Abstract

Purpose: The present study was conducted to determine oxidative stress and cell viability after contact with resin composites polymerized for different times.

Methods: Disk-shaped specimens of Admira Fusion, Ceram X One Universal, Solare x and Filtek Z550 (n = 12) were prepared, and two subgroups with polymerization times of 20 and 40 s were employed. The specimens were incubated with mouse fibroblast cells for 48 and 72 h, and changes in reactive oxygen species (ROS) production and cellular viability were determined by an assay with a cell-permeable fluorescent dye, 2',7'-dichlorodihydrofluorescein diacetate (H2DCF-DA), and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, respectively.

Results: At 72 h, ROS production in the presence of Admira Fusion polymerized for 40 s was reduced relative to that in the presence of Admira Fusion polymerized for 20 s (P < 0.05). Cell viability was maximal in the Admira Fusion and Solare x groups and there was no difference relative to the control group at 48 h. Cell viability was higher in the Admira Fusion and Solare x groups polymerized for 40 s than for the same materials polymerized for 20 s at 72 h (P < 0.05).

Conclusion: Extension of the polymerization time has a material-specific effect and may be used as a strategy to increase the biocompatibility of resin composites.

Keywords: cell viability, polymerization time, reactive oxygen species, resin composite

Introduction

Resin composites have widespread usage in dental practice because of their advanced aesthetic and mechanical properties in the oral environment. In recent years, a number of studies have focused on the biocompatibility of resin-based materials in order to overcome the adverse effects of residual monomers released from the resin composites [1]. These monomers, as well as the initiators and fillers, have an impact on the biocompatibility of resin composites [2]. The degree of conversion after polymerization, the release of free monomers and degradation of the resin matrix result in various degrees of cytotoxicity [3]. Therefore the curing kinetics and the polymerization time are critical points affecting the degree of conversion, the amount of elutable substances, and consequently the biocompatibility of light-cured resin composites [4-7].

The type of monomer also has impact on biocompatibility, and the main monomers bisphenol A-glycidylmethacrylate (Bis-GMA), triethylene glycol dimethacrylate (TEGDMA) and urethane dimethacrylate (UDMA) have been reported to be cytotoxic [8,9]. The cytotoxicity ranking of the most widely used monomers has been reported to be Bis-GMA > UDMA > TEGDMA > hydroxyethylmethacrylate (HEMA) > methyl methacrylate (MMA) [10]. Residual monomers released from resin-based restorations as a result of incomplete polymerization enhance the formation of reactive oxygen species (ROS), which are most likely the cause of detrimental biological reactions [11]. From this viewpoint, it is suggested that increasing the polymerization time may enhance the degree of conversion of resin composites, decrease the amount of residual monomers, and thus diminish any toxic biological reactions. The aim of the present study was to examine the aspects of cellular metabolism, including oxidative stress and cell viability, affected by resin composites after polymerization for different irradiation times.

Materials and Methods

Four different materials were tested: nanohybrid ormocer restorative material (Admira Fusion, VOCO, Cuxhaven, Germany), nanoceramic restorative (Ceram X One Universal, Dentsply, Konstanz, Germany), composite restorative (Solare x, GC, Tokyo, Japan) and nanohybrid universal restorative (Filtek Z550, 3M ESPE, St. Paul, MN, USA). Details of these restorative materials are listed in Table 1.

Specimen preparation

Standardized disk-shaped specimens 6 mm in diameter and 2 mm height were prepared in plastic blocks placed between two glass surfaces via an incremental technique as a 2-mm layer in accordance with the manufacturers’ instructions (n = 12). Two subgroups were created, with polymerization times of 20 s and 40 s (LED 1,200 mw/cm2, Elipar S10, 3M ESPE). The tip of the curing unit (8 mm in diameter) was placed in contact with the glass surface to minimize the distance between the curing unit and the specimen. The finishing procedure was performed with the discs (Super-Snap Rainbow Technique Kit, Shofu, Kyoto, Japan) in order, and then a PoGo and Enhance system (Dentsply) was used for polishing. Each instrument was applied for 10 s to each specimen under water cooling.

