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Cytotoxicity and Oxidative Stress Induction by Root Canal Sealers in Human Periodontal Ligament Fibroblasts: An *in vitro* Study

Malgorzata Pawinska [a*], Grzegorz Szczurko [a], Elzbieta Luczaj-Cepowicz [b], Grazyna Marczuk-Kolada [b], Adam Holownia [c]

a Department of Integrated Dentistry, Medical University of Bialystok, Poland; b Department of Pedodontics, Medical University of Bialystok, Poland; c Department of Pharmacology, Medical University of Bialystok, Poland

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**Corresponding author*: Małgorzata Pawińska, Department of Integrated Dentistry, Medical University of Bialystok, Poland.

E-mail: malgorzata.pawinska@umb.edu.pl

ABSTRACT

Introduction: The aim of the study was to investigate the *in vitro* cytotoxicity, the profile of cell death, and the level of oxidative stress in human periodontal ligament fibroblasts (HPdLFs) after exposure to selected root canal sealers. **Methods and Materials:** Freshly mixed or set Endomethasone N (EN), RealSeal (RSEAL), Roeko Seal Automix (RSA), and Sealapex (SP) were incubated with HPdLFs. Fluorescein isothiocyanate (FITC)-annexin V (AnV) and propidium iodide (PI) staining followed by flow cytometry was used to identify the effects of the materials on cell viability and the profile of cell death. 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) with fluorescence-activated cell sorting was used to determine reactive oxygen species (ROS) formation in HPdLFs. Statistical analyses were performed using one-way ANOVA followed by post hoc tests, and significance was determined at $P<0.05$. **Results:** All materials reduced the viability of the cultured cells compared with the controls ($P<0.05$). Fresh SP and EN, and set RSA generated an increase of necrotic cells ($P<0.05$), whilst fresh RSEAL and RSA induced an elevation of apoptotic cells ($P<0.001$). Set RSEAL caused a rise in both apoptotic and necrotic cells compared with the controls ($P<0.05$). Fresh EN, RSEAL, and SP resulted in increased intracellular ROS generation compared with the negative control ($P<0.001$), whilst fresh RSA and all set materials were ineffective. **Conclusions**: This *in vitro* study showed us the materials tested were characterized by differentiated cytotoxic effects on HPdLFs. The fresh and set forms of sealers were capable of eliciting toxic action, inducing apoptosis and/or necrosis in HPdLFs. The toxic effects of fresh EN, RSEAL, and SP might have been due to the induction of oxidative stress in human periodontal fibroblasts. The cytotoxicity of RSA seemed to be related to the involvement of other mechanisms.

Keywords: Cytotoxicity; Endodontics; Periodontal Ligament; Root Canal Sealer

Introduction

The essential components of root canal obturation to establish a fluid tight seal are gutta-percha and an endodontic sealer . The main role of a sealer is to fill the irregularities of the root canal system and to bond the core material to the root canal dentin. The sealers currently used in clinical practice are based on various formulations such as zinc oxide-eugenol, calcium hydroxide, epoxy and methacrylate resin, silicone and bioceramic. Zinc oxide-eugenol based sealers [Pulp Canal Sealer (Kerr Dental EMEA, Bioggio, Switzerland), Endomethasone N (Septodont, Cedex, France), Tubliseal (Kerr Dental EMEA, Bioggio, Switzerland)] have been a standard in endodontics since their development, based on their long-term success. Calcium hydroxide materials [SealapexKerr (Dental EMEA, Bioggio, Switzerland), Apexit (Ivoclar Vivadent, Schaan,

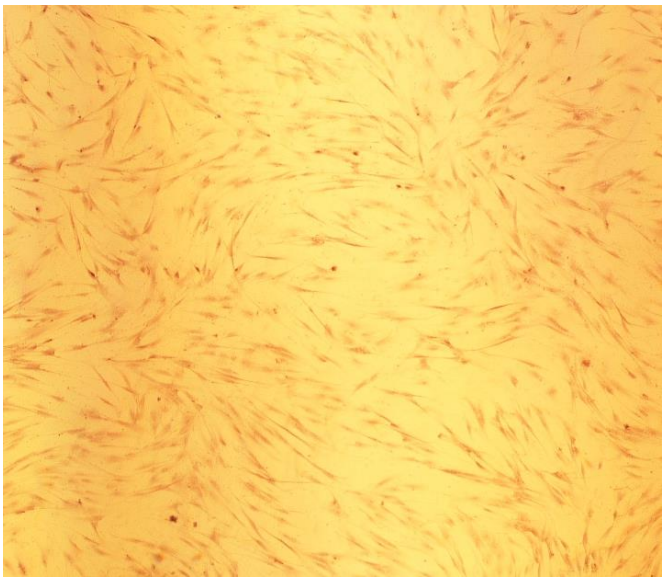


Figure 1. Human periodontal ligament fibroblasts cultured in Dulbecco's modified Eagle's medium (200× magnification)

Lichtenstein)] may promote hard tissue formation, but they tend to dissolve over time and may thus compromise the endodontic seal. Silicone-based sealers [Roeko Seal Automix (Roeko, Langenau, Germany), GuttaFlow (Coltene/Whaledent GmbH+Co. KG, Langenau, Germany)] may expand upon setting which may be beneficial. However, their sealing abilities are only mechanical and do not create a uniform bond at the dentin-sealer interface [1].

Methacrylate-based materials [RealSeal (SybronEndo Co.,West Collins, Orange, USA), Epiphany (Pentron, Wallingford, USA)] have been developed to improve adhesion and bondability of the core material to the root canal dentin. The application of these sealers in combination with a self-etching primer and Resilon (Pentron, Wallingford, USA), a thermoplastic polymer (polycaprolactone) with handling properties similar to gutta-percha, was to ensure the generation of a monoblock with the root canal walls [2]. Unfortunately, several undesirable properties of Resilon that have been discovered over time, including its susceptibility to degradation-hydrolysis by bacterial enzymes, lack of a true monoblock and sealer shrinkage, resulted in the material being withdrawn from the market [3]. Nevertheless, methacrylate-based sealers are still used and modified constantly.

