

In vitro evaluation of pediatric restorative materials on human dental pulp stem cells

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ABSTRACT

This in vitro study aimed to compare the cellular viability of four different restorative materials on human dental pulp stem cells (hDPSCs). The necessary tissues of isolated hDPSCs were obtained from 10 impacted third molars of healthy individuals 19–25 years old undergoing surgical extraction. The effects of GC Fuji IX GP, GC EQUIA Forte, APA Glass Carbomer, and APA Esthetic Fill eluates on hDPSCs' viability on the first, third, and seventh days were examined by flow cytometry using an Annexin V binding assay. The cell viability ratios differed significantly between the restorative material groups ($p < 0.001$). When the restorative materials and the control group were compared, the control group had the highest cell viability on all experiment days, while the Glass Carbomer group had the highest percentage of viable cells of all the restorative material groups. A significant difference between days in terms of cellular viability ($p < 0.001$) was found. Considering the early apoptotic cell ratios, no significant difference was found between the groups and days ($p > 0.05$). Furthermore, based on time, except for the first day of the GC Fuji IX GP group, no statistically significant difference was observed in the early apoptotic cell ratios in each group ($p > 0.05$). When the rates of late apoptotic cells were compared between the groups, no significant difference was observed, except for with the control group, which had the lowest rate of late apoptotic cells ($p < 0.001$). However, there was a significant difference in necrotic cell ratios between groups and days ($p < 0.001$; $p = 0.001$). The lowest rates of necrotic cells were found in the control group and Glass Carbomer groups, while the rates of necrotic cells were similar among the other groups. Therefore, we concluded that APA Glass Carbomer presents the highest cellular viability among the materials.

1. Introduction

Many kinds of restorative materials are used in pediatric dentistry. Of them, glass ionomer cements have been extensively used in clinical practice for more than 30 years (Hii et al., 2020). Conventional glass ionomer cements (CGICs) have several advantageous properties, including the ability to release fluoride, chemical adhesion to enamel and dentin, a modulus of elasticity similar to dentin, and antibacterial properties. However, they also have some unfavorable properties, such as a long setting time, low bonding strength, high solubility, and moisture sensitivity in the initial setting process (Kanjevac et al., 2015; Noorani et al., 2017; Hii et al., 2020). To address the negative features of CGICs, high-viscosity GICs (HVGICs) were developed by changing the

size distribution of the particles, creating a cross-linked matrix that hardened the CGICs (Ersahan et al., 2019). Previous studies have recommended the use of HVGICs as an alternative to composite resins due to their long-term mechanical and wear resistance (Yap et al., 2003; Van Duinen et al., 2005). Glass carbomer cement is a GIC-based restorative material containing nano-sized particles and fluorapatite, which helps to improve its physical properties and to form an enamel-like structure (Cehreli et al., 2013).

Since the restorative materials come into direct or indirect contact with the surrounding tissues, the substances released from the restorative materials can affect the pulp tissue from the dentinal tubules due to the osmotic pressure of the dentin fluid (Rodríguez-Lozano et al., 2013, 2021). This issue necessitates evaluating the appropriate tissue response

Abbreviations: hDPSC, human dental pulp stem cell; CGIC, conventional glass ionomer cements; HVGIC, high-viscosity glass ionomer cement; DPBS, Dulbecco's phosphate buffered saline; DMEM, Dulbecco's modified eagle medium; CDMEM, Complete Dulbecco's Modified Eagle Medium.

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to the cellular viability of the restorative materials, as well as their physical, chemical, and mechanical properties (Murray et al., 2007; Marczuk-Kolada et al., 2017). In vitro cell culture tests, which are used as the first step in assessing biocompatibility, are easy, fast, and standardized tests that can reflect the physiological status of living tissues (Noorani et al., 2017). To evaluate cellular response, these tests use primary and permanent cell lines. Although primary cell culture isolation and passages are sensitive and difficult to work with, they are preferred in cytotoxicity studies because they can create an experimental environment that imitates the physiological condition and better reflects in vivo conditions (Pizzoferrato et al., 1994; Marczuk-Kolada et al., 2017).

Human dental pulp stem cells (hDPSCs) are multipotent and self-renewable cells that have high proliferation and plasticity. These cells, showing mesenchymal stem cell characteristics, have a fibroblast-like morphology, are capable of multilineage differentiation, and can adhere to plastic surfaces (Todorović et al., 2008). Noorani et al. reported that hDPSCs play an important role in the continuity of healthy dental pulp tissue; because substances leaking from the restorative materials affect cell viability in the pulp tissue through the dentinal tubules, they should not be cytotoxic, especially for these cells (Noorani et al., 2017; López-García et al., 2019).

Studies evaluating cellular viability act as the preliminary assessment of the biological responses of various dental materials. The advantage of these studies is that they present a first line assessment of the materials' biological properties, while evaluating their possible health risks (Peters, 2013). Accordingly, this in vitro study aimed to compare the cellular viability of four different restorative materials on hDPSCs. The null hypothesis was that there would be no difference between the restorative materials in relation to their cellular viability on hDPSCs.

2. Materials and methods

All procedures were approved by the Clinical Research Ethics Committee, Faculty of Dentistry, Istanbul University (2018/93) and followed the Helsinki Declaration guidelines.

2.1. Isolation and culture of hDPSCs

Human dental pulp tissues were obtained with written informed consent from healthy subjects ($n = 10$) between 19 and 25 years old who had been scheduled for the extraction of 10 impacted third molars for orthodontic or surgical reasons. The extracted third molars were collected antiseptically and placed into a physiological solution containing antibiotics (1 % penicillin/streptomycin (Gibco)) to prevent contamination and delivered to a laboratory in the Marmara University Research Hospital within two hours. After cleaning of the teeth's surfaces, the teeth were mechanically broken, and the pulp tissues were gently removed using an excavator, without any drilling, which could have adverse effects on the hDPSCs' viability.

The pulp tissues were mechanically cut into pieces approximately 1 mm^3 in size with a scalpel blade. After micro-mechanical digestion, a collagenase solution containing 1 ml of Dulbecco's Phosphate Buffered Saline (DPBS; Gibco, Grand Island, NY, USA) and 3 mg/ml of Type 1 collagenase (Gibco) was prepared in a 15-ml sterile falcon tube. Then, 2 ml of the preparation were added to the homogenized tissues, and the enzymatic digestion process was started. To ensure equal tissue distribution, pipetting was performed in Dulbecco's Modified Eagle Medium (DMEM; Gibco) and the tubes were placed in an incubator at 37°C and 5 % CO_2 for 45 min. Upon formation of a fibrous structure, the tubes were removed, and the cells were washed two or three times with DMEM (Gibco) to inactivate collagenase enzymes and were then centrifuged at 1500 rpm for 5 min. After centrifugation, the supernatant at the top of the tube was discarded.

The cells were placed in a T-25 cm^2 cell culture flask with a cell

culture medium containing DMEM (Gibco) supplemented with 15 % Fetal Bovine Serum (FBS; Gibco) and 1 % penicillin/streptomycin (Gibco) and transferred to an incubator. The growth medium was replaced every three days, and the cell cultures were observed regularly using an inverted phase-contrast microscope (EVOS-AMG, Thermo Fisher Scientific, Waltham, MA, USA). The adherent cells reaching 70–80 % confluence were identified as passage zero (P0). For subsequent passaging, adherent cells were trypsinized with 0.25 % Trypsin/EDTA (Sigma-Aldrich, St. Louis, MO, USA) and washed with DPBS. The cell subculturing procedures were repeated until the cells reached the third passage. The characterization of isolated and cultured hDPSCs involving specific surface antigens and multiple differentiation capacities was analyzed with a flow cytometer. In this study, hDPSCs from passage 3 were used.

