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Impact of various endodontic sealers on HPDLF Cell viability and apoptosis

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This study aimed to investigate the cytotoxicity and apoptotic activity of different endodontic sealers: Sealapex, Apexit Plus, AH Plus, MTA-Fillapex and TotalFill BC Sealer in the culture of human periodontal ligament fibroblast (HPDLF) cells. The sealers were mixed, set for 24 h, and then covered with culture medium to obtain extracts, which were diluted to 1:0, 1:1, 1:2, 1:4, and 1:8. Simultaneously, HPDLF cells (1×10^4) were seeded in 96-well plates and incubated for 24 h at 37 °C 5% CO_2 conditions. The cells were then exposed to 100 µL of diluted extract medium. Cytotoxicity was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to check cell viability, while apoptosis was assessed by TUNEL assay. Statistical analyses were performed using Kruskal-Wallis and Dunn post hoc tests with Mann Whitney U. In MTT and TUNEL assay cells were treated with sealers both 24 and 72 h. All materials showed higher toxicity at 72 h compared to 24 h. AH Plus exhibited the highest cytotoxicity, followed by MTA-Fillapex, Apexit Plus, Sealapex, while TotalFill BC Sealer had the lowest cytotoxicity. Consuquently, it was considered that TotalFill BC Sealer had the lowest cytotoxic potency when compared to other sealers, so it can be considered biocompatible.

Keywords Biocompatibility, Cytotoxicity, Apoptosis, MTA-Fillapex, TotalFill BC Sealer, Human periodontal ligament fibroblast cells

Tissue loss in teeth caused by trauma, disease, or congenital abnormalities poses a significant global health challenge as it can lead to functional and aesthetic complications. One of the primary treatments for managing tooth damage and preventing tooth loss is root canal therapy[1]. The success of root canal treatment depends on three key factors: thorough removal of debris from the canals, effective disinfection of the canals, and three-dimensional hermetic filling with a dimensionally stable and biocompatible canal filling material which prevents reinfections and supports tissue healing[2,3]. To achieve a completely sealed and impermeable filling of the canals, gutta-percha is the primary material utilized, while endodontic sealer is used for areas that are difficult to access, such as the gaps between the walls of the root canal and the gutta-percha, besides apical deltas, branchings, and accessory canals[4,5]. The materials required for root canal filling must have adequate physicochemical and biological characteristics, including a capacity to form an appropriate seal, minimal cytotoxicity, the capacity to create an optimal environment for the healing of periapical tissues, antibacterial performance, and tissue tolerance[5]. Endodontic sealers are categorized based on their setting reaction and composition, and some of the varieties included in this classification are zinc oxide eugenol, salicylate, silicone, epoxy resin, and tricalcium silicate sealers[6]. Each of these types possesses distinct characteristic that influence their clinical performance and biocompatibility[5,6].

Calcium hydroxide based sealers have been manufactured because of the osteogenic and cementogenic characteristics of calcium hydroxide-containing cavity liners and bases, as well as their antibacterial properties[6,7]. Two well-known examples of calcium hydroxide-based sealers are Sealapex and Apexit Plus[8]. Initially marketed as MTA-based sealer, MTA-Fillapex is a salicylate resin-based sealer that contains mainly resin in addition to 15% MTA powder[5]. AH Plus is an epoxy resin based sealar that is cytotoxic when freshly mixed and whose toxicity decreases after the curing period is completed. AH Plus is widely regarded as the gold standard in endodontics due to its excellent physicochemical, biological, and antimicrobial properties and is frequently used as a reference material in comparative studies[5,9].

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Tricalcium silicate-based materials, also known bioceramics, have gained popularity in various dental applications, including vital pulp therapy, perforation repair, root end filling, and root canal sealer. This is primarily due to their apatite-like crystal formation, bioactivity and ease of use[10]. EndoSequence BC Sealer, introduced as TotalFill BC Sealer in Europe, is an example of a tricalcium silicate based sealer[11].

One challenge associated with endodontic sealers is their potential for extrusion beyond the apical foramen due to their viscous nature[12]. Most endodontic root canal sealers are known to have some toxic properties or might be exhibiting different levels of cytotoxicity on tissues, which would result in inflammation, prolonged wound healing, and bone resorption in the periapical tissues[13,14]. The chemical composition of these biomaterials can significantly influence cell death pathways, mediated by various extracellular signals[15]. To minimize the risk of local and systemic side effects, it is essential that the biological properties and biocompatibility of dental materials are thoroughly and independently evaluated through in vitro testing to ensure their suitability for clinical use. Two-dimensional (2D) culture is frequently employed to evaluate biocompatibility for that purpose[16]. Human periodontal ligament fibroblast (HPDLF) cells are specialized cells found within the periodontal ligament, a connective tissue structure that anchors the tooth to the surrounding alveolar bone[17]. Due to the continuation of the root canal pulp structure, the HPDLF cell is a preferred cell group in cell culture studies evaluating the cytotoxicity of endodontic sealers[18,19].

In cell culture studies, the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay is commonly used to assess cell viability and proliferation, providing a measure of metabolic activity[20]. Complementing this, the TUNEL assay is a histological method that detects DNA fragmentation, a hallmark of apoptosis, by marking fragmented DNA[21]. Together, these assays offer a comprehensive approach to evaluating both cell health and the occurrence of programmed cell death in response to various treatments[22].

The objective of this in vitro investigation is to evaluate the cytotoxic and apoptotic effects of various endodontic sealaers, including Sealapex, Apexit Plus, MTA-Fillapex, AH Plus, and Totalfill BC Sealer, on HPDLF cells. The novelty of this research lies in its comprehensive assessment of the apoptotic features of these sealers, which offers new insights into their potential cytotoxic effects.

Material method

This study was confirmed by the Ethics Committee of Istanbul University Faculty of Dentistry, protocol number 2017/47/202.

Preparation of extracts

This study was conducted in accordance with the standards of the International Organization for Standardization (ISO) 10993-5:20097 and 10993-12:2021.

This study aimed to assess the cytotoxicity and biocompatibility characteristics of Sealapex (SybronEndo, Romulus, MI, USA), Apexit Plus (Ivoclar Vivadent, Schaan, Liechtenstein), AH Plus (Dentsply DeTrey, Konstanz, Germany), Totalfill BC Sealer (FKG Dentaire, La-Chaux-de-Fonds, Switzerland), and MTA-Fillapex (Angelus Industria de Produtos Odontologicos S/A, Londrina, PR, Brazil) provides details regarding the manufacturers and compositions of these sealers (Table 1). The endodontic sealers were prepared following the instructions provided by the manufacturers, with each sealer weighing 0.5 g. Apexit Plus and TotalFill BC Sealer were directly applied from their containers, while Sealapex, AH Plus, and MTA-Filapex were mixed in a sterile petri dish under aseptic conditions and then transferred to 6-well plates. Following mixing, sealers were incubated at 37 °C under humidified conditions for 24 h. Subsequently, the materials underwent sterilization using ultraviolet irradiation for 20 min. Specimens were covered with 2.5 mL of cell culture Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% penicillin-streptomycin, 1% gentamicin, and 0.4% amphotericin B, and incubated in the dark for 24 h at 37 °C under humidified conditions. The extraction ratio used was 0.2 g of sealer per 1 mL of the culture medium[23]. After incubation, the original extracts (conditioned medium) were utilized for further analyses.