Cell culture

L929 mouse fibroblast cells (HUKUK; Foot and Mouth Disease Institute, Animal Cell Culture Collection, Ankara, Turkey) were seeded in 48-well plates (3 × 103 cells/well). The culture medium contained RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA), 10% fetal bovine serum (Gibco Invitrogen, Grand Island, NY, USA), 1% L-glutamine (Sigma-Aldrich) and 100 units/ml penicillin/streptomycin (Gibco Invitrogen) [12]. The prepared specimens were stored in 70% ethanol for 5 min and then dried and placed in 48-well plates. Each well contained only one specimen. Each specimen in each well was incubated with a cell suspension (1 × 104 cells in 500 µl RPMI 1640) for 48 or 72 h. Wells containing cells without restorative material specimens served as controls.

ROS production assay

ROS production was measured using a cell-permeable fluorescent dye, 2',7'-dichlorodihydrofluorescein diacetate (H2DCF-DA). The cells were incubated with 10 µM H2DCF-DA for 45 min at 37°C in the dark following the exposure. The dye-loaded cells were washed with Krebs-Ringer buffer including 130 mM NaCl, 5 mM KCl, 10 mM glucose, 2 mM MgCl2, 1 mM CaCl2, 20 mM HEPES. The cells were then kept at room temperature in the dark for 10 min and the fluorescence intensity was measured.
The changes in ROS production in response to different treatments were estimated in terms of the fluorescence intensity (excitation wavelength 485 nm, emission wavelength 535 nm) using a Varioskan Flash Multimode Reader (Thermo Scientific, Waltham, MA, USA). The production of ROS was evaluated as a percentage relative to the control group.

**Cell viability assay**

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The cells were grown to 90% confluence for about 24 h in culture medium and then treated with restorative materials for 48 or 72 h in 48-well cell culture plates. MTT (final concentration 0.5 mg/mL) was added to each well, and then the cells were grown to confluence for about 24 h in culture medium and then treated with restorative materials for 48 or 72 h. The absorbance was measured at 570 nm and 630 nm via a microplate reader (VersaMax, Molecular Devices, Sunnyvale, CA, USA). Percentage survival was interpreted relative to the control for each group (normalized to 100%).

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**Statistical analysis**

GraphPad Prism software (GraphPad Prism version 7.00 for Windows, GraphPad software, La Jolla, CA, USA) was used for statistical analysis. Data are presented as mean ± S.D. (standard deviation) for six independent experiments. The normality of the data distribution was determined using the Shapiro-Wilk test (*P* > 0.05). Homodispersity was tested by the Bartlett test (*P* > 0.05). The production of ROS was lower than in the control group for Solare x polymerized for 20 s and Filtek Z550 polymerized for 40 s relative to the same treatment.

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**Table 1**

| Restorative material | Product description | Composition | Advised irradiation time for polymerization | Lot number |
|----------------------|---------------------|-------------|--------------------------------------------|------------|
| Admira Fusion        | Light-curing, radiopaque nanohybrid ORMOCER restorative material | 84% w/w inorganic fillers. Aromatic and aliphatic dimethacrylates, methacrylate-functionalized polysiloxane, barium-aluminum-glass, pyrogeneric silicon dioxide | 20 s (minimum 500 mW/cm²) | 1817656 |
| Ceram X One Universal | Universal nanocomposite restorative | Polysiloxane matrix, poly-urethane-methacrylate, Bis-EMA, TEGDMA, SpheriTEC fillers, barium glass, ytterbium fluoride, methacrylate polysiloxane nanoparticles, filler load 77.79% by weight, 59-61% by volume | 20 s (500 mW/cm²) | 1802001050 |
| Solare x             | Light-cured composite restorative | A glassy inorganic solid in powdered form. The porcelian is formed by melting together a mixture of inorganic oxides in various proportions. Colouration is achieved by the addition of various inorganic pigments. 7,7,9(9H-trimethyl-1,3-dioxo-4,13-dioxo-5,12-diazahexadecane-1,16-diy1 bismethacrylate, Ytterbium trifluoride, (octahydro-4,7-methano-1H-indenediy1)bismethylene bismethacrylate, silica fillers, lanthanoid fluoride fillers, filler content 77% by weight and 65% by volume | 10/20 s (1,200 mW/cm²) | 1707086 |
| Filtek Z550          | Nanohybrid universal restorative | Surface modified zirconia/silica, surface modified silica, inorganic filler 81.8% by weight, 67.8% by volume, Bis-GMA, UDMA, Bis-EMA, PEGDMA, TEGDMA | 20 s (minimum 400 mW/cm²) | N751485 |