2.2. Flow cytometry analysis of hDPSCs' surface marker expression

The immunophenotype of isolated hDPSCs was identified by a flow cytometric analysis that determined the expression of mesenchymal stem cell surface markers. CD73 phycoerythrin (PE), CD90 PE, CD105 PE, CD29 allophycocyanin (APC), CD146 fluorescein isothiocyanate (FITC), CD45 FITC, CD28 PE, CD14 PE, CD34 PE, CD 25 APC (BD Biosciences, CA, USA), and the cells (5×10^6) were incubated for 15 min against isotype controls at room temperature in the dark. A flow cytometry instrument (BD, FACS Calibur, San Jose, CA, USA) was used to perform the flow cytometry analysis.

2.3. Analysis of hDPSCs' differentiation potential

Analysis of the trilineage differentiation potential of hDPSCs, including their osteogenic, chondrogenic, and adipogenic potential, was determined according to the International Society for Cellular Treatment (ISCT) criteria. In order to assess the cells' differentiation potentials, osteogenic, adipogenic, and chondrogenic differentiation kits (Thermo Fisher, USA) were used. The hDPSCs were seeded into a six-well plate at a concentration 5×10^4 cell/well and incubated with the differentiations medium, which was replaced every three days. After 28 days, the cultured cells were stained with Alizarin Red (Sigma-Aldrich) for 45 min in the dark at room temperature to detect osteogenic differentiation, as well as the presence of extracellular calcifications and calcium deposits. After 14 days of the chondrogenic differentiation, the cultured cells were stained with Alcian Blue (Sigma-Aldrich) to detect chondrocyte-like cells and proteoglycans. Lipid droplets were confirmed with an Oil Red O (Sigma-Aldrich) staining assay conducted following 14 days of the adipogenic differentiation. A binocular microscope (Olympus, BH2-RFCA, Olympus, Tokyo, Japan) was used to detect the differentiation potential.

2.4. Preparation of the restorative material eluates

The restorative materials used in this study were Glass Carbomer (APA Dental, Netherlands), Esthetic Fill (APA Dental, Netherlands), EQUIA Forte (GC, Japan), and Fuji IX GP (GC, Japan). Complete Dulbecco's Modified Eagle Medium (CDMEM; Gibco) was used as the control group. The materials' main components are shown in Table 1. All restorative materials were mixed under aseptic conditions according to the manufacturers' instructions, as shown in Table 2. Twenty discs of each material ($n: 20$ samples per group) were shaped in sterile Teflon molds with a diameter of 8 mm and depth of 2 mm. After polymerization, the samples were removed from the molds and were weighed and subjected to ultraviolet (UV) light for 20 min for sterilization.

The material extraction fluid was obtained by using a direct contact test. All procedures were performed according to International Organization for Standardization (ISO) 10993–12 standards. The discs of each material were immersed in 15 ml of DMEM and incubated for 72 h at 37°C with 5 % CO_2 . After incubation, sterile material extracts were

Table 1
Compositions of the restorative materials.

Materials	Composition	Manufacturer
APA Glass Carbomer	Fluoroaluminosilicate, polyacrylic acid, nanofluoro / hydroxyapatite, distilled water	GCP Dental, Netherlands
APA Esthetic Fill	Fluoroaluminosilicate, polyacrylic acid, nanofluoro / hydroxyapatite, phosphate	GCP Dental, Netherlands
GC EQUIA Forte	Fluoro-aluminosilicate glass, polycarboxylic acid, carboxylic acid	GC Corp. Japan
GC Fuji IX GP	Fluoroaluminosilicate glass, polyacrylic acid, distilled water	GC Corp. Japan
Complete DMEM (CDMEM)	DMEM (Dulbecco's Modified Eagles Medium) with 10 % FBS (Fetal bovine serum), 1 % penicillin / streptomycin added	Gibco, Grand Island, USA

Table 2
Application procedures of the restorative materials.

Materials	Application	Setting Time
APA Glass Carbomer	Mixing the capsule for 10–15 s with a mixer and applying with a gun	60 s led light applicaiton
APA Esthetic Fill	Mixing the capsule for 10 s with a mixer and applying with a gun	60 s led light application
GC EQUIA Forte	Mixing the capsule for 10 s with a mixer and applying with a gun	60 s led light application
GC Fuji IX GP	Hand mixing of powder and liquid on mixing paper with plastic spatul	3 min at room temperature

collected using a 0.22- μ m filter (Merck Millipore, Billerica, MA, USA).

2.5. Analysis of cell viability by flow cytometry (Annexin V/7-AAD staining)

The hDPSCs were seeded in 48-well plates (5×10^5 cells/well) with a culture medium. After 24 h, the culture medium was removed from the wells for an analysis of cell viability. The four different restorative materials and the control group were exposed to the extraction fluid (0.5 ml in each well). After incubation with the eluates, the cells were double stained with Annexin-V (BD Pharmingen, USA) and 7-AAD (BD Pharmingen, USA). Viable (Annexin-V⁻/7-AAD⁻), early apoptosis (Annexin-V⁺/7-AAD⁻), late apoptosis (Annexin-V⁺/7-AAD⁺), and necrosis cell (Annexin-V⁻/7-AAD⁺) measurements of the restorative materials on the stem cells were taken using flow cytometry. The cells were subjected to extraction fluid on the first, third, and seventh days. Cell viability measurements were repeated five times for each group, and mean values were used for the results.

2.6. Statistical analysis

IBM SPSS V23 was used to perform the statistical analyses. Compatibility with a normal distribution was analyzed by the Shapiro-Wilk test. The effects of time and interactions of the group parameters were examined with a generalized linear model, and the Wald Chi-squared test was used. The level of significance was set at $p < 0.05$.

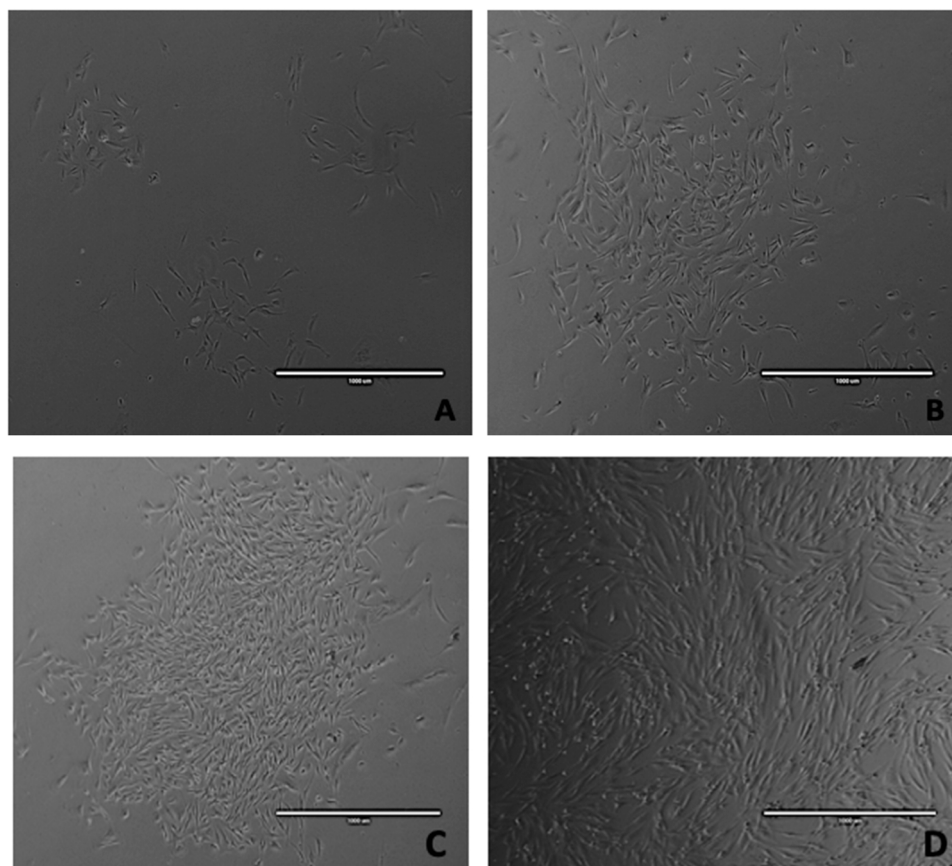


Fig. 1. Morphological appearance of hDPSCs: (x10), A) hDPSCs on 0th passage B) hDPSCs on 1st passage C) hDPSCs on 2nd passage, D) hDPSCs on 3rd passage.