Cell culture

Primary HPDLF cells were used in this study. These cells were sourced from previously extracted primary teeth by Islam A., et al. with the Near East University Scientific Research Evaluation Ethics Committee, under decision

Materials	Composition	Manifacturer	Batch Number
Sealapex	Calcium oxide, trimetilol provan, Neopentil Glikol, salicylate resin, isobutyl salicylate	SybronEndo, Romulus, MI, USA	18,432
Apexit Plus	Calcium salts (hydroxide, oxide, phosphate), hydrogenised colophony, disalicylate, bismuth salts (oxide, carbonate), highly dispersed silicon dioxide, alkyl ester of phosphoric acid	Ivoclar Vivadent, Schaan, Liechtenstein	595,377
AH Plus	Paste A: bisphenol-A, epoxy resin, bisphenol-F epoxy resin, calcium tungstate, zirconium oxide, silica, iron oxide pigments Paste B: dibenzyldiamine, aminoadamantane, tricyclodecane-diamine, calcium tungstate, zirconium oxide, silica, silicone oil	Dentsply DeTrey, Konstanz, Germany	60,620,110
TotalFill BC Sealer	Zirconium oxide; calcium silicate; monobasic calcium phosphate, calcium hydroxide	FKG Dentaire, La-Chaux-de-Fonds, Switzerland	5023102EU
MTA-Fillapex	Paste A: salicylate resin, bismuth trioxide, fumed silica Paste B: fumed silica, titanium dioxide, mineral trioxide aggregate, base resin	Angelus Industria de Produtos Odontologicos S/A, Londrina, PR, Brazil	8288

Table 1. Manufacturer and composition of endodontic sealers used in this study.

number YDU/2015/34/243 [24]. The deciduous teeth used in this study were collected from 10 healthy children aged 6 to 11 years. Informed consent was obtained from a parent and/or legal guardian for the participation of all minors included in this study. All participants were provided with detailed information about the study, and consent was given voluntarily by the parents or legal guardians before the study commenced. The selection criteria ensured that the samples were obtained from non-decayed deciduous teeth, free from abscesses, fistulas, or periapical lesions. After derivation of HPDLF cells, they underwent two passages, and were preserved in the Histology and Embryology Department of Manisa Celal Bayar University in Manisa.

The cells were cultured in DMEM with high glucose (Capricorn Scientific, DMEM-HPXA, Ebsdorfergrund, Germany), containing 10% fetal bovine serum (Capricorn Scientific, FBS-12B, Ebsdorfergrund, Germany), 1% L-glutamine (Biochrom GmbH, K0283, Germany), 1% penicillin/streptomycin (Biosera, XC-A4122, Nuaille, France), 1% gentamicin (Gibco, 15710-064, Grand Island, USA), and 0.4% amphotericin-B (Capricorn Scientific, AMP-B, Ebsdorfergrund, Germany). The cells were maintained under culture conditions of 37 °C and 5% CO2 until they get confluent. Cell viability was assessed using the trypan blue staining protocol in a hemocytometer (Biological Industries, 03-102-1B, Kibbutz Beit-Haemek, Israel).

Characterization of HPDLF cells

Indirect immunoperoxidase staining was used to analyze the distributions of CD34, CD90, and collagen1 for characterization. Initially, cells were fixed in 4% paraformaldehyde (1.04004.0800, Merck) for 30 min, followed by triple washing with phosphate buffer saline (PBS). Subsequently, permeabilization was achieved by incubating the cells with a 0.1% Triton-X-100 (A4975,0100, Applichem) solution on ice for 15 min. A 5-minute treatment with 3% hydrogen peroxide (H2O2, 1.08597.2500, Merck) was performed after PBS washing. The cells were then blocked with blocking solution (TA-125-UB, ThermoFisher) for 1 h at room temperature and subsequently incubated overnight at 4 °C with anti-CD34 (Santa Cruz Biotechnology, sc-74499, Texas, USA), anti-CD90 (Santa Cruz Biotechnology, sc-53456, Texas, USA) and anti-Collagen 1 (Millipore, 08-115, Darmstadt, Germany) antibodies. After washing with PBS, the cells were incubated with a biotinylated rabbit anti-mouse secondary antibody (TP-125-UB, ThermoFisher) for 30 min. Following another PBS wash, streptavidin-hydrogen peroxidase (TP-125-UB, ThermoFisher) was added for 30 min. Immunocytochemical reaction development was initiated by applying diaminobenzidine (DAB, 38611, ScyTek Laboratories) for 5 min. Post-DAB treatment, counterstaining was performed using Mayer's hematoxylin (Bio-Optica, 05-06002/L Milano, Italy), followed by mounting with a mounting medium (DMM-125, Spring Bioscience). Immunolabeling intensity was assessed by two investigators at various time points using light microscopy (BX40, Olympus). Immunoreactivities were categorized as negative (-), weak (+), moderate (++), and strong (+++).

In vitro cytotoxicity analysis

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test is a standard colorimetric test that measures cellular metabolic activity and provides information about cell viability, proliferation, and cytotoxicity. For the cell viability assay, adherent HPDLF cells in a logarithmic growth phase were seeded (100 mL/well) in 96-well flat-bottom microtiter plates at a concentration of 1×10^3 cells/well. Then, the sealers' extracts were applied to the cells at 1:0, 1:1, 1:2, 1:4, and 1:8 dilutions (100 µL/well) for 24–72 h using the culture medium as the dilution material. Cells were cultured with 100 µL of culture medium, and it was used as a negative control. All experiments were performed at least in 6 replicates and 3 independent experiments. The MTT solution (Sigma, M5655, Darmstadt, Germany) was prepared with PBS (in 5 mg/mL) just before use. Next, 10 µl of MTT solution and 90 µl of cell culture medium were added to each well and incubated at 37 °C for 4 h. The process was stopped by adding 50 µl of dimethyl sulfoxide (DMSO, Applichem, A3672, 0250, Darmstadt, Germany) to each well. Finally, reaction was detected at 570 nm (Abs570, Biotek ELX800UV) absorbance. The absorbance value readings were normalized to untreated control cultures (100%).