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**Fig. 1** ROS production after 48 h of incubation. Intracellular ROS accumulation was assayed using DCF-DA fluorescent dye following treatment with restorative materials for 48 h. All data were expressed as means ± S.D. for six independent experiments. *P* < 0.05 indicates significant difference between samples of Admira Fusion polymerized for 40 s compared to the control. **P* < 0.05 indicates significant difference between samples of Solare x polymerized for 20 s and Admira Fusion polymerized for 40 s compared to the control. ***P* < 0.05 indicates significant difference between samples of Filtek Z550 polymerized for 40 s and Admira Fusion polymerized for 40 s. ****P* < 0.05 indicates significant difference between samples of Filtek Z550 polymerized for 40 s compared to the control.

**Fig. 2** ROS production after 72 h of incubation. Intracellular ROS accumulation was assayed using DCF-DA fluorescent dye following treatments with restorative materials for 72 h. All data were expressed as means ± S.D. for six independent experiments. *P* < 0.05 indicates significant difference between samples of Admira Fusion polymerized for 20 s compared to the control. **P* < 0.05 indicates significant difference between samples of Admira Fusion polymerized for 40 s compared to the control. ***P* < 0.05 indicates significant difference between samples of Filtek Z550 polymerized for 40 s and Admira Fusion polymerized for 40 s compared to the control. ****P* < 0.05 indicates significant difference between samples of Filtek Z550 polymerized for 40 s compared to the control. **P* < 0.05 indicates significant difference between samples of Filtek Z550 polymerized for 40 s compared to the control. **P* < 0.05 indicates significant difference between samples of Filtek Z550 polymerized for 40 s compared to the control.
materials polymerized for 20 s at 48 h ($P < 0.05$). After 72 h of incubation, there was no significant difference in ROS production among Ceram X One Universal polymerized for 20 s and 40 s and Solare x polymerized for 20 s and 40 s relative to the control. ROS production was lower compared to the control for Filtek Z550 polymerized for 20 s ($P < 0.05$). Production of ROS was decreased for Admira Fusion polymerized for 40 s relative to the same material polymerized for 20 s at 72 h ($P < 0.05$). ROS production was increased for Filtek Z550 polymerized for 40 s relative to the same material polymerized for 20 s at 72 h ($P < 0.05$).

After 48 h of incubation (Fig. 3), cell viability was maximal in the Admira Fusion and Solare x groups and there was no significant difference relative to the control group. The lowest cell viability was found for Ceram X One Universal (30.61 ± 0.48% for 20 s and 38.78 ± 1.92% for 40 s, $P < 0.05$) at 48 h. There was no significant difference in cell viability for the same material groups irrespective of polymerization time at 48 h. The percentage cell viability relative to the control was higher after 48 h than after 72 h of incubation for all materials (Figs. 3, 4). Cell viability appeared to be higher for specimens polymerized for 40 s after 72 h, but this was not obvious after 48 h. After 72 h of incubation (Fig. 4), the decrease in cell viability was remarkable in all experimental groups except for Solare x polymerized for 40 s compared with the control group ($P < 0.05$). There was no significant difference between Solare x polymerized for 40 s and the control. The highest cell viability was observed in the Admira Fusion and Solare x groups polymerized for 40 s. Admira Fusion polymerized for 40 s and Solare x polymerized for 40 s showed a reversal of the decrease in cell viability from 35.34 ± 2.38% to 69.04 ± 9.36% and from 48.61 ± 3.35% to 84.10 ± 1.46% when compared to polymerization for 20 s, respectively ($P < 0.05$) (Fig. 4).

Discussion

It has been reported that resin monomers are cytotoxic for different cell types [13,14]. For this in vitro study, L929 mouse fibroblasts were chosen consistent with the ISO standards, as these cells are derived from gingival and pulpal tissues, which would likely be exposed to any toxic substances released from resin-based materials in the oral environment [15].