3. Results

3.1. Isolation and culture of the hDPSCs

The hDPSCs were monitored using an inverted microscope, through which spindle-shaped fibroblast-like cells were clearly seen adhering to the plate surface. The cells had become confluent, completely covering the surface due to their plasticity capabilities in the third passage. Cells were seen to be in contact with each other from the first passage to the third passage (Fig. 1A–D).

3.2. Characterization of the hDPSC

In order to confirm the mesenchymal phenotype of the stem cells, cell surface antigens were analyzed by flow cytometry. The analysis results showed that mesenchymal stem cell markers CD73 (92.30 %), CD90 (94.68 %), CD105 (89.07 %), CD146 (97.61 %), and CD29 (92.2 %) were expressed strongly positively, whereas the expression of the hematopoietic markers, CD14 (5.99 %), CD28 (11.56 %), CD34 (0.33 %), CD25 (2.67 %), and CD45 (3.5 %), was negative (Fig. 2A–B).

The hDPSCs exhibited trilineage differentiation potential, including osteogenic, chondrogenic, and adipogenic potential, after induction of the differentiation medium. Stimulation of the hDPSCs with the osteogenic differentiation medium resulted in the formation of calcified nodules and osteocyte-like cells colored in red (Fig. 3A). The presence of proteoglycan and chondrocyte-like cells colored in blue were monitored in the chondrogenic differentiation experiment (Fig. 3B), while in the adipogenic differentiation, the formation of lipid droplets and adipocyte-like cells stained with the pinkish color were observed (Fig. 3C).

3.3. Analysis of cell viability by flow cytometry (Annexin V/7-AAD staining)

The viability values of cells exposed to different restorative material eluates and the control group on the first, third and seventh are shown in the flow cytometry graphics (Figs. 4–8). In the graphics, viable cell ratios are displayed in the lower-left quadrant, early apoptotic cell ratios in the lower-right quadrant, late apoptotic cell ratios in the upper-right quadrant, and necrotic cell ratios in the upper-left quadrant.

In the GC EQUIA Forte group, no significant difference was found ($p > 0.05$) when the early apoptotic cell ratios and live cell ratios were compared for all experiment days. Considering the late apoptotic cell ratios, no significant difference was observed between the first and third days ($p > 0.05$), while there was a significant difference between the third and seventh days. ($p < 0.001$). The highest ratios of late apoptotic

cells were found on the seventh day, while the lowest rate of late apoptotic cells was found on the third day. The ratio of the cell viability of the GC EQUIA Forte group was mathematically similar to those of the GC Fuji IX GP and APA Esthetic Fill groups (Tables 3–6).

Regarding the GC Fuji IX GP group, no significant difference was found between the different experiment days for the live, late apoptotic, and necrotic cell groups ($p > 0.05$). While the early apoptotic cell ratios were found to be lowest on the first day ($p < 0.001$), no significant difference was found between the third and seventh days ($p > 0.05$). The lowest cell viability among the groups was observed in the GC Fuji IX GP group (Tables 3–6).

When the early apoptotic cell ratios and the late apoptotic cell ratios in the APA Glass Carbomer group were compared between the first, third and seventh days, no significant difference was observed ($p > 0.05$). The cell viability ratios were highest on the first day, and no significant difference was found between the third and seventh days ($p > 0.05$), and the necrotic cell ratios were lowest on the first day. The rates of the cell viability of the APA Glass Carbomer group were mathematically higher than those of the other experimental groups (Tables 3–6).

For the APA Esthetic Fill group, no significant difference was found between the live, early apoptotic, late apoptotic, and necrotic cell ratios on the first, third, and seventh days ($p > 0.05$). The early and late apoptotic cell ratios were highest on the third day. The rates of the late apoptotic cells in the APA Esthetic Fill group were mathematically lower than those in the other experimental groups (Tables 3–6).

Concerning the control group, no significant difference was found in the early apoptotic cell ratios, the late apoptotic cell ratios and the necrotic cell ratios on the first, third, and seventh days ($p > 0.05$). When the cell viability ratios were analyzed, there was a statistically significant difference between the first and seventh days ($p < 0.001$). Of all the groups, the control group had the highest live cell ratios and the lowest necrotic cell ratios (Tables 3–6).

The cell viability ratios differed significantly between the restorative material groups ($p < 0.001$). When the restorative material groups and the control group were compared, the control group had the highest cell viability on all experiment days, with the APA Glass Carbomer group having the highest percentage of viable cells of all the restorative material groups. In regard to the early apoptotic cell ratios, no significant difference was found between the groups. Furthermore, based on time, except for the GC Fuji IX GP group's first day, no statistically significant difference was observed in the early apoptotic cell ratios of any group ($p > 0.05$). When the rates of late apoptotic cells were compared between the groups, no significant difference was observed, except for the control group, which had the lowest rate of late apoptotic cells. However, there was a significant difference in necrotic cell ratios between

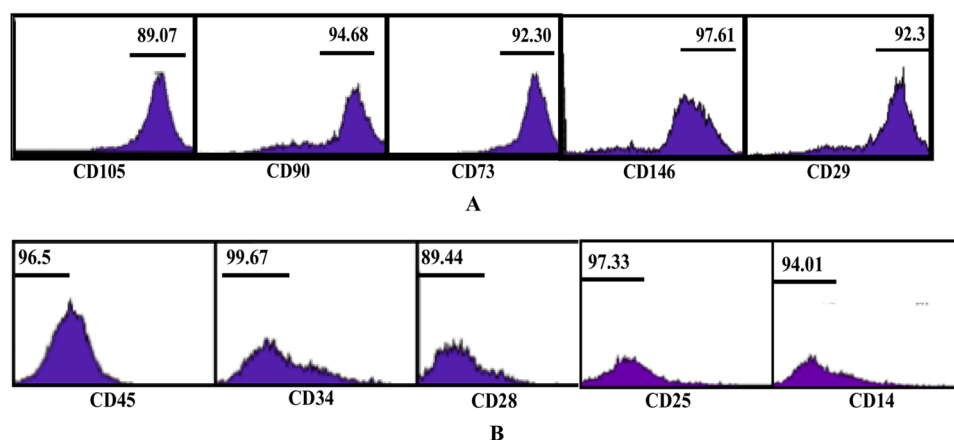


Fig. 2. A) Determination of the mesenchymal cell surface markers on hDPSCs by flow cytometry, B) Determination of the hematopoietic cell surface markers on hDPSCs by flow cytometry.

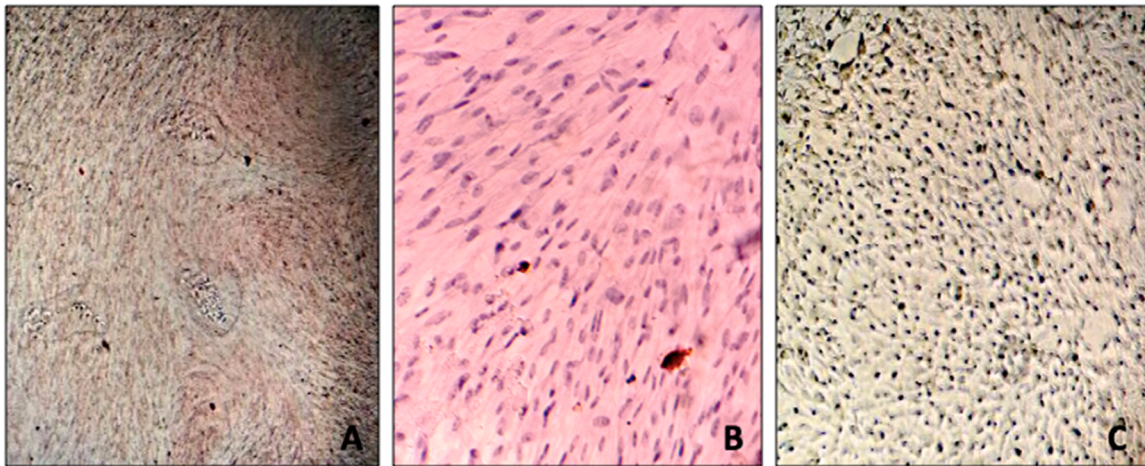


Fig. 3. Differentiation analysis in hDPSCs: A) Differentiation of hDPSCs into osteoblasts was confirmed by Alizarin Red staining, (x40), B) Differentiation of hDPSCs into adipocytes was confirmed by Oil Red O staining, (x40), C) Differentiation of hDPSCs into chondrocytes was confirmed by Alcian Blue staining, (x40).