Materials for permanent canal filling remain in contact with the periapical tissues for a long time; therefore, in addition to their sealing capacity, the biocompatibility of the obturating materials is decisive in the success of endodontic treatment [4, 5]. Even when the canal is properly filled, the sealer has contact with the adjacent tissues through the anatomical foramen, and sometimes the material penetrates the periapical tissues.

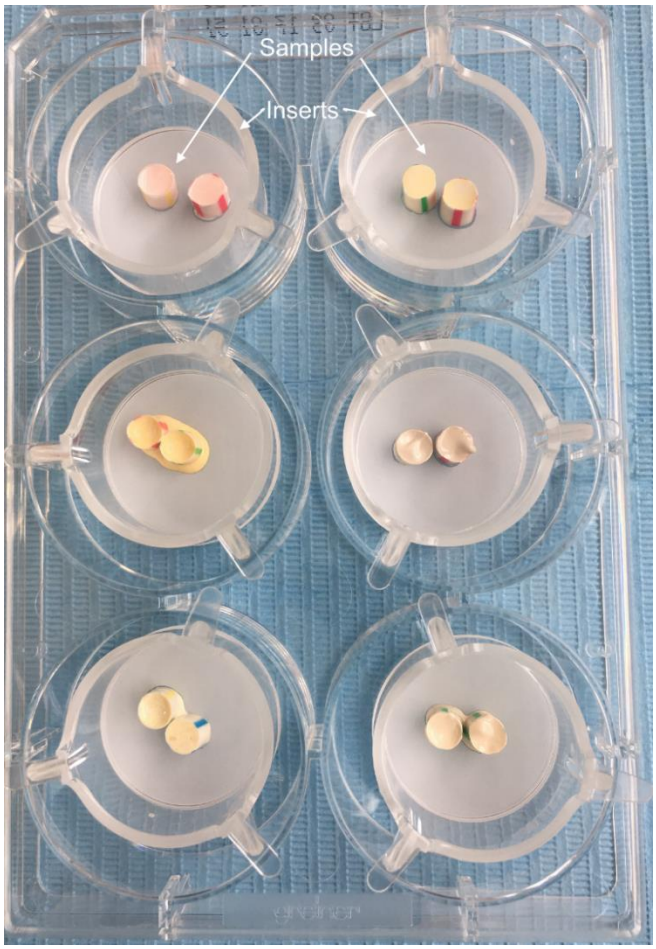


Figure 2. Fresh and set samples transferred into inserts, separating the sealer and establishing indirect contact with the material and the cells

Materials with toxic activity may damage tissues or hinder the healing of inflamed periapical structures [6, 7]. Thus, biocompatibility, which is the ability to trigger the proper histological and immune response of host tissues after the application of filling materials, is the main factor taken into account during material refinement.

One of the possible mechanisms that plays a role in the phenomenon of cytotoxicity of obturating materials is the induction of oxidative stress in tissues adjacent to the applied material [4, 5, 8]. Oxidative stress is caused by an imbalance between the formation of reactive oxygen and nitrogen species (RONS) and the body's antioxidant mechanisms. RONS released in physiological quantities act as mediators and regulators. Playing an important role in the modulation of intercellular communication processes, they participate in the processes of cell growth, proliferation, and differentiation, and thus ensure their proper functioning. However, RONS activity depends on their concentration and duration of action. In homeostasis conditions, RONS production and deactivation processes are under the body's

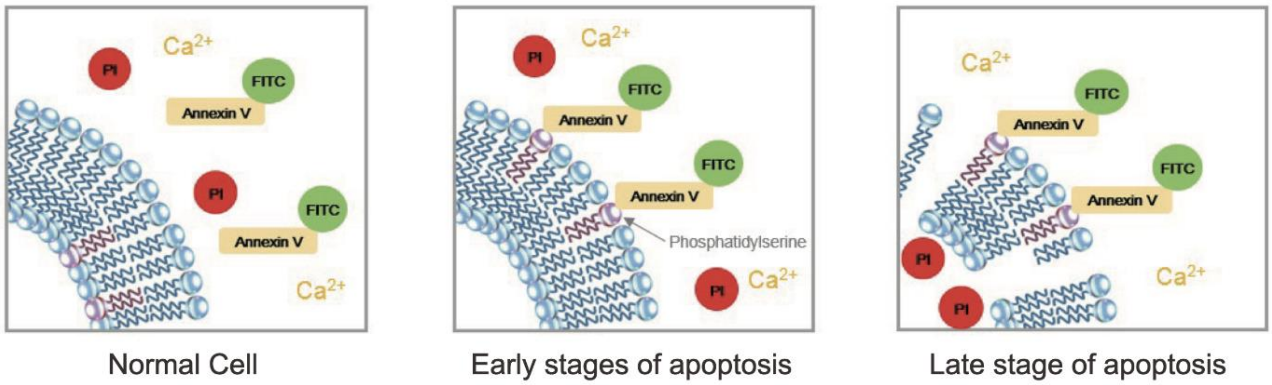


Figure 3. Analysis of the mode of cell death (apoptosis/necrosis) assessed by annexin V–FITC/PI apoptosis assay. In normal cells, phosphatidylserines (PS, membrane phospholipids) are held on the inner layer of the cell membrane, so Annexin V does not attach to the cells. During early apoptosis, the PS are exposed on the outer layer, where they attach to the FITC-labeled Annexin V and stain the cell surface green. During late apoptosis, propidium iodide (PI) enters the cell and stains the contents red [16]

strict control, as a result of enzymatic and non-enzymatic defense mechanisms [9]. A short-term increase in RONS production is well tolerated by cells due to antioxidant system activity. However, prolonged oxidative stress causes damage to cellular structures and can lead to cell death [9, 10]. The destructive action of reactive oxygen species can include all of the biomolecules found in the body, causing damage at the molecular and cellular level. Free radicals induce chemical modifications and damage of proteins (aggregation and denaturation), lipids (peroxidation), carbohydrates, and nucleotides, resulting in changes in the DNA structure leading to mutations or cytotoxic effects [9]. Additionally, it was proved that an increase in the level of oxygen free radicals induced by toxic substances can modulate the cell cycle and cause the activation of genes responsible for the production of proinflammatory cytokines, contributing to the progressive destruction of tissues [11, 12].

