Selective Apoptotic Effects of Resilon and Epiphany Se: In Vitro Investigation in Cell Cultures

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Abstract

Objective: The aim of this investigation is to examine the programmed cell death mechanism using Resilon, a root canal filling material, and Epiphany SE, a root canal sealant, in a microenvironment of cultured epithelial-like cells.

Methods: Vero cells were distributed into a 24-well plate at a density of 2 x 105 cells/ml per well. Following an incubation period of 18 hours, Resilon was applied to the cell monolayer. The monolayer was combined with Resilon and covered with a pre-warmed medium before being incubated at 37 ^{0} C in a 5% CO_2 incubator. Following this, the wells were treated with a 50% diluted eluate of Epiphany SE. Resilon and Epiphany SE were then left to incubate for 0.5, 1, 3, 6, 12, 18 and 24 hours at 37 ^{0}C. Data were analyzed in IBM SPSS V23. Statistical analysis involved determining the differences in means of the specimens using a one-way ANOVA, followed by Duncan's multiple range test to assess specific statistical differences across various time periods.

Results: It was found that between 0 and 6 hours, it was unclear whether Resilon and Epiphany SE induce apoptosis in Vero cells. Significant differences were observed 12 hours after examination comparing both the Resilon and Epiphany SE groups to the examination time prior to 12 hours ($p < 0.05$). These cells decreased at 18 and 24 hours. However, necrotic cells increased at each stage of the study period. The increase in the number of necrotic cells was statistically significant at 18 and 24 hours ($p < 0.05$).

Conclusion: It is inferred that necrosis of fibroblastic cells is caused by the cytotoxic effects of both Resilon and Epiphany SE. Hence, it can cause problems with root canal over obturation.

Keywords: Apoptosis, Resilon, Epiphany

IRB: In Vitro Study exempted from ethical approval by Zongul Bulent Ecevit university.

Citation: Hazar Bodrumlu E, Bodrumlu E, Yazc Z. Selective Apoptotic Effects of Resilon and Epiphany Se: In Vitro Investigation in Cell Cultures [Online]. Annals ASH & KMDC 2024;29(1): 19-26

(ASH & KMDC 2024;29(1): 19-26)

Introduction

One of the objectives of endodontic therapy is to fill the root canal system with a filling material that is impervious, biocompatible, and dimensionally stable[1]. The apical constriction of the root canal could be affected as a result of inflammatory resorption of the root apex or an incompletely developed root apex. Overfilling the root canals is a frequent complication and is related to overinstrumentation. Despite being biocompatible, overfilled materials can act as a foreign body and cause irritation of the periapical tissues, leading to failure of the endodontic treatment and severe postoperative complications and persistent inflammation[2-6]. Both sealers and endodontic filling materials have the potential to interact with periapical tissues[7].

Gutta-percha and a root canal sealer are frequently employed in the process of root canal filling. In contrast, Resilon core material and Epiphany SE, developed by Pentron Clinical Technologies in Wallingford, CT, USA, are commonly chosen as substitutes for gutta-percha and conventional root

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Date of Submission: 6th December 2024
Date of First Revision: 6th February 2024
Date of Acceptance: 26th February 2024

canal sealers. Resilon core material, characterized by its synthetic polymer composition, serves as a thermoplastic root canal filling material. On the other hand, Epiphany SE functions as a self-etched resin-based sealer, eliminating the requirement for the priming step. The response of the tissue to these materials bears significance as it could impact the long-term result of the endodontic procedure[1].

Several studies have investigated the cytotoxic impacts of root canal sealers on gingival and periodontal fibroblasts, osteoblasts, subcutaneous tissues, macrophages, and nerve cells using different techniques, such as cell culture, in vivo implantation in the subcutaneous tissues, in vitro nerve electrophysiological research, and histological analysis of periradicular tissues[8, 9].

Programmed cell death (PCD), or “apoptosis,” is the process by which individual cells in a multicellular organism undergo self-destruction in response to various stimuli[10].

Apoptosis also occurs in response to exposure to toxic dental materials. Hence, the analysis of apoptosis can be used as an additional surrogate marker for the cytotoxicity of dental materials[11].

This study aims to examine the PCD process in a microenvironment of cultured epithelial-like cells using Resilon, a root canal filling material, and Epiphany SE, a root canal sealant.