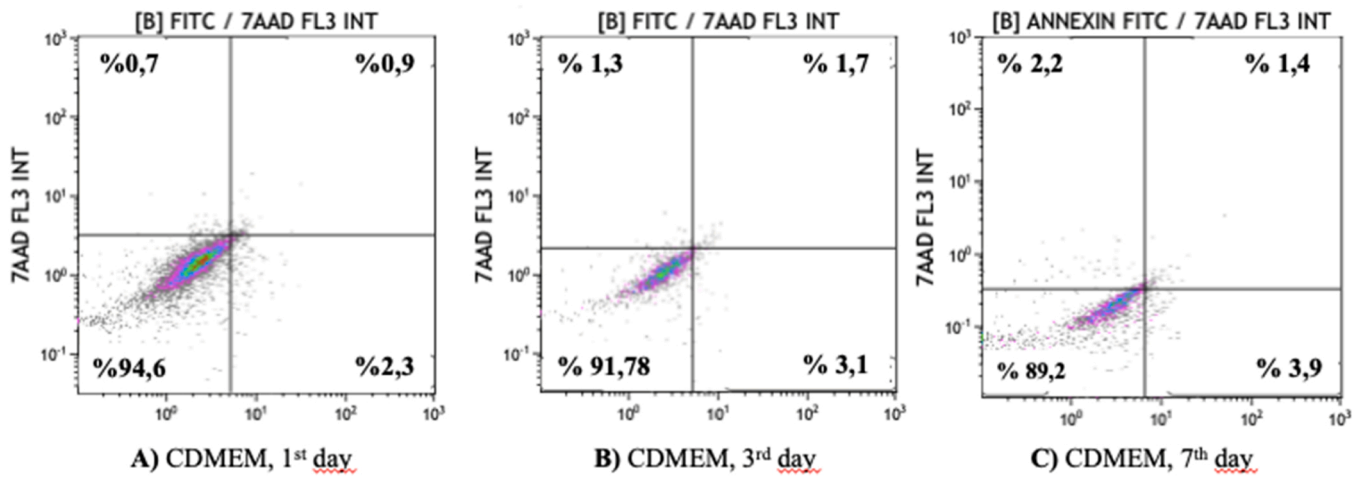


Fig. 4. Flow cytometry graph of hDPSc after incubation in CDMEM for different day intervals: (A) 1 day, (B) 3 days, (C) 7 days.

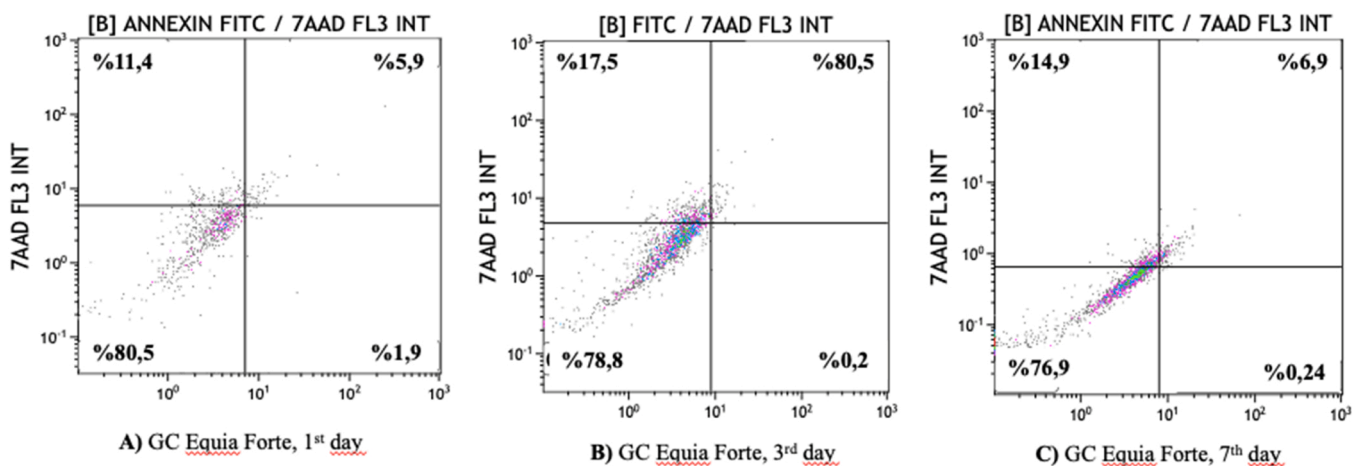


Fig. 5. Flow cytometry graph of hDPSc after incubation in GC Equia Forte for different day intervals: (A) 1 day, (B) 3 days, (C) 7 days.

groups. The lowest rates of necrotic cells were found in the control group and APA Glass Carbomer groups, while the rates of necrotic cells were similar among the other groups (Tables 3–6).

4. Discussion

Currently, many materials are used for restorative purposes in pediatric dentistry. When determining the ideal materials for restorations,

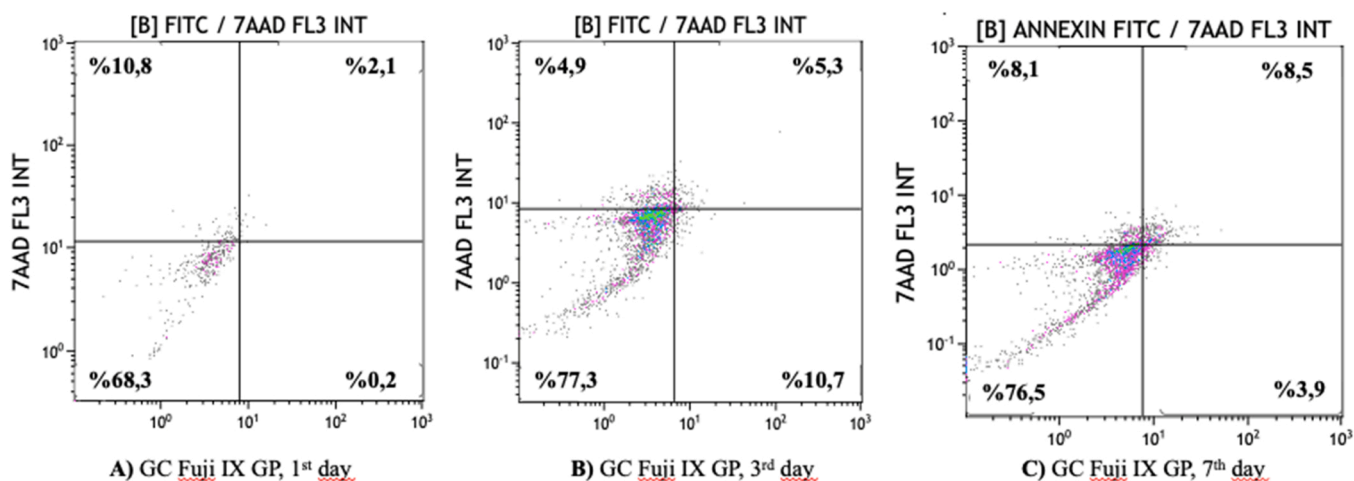


Fig. 6. Flow cytometry graph of hDPSC after incubation in GC Fuji IX GP for different day intervals: (A) 1 day, (B) 3 days, (C) 7 days.