Detection for apoptosis

The optimal concentration of each root canal filling material for the detection of apoptosis cells was determined by assessing concentrations that affect 50% of the cells via the MTT test. Subsequently, based on the dosage and timing outcomes of the MTT assay, extracts of root canal filling materials were administered to HPDLF cells. The culture medium was used as the control group.

The in situ apoptosis detection kit (ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit, Millipore, MA, USA) was used. The cells were fixed with 4% paraformaldehyde for 30 min and washed twice with PBS. They were then incubated for 10 min with a 0.1% Triton X-100 solution on ice for permeabilization, and endogenous peroxidase activity was inhibited with 3% H_2O_2. The cells were then incubated with equilibration buffer for 10–15 s and TdT-enzyme in a humidified atmosphere at 37 °C for 60 min. They were subsequently put into pre-warmed working strength stop wash buffer at room temperature for 10 min and incubated with anti-digoxigenin conjugate for 45 min. Each step was separated by careful washing in PBS. Next, DAB staining was conducted, followed by counterstaining with Mayer's hematoxylin. All slides were then evaluated under a light microscope. To quantify the number of TUNEL positive (brown) cells, at least 3 random fields at 20 µm magnification, were counted and reported as percent of TUNEL positive cells.

Statistical analysis

IBM SPSS Statistics 22 (IBM SPSS, Turkey) program was used for statistical analysis. While evaluating the study data, Kruskal Wallis test was used to compare the parameters between groups, and Mann Whitney U test was used to determine the group that caused the difference. Mann Whitney U test was used for the comparison of parameters between two groups. Significance was evaluated at the $p < 0.05$ level.

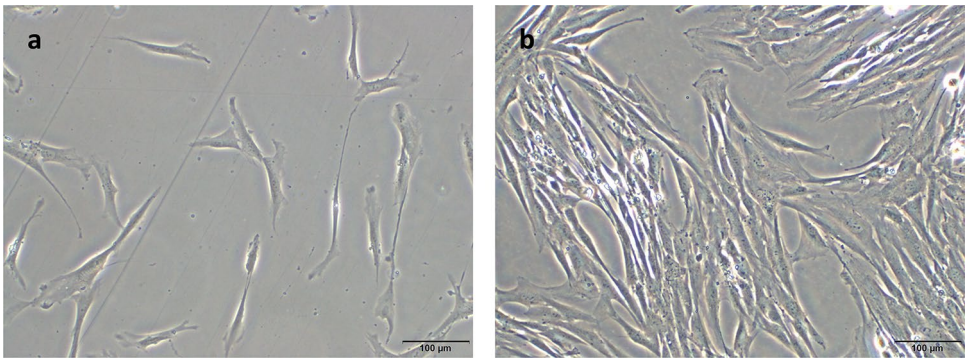

Fig. 1. Cell culture of HPLDF cells. Cell culture photographs of HPDLF cells at third passage (**a**) and seventh passage (**b**). Scale bars: 100 µm.

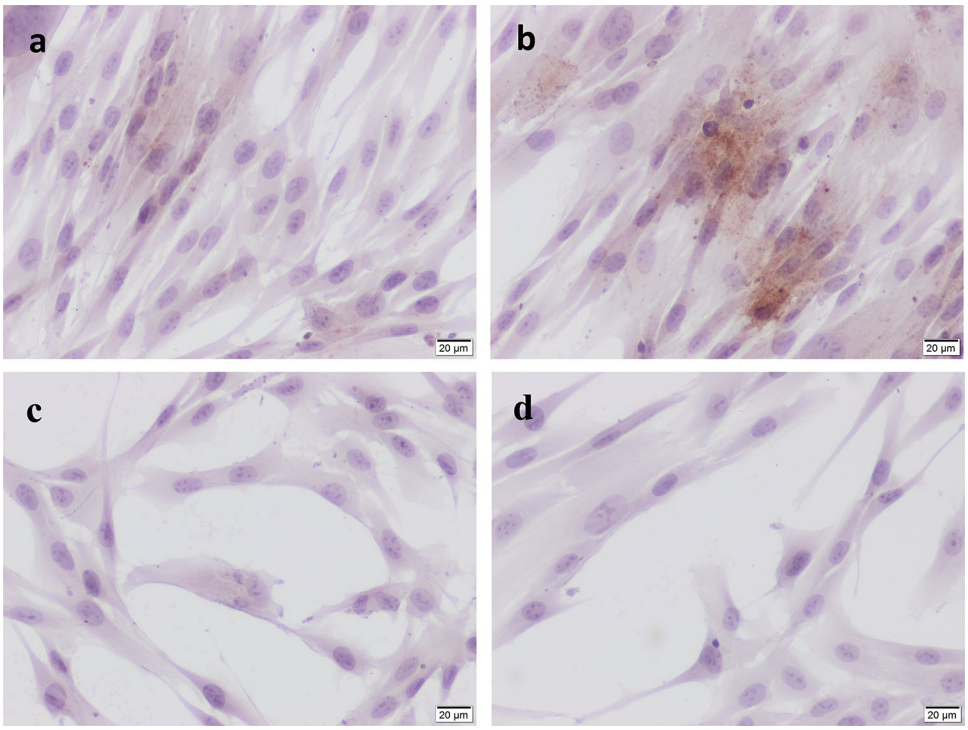

Fig. 2. Distribution of CD90 (**a**), collagen 1 (**b**), and CD34 (**c**) after immunocytochemistry in the HPDLF cells. Control immunocytochemistry staining was demonstrated in (**d**). Scale bars: 20 µm.

Results

Cell Culture and characterization of HPDLF cells

HPDLF cells were transferred to a culture dish, and the cells were observed to have a fibroblast-like structure, with flat, elongated, spindle-shaped cytoplasmic extensions (Fig. 1a). When the cells reached 80% confluency, they underwent several passages, and cells from the seventh passage (P7) were used for the further experiments (Fig. 1b).

The characterization of HPDLF cells was performed through the distribution of CD90, collagen 1, and CD34. According to the results of the immunocytochemical analysis, the isolated HPDLF cell samples showed positive labeling for CD90(+) and collagen 1 (+) (Fig. 2a and b respectively), while CD34 (-), a hematopoietic stem cell marker, exhibited negative labeling (Fig. 2c). The negative control staining was negative (-) (Fig. 2d).