The issue of the toxicity of root canal filling materials seems to be interesting due a lack of detailed information on the mechanisms of their harmful effects on tissues in the literature.

The objective of the present study was to investigate the *in vitro* cytotoxicity, the mode of cell death, and the level oxidative stress exhibited by human periodontal ligament fibroblasts after exposure to selected root canal sealers.

Methods and Materials

Cell culture

Human periodontal ligament fibroblasts (HPdLFs) (Cell System HPdLF Cloneticsᵀᴹ, Lonza Walkersville, Inc., Walkersville, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Merk Life Science, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS) (Merk Life Science, Darmstadt , Germany), 100 µg/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO_2 (Figure 1). Confluent cells were detached with 0.25% trypsin solution supplemented with 0.53 mM ethylenediaminetetraacetic acid (EDTA). Enzyme activity was stopped by adding medium with 10% FBS. The cell suspension was diluted in fresh medium, seeded into 6-well culture plates, and incubated for 24 h.

Table 1. Root canal sealers used and their composition

Product	Manufacturer	Active ingredients
Endomethasone N (EN) Eugenol	Septodont, Cedex, France Chema –Elektromet, Rzeszów, Poland	zinc oxide, hydrocortisone octane, thymol iodide, barium sulphate, magnesium stearate eugenol
Roeko Seal Automix (RSA)	Coltene/WhaledentGmbH+Co. KG, Langenau, Germany	polydimethylsiloxane, silicone oil, paraffin oil, platinum catalyst, zirconia
RealSeal (RSEAL)	SybronEndo Co., West Collins, Orange, USA	Urethane dimethacrylate (UDMA), ethylene glycol polydimethacrylate (PEGDMA), ethoxyethylenebisphenol A dimethacrylate (EBPADMA), bisphenol A methacrylate (BIS-GMA), barium borosilicate, barium sulphate, bismuth oxychloride, calcium hydroxide, photoinitiators, diluted resins
Sealapex (SP)	Kerr Italia S.p.A., Salerno, Italy	calcium oxide, bismuth trioxide, zinc oxide, silica, titanium dioxide, zinc stearate, tricalcium phosphate, isobutyl salicylate, methyl salicylate

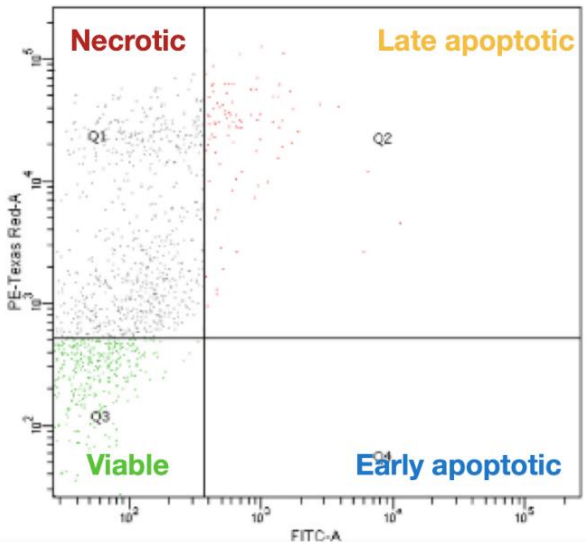


Figure 4. In a dual-parameter flow cytometry scatter diagram, if the X axis indicates Annexin V signal and the Y axis indicates PI signal, then the bottom left panel (Annexin V-/PI-) indicates live cells; the bottom right panel (Annexin V+/PI-) indicates early-stage apoptotic cells; the top right panel (Annexin V+/PI+) indicates late-stage apoptotic cells and necrosis cells; the top left panel (Annexin V-/PI+) is considered a detection error in the permissible range