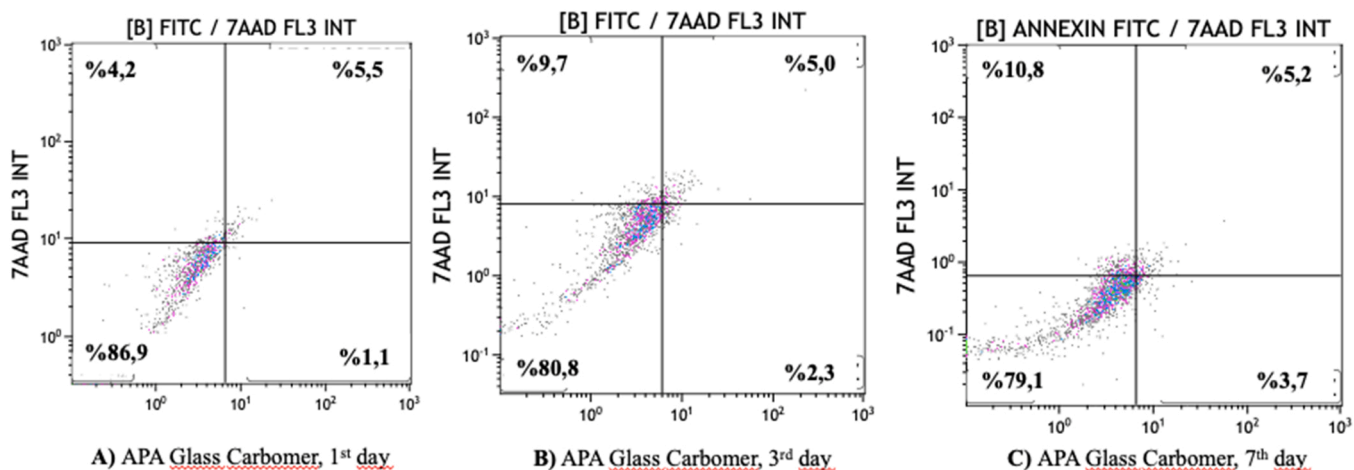


Fig. 7. Flow cytometry graph of hDPSC after incubation in APA Glass Carbomer for different day intervals: (A) 1 day, (B) 3 days, (C) 7 days.

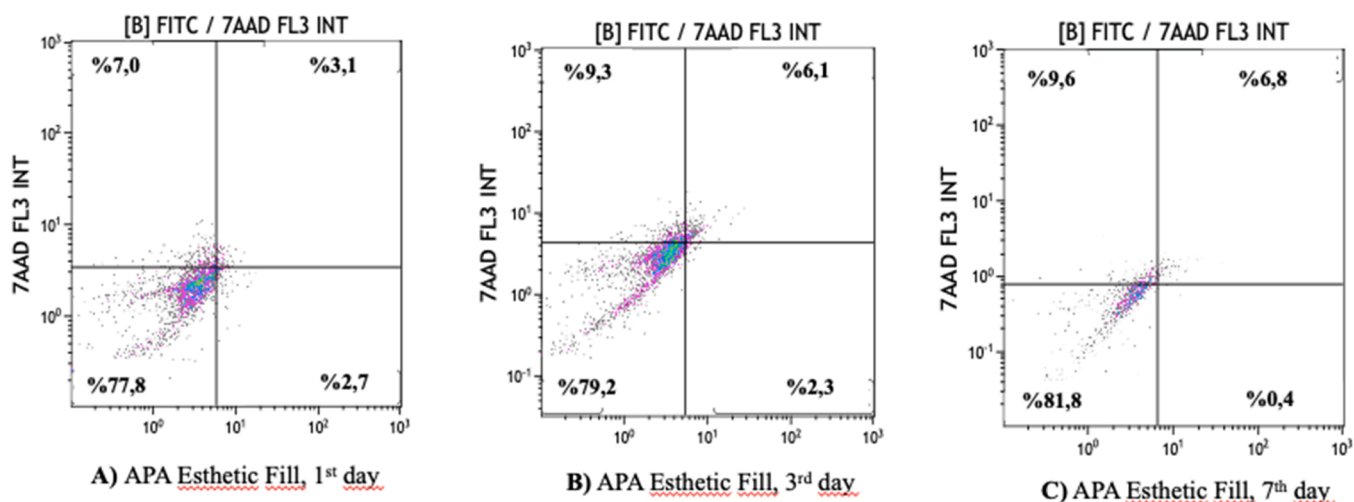


Fig. 8. Flow cytometry graph of hDPSC after incubation in APA Esthetic Fill for different day intervals: (A) 1 day, (B) 3 days, (C) 7 days.

it is important to evaluate their biocompatibility, as well as their mechanical and physical properties (Sisman et al., 2016; Bapat et al., 2021). To date, many studies have employed in vitro cell culture tests, because they reflect the physiological state of living tissues in assessing

the cytotoxic effects of dental materials and can thus be used as an initial screening to evaluate the biological and toxicological properties of dental materials (Huang and Chang, 2002; Cao et al., 2005; Sisman et al., 2016). Stanislawski et al. earlier reported that in vitro cell culture

Table 3
Evaluation of cell viability ratios on 3 different days and among 5 different groups.

	Cell Viability			
	1st day	3rd day	7th day	Total
	Mean±SD (median)	Mean±SD (median)	Mean±SD (median)	
Control (CDMEM)	94,91 ± 1,31 ^E	93,74 ± 0,65 ^{DE}	90,58 ± 1,71 ^{BD}	93,07 ± 2,24 ^c
GC EQUIA Forte	81,39 ± 2,2 ^{AC}	79,8 ± 1,14 ^{AC}	78,55 ± 1,5 ^C	79,91 ± 1,96 ^a
GC Fuji IX GP	81,02 ± 3,89 ^{AC}	79,94 ± 1,96 ^{AC}	78,35 ± 1,24 ^C	79,77 ± 2,67 ^a
APA Glass Carbomer	87,97 ± 1,97 ^B	82,33 ± 2,48 ^{AC}	80,3 ± 1,86 ^{AC}	83,53 ± 3,89 ^b
APA Esthetic Fill	82,87 ± 2,86 ^A	80,9 ± 1,91 ^{AC}	79,66 ± 2,04 ^{AC}	81,14 ± 2,53 ^a
Total	85,63 ± 5,87 ^a	83,34 ± 5,62 ^b	81,49 ± 4,94 ^c	83,49 ± 5,68

a-b-c-d-e-f: There is no difference between the groups with the same
A-B-C-D-E-F: There is no difference between the groups with the same
Wald-chi square test

Table 4
Evaluation of early apoptotic cell ratios on 3 different days and among 5 different groups.

	Early Apoptotic Cell Ratios			
	1st day	3rd day	7th day	Total
	Mean±SD (median)	Mean±SD (median)	Mean±SD (median)	
Control (CDMEM)	2,18 ± 0,73 ^{ABC}	2,67 ± 0,64 ^{ABC}	3,15 ± 1,33 ^{ABC}	2,67 ± 0,97
GC EQUIA Forte	3,28 ± 3,99 ^{ABC}	0,67 ± 0,75 ^A	0,94 ± 0,65 ^{AB}	1,63 ± 2,51
GC Fuji IX GP	0,85 ± 0,64 ^A	3,79 ± 4,06 ^C	3,75 ± 1,14 ^C	2,8 ± 2,69
APA Glass Carbomer	1,68 ± 1,15 ^{ABC}	3,67 ± 1,76 ^{BC}	2,49 ± 1,23 ^{ABC}	2,61 ± 1,55
APA Esthetic Fill	2,27 ± 0,77 ^{ABC}	3,33 ± 1,96 ^{ABC}	1,74 ± 1,86 ^{ABC}	2,45 ± 1,65
Total	2,05 ± 1,95	2,83 ± 2,33	2,41 ± 1,56	2,43 ± 1,97

a-b-c-d-e-f: There is no difference between the groups with the same
A-B-C-D-E-F: There is no difference between the groups with the same
Wald-chi square test

Table 5
Evaluation of late apoptotic cell ratios on 3 different days and among 5 different groups.