Cell viability and cytotoxicity

When examining the 24 h results, it was found that in all dilutions, Totalfill BC Sealer had significantly higher cell viability than the other sealer groups (Fig. 3a). Also, it is observed that across all dilutions, AH Plus exhibited higher toxicity than all the other sealers, with the exception of MTA-Fillapex (Fig. 3a). The cell viability of the Sealapex in a 1:0 dilution was statistically higher than that of the Apexit Plus ($p = 0.010$) (Table 2a). Apart from

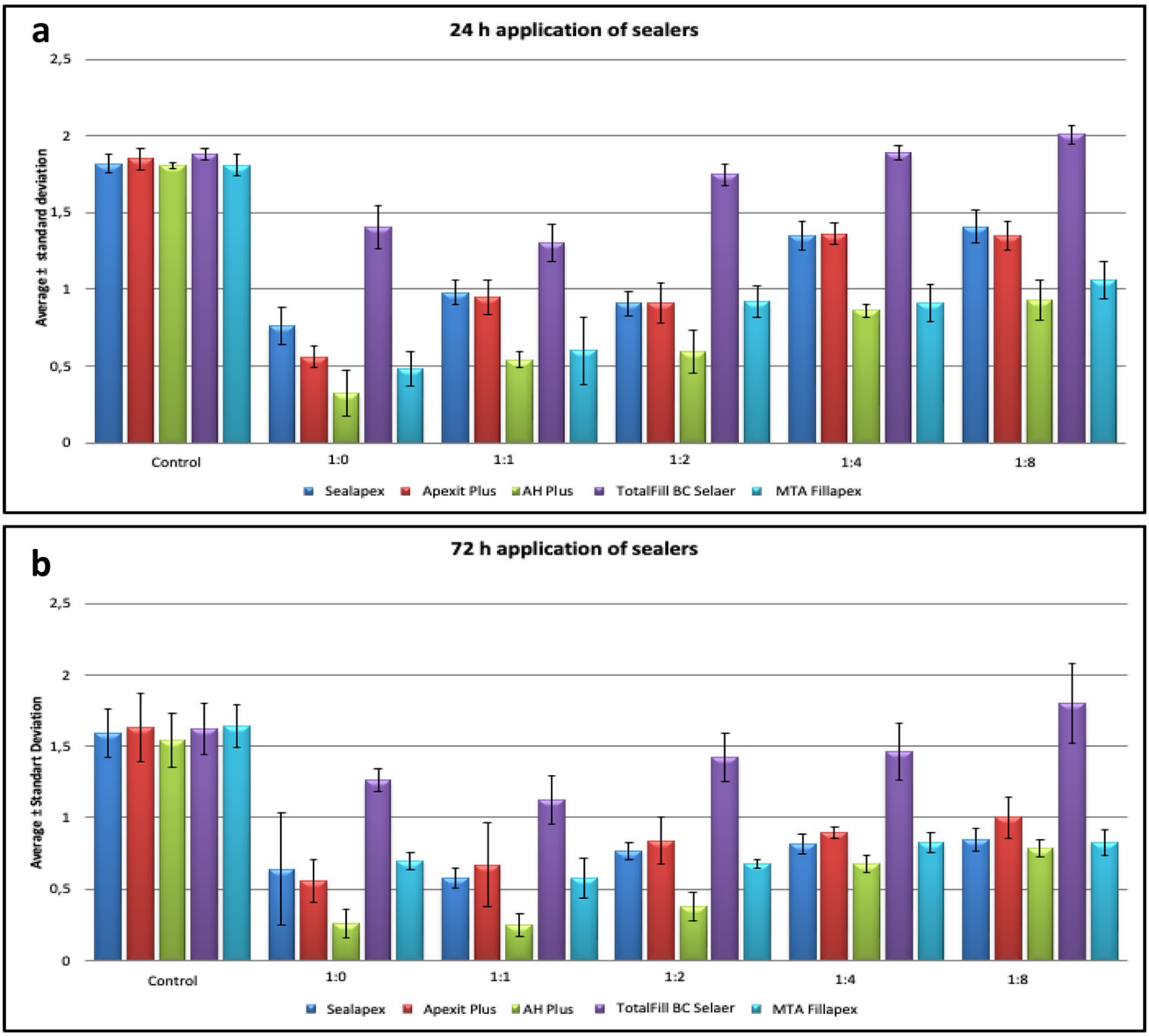

Fig. 3. Cell viability assay of different root canal sealers in HPDLF cells at 24 (**a**) and 72 (**b**) hours. Dose-response columns of different root canal sealers. Cell viability was quantitated by the MTT assay. HPDLF cells were incubated to different concentrations (1:0, 1:1, 1:2, 1:4 and 1:8) of Sealapex, Apexit Plus, AH Plus, TotalFill BC Sealer and MTA-Fillapex for 24 (**a**) and 72 (**a**) hours. Control group: Cells + Culture medium, MTT: 2,5-diphenyl-2 H-tetrazolium bromide.

this, Sealapex and Apexit Plus showed similar percentages of cell viability (Fig. 3a). MTA-Fillapex in the 1:2 and 1:8 groups had significantly higher cell viability than the AH Plus (respectively, $p = 0.006$ and $p = 0.045$, Table 2a). In other dilutions, the closest group to the high toxicity of AH Plus was MTA-Fillapex, which in most dilutions had significantly lower cell viability compared to the other sealers (Fig. 3a, Table 2a).

At 72 h, it was found that in all dilutions, the cell viability of the Totalfill BC sealer was significantly higher than that of other sealer groups ($p < 0.05$, Fig. 3b, Table 2b). Also, at 72 h, except for the 1:8 dilution of Sealapex and MTA-Fillapex, the AH Plus was more toxic than the other sealers in all dilutions (Fig. 3b, Table 2b). At 72 h, the Sealapex group showed similar viability percentages as both Apexit Plus and MTA-Fillapex (Table 2b). Apexit Plus exhibited higher viability in the 1:0 ($p = 0.037$), 1:2 ($p = 0.010$), and 1:8 ($p = 0.037$) dilutions compared to MTA-Fillapex, although in other dilutions, the cell viability appeared similar (Fig. 3, Table 2b).

As a result of the MTT assay, optimal dilutions for each sealer were determined for application in the TUNEL assay at 24 and 72 h. Accordingly, the concentrations were set as follows: 1:2 for Sealapex, 1:2 for Apexit Plus, 1:8 for AH Plus, 1:0 for Totalfill BC Sealer, and 1:4 for MTA-Fillapex.