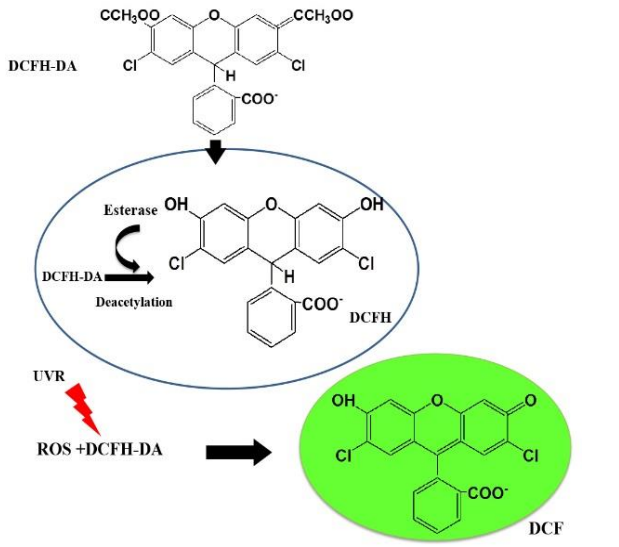


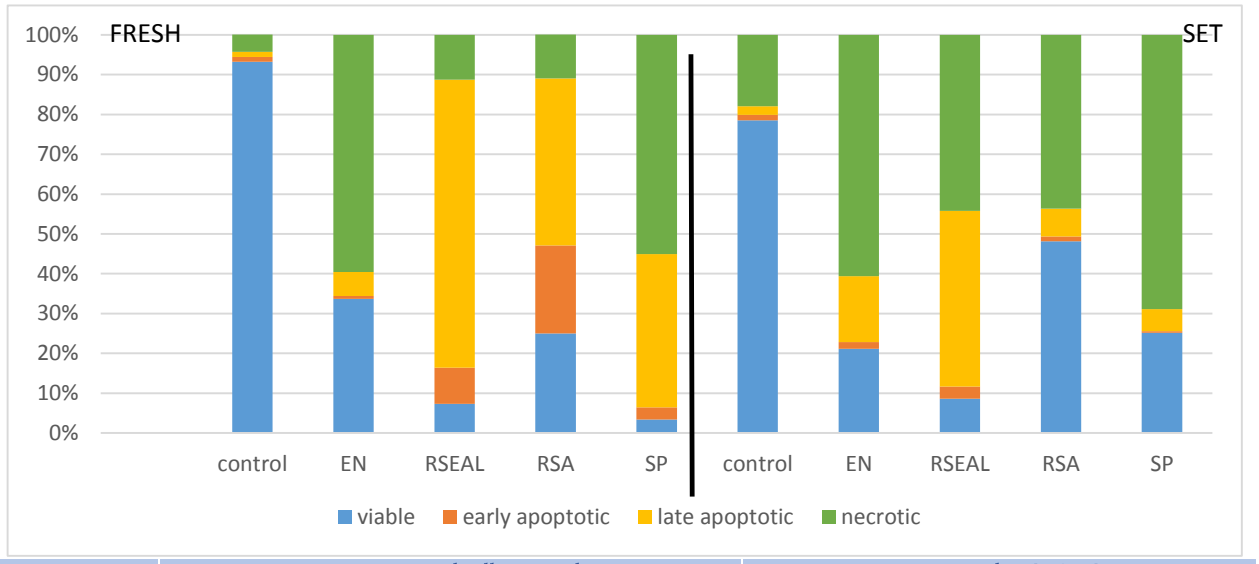
Figure 5. Mechanism of action of 2'7'-dichlorodihydrofluorescein diacetate (DCFH-DA) probe inside the cell (Adapted from He and Häder, 2002)

Sample preparation

Four root canal sealers were evaluated. The materials, product names, manufacturers, and components are listed in Table 1 [2, 13-15]. Under aseptic conditions, the sealers were mixed according to the manufacturers' instructions. Immediately after preparation, the materials were applied into plastic rings 5 mm (diameter) × 5 mm (height) in size to maintain equal volumes. Rings containing materials intended for setting (set samples) were stored at 37°C, 5% CO2, and 95% humidity for 24 h. Fresh formulations were mixed immediately before the experiment (fresh samples). Next, both groups of materials were transferred into inserts (surface area of 3.14 cm^2; 0.4-µm pore size) (Greiner Bio-One, Kremsmünster, Austria), separating the sealer and establishing indirect contact with the material and the cells (Figure 2). Then, the inserts were placed into 6-well culture plates (Nunc Biokom, Warsaw, Poland) and incubated with HPdLFs. Two samples were prepared for each material.

Cytotoxicity assay

Root canal material cytotoxicity and an analysis of the mode of cell death (apoptosis/necrosis) were assessed by annexin V–FITC/PI apoptosis assay and flow cytometry according to the manufactures' protocols. In normal cells, phosphatidylserines (PS) (membrane phospholipids) are held on the inner layer of the cell membrane, so Annexin V does not attach to the cells. During early apoptosis, the PS are exposed on the outer layer, where they attach to the FITC-labeled Annexin V and stain the cell surface green. During late apoptosis, propidium iodide (PI) enters the cell and stains the contents red [16] (Figure 3). HPdLFs were incubated with fresh and set root canal sealers, and core materials for 24 h. After incubation, the medium was aspirated using a Pasteur pipette (Sarstedt, Inc., Newton, NC, USA), and the cells were washed three times (3×1 mL) with phosphate-buffered saline (PBS) without calcium or magnesium (Polfa Lublin, Warsaw, Poland). HPdLFs were mechanically separated from the medium. The cells were suspended in buffer (HEPES/NaOH 10 mM, pH 7.4; 140 Mm NaCl; 2.5 mM $CaCl_2$), and 5 µL of Annexin-V-FITC and 10 µL of propidium iodide (Annexin V-FITC Apoptosis Detection Kit, Merk Life Science, Darmstadt, Germany) were added (Figure 3). Then the cells were incubated for 15 min at room temperature in the dark and subjected to fluorescence-activated cell sorting by using flow cytometry to determine the percentage distribution of cell populations [17, 18] (Figure 4). Untreated cell cultures served as the negative controls. FACS Canto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) equipped with two filters (488 nm excitation and 633 nm emission) was used for the dyes. At least 1000 counts were performed for each measurement [19]. The experiment was repeated twice. The flow cytometer was provided by the Faculty of Pharmacy with the Division of Laboratory Medicine at the Medical University of Bialystok.



Viable cells	*P*<0.001 control- all materials; *P*<0.05 EN- RSEAL, SP	*P*<0.001 control- RSEAL,SP,EN *P*<0.05 control-RSA, RSA-RSEAL,EN
Early apoptotic	*P*<0.05 control- RSA; RSA- EN,SP	No significant differences
Late apoptotic	*P*<0.001 control- RSEAL, RSA; RSEAL-EN *P*<0.05 control- SP; RSEAL-SP,RSA; EN-SP, RSA	*P*<0.001 control- RSEAL; RSEAL- SP,RSA *P*<0.05 RSEAL -EN
Necrotic	*P*<0.001 control- SP,EN; RSEAL-SP,EN; RSA-SP,EN	*P*<0.001 control-SP,EN; *P*<0.05 control- RSEAL,RSA; RSEAL-SP; SP-RSA
P<0.001; e *P*<0.05 statistically significant differences when compared fresh with set root canal sealers		

Figure 6. Effects of fresh and set root canal sealers on the viability of HPdLFs assessed using flow cytometry. The cytotoxicity was determined based on a comparison between the proportions of apoptotic, necrotic and viable cell fractions, following the exposure of HPdLFs to the tested materials. The cumulative diagram shows the percentage of necrotic, early and late apoptotic, and viable cells (with standard deviation; SD) EN: Endomethasone N, RSEAL: RealSeal, RSA: Roeko Seal Automix, SP: Sealapex, HPdLFs: Human Periodontal Ligament Fibroblasts

Measurement of reactive oxygen species (ROS)

The level of reactive oxygen species was measured by flow cytometry using the oxidation-sensitive fluorescent probe 2'7'-dichlorodihydrofluorescein diacetate (DCFH-DA) [20] (Figure 5). HPdLFs were incubated with fresh and set root canal sealers and core materials for 2 h. Then, the cells were stained with 20 µM of 2', 7'DCFH-DA reagent for 120 min at 37 ˚C in the dark, mechanically detached, washed twice with PBS, and suspended in buffered saline. Immediately after suspension, DCF fluorescence was determined by flow cytometry (COULTER® EPICS® XL™ Flow Cytometer) at an excitation wavelength of 495 nm and an emission wavelength of 525 nm. Mean DCF fluorescence intensities were obtained by histograms and used for data presentation [4]. At least 1000 events were acquired for each sample. Untreated cell cultures were used as the negative controls. HPdLF cells incubated for 24 h with arachidonic acid (60 µM) were used as the positive controls [21]. The experiment was repeated twice.