	Late Apoptotic Cell Ratios			
	1st day	3rd day	7th day	Total
	Mean±SD (median)	Mean±SD (median)	Mean±SD (median)	
Control (CDMEM)	1,29 ± 0,56 ^F	2,18 ± 0,57 ^{EF}	2,51 ± 0,74 ^{DEF}	1,99 ± 0,79 ^a
GC EQUIA Forte	5,46 ± 1,16 ^{ABCD}	4,21 ± 1,22 ^{ABDEF}	8,14 ± 1,08 ^C	5,94 ± 2 ^b
GC Fuji IX GP	5,64 ± 3,6 ^{ABC}	5,32 ± 1,44 ^{ABCD}	7,07 ± 2,21 ^{BC}	6,01 ± 2,51 ^b
APA Glass Carbomer	5,04 ± 0,91 ^{ABDEF}	6,14 ± 1,94 ^{ABC}	5,67 ± 1,69 ^{ABC}	5,62 ± 1,53 ^b
APA Esthetic Fill	4,01 ± 0,62 ^{ADE}	6,66 ± 0,97 ^{ABC}	6,02 ± 1,06 ^{ABC}	5,56 ± 1,44 ^b
Total	4,29 ± 2,3^a	4,9 ± 2,02^a	5,88 ± 2,35 ^b	5,02 ± 2,29

a-b-c-d-e-f: There is no difference between the groups with the same
A-B-C-D-E-F: There is no difference between the groups with the same
Wald-chi square test

Table 6
Evaluation of necrotic cell ratios on 3 different days and among 5 different groups.

	Necrotic Cell Ratios			
	1st day	3rd day	7th day	Total
	Mean±SD (median)	Mean±SD (median)	Mean±SD (median)	
Control (CDMEM)	1,62 ± 1,32 ^A	1,41 ± 0,97 ^A	3,77 ± 1,45 ^{AB}	2,26 ± 1,61 ^c
GC EQUIA Forte	9,87 ± 3,6 ^{CD}	15,32 ± 2,26 ^E	12,37 ± 1,8 ^{DE}	12,52 ± 3,38 ^a
GC Fuji IX GP	12,5 ± 1,68 ^{DE}	10,94 ± 3,44 ^{DE}	10,83 ± 1,9 ^{DE}	11,42 ± 2,42 ^a
APA Glass Carbomer	5,31 ± 2,38 ^{ABC}	7,86 ± 2,03 ^{BCD}	11,47 ± 2,05 ^{DE}	8,21 ± 3,29 ^b
APA Esthetic Fill	10,85 ± 2,71 ^{DE}	9,11 ± 3,47 ^{CD}	12,58 ± 2,44 ^{DE}	10,85 ± 3,06 ^a
Total	8,03 ± 4,66^a	8,93 ± 5,2^{ab}	10,2 ± 3,79^b	9,05 ± 4,62

a-b-c-d-e-f: There is no difference between the groups with the same
A-B-C-D-E-F: There is no difference between the groups with the same
Wald-chi square test

tests are reproducible, easy, fast, and enable the independent study parameters (Stanislawski et al., 1999). Since it affects the reaction of cultured cells on restorative materials, appropriate cell line selection plays a crucial role in in vitro cytotoxicity studies (Ersahan et al., 2019). Primary cells obtained from gingiva, dental pulp, and periodontium or permanent cell lines are used in cytotoxicity studies of dental materials. Permanent cell lines consist of cells that have a stable phenotype, are morphologically and physiologically uniform, and are used to evaluate changes independently of many parameters, such as age and metabolism (Murray et al., 2007; Schmalz and Arenholt-Bindslev, 2009). Despite this, Huang et al. emphasized that in vitro cell viability tests should be carried out with cell lines that are homologous to human tissues and primary cells. Although they have lower viability and are more difficult to obtain than permanent cell lines, they better mimic clinical conditions (Geurtsen et al., 1998; Huang and Chang, 2002). The present study was conducted using primary cells collected from human dental pulp tissue. Primary pulp cells are closely related to their original tissues and have an almost unchanged metabolic status in regard to their original tissues (Kong et al., 2009). Therefore, in the current study, cultured human pulp cells were used to evaluate the cellular viability of restorative materials.

hDPSCs, are self-renewable cells possessing multiple differentiation potential and immunomodulatory features. In the event of pulp damage, dental pulp stem cells differentiate into odontoblasts by stimulating a local response, through which they provide pulpal regeneration (Huang et al., 2010; Chen et al., 2019). hDPSCs are frequently used in cytotoxicity studies of dental materials because of their easy availability from extracted teeth, lack of ethical problems, and long life span (Todorović et al., 2008; Sisman et al., 2016). Since restorative dental materials have close contact with the dental pulp tissue, the continuity of the healthy pulp tissue and the biocompatibility of restorative materials with the dental pulp tissue are consequential issues (Huang and Chang, 2002). Under normal circumstances, few pulp cells proliferate in normal pulp tissue; hence, cells in the resting position seem to reflect the in vivo condition more closely than cells in the growing position (Huang and Chang, 2002). For this reason, the cellular viability of restorative materials was examined on confluent cells in the study.

The International Society for Cellular Therapy (ISCT) has established minimal criteria for the confirmation of mesenchymal stem cells (MSCs) that can be cultured from various tissues, such as skin, adipose tissue, bone marrow, and dental tissues. According to these criteria, MSCs should be able to adhere to plastic surfaces, differentiate into three cell types (osteoblasts, adipocytes, and chondroblasts), and express specific cell surface antigens (Gronthos et al., 2000; Seo et al., 2004; Dominici et al., 2006). Our findings show that cultured hDPSCs have a

fibroblast-like morphology, immunophenotypically express specific CD105, CD146, CD90, CD29, and CD73 MSC surface antigens, and exhibit osteogenic, chondrogenic, and adipogenic differentiation potential. These results prove that these cells are MSCs.

According to ISO 10993-5 standards (2009), a minimum incubation time of 24 h at 37 °C is recommended for materials used for in vitro cytotoxicity tests. In our study, cellular viability was assessed at the end of the seventh day to determine the long-term effects of dental materials on hDPSCs. In addition, although previous studies have evaluated cellular viability using different methods, flow cytometry was used in this study because it is faster and more sensitive, can distinguish apoptotic and necrotic cells, and can recognize changes in the early period (Barbosa et al., 2008). Flow cytometry enabled the identification of all viable, early apoptotic, late apoptotic, and necrotic cells in the analysis.

In the literature, different types of glass ionomer cements have been evaluated in terms of cellular viability. In this study, unlike in others, a newly developed material, Esthetic Fill, was used. Esthetic Fill, which is specified as a glass phospho-carbomer cement, is a monomer-free material containing nanoapatite particles. There are no studies reported of this material; therefore, the current study will be the first to assess the cellular viability of Esthetic Fill on primary stem cells. To our knowledge, this is the first study in which viable, early apoptosis, late apoptosis, and necrotic cellular ratios of different categories of glass ionomer cements have been demonstrated in vitro on primary cell culture by flow cytometry.

Cosgun et al. researched the effects of five different GICs on Vero cells after the 24 h, 48 h, and 72 h incubation periods using an MTT test. They showed that extracts of Zirconomer, EQUIA Forte, Fuji II LC, and Fuji IX GP materials led to a reduction in cell viability in relation to incubation time, similar to our study (Cosgun et al., 2019). However, in contrast with the our study, they observed no significant difference between the EQUIA Forte and control groups at the end of the first day. In our study, while the control group showed the highest cellular viability, no significant difference was observed between the EQUIA Forte and Fuji IX GP.