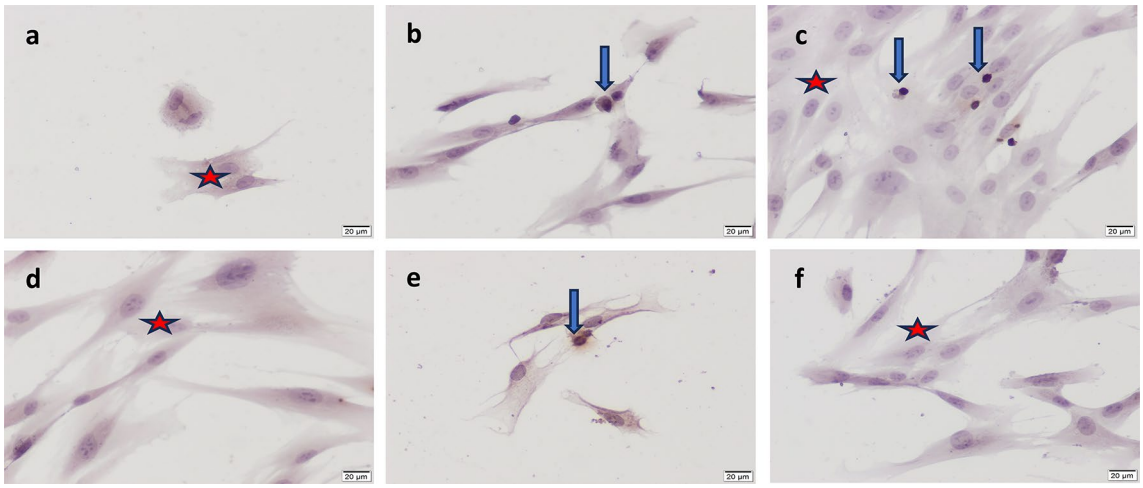

Fig. 4. Representative images of TUNEL staining HPDLF cells after Sealapex (**a**), Apexit Plus (**b**), AH Plus (**c**), TotalFill BC Sealer (**d**), MTA-Fillapex (**e**) sealers application for 24 h. Control TUNEL staining was demonstrated in (**f**). (arrow: apoptotic cells, star: healthy cells). Scale bars: 20 µm.

TUNEL assay evaluation

TUNEL positive cells were detected by brown staining of the nucleus of the cells. Statistically significant differences were found between the groups in terms of the 24 h TUNEL positive cell percentage of HPDLF cells (p:0.000; $p < 0.05$) (Fig. 4; Table 3). After conducting pairwise comparisons to determine the source of these significant differences, it was found that the 24 h TUNEL positive cell percentage levels of the AH Plus group was significantly higher compared to that of the Sealapex, Apexit Plus, TotalFill BC Sealer, MTA-Fillapex, and Control groups ($p < 0.05$), (Table 3). The 24 h TUNEL positive cell percentage of the control group were significantly lower than those of the Sealapex, TotalFill BC Sealer, and MTA-Fillapex groups ($p < 0.05$) (Table 3). No significant differences were found between the other groups ($p > 0.05$) (Fig. 4; Table 3).

Statistically significant differences were found in the 72 h TUNEL positive cell percentage of HPDLF cells between the groups (p:0.000; $p < 0.05$) (Fig. 5; Table 3). Following pairwise comparisons to identify which groups contributed to these significant differences, it was observed that the 72 h TUNEL positive cell percentage of the AH Plus group was significantly higher than that of the Sealapex, Apexit Plus, TotalFill BC Sealer, MTA-Fillapex, and control groups ($p < 0.05$) (Fig. 5; Table 3). The 72 h TUNEL positive cell percentage of the control group was significantly lower compared to the Sealapex, Apexit Plus, TotalFill BC Sealer, and MTA-Fillapex groups ($p < 0.05$) (Table 3). Additionally, the 72 h TUNEL positive cell percentage of the Apexit Plus group was significantly lower than that of the Sealapex group ($p < 0.05$). No significant differences were found between the other groups ($p > 0.05$) (Fig. 5; Table 3).

Discussion

The existence of HPDLF near the root suggests that the endodontic sealer plays a role in enhancing repair and biological sealing through mineralized tissue deposition in the apical foramen[25]. Therefore, to more accurately mimic the clinical environment in vitro, human periodontal ligament fibroblasts (HPDLFs) derived from primary teeth were selected as the cell type, considering their potential interaction with endodontic sealers in vivo. Additionally, the study by Khoshhal et al. indicated minor differences between the periodontal ligament cells of permanent and deciduous teeth[26]. As a result, we chose to use periodontal ligament cells derived from deciduous teeth. To confirm the mesenchymal origin of the cells utilized in this study as periodontal stem cells, surface antigens including CD34, CD90, and collagen 1 were assessed. Immunohistochemical analysis revealed positive staining for CD90 and collagen 1, while CD34 staining was negative. Based on these results, we conclude that the cells utilized in the study are periodontal ligament fibroblast cells of mesenchymal origin.

In cell culture studies, the most common test used for assessing cell viability and proliferation is the MTT assay[20]. Our study assessed the cytotoxicity of two different calcium hydroxide based sealers, Sealapex and Apexit Plus, using the MTT test. Both sealers revealed reduced viability in comparison to the control group regardless of dilutions and exposure times, giving similar results. The high pH of hydroxyl ions released by calcium hydroxide induces an initial degenerative response in cells, which is subsequently followed by the processes of mineralization and calcification[27]. The potential cause of the calcium hydroxide based sealer's initial moderate to severe cytotoxic effect could be attributed to the alkaline pH level of the calcium hydroxide[28]. The presence of amines in the epoxy base of calcium hydroxide based sealers could also potentially cause toxicity[29]. Specifically, the toxicity of these sealers may be related to isobutyl salicylate, polymethylene methyl salicylate in Sealapex, and silicone oil in Apexit Plus[30,31].

The MTT test results showed that AH Plus was found to have a considerably larger toxic effect than the groups of Sealapex, Apexit Plus, MTA-Fillapex, and TotalFill BC Sealer. This finding is consistent with previous studies, and the high toxicity of AH plus is likely related to its resin component, which is known to be highly