Statistical analysis

The obtained results were analyzed using the SPSS Statistics 21.0 (IBM) statistical package. One-way analysis of variance (ANOVA) supplemented with Tukey's post hoc test to assess the cytotoxicity of materials by flow cytometry (comparison of materials within each group) and Student's t-test for independent samples (comparison of materials between two groups) were used. In the statistical analysis of the level of intracellular reactive oxygen species, one-way ANOVA supplemented with the Kruskal-Wallis test for independent samples was used (comparison of materials within each group), while Student's t test for two averages was used to compare materials between two groups and to compare materials with the positive and the negative controls. A significance level of *P*<0.05 was set.

Results

Cytotoxicity assay

The results are presented in Figure 6 (fresh and set materials) and Figure 7 (dot plots of fresh and set materials).

Fresh materials (group I)

The 24-h exposure of HPdLFs to fresh root canal sealers resulted

in a significant reduction in the percentage of viable cells in all sealers compared with the control group (P<0.05). The lowest percentage of living cells was observed for Sealapex (SP) (3.4±0.28%), RealSeal (RSEAL) (7.35±4.17%), and Roeko Seal Automix (RSA) (25±1.56 %), with no significant differences between them (P>0.05). The highest percentage of living cells was noted for Endomethasone N (EN) (33.65±1.63%), and it was statistically significant in relation to RSEAL and SP (P<0.05). RSEAL and RSA induced significant growth in the percentage of apoptotic cells (P<0.05), while EN and SP significantly increased the proportion of necrotic cells in the cell culture compared with the other materials and the controls (P<0.001). Detailed statistical analysis of the results is presented in Figure 6.

Set materials (group II)

The 24-h incubation of HPdLFs with set materials resulted in a significant reduction in the proportion of viable cells compared with the control group, following the use of all formulations (P<0.05). The lowest cell survival was reported for RSEAL (8.6±7.07%), and then in ascending order for EN (21.15±9.55%) and SP (25.2±3.54%), with no significant differences between them (P>0.05). The highest percentage of viable cells, similar to that in the control group, was observed for RSA (48.15±0.78%), and it was statistically significant compared with RSEAL and EN (P<0.05). RSEAL induced a significant growth in the percentage of both apoptotic and necrotic cells (P<0.05). SP, EN, and RSA produced a significant increase in the proportion of necrotic cells in the cell culture compared with the controls (P<0.05). Detailed statistical analysis of the results is presented in Figure 6.

Comparison of fresh and set materials

The exposure of HPdLFs to the fresh materials resulted in a lower percentage of viable cells compared to the set materials with respect to RSEAL, SP and RSA, but the differences were statistically insignificant (P>0.05). A reverse, but also statistically insignificant, tendency was observed for EN (P>0.05). RSEAL, SP, and RSA generated a statistically significant percentage of apoptotic cells immediately after mixing and setting (P<0.05). A reverse relationship was found after the exposure of HPdLFs to EN in the necrotic cell population; however, the differences were statistically insignificant (P>0.05). Set RSEAL and RSA induced a significantly higher percentage of necrotic cells than fresh ones (P<0.001) (Figure 6).

Measurement of reactive oxygen species

The results of oxidative stress are presented in Figure 8 (fresh and set materials) and Figure 9 (dot plots of fresh and set materials).

Fresh materials (group I)

Exposure of HPdLFs to EN, RSEAL, and SP resulted in a statistically significant increase in intracellular ROS generation, detected by DCF fluorescence, in comparison with the negative control (P<0.001). Simultaneously, intracellular ROS level with EN, RSEAL, and SP was lower and statistically insignificant compared with the positive control (P>0.05). The largest elevation of ROS, by a factor of 2.7 (in relation to the negative control), was detected after cell exposure to EN, and it was statistically significant compared with RSA (P<0.05). There were no statistically significant differences in ROS induction between RSA and the negative control (P>0.05). However, there was a significantly lower increase of ROS in cell cultures incubated with RSA compared with the positive control (P<0.001) (Figure 8).

Set materials (group II)

HPdLFs exposure to all set materials produced significantly lower amounts of ROS from those found in the positive control (P<0.001). There were also no statistically significant differences compared with the negative control (P>0.05). Moreover, there were no statistically significant differences in intracellular ROS induction between individual sealers (P>0.05) (Figure 8).

Comparison of fresh and set materials

The results are shown in Figures 8. Fresh EN, RSEAL, and SP resulted in a statistically significant rise in DCF fluorescence, which is indicative of ROS generation in comparison with the set sealers (P<0.001). There were no statistically significant differences in intracellular ROS induction between fresh and set RSA (between RSA in fresh and set form) (P>0.05).

Discussion

In the present study, exposure of HPdLFs to both groups of materials resulted in a reduction in the percentage of viable cells compared with the control group for all preparations. Fresh SP and EN generated a rise in necrotic cells, while RSEAL and RSA induced an increase in the percentage of apoptotic cells compared with the control group and other materials. After setting, changes in the cell death profile were observed with regard to RSEAL and RSA.

Very few studies have pondered the question of whether the cytotoxic potential of root canal filling materials is related to apoptosis or necrosis. Therefore, it is difficult to compare our results with those of other authors.