The results of the study confirmed material-specific cellular responses to the four tested resin composites, which are commercially available and widely used for their improved properties. All of the four light-cured resin composites have an increased filler content (above 75% by weight) and two of them have novel matrices without any conventional monomers. Although some of the resin composites include the same monomers as declared, ROS production and cell viability changes were found to be distinct, as reported previously for different dentin bonding agents [12]. These findings are consistent with previous studies showing that the cytotoxic effect depends not only on the monomers and co-monomers, but also reactions between the components, degradation by products, additives and fillers, which all elicit cytotoxic and inflammatory responses to various degrees [16-18].

The light transmission characteristics, the released energy during polymerization, and the amount and type of the toxic substances released from unpolymerized resins may alter according to the polymerization source and time. Therefore the period of photoactivation has been reported to be related to the cytotoxic effects [19]. Deficient polymerization causes dissolution of the residual monomers [20]. The photoinitiators included in resin composite formulations have been reported to be unsatisfactory for obtaining low cytotoxicity [20]. In dental practice it is not possible to change the contents of marketed products, but it may be feasible to decrease the cytotoxic potential by reducing any residual monomers via extended polymerization. From this viewpoint, the present results suggested that although the biological reactions caused by resin composites exhibited a material-specific profile, the extension of the polymerization time to 40 s resulted in a tendency for a decrease in ROS production, especially in the Admira Fusion group at 72 h ($P < 0.05$). The positive effect of extended polymerization time was evidenced as higher cell viability for specimens polymerized for 40 s after 72 h, but this was not obvious after 48 h. This findings suggested the possible complex effects of cellular metabolism in the early cell growth phase at 48 h. This phenomenon should be analyzed in detail in further studies. The increased cell viability in the Admira Fusion and Solare x groups polymerized for 40 s at 72 h was consistent with a previous study showing that cellular toxicity was decreased by extending the polymerization time [1]. The positive effect of extended polymerization time on cell viability was clear for both Admira Fusion and Solare x, the formulations of which both lack conventional monomers such as Bis-GMA, UDMA and TEGDMA. The oxidative stress and ROS production induced by residual resin monomers have significant effects on the balance between cell viability and apoptosis [21]. The present data showed that ROS production was minimally altered in the Solare x groups at 48 h when compared to untreated cells, and as expected the cell viability was close to that in the control group. On the other hand, the adaptive cell responses to oxidative stress caused by monomers may differ [11]. Although ROS production was increased in the Admira Fusion and Filtek Z550 groups, cell viability was higher than expected. ROS production after 48 h of incubation with Admira Fusion polymerized for 40 s was higher than that after polymerization for 20 s, but the increase in ROS was not evidenced as a decrease in cell viability considering the results at 48 h. These findings suggest that oxidative stress and ROS production caused by these materials remain within the limits of intracellular adaptive responses and can be tolerated. However, this study was limited to cell viability analysis as a cellular reaction, and thus the effects of ROS on cellular responses should be investigated in more detail to clarify the specific adaptive responses to ROS-related cellular damage for each material. Another notable finding
of this study was the lower cell viability in the Ceram X One Universal groups, despite their relatively lower ROS production. In this in vitro study, no extraction was employed and cell viability was assessed after direct contact of the cells with the materials. As cultured fibroblast cells are known to show greater adhesion to plastic than to glass surfaces, the lower cell viability in the Ceram X One Universal groups may have been influenced by the glass content of the material. Direct contact of the cells with the materials may have resulted in increased cell toxicity over time, and the decreased cell viability in the later stages may have been correlated with the sustained release of monomers from the materials [1].

Within the limitations of this in vitro study, it can be concluded that 1) the biological reactions including cellular damage and ROS production caused by resin composites exhibit a material-specific profile. 2) extension of the polymerization time shows a material-specific profile and may be used as a strategy to increase the biocompatibility of resin composites. 3) further detailed studies will be necessary to analyze and monitor changes in the biological effects of resin composites via extended polymerization time.

Conflict of interest

The authors declare that they had no actual or potential conflict of interest, including financial, personal or other relationships with third parties or organizations within three years of beginning the submitted work that could have inappropriately influenced, or been perceived to influence, the results.

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