Ersahan et al. evaluated the cytotoxic effects of different GICs on dental pulp and L929 mouse fibroblast cell cultures using an MTT test. The EQUIA Forte material increased the cellular viability of both the L929 mouse fibroblast cells and dental pulp cells compared with the control group. EQUIA Forte is a high-viscosity glass ionomer cement, and it is thought that the good biocompatibility of these cements may be due to the small particle size of the glass fillers, their low exothermic reaction, rapid neutralization, and the release of benign ions (Ersahan et al., 2019). In contrast to Ersahan et al., our results showed that while the EQUIA Forte material showed lower cellular viability on dental pulp MSCs compared to the control group and Glass Carbomer material at the end of the first day, there was no statistical difference with Fuji IX GP, a CGIC.

Hii et al. stated that as the concentration value of the CGIC Fuji IX GP and nano-HA-silica-GIC is increased, the components released from the restorative materials may cause more cytotoxic effects on hDPSCs (Hii et al., 2019). The findings of that study conflict with those of Noorani et al., who evaluated the same materials. This may be due to their use of different culture mediums (Noorani et al., 2017). However, in our study, the viability of restorative materials on cells was compared between different days instead of between different concentrations.

Another study reported that higher cellular viability of the GC Fuji II LC material at the end of 24 h compared to the GCP Glass Carbomer material may be related to surface topography and surface roughness, two factors which can play an important role in cellular interaction (Michel et al., 2017). A study by Schweikl et al. revealed that surface topography is a factor affecting the functional activity of cells in contact with a biomaterial, findings that cells would spread better on smooth surfaces and that they might have a smaller appearance on rough ones (Schweikl et al., 2007). In the present study, Glass Carbomer showed

higher cellular viability on hDPSCs at the end of the first, third, and seventh days compared to the other restorative materials. The higher viability values of the Glass Carbomer material may be due to the components being released after polymerization affecting cellular viability less negatively than other restorative materials or because its particles have smoother surfaces.

Previous studies have indicated that the pH levels, types, and concentration of ions of restorative materials are important in the evaluation of their cytotoxicity to cells (Consiglio et al., 1998; Schmid-Schwab et al., 2009; Kanjevac et al., 2015). Consiglio et al. reported that a decrease in pH level has a detrimental effect on cellular protein synthesis, while Hirose et al. stated that acidic extracellular pH status was associated with cellular death and the cessation of dental pulp stem cells proliferation (Consiglio et al., 1998; Hirose et al., 2016). Similarly, Stanislawski et al. reported that the pH level of CGICs decreases due to the polyacrylic acid content and damages dental pulp cells (Stanislawski et al., 1999). Our results showed that the cellular viability of the Fuji IX GP material, which is a CGIC, is lower than that of other restorative materials. Regarding the ion release of restorative materials, Kanjevac et al. reported that Fuji Plus, Vitrebond, and Fuji VIII materials, which have the most cytotoxic effects on human exfoliated deciduous stem cells and human dental pulp stem cells, released the highest amount of fluoride. No correlation was found between aluminum and strontium ion concentrations and cytotoxicity (Kanjevac et al., 2015). This is in line with the findings of Consiglio et al. who evaluated the effects of GICs on protein synthesis in human gingival fibroblast cells and reported that fluoride concentration was one of the factors that had an inhibitory effect on protein synthesis (Consiglio et al., 1998). Similarly, a study by Chang et al. demonstrated that fluoride inhibits the growth, proliferation, mitochondrial activity, and protein synthesis of human dental pulp stem cells (Chang and Chou, 2001). Cosgun et al. also found that the concentrations of ions, such as F⁻, Al⁺³ and Sr⁺², released from glass ionomer restorative materials determine their cytotoxic potential (Cosgun et al., 2019). Unlike the above studies, Chen et al. reported that the cytotoxic effects of conventional GICs are not caused by ions such as Ca, Al, Si, and Sr in their content, that instead a toxic effect may be caused by the release of organic compounds (Chen et al., 2016).

A limitation of this study is that only cellular viability was evaluated. Since the chemical contents, ion concentrations, fluoride release, and pH levels of the restorative materials, were not assessed, the reasons for higher or lower cellular viability could not be explained. Thus, further studies are needed in which the chemical contents, ion concentrations, and pH levels of the restorative materials will be correlated with the cytotoxicity findings of the materials. Additionally, since in vitro cell culture studies do not consider the protective effect of dentin, the immunological properties of pulp, and the absence of saliva, the results of this study need to be supported by in vivo studies.

5. Conclusion

This study contributes to the relevant literature concerning the biological response of restorative materials on hDPSCs. Despite the limitations of this in vitro study, clinicians should find the results interesting. The results indicated that Glass Carbomer was the material with the highest cellular viability, while Fuji IX GP was the material with the lowest cellular viability. In terms of early and late apoptosis findings, no significant difference was found between the materials. Glass Carbomer had the lowest rate of necrotic cells, while EQUIA Forte had the highest rate of such cells. According to the results, Glass Carbomer had better cellular response on hDPSCs than others. Although all the evaluated restorative materials led to positive results, further in vitro and in vivo studies are needed.

CRedit authorship contribution statement

Designing the study by Figen Seymen, S. Ceren Ilisulu; generating the

data by S. Ceren Ilisulu; analyses the data by Nur Ecem Oztop, Tunc Akkoc; writing the paper by S. Ceren Ilisulu; approved the final version of this paper by S. Ceren Ilisulu, Nur Ecem Oztop, Tunc Akkoc, Figen Seymen.

Declaration of Competing Interest

The authors have no conflict of interest to declare.

Data availability

No data was used for the research described in the article.