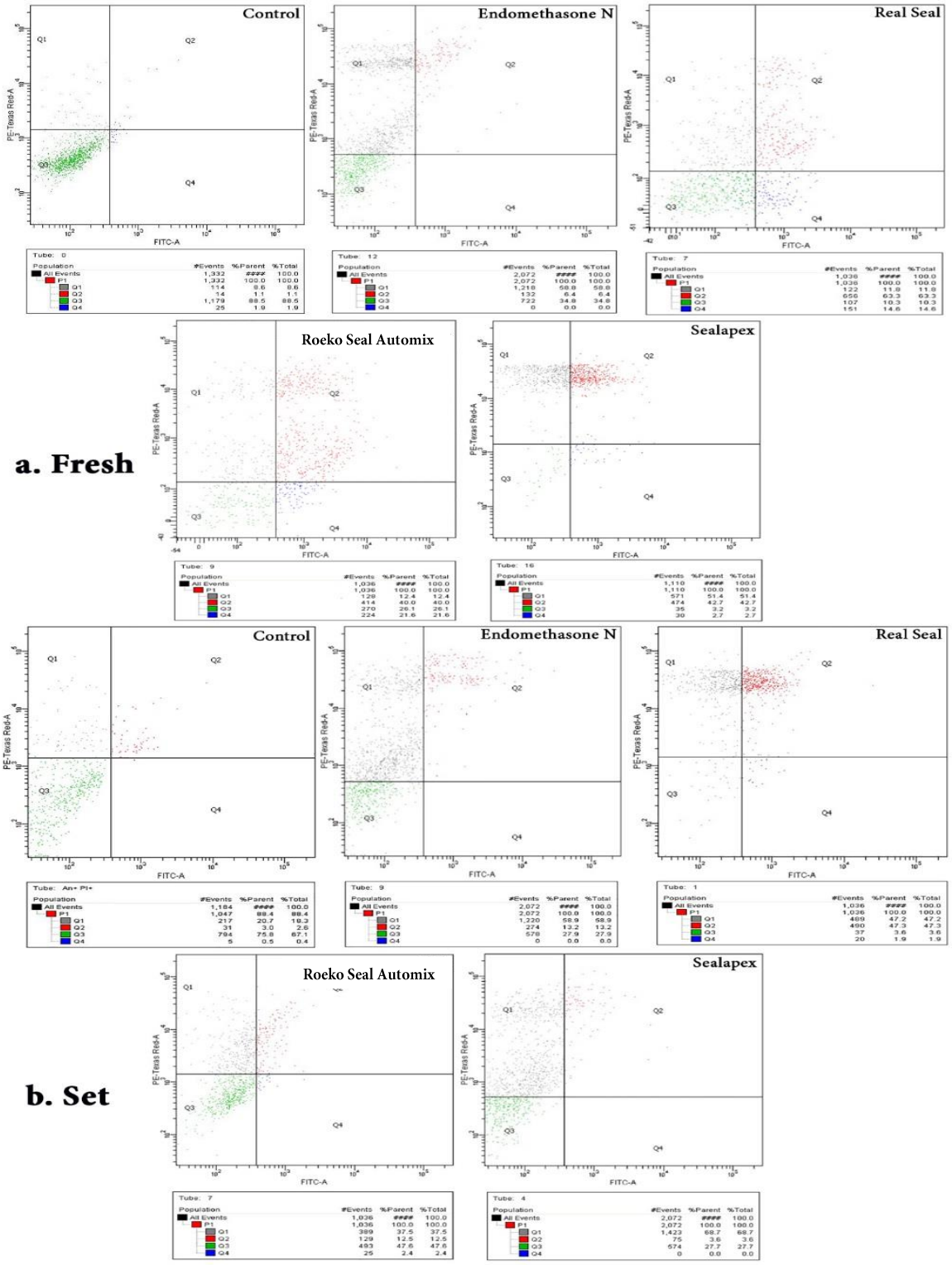


Figure 7.Representative 2-dimenshional dot plots of the flow cytometry data derived from FITC-AnV and PI-stained HPdLFs after 24-hour exposure to fresh materials (a) and set materials (b). The dot plot represented the distribution of viable (lower left), early apoptotic (lower right), late apoptotic (upper right), and necrotic (upper left), respectively. FITC-AnV: Annexin V bound to fluorescein isothiocyanate, PI: Propidium iodide, HPdLFs: Human Periodontal Ligament Fibroblasts

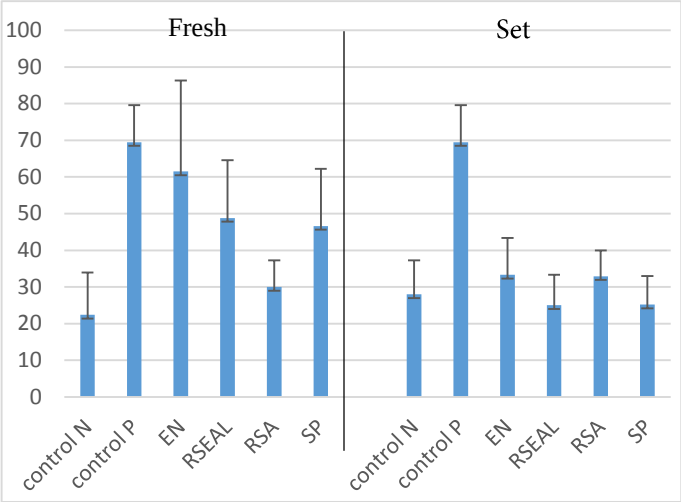


Figure 8. Effect of fresh and set materials on the mean percentage of intracellular ROS production in Human Periodontal Ligament Fibroblasts

Silva *et al.* [22] assessed the inflammatory response to subcutaneous Sealapex implantation in Balb/c mice, which corresponds to our *in vitro* test results. The authors observed necrosis of tissue directly in contact with SP, but only in the initial stages of the experiment. Al-Awadhi *et al.* [23] evaluated the effect of SP and RSA eluates on caspase-3 activity in osteoblasts in rats. The researchers did not notice a statistical difference in caspase activity, using a colorimetric method to identify apoptosis-specific enzymes. However, it is difficult to relate these results to your own experiment due to a completely different methodology.