		Control	1:0	1:1	1:2	1:4	1:8
		p	*p*	*p*	*p*	*p*	*p*
a							
Sealapex	Apexit Plus	0.200	0.010*	0.522	0.873	0.423	0.337
	AH Plus	0.873	0.004*	0.004*	0.006*	0.004*	0.004*
	TotalFill BC Sealer	0.109	0.004*	0.004*	0.004*	0.004*	0.004*
	MTA-Fillapex	0.749	0.010*	0.025*	0.631	0.004*	0.004*
Apexit Plus	AH Plus	0.055	0.006*	0.004*	0.010*	0.004*	0.004*
	TotalFill BC Sealer	0.631	0.004*	0.004*	0.004*	0.004*	0.004*
	MTA-Fillapex	0.150	0.109	0.016*	0.873	0.004*	0.006*
AH Plus	TotalFill BC Sealer	0.060	0.004*	0.004*	0.004*	0.004*	0.004*
	MTA-Fillapex	0.522	0.109	0.337	0.006*	1.000	0.045*
Total Fill BC Sealer	MTA-Fillapex	0.055	0.004*	0.004*	0.004*	0.004*	0.004*
b							
Sealapex	Apexit Plus	0.810	0.337	0.262	0.522	0.055	0.037
	AH Plus	0.378	0.005*	0.004*	0.004*	0.010*	0.200
	TotalFill BC Sealer	0.873	0.045*	0.004*	0.004*	0.004*	0.004*
	MTA-Fillapex	0.631	0.055	1.000	0.078	0.748	0.749
Apexit Plus	AH Plus	0.200	0.025*	0.037*	0.004*	0.004*	0.016*
	TotalFill BC Sealer	0.873	0.004*	0.013*	0.004*	0.004*	0.004*
	MTA-Fillapex	1.000	0.037*	0.200	0.010*	0.055	0.037*
AH Plus	TotalFill BC Sealer	0.262	0.004*	0.004*	0.004*	0.004*	0.004*
	MTA-Fillapex	0.262	0.004*	0.004*	0.004*	0.010*	0.423
TotalFill BC Sealer	MTA-Fillapex	0.873	0.004*	0.004*	0.004*	0.004*	0.004*

Table 2. Post hoc evaluation results of 24 (a) and 72 (b) hours MTT levels in HPDLF cells of endodontic sealer groups and control group (cells + culture medium) at different dilutions. Mann Whitney U Test $*p < 0.05$.

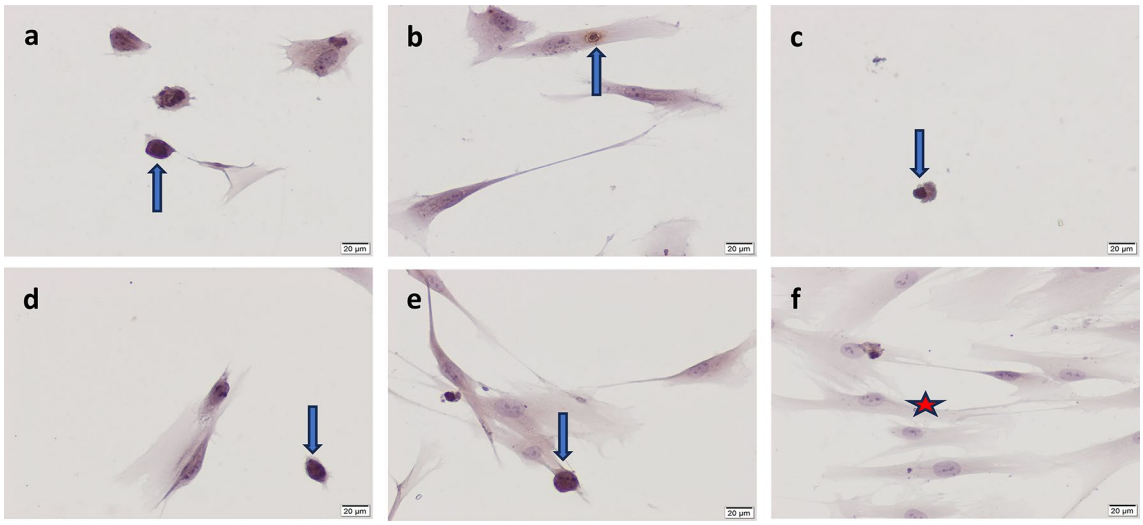

Fig. 5. Representative images of TUNEL staining HPDLF cells after Sealapex (**a**), Apexit Plus (**b**), AH Plus (**c**), TotalFill BC Sealer (**d**), and MTA-Fillapex (**e**) sealers application for 72 h. Control TUNEL staining was demonstrated in (**f**). (arrow: apoptotic cells, star: healthy cells). Scale bars: 20 µm.

toxic[9,32]. Additionaly, the presence of bisphenol-A and the low formaldehyde generated during polymerization may contribute to its toxic effects[9,23].

At all dilutions and contact periods in this investigation, MTA-Fillapex revealed lower viability than the control group. When the time-dependent cell viability of MTA-Fillapex was evaluated for significance, cell viability at 24 h was found to be lower than at 72 h at a dilution of 1:0. However, at dilutions of 1:2 and 1:8, cell viability at 24 h was found to be higher than at 72 h. Previous studies have also reported high cytotoxicity for MTA-Fillapex, including one that used L929 mouse fibroblasts[33,34].

The TUNEL assay, which marks DNA fragmentation associated with apoptosis, showed that the percentage of TUNEL-positive cells was significantly higher for the calcium hydroxide-based sealers Sealapex and Apexit Plus compared to the control group, although it did not exceed 50%. Studies have established that both calcium hydroxide and calcium ions have the ability to induce programmed cell death, also known as apoptosis[35,36]. Calcium hydroxide, with its elevated pH, destroys the cytoplasmic membrane, organic components, and nutritional mechanism in the early stages, resulting in cell death in surrounding tissue and the formation of a necrotic zone[37]. In our study, there may be a correlation between the moderate apoptotic effect of calcium hydroxide based sealers and the dual ability of calcium hydroxide to induce apoptosis and create necrotic regions[36,38].

TUNEL results also indicated that the AH Plus paste had the highest level of apoptotic staining in our study, which investigated relatively early effects like the 24th and 72nd hours. Long-term cytotoxicity studies revealed that AH Plus had a high initial cytotoxic effect, which decreased over time[39,40]. The diminishing toxic effects of AH Plus over time may be associated with the mechanism of cell death through apoptosis, because it is accepted that apoptosis does not cause an immunological response and that the tissue adapts to changes more quickly[22].

MTA-Fillapex was also highly apoptotic, second only to AH Plus in our study. Previous research using Annexin V analysis on the HT-1080 human fibrosarcoma cell line showed that calcium salicylate in MTA-Fillapex induced apoptosis within 24 h, with histological evidence of characteristic apoptotic features including cell blebbing, shrinkage, and the presence of fragmented genetic material[41]. The reason for the high cytotoxic and apoptotic effects observed in MTAFillapex can be explained by the fact that it is a resin salicylate-containing sealer[32,41].

It has been shown that the calcium phosphate components contained in TotalFill BC Sealer significantly change the physico-chemical properties of the tooth, developing a cellular response for the formation of bone-like mineral structures and increasing the ability to form hydroxyapatite[42]. In our study; TotalFill BC Sealer caused less cell death and apoptosis than all other endodontic sealers after 24 and 72 h and gave results close to the control group. Regarding biocompatibility, it performed the best when compared to other materials we tested in the study.