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References

- Bapat, R.A., Parolia, A., Chaubal, T., Dharamadhikari, S., Abdulla, A.M., Sakkir, N., Kesharwani, P., 2021. Recent update on potential cytotoxicity, biocompatibility and preventive measures of biomaterials used in dentistry. *Biomater. Sci.* 9, 3244–3283.
- Barbosa, J., Costa-de-Oliveira, S., Rodrigues, A.G., Pina-Vaz, C., et al., 2008. Optimization of a flow cytometry protocol for detection and viability assessment of *Giardia lamblia*. *Travel Med. Infect. Dis.* 6, 234–239.
- Cao, T., Saw, T.Y., Heng, B.C., Liu, H., Yap, A.U.J., Ng, M.L., 2005. Comparison of different test models for the assessment of cytotoxicity of composite resins. *J. Appl. Toxicol.* 25, 101–108.
- Cehreli, S.B., Ebrü, T.R., Yalcinkaya, Z., Cehreli, Z.C., 2013. Microleakage of newly developed glass carbomer cement in primary teeth. *Eur. J. Dent.* 7, 15–21.
- Chang, Y.C., Chou, M.Y., 2001. Cytotoxicity of fluoride on human pulp cell cultures in vitro. *Oral Surg. Oral. Med. Oral. Pathol. Oral. Radiol. Endod.* 91, 230–234.
- Chen, C.A., Chen, Y.L., Huang, J.S., Huang, G.T.J., Chuang, S.F., et al., 2019. Effects of restorative materials on dental pulp stem cell properties. *J. Endod.* 45, 420–426.
- Chen, S., Mestres, G., Lan, W., Xia, W., Engqvist, H., 2016. Cytotoxicity of modified glass ionomer cement on odontoblast cells. *J. Mater. Sci. Mater. Med.* 27, 116.
- Consiglio, R., Rengo, S., Liguoro, D., Riccitiello, F., Formisano, S., Palumbo, G., Di Jeso, B., 1998. Inhibition by glass-ionomer cements of protein synthesis by human gingival fibroblasts in continuous culture. *Arch. Oral. Biol.* 43, 65–71.
- Cosgun, A., Bolgul, B., Duran, N., 2019. In vitro investigation of antimicrobial effects, nanohardness, and cytotoxicity of different glass ionomer restorative materials in dentistry. *Niger. J. Clin. Pract.* 22, 422.
- Dominici, M.L.B.K., Blanc, L., Mueller, K., Slaper-Cortenbach, I., Marini, I., Krause, F.C., Horwitz, D.S., M, E., et al., 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 8, 315–317.
- Ersahan, S., Oktay, E.A., Sabuncuoglu, F.A., Karaoglanoglu, S., Aydin, N., Suloglu, A.K., 2019. Evaluation of the cytotoxicity of contemporary glass-ionomer cements on mouse fibroblasts and human dental pulp cells. *Eur. Arch. Paediatr. Dent.* 21, 1–8.
- Geurtsen, W., Lehmann, F., Spahl, W., Leyhausen, G., 1998. Cytotoxicity of 35 dental resin composite monomers/additives in permanent 3T3 and three human primary fibroblast cultures. *J. Biomed. Mater. Res. A* 41, 474–480.
- Gronthos, S., Mankani, M., Brahimi, J., Robey, P.G., Shi, S., 2000. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proc. Natl. Acad. Sci. U. S. A.* 97, 13625–13630.
- Hii, S.C., Luddin, N., Kannan, T.P., Ab Rahman, I., Ghani, N.R.N.A., 2019. The biological evaluation of conventional and nano-hydroxyapatite-silica glass ionomer cement on dental pulp stem cells: a comparative study. *Contemp. Clin. Dent.* 10, 324–332.
- Hirose, Y., Yamaguchi, M., Kawabata, S., Murakami, M., Nakashima, M., Gotoh, M., Yamamoto, T., 2016. Effects of extracellular pH on dental pulp cells in vitro. *J. Endod.* 42, 735–741.
- Huang, F.M., Chang, Y.C., 2002. Cytotoxicity of resin-based restorative materials on human pulp cell cultures. *Oral Surg. Oral. Med. Oral. Pathol. Oral. Radiol. Endod.* 94, 361–365.
- Huang, G.T.J., Yamaza, T., Shea, L.D., Djouad, F., Kuhn, N.Z., Tuan, R.S., Shi, S., et al., 2010. Stem/progenitor cell-mediated de novo regeneration of dental pulp with newly deposited continuous layer of dentin in an in vivo model. *Tissue Eng. Part A* 16, 605–615.
- ISO 10993-5: 2009. Biological Evaluation of Medical Devices-part 5: Tests for in Vitro Cytotoxicity. International organization for standardization, Geneva, Switzerland.
- Kanjevac, T.V., Milovanovic, M.Z., Milosevic-Djordjevic, O., Tesic, Z.L., Ivanovic, M., Lukic, A., 2015. Cytotoxicity of glass ionomer cement on human exfoliated deciduous teeth stem cells correlates with released fluoride, strontium and aluminum ion concentrations. *Arch. Biol. Sci.* 67, 619–630.
- Kong, N., Jiang, T., Zhou, Z., Fu, J., et al., 2009. Cytotoxicity of polymerized resin cements on human dental pulp cells in vitro. *Dent. Mater.* 25, 1371–1375.
- López-García, S., Pecci-Lloret, M.P., Pecci-Lloret, M.R., Onate-Sánchez, R.E., García-Bernal, D., Castelo-Baz, P., Guerrero-Gironés, J., 2019. In vitro evaluation of the biological effects of ACTIVA Kids BioACTIVE restorative, Ionolux, and Riva Light Cure on human dental pulp stem cells. *Materials* 12, 3694.
- Marczuk-Kolada, G., Łuczaj-Cepowicz, E., Pawińska, M., Hołownia, A., 2017. Evaluation of the cytotoxicity of selected conventional glass ionomer cements on human gingival fibroblasts. *Adv. Clin. Exp. Med.* 26, 1041–1045.
- Michel, A., Erber, R., Frese, C., Gehrig, H., Saure, D., Mente, J., 2017. In vitro evaluation of different dental materials used for the treatment of extensive cervical root defects using human periodontal cells. *Clin. Oral. Invest.* 21, 753–761.
- Murray, P.E., García Godoy, C., García Godoy, F., 2007. How is the biocompatibility of dental biomaterials evaluated? *Med. Oral. Patol. Oral. Cir. Bucal* 12, 258–266.
- Noorani, T.Y., Luddin, N., Rahman, I.A., Masudi, S.M., 2017. In vitro cytotoxicity evaluation of novel nano-hydroxyapatite-silica incorporated glass ionomer cement. *J. Clin. Diagn. Res.* 11, 105–109.
- Peters, O.A., 2013. Research that matters—biocompatibility and cytotoxicity screening. *Int. Endod. J.* 46, 195–197.
- Pizzoferrato, A., Ciapetti, G., Stea, S., Cenni, E., Arciola, C.R., Granchi, D., 1994. Cell culture methods for testing biocompatibility. *Clin. Mater.* 15, 173–190.
- Rodríguez-Lozano, F.J., Serrano-Belmonte, I., Pérez Calvo, J.C., Coronado-Parra, M.T., Bernabeu-Esclapez, A., Moraleda, J.M., 2013. Effects of two low-shrinkage composites on dental stem cells (viability, cell damaged or apoptosis and mesenchymal markers expression). *J. Mater. Sci. Mater. Med.* 24, 979–988.
- Rodríguez-Lozano, F.J., López-García, S., García-Bernal, D., Sanz, J.L., Lozano, A., Pecci-Lloret, M.P., Forner, L., 2021. Cytocompatibility and bioactive properties of the new dual-curing resin-modified calcium silicate-based material for vital pulp therapy. *Clin. Oral. Investig.* 25, 5009–5024.
- Schmalz, G., Arenholt-Bindslev, D., 2009. Basic aspect. Biocompatibility of Dental Materials. Springer, Berlin, pp. 1–12.
- Schmid-Schwab, M., Franz, A., König, F., Bristela, M., Lucas, T., Piehlinger, E., Schedle, A., 2009. Cytotoxicity of four categories of dental cements. *Dent. Mater.* 25, 360–368.
- Schweikl, H., Müller, R., Englert, C., Hiller, K.A., Kujat, R., Nerlich, M., Schmalz, G., 2007. Proliferation of osteoblasts and fibroblasts on model surfaces of varying roughness and surface chemistry. *J. Mater. Sci. Mater. Med.* 18, 1895–1905.
- Seo, B.M., Miura, M., Gronthos, S., Bartold, P.M., Batouli, S., Brahimi, J., Shi, S., 2004. Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet* 364, 149–155.
- Sisman, R., Aksoy, A., Yalçın, M., Karaöz, E., 2016. Cytotoxic effects of bulk fill composite resins on human dental pulp stem cells. *J. Oral. Sci.* 58, 299–305.
- Stanislawski, L., Daniau, X., Lautié, A., Goldberg, M., 1999. Factors responsible for pulp cell cytotoxicity induced by resin-modified glass ionomer cements. *J. Biomed. Mater. Res. A* 48, 277–288.
- Todorović, V., Marković, D., Milošević-Jovčić, N., Petakov, M., Balint, B., Čolić, M., Milenković, A., Čolak, I., Jokanović, V., Nikolić, N., 2008. Dental pulp stem cells: potential significance in regenerative medicine. *Stomatol. Glas. Srb.* 55, 170–179.
- Van Duinen, R.N., Kleverlaan, C.J., de Gee, A.J., Werner, A., Feilzer, A.J., 2005. Early and long-term wear of “fast-set” conventional glass-ionomer cements. *Dent. Mater.* 21, 716–720.
- Yap, A.U., Pek, Y.S., Cheang, P., 2003. Physico-mechanical properties of a fast-set highly viscous GIC restorative. *J. Oral. Rehabil.* 30, 1–8.