A high percentage of necrotic HPdLFs occurred after contact with EN. The issue of ZnO/E sealer harmfulness is quite often addressed in the literature [6, 23, 24]. Eugenol [23, 25] and zinc oxide [26] are common components of sealers with documented cytotoxicity. The damaging effect of eugenol was confirmed by Araki *et al.* [27] , who showed that canals mixed with eugenol reduced the viability of human periodontal fibroblasts to a much higher degree than canals mixed with fatty acids. The cause of the harmful effects of zinc oxide is the release of Zn^{2+} ions, which induce an inflammatory reaction in the connective tissues and are already toxic at a concentration of 10 µg/mL [28]. Javidi *et al.* [29] suggested incorporating zinc oxide nanoparticles to decrease the cytotoxicity of zinc oxide-eugenol based sealers.

REAL Seal (also sold as Epiphany), which contains methacrylate resins, generated a high percentage of apoptotic cells (both in groups I and II) in our study. *In vitro* studies confirmed the damaging effects of the sealer on human and animal cell cultures [24, 30-32], which can last up to 6 weeks [33]. Bis-GMA(bisphenol A-glycidyl methacrylate) and UDMA (urethane dimethacrylate) monomers released from the material matrix, as well as incomplete polymerization of resin, may contribute to this state [34]. Silva *et al.* [18] assessed the cytotoxicity of RSEAL XT (SybronEndo, Orange, USA) and Sealapex Xpress (SybronEndo, Orange, USA) (an evolution of the well-known RSEAL and SP available as a base/catalyst system in automix syringes) 24 h after mixing using RAW 264.7 cells of the macrophage lineage. In a dilution of 1:20 for both materials, they noted a high percentage of apoptotic cells (91.5%), close to those obtained when the cells were kept in contact with a positive control (Staurosporine). At higher dilutions, the percentage of apoptotic cells decreased.

The fresh form of RSA induced an increase in the percentage of apoptotic cells. There are no studies in the literature describing the cell death profile after exposure to RSA, and the literature indicates the biocompatibility of the preparation [23, 34-36].

Toxic chemicals released from endodontic materials can affect the viability of periodontal ligament cells in the periradicular area and cause death through apoptosis and/or necrosis [37]. As a consequence of the increased number of cells in a necrosis state, modification of the inflammatory response occurs, leading to defects in the periapical tissues and an impairment of the healing process.The cytotoxicity of most materials is not permanent and decreases after setting or with time [38-42]. The components of freshly mixed sealers have a higher diffusion capacity than set ones [24]. However, materials used for filling root canals produce cytotoxic effects also in set form [25, 34], which was confirmed by our study.

The method considered the standard in the evaluation of material cytotoxicity in cell cultures is methylthiazol-tetrazolium colorimetric assay (MTT), which determines the state of cellular metabolism on the mitochondrial level. The MTT assay is crucial for comparing the strength of various materials in eliciting cell death. Nevertheless, it reveals cell death at the apoptosis stage when cell metabolism is significantly decreased [17]. Moreover, this assay is characterized by inadequate linearity with cell number and low reactivity to environmental conditions [43]. To identify the cytotoxicity of dental materials, flow cytometry (FC) has been used [17, 18]. FC provides a rapid and reliable method for quantifying viable and dead cells. It enables more precise assessment of the cytotoxicity of materials, with a description of the damaged cell death profile [17].

Cell death is controlled by numerous intracellular factors involving disturbing the cell oxidation-reduction balance [4, 5, 8]. According to Mouthuy *et al.* [10], the properties of materials, such as molecule size and shape, chemical composition, surface, porosity, and mechanical properties, can affect stress induction, but can also be modified by oxidative stress. Evaluation of the formation of intracellular ROS can help explain the cellular mechanisms of the cytotoxicity of materials for permanent root canal filling.