The limitation of this study is that in vitro experiments, which may not fully replicate the complex in vivo environment. Differences in setting times, drug concentrations, and cellular responses between in vitro and in vivo conditions can affect the generalizability of the results.

Conclusion

In our study, the following is the ranking of the lowest to most toxic endodontic sealers when compared generally in terms of toxicity: TotalFill BC Sealer, Sealapex, Apexit Plus, MTA-Fillapex, AH Plus. Nevertheless, in vitro study results may not always be parallel to in vivo studies and results obtained from clinical use. Choosing the material to be applied as an endodontic sealer requires not only knowledge of its biological characteristics but

TUNEL %			
		24 h	72 h
		p	*p*
Sealapex	Apexit Plus	0.199	0.024*
	AH Plus	0.004*	0.020*
	TotalFill BC Sealer	0.807	0.170
	MTA-Fillapex	0.411	0.376
	Control	0.045*	0.004*
Apexit Plus	AH Plus	0.004*	0.004*
	TotalFill BC Sealer	0.053	0.077
	MTA-Fillapex	0.076	0.128
	Control	0.514	0.035*
AH Plus	TotalFill BC Sealer	0.004*	0.004*
	MTA-Fillapex	0.003*	0.005*
	Control	0.004*	0.004*
TotalFill BC Sealer	MTA-Fillapex	0.310	0.627
	Control	0.023*	0.004*
MTA-Fillapex	Control	0.016*	0.006*

Table 3. Results of the post hoc evaluation for TUNEL positive cells after various endodontic sealer's application to HPDLF cells at 24 and 72 h. control group (cells+ culture medium). Mann Whitney U Test $*p < 0.05$.

also knowledge of its physical and chemical properties. More in-vivo and in-vitro research is required for reliable results.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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References

1. Galler, K. M., Grätz, E. M., Widbiller, M., Buchalla, W. & Knüttel, H. Pathophysiological mechanisms of root resorption after dental trauma: A systematic scoping review. *BMC Oral Health*. **21**, 1–14. https://doi.org/10.1186/s12903-021-01707-1 (2021).
2. Li, G. H. et al. Ability of new obturation materials to improve the seal of the root canal system: A review. *Acta Biomater.* **10**(3), 1050–1063. https://doi.org/10.1016/j.actbio.2013.10.017 (2014).
3. Hosseinpour, S., Gaudin, A. & Peters, O. A. A critical analysis of research methods and experimental models to study biocompatibility of endodontic materials. *Int. Endod. J.* **55**, 346–369. https://doi.org/10.1111/iej.13668 (2022).
4. Camilleri, J. et al. Sealers and warm gutta-percha obturation techniques. *J. Endod.* **41**(1), 72–78. https://doi.org/10.1016/j.joen.2014.10.002 (2015).
5. Vertuan, G. C. et al. Evaluation of physicochemical properties of a new root canal sealer. *J. Endod.* **44**(3), 501–505. https://doi.org/10.1016/j.joen.2017.11.015 (2018).
6. Komabayashi, T. et al. Comprehensive review of current endodontic sealers. *Dent. Mater. J.* **39**(5), 703–720. https://doi.org/10.4012/dmj.2020-012 (2020).
7. Shandilya, A. et al. Evaluation of the sealing ability of gutta percha with bioroot RCS, MTA fillapex and sealapex – an SEM study. *J. Pharm. Bioallied Sci.* **14**, 893–896. https://doi.org/10.4103/jpbs.JPBS_191_22 (2022).
8. Hoshino, R. A. et al. Physical properties, antimicrobial activity, and in vivo tissue response to Apexit Plus. *Materials*. **13**(5), 1171. https://doi.org/10.3390/ma13051171 (2020).
9. Erdogan, H., Yildirim, S. & Cobankara, F. K. Cytotoxicity and genotoxicity of salicylate- and calcium silicate-based root canal sealers on primer human periodontal ligament fibroblasts. *Australian Endodontic J.* **47**(3), 645–653. https://doi.org/10.1111/aej.12507 (2021).
10. Sheela, S., Nassar, M., AlGhalban, F. M. & Gorduysus, M. O. In vitro cytotoxicity and mineralization potential of an endodontic bioceramic material. *Eur. J. Dentistry*. **17**(02), 548–555. https://doi.org/10.1055/s-0042-1750358 (2023).
11. Agrafioti, A., Koursoumis, A. D. & Kontakiotis, E. G. Re-establishing apical patency after obturation with Gutta-Percha and two novel calcium silicate-based sealers. *Eur. J. Dentistry*. **9**(04), 457–461. https://doi.org/10.4103/1305-7456.149646 (2015).
12. Aminoshariae, A. & Kulild, J. C. The impact of sealer extrusion on endodontic outcome: A systematic review with meta-analysis. *Australian Endodontic J.* **46**(1), 123–129. https://doi.org/10.1111/aej.12401 (2020).
13. Drumond, J. P. S. Comparison of Postobturation Pain experience after apical extrusion of Calcium Silicate–and Resin–Based Root Canal Sealers. *J. Endod.* **47**(8), 1278–1284. https://doi.org/10.1016/j.joen.2021.05.012 (2021).
14. Özdemir, O. & Kopac, T. Cytotoxicity and biocompatibility of root canal sealers: A review on recent studies. *J. Appl. Biomater. Funct. Mater.* **20**, 22808000221076325. https://doi.org/10.1177/22808000221076325 (2022).
15. Souza, G. L. et al. Evaluation of apoptosis/necrosis and cytokine release provoked by three root canal sealers in human polymorphonuclears and monocytes. *Int. Endod. J.* **52**(5), 629–638. https://doi.org/10.1111/iej.13035 (2019).
16. da Silva, E. J. N. L., Zaia, A. A. & Peters, O. A. Cytocompatibility of calcium silicate-based sealers in a three-dimensional cell culture model. *Clin. Oral Invest.* **21**, 1531–1536. https://doi.org/10.1007/s00784-016-1955-1 (2017).
17. Naruishi, K. Biological roles of fibroblasts in periodontal diseases. *Cells*. **11**(21), 3345. https://doi.org/10.3390/cells11213345 (2022).
18. Jimenez-Bueno, I. et al. Cytotoxicity, differentiation, and biocompatibility of root-end filling: A comprehensive study. *Biomimetics*. **8**(7), 514. https://doi.org/10.3390/biomimetics8070514 (2023).
19. de Souza, L. C., Neves, G. S. T., Kirkpatrick, T., Letra, A. & Silva, R. Physicochemical and biological properties of AH plus bioceramic. *J. Endod.* **49**(1), 69–76. https://doi.org/10.1016/j.joen.2022.10.006 (2023).
20. Präbst, K., Engelhardt, H., Ringgeler, S. & Hübner, H. Basic colorimetric proliferation assays: MTT, WST, and resazurin. *Cell viability assays: methods and protocols*, 1–17 (2017). https://doi.org/10.1007/978-1-4939-6960-9_1
21. Gavrieli, Y., Sherman, Y. & Ben-Sasson, S. A. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* **119**(3), 493–501. https://doi.org/10.1083/jcb.119.3.493 (1992).
22. Mirzayans, R. & Murray, D. Do TUNEL and other apoptosis assays detect cell death in preclinical studies? *Int. J. Mol. Sci.* **21**, 9090. https://doi.org/10.3390/ijms21239090 (2020).
23. Candeiro, G. D. M. et al. Cytotoxicity, genotoxicity, and antibacterial effectiveness of a bioceramic endodontic sealer. *Int. Endod. J.* **49**(9), 858–864. https://doi.org/10.1111/iej.12519 (2016).
24. Islam, A. et al. In vitro cultivation, characterization and osteogenic differentiation of stem cells from human exfoliated deciduous teeth on 3D printed polylactic acid scaffolds. *Iran. Red Crescent Med. J.* **19**(8). https://doi.org/10.5812/ircmj.55593 (2017).
25. Rodríguez-Lozano, F. J. et al. Evaluation of cytocompatibility of calcium silicate-based endodontic sealers and their effects on the biological responses of mesenchymal dental stem cells. *Int. Endod. J.* **50**(1), 67–76. https://doi.org/10.1111/iej.12595 (2017).
26. Khoshhal, M., Amiri, I. & Gholami, L. Comparison of in vitro properties of periodontal ligament stem cells derived from permanent and deciduous teeth. *J. Dent. Res. Dent. Clin. Dent. Prospects*. **11**(3), 140. https://doi.org/10.15171/joddd.2017.026 (2017).
27. Desai, S. & Chandler, N. Calcium hydroxide–based root canal sealers: a review. *J. Endod*. **35**, 475–480. https://doi.org/10.1016/j.joen.2009.01.005 (2009).
28. Okamura, T. Biocompatibility of a high-plasticity, calcium silicate-based, ready-to-use material. *Mater*. **13**, 4770. https://doi.org/10.3390/ma13214770 (2020).
29. Eldeniz, A. U., Mustafa, K., Ørstavik, D. & Dahl, J. E. Cytotoxicity of new resin-, calcium hydroxide- and silicone-based root canal sealers on fibroblasts derived from human gingiva and L929 cell lines. *Int. Endod J.* **40**, 329–337. https://doi.org/10.1111/j.1365-2591.2007.01220.x (2007).
30. Yang, C. S., Chen, K. H., Hsu, W. M. & Li, Y. S. Cytotoxicity of silicone oil on cultivated human corneal endothelium. *Eye*. **22**, 282–288. https://doi.org/10.1038/sj.eye.6702591 (2008).
31. Chang, M. C. et al. Comparative cytotoxicity of five root canal sealers on cultured human periodontal ligament fibroblasts. *Int. Endod J.* **43**, 251–257. https://doi.org/10.1111/j.1365-2591.2010.01676.x (2010).
32. Sanz, J. L. et al. Cytocompatibility and bioactive potential of AH Plus Bioceramic Sealer: An in vitro study. *Int. Endod J.* **55**, 1066–1080. https://doi.org/10.1111/iej.13787 (2022).

