observed after 24 hour. Compomer and bulk-fill composites are frequently used in dental restorations of paediatric patients, and the restorations are expected to function in the oral cavity for long years. However long term biological effects of these materials are still unclear. Thus, finishing and polishing procedures play an important role in eliminating unreacted monomers as much as possible to prevent damages due to long term exposure. It is recommended to use finishing and polishing agents even if a smooth restoration surface is obtained by using matrix strips.

According to the in vitro test criteria specified in the ISO 10993-5 standard, it has been reported that the cell viability should be more than 70% for a material to be accepted biocompatible. [26] Cell viability remained below 70% in all time intervals in the Dyract XP- group. Although the normalized CI values of Dyract XP+ exhibited a significant decrease compared to the control group at the 24-hour interval, the percentages of cell viability were above 70% for all time intervals. This indicates that the finishing and polishing procedures have a significant effect on biocompatibility with Dyract XP compomer.

The present study has several limitations. Firstly, the biological situation of human gingival fibroblast cells was monitored for 72 hours. However, dental restorations are in interaction with the oral tissues dynamically for many years. Therefore, it is necessary to evaluate the cell responses in long term periods to simulate actual clinical conditions. Secondly, only a single brand of compomer and bulk-fill composite were used for the assessment of biocompatibility. The differences in the composition of resin-based restorative materials could result in variability in potential toxicity. Further in-vitro research studies may focus on biocompatibility studies by using different materials and for longer time periods.

CONCLUSIONS

Within the limits of our study, we consider that finishing and polishing procedures are necessary when using Dyract XP compomer, as it significantly improved the biocompatibility of the material. It is well-known that finishing and polishing procedures are required to increase clinical success, such as microhardness and surface roughness. Therefore, it is recommended to apply finishing and polishing procedures for Filtek™ Bulk Fill composite, even though biocompatible results were obtained without finishing and polishing procedures.

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Conflict of Interests

The authors confirm that there are no conflicts of interest.

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