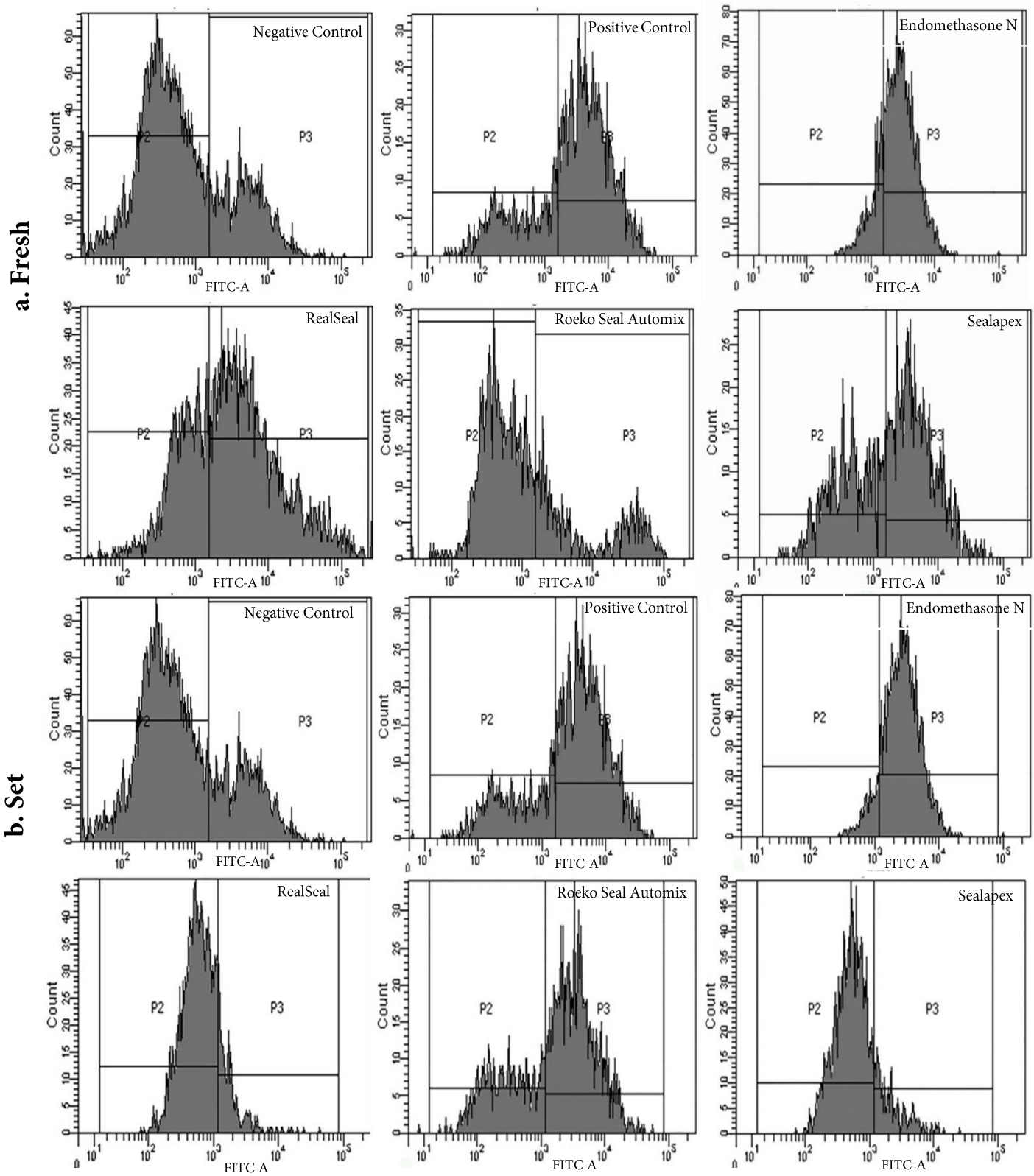


Figure 9. Representative histograms showing DCF distribution in HPdLFs after a 2-hour exposure to (a) fresh and (b) set materials. In most of the tested materials, a 2-population DCF fluorescence distribution was obtained. The number of cells in the right part of the histogram, in the P3 field, in comparison with the number of the control subpopulation, reflects the quantitative changes obtained as a result of the experiment, HPdLFs: Human Periodontal Ligament Fibroblasts, DCF: Dichlorodihydro fluorescence

In the conducted experiment, fresh materials based on methacrylate resins induced an increase in fibroblasts with Dichlorofluorescein (DCF) fluorescence, which was statistically significant. In the case of the fresh form of RSEAL, containing UDMA and BisGMA, a 2.7-fold increase in ROS level was observed with respect to the negative control group. These reports correspond with data available in the literature. Chang *et al.* [44] found that the exposure of pulp cells to the BisGMA monomer lead to an increase in the intracellular oxygen free radicals level. In addition, catalase, an enzyme that breaks down hydrogen peroxide, may prevent PGE_2 production induced by BisGMA. This suggests induction by BisGMA of the expression of COX-2 cyclooxygenase-2 (an enzyme involved in the production of mediators involved in the course of inflammation), the production of PGE_2 by the formation of hydrogen peroxide or other oxygen radicals arising in the pulp cells [44]. Di Nisio *et al.* [12] showed that HEMA (hydroxyethylmethacrylate) induced an inflammatory response in human gingival fibroblasts, modulated by ROS production and increased expression of TNF-α and COX-2 genes, and release of PGE_2.

Arun *et al.* [45] investigated the effect of the antioxidant, pachymic acid, on the cytotoxicity of four different sealers using the MTT assay. The addition of an antioxidant to L929 murine fibroblasts reduced the cytotoxicity of all materials (Tubliseal, AH-Plus, Sealapex), with the exception of EndoREZ containing methacrylates. In another study investigating mouse osteoblasts (MC-3T3 E1), Kim *et al.* [5] concluded that the use of pachymic acid also restored cell viability to the limit from before exposure to AH-Plus. (Dentsply Sirona,Konstanz, Germany)

Moreover, according to a study by Camargo *et al.* [4], exposure of pulp cells to extracts obtained from set resin-containing sealers, Epiphany and AH-Plus, increased oxygen free radical production, causing oxidative stress and disrupting cell function. Studying the effects of exposure to setting composites on pulp cells, Demirci *et al.* [46] registered a five-fold increase in the ROS amount, with greater stress being generated by bonds with enamel than dentin.

In the conducted study, apart from methacrylate sealers, the representative of zinc oxide-eugenol materials (EN) also induced a significant increase in the fibroblasts with DCF fluorescence. Eugenol has been described as a factor inducing oxidative stress in human submandibular gland cells (HSG) [47] and leukemia cells (HFL-60) [48]. Whereas, another study showed that eugenol (10-100 μmol/L) inhibited ROS induction in liver cells [49]. Discrepancies may result from the use of different cell populations, activation times, and incubation media in the experiments. To clarify the mechanism of eugenol' s effect on the intracellular production of reactive oxygen species, further research should be undertaken.

In this study, it was proved that in exposed cells fresh sealers induce a much higher level of ROS compared with set materials. Unfortunately, there are no publications focusing on this issue in the available literature. However, there are reports that oxidative stress induces apoptotic processes after exceeding the ability to maintain an oxidative balance [50, 51]. Beak *et al.* [52] confirmed the effect of nitric oxide, which induced apoptosis in human gingival fibroblasts. The setting resin-based endodontic sealers (Epiphany and RealSeal) also increased the cytotoxicity of human leukocytes, inducing apoptosis [32]. In our study, fresh and set forms of methacrylate sealer (RSEAL) generated apoptosis, but a statistically significant increase in the ROS amount, relative to the negative control, was observed only in contact with fresh materials. In contrast, EN and SP, which induced oxidative stress in the fresh forms, significantly increased the percentage of necrotic cells compared with the control group and other materials. This may indicate the participation of reactive oxygen species in the cytotoxicity process of some root canal sealers, mainly before they set.

Conclusions

Under the conditions of the present study, the root canal sealers were characterized by cytotoxic effects on human periodontal fibroblasts. The fresh and set forms of root canal sealers were capable of eliciting toxic action, inducing apoptosis and/or necrosis in human periodontal fibroblasts. The toxic effects of Endomethasone N, RealSeal, and Sealapex, tested immediately after mixing, might have been due to the induction of oxidative stress in human periodontal fibroblasts. The cytotoxicity of Roeko Seal Automix and materials after setting seemed to be related to the involvement of other mechanisms. Due to the potential cytotoxicity risk of the root canal sealers, clinicians should avoid apical overpreparation and subsequent root canal overfilling in obturation procedures to prevent or reduce possible periapical reactions.

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