33. Collado-González, M., Tomás-Catalá, C. J., Oñate-Sánchez, R. E., Moraleda, J. M. & Rodríguez-Lozano, F. J. Cytotoxicity of GuttaFlow Bioseal, GuttaFlow2, MTA Fillapex, and AH Plus on human periodontal ligament stem cells. *J. Endod*. **43**, 816–822. https://doi.org/10.1016/j.joen.2017.01.030 (2017).
34. Saygili, G., Saygili, S., Tuglu, I. & Capar, I. D. In vitro cytotoxicity of guttaflow bioseal, guttaflow 2, ah-plus and mta fillapex. *Iran. Endod J.* **12**, 354. https://doi.org/10.22037/iej.v12i3.15675 (2017).
35. Satchell, P. G., Gutmann, J. L. & Witherspoon, D. E. Apoptosis: An introduction for the endodontist. *Int. Endod J.* **36**, 237–245. https://doi.org/10.1046/j.1365-2591.2003.00638.x (2003).
36. Szczurko, G. et al. Effect of root canal sealers on human periodontal ligament fibroblast viability: Ex vivo study. *Odontology*. **106**, 245–256. https://doi.org/10.1007/s10266-017-0325-4 (2018).
37. Fridland, M. & Rosado, R. MTA solubility: a long term study. *J. Endod*. **31**, 376–379. https://doi.org/10.1097/01.don.0000157990.89594.54 (2005).
38. Zhivotovsky, B. & Orrenius, S. Calcium and cell death mechanisms: a perspective from the cell death community. *Cell. Calcium*. **50**, 211–221. https://doi.org/10.1016/j.ceca.2011.03.003 (2011).
39. Kumar, A. et al. Cytotoxicity evaluation of Bio-C, CeraSeal, MTA-Fillapex, and AH Plus root canal sealers by microscopic and 3-(4, 5 dimethythiazol-2yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. *J. Conserv. Dent.* **26**, 73–78. https://doi.org/10.4103/jcd.jcd_62_22 (2023).
40. Oh, H. et al. Comparison of biocompatibility of calcium silicate-based sealers and epoxy resin-based sealer on human periodontal ligament stem cells. *Mater*. **13**, 5242. https://doi.org/10.3390/ma13225242 (2020).
41. Mahdi, J. G., Alkarrawi, M. A., Mahdi, A. J., Bowen, I. D. & Humam, D. Calcium salicylate-mediated apoptosis in human HT-1080 fibrosarcoma cells. *Cell. Prolif.* **39**, 249–260. https://doi.org/10.1111/j.1365-2184.2006.00379.x (2006).
42. Al-Haddad, A., Che, A. & Aziz, Z. A. Bioceramic-based root canal sealers: A review. *Int. J. Biomater.* (2016). https://doi.org/10.1155/2016/9753210 (2016).

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Author contributions

İSCK.: writing—review and editing, writing—original draft, methodology, investigation, funding acquisition, validation. HK: formal analysis, methodology, writing—review and editing. HSV.: supervision, methodology, writing—original draft, funding acquisition, project administration, conceptualization, resources, visualization. FRE.: supervision, methodology, writing—original draft, funding acquisition, project administration, conceptualization, resources, visualization.

Declarations

Competing interests

The authors declare no competing interests.

Ethics declarations

This study was confirmed by the Ethics Committee of Istanbul University Faculty of Dentistry, protocol number 202.

Additional information

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