Methodology

African green monkey kidney-derived Vero cell lines were grown in Dulbecco’s minimum essential medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin. The cells were cultured under standard conditions at 37°C in a 5% CO2 atmosphere with 95% humidity. Experiments were conducted only with cultures that attained a minimum of 80% cell density, excluding those that did not meet this criterion.

The components of Resilon comprise polyester, methacrylate resin, 65% bioactive glass, bismuth oxychloride, and barium sulfate fillers. Epiphany SE is composed of a combination of HEMA, BISGMA, EBPADMA, and acidic methacrylate resins, silane-treated barium borosilicate glas-ses, Ca-Al-F-silicate, peroxide, photoinitiator, and pigment.

Epiphany SE was used in a freshly mixed state, following the usage guidelines provided by the manufacturer. Resilon points were added to the culture medium to achieve a concentration of 6 mg of Resilon/ml culture medium. Stainless steel devices (3 mm diameter and 1 mm high) (mold + extractor) specially designed for this study were used to place root canal sealer samples. These devices were sterilised and filled with freshly mixed sealer as per the manufacturer’s instructions. The number of samples to be used in the present study was determined to have 95% test power (1 - â), 95% confidence (1 - á), f = 0.677 effect size, and at least 10 samples in each of the subgroups with statistical power analysis calculated using the G *Power program. Vero cells were seeded into 24-well tissue culture plates (Greiner, Germany) at a concentration of 2 x 10^5 cells/ml per well. After 18 hours of incubation, Resilon was introduced into the monolayer of cells. The combination of the monolayer with Resilon was then covered with pre-warmed medium (DMEM containing 2 mM L-Glutamine and 2% FCS) and incubated in a 5% CO_2 incubator at 37 ^{0}C. The Resilon cell combination was kept in the medium for 0,5, 1, 3, 6, 12, 18, and 24 hours during incubation at 37 ^{0}C.

Additionally, a 50% diluted eluate in the cell culture medium was obtained from Epiphany SE in a parallel experiment and passed through a 0.45 nm Millipore filter. Vero cells were then seeded into a 24 - well plate. Then, 50% diluted Epiphany SE eluate was added to the wells and kept in the medium for 0,5 , 1, 3, 6, 12, 18, and 24-hour incubation times at 37^{0}C.

Cell death was determined and quantified using the traditional morphological criteria alongside functional vital dyes such as DAPI (4'-6-Diamidino-2-phenylindole, Serva, Germany) and PI (propidium iodide, Sigma, USA). DAPI is a stain that specifically binds to AT- and AU-rich regions of double-stranded DNA. This technique is based on time-sen

sitive factors and the varied permeability of cell membranes in living, deceased and apoptotic cells to two DNA dyes; propidium iodide (Sigma) and DAPI. Propidium iodide and DAPI are present in both dead cells, whereas living cells have the ability to expel both dyes, and apoptotic cells can reject propidium iodide but not DAPI. Cells were stabilized using a 96% ethyl alcohol solution at ambient temperature for five minutes. The fixing solution was removed and the cells were washed thrice using PBS. Afterward, the cells were stained with 0.5 µg/mL DAPI for five minutes at a temperature of 37°C and again washed three times utilizing PBS. Subsequent to DAPI staining, propidium iodide (50 µg/mL from a 1 mg/ml stock in PBS) was introduced right before viewing with fluorescent microscopy. Cells were viewed through a fluorescent light microscope (Olympus BHZ, RFCA, Japan) with an excitation wavelength of 330 nm and a barrier filter of 420 nm[12-14].

At least 200 cells were tallied in five distinct regions within each well.

The experiment was replicated thrice and categorized based on prior descriptions[15].

· Chromatin condensation, nuclear fragmentation, and smaller nuclear bodies are indicative of apoptotic cells whilst normal

· Nuclei and green pale chromatin are characteristic of live cells.

· Damaged cytoplasmic membranes and enlarged red nuclei, on the other hand, are indicative of necrotic cells.

The analysis of data was conducted using IBM SPSS V23. To assess the normal distribution of the data, the Shapiro-Wilk test was employed. Descriptive statistics, such as the percentage mean and standard deviation, are provided in the study. Statistical analysis included the use of a one-way ANOVA to determine differences in means among the specimens. Subsequently, Duncan's multiple range test was applied to assess specific statistical differences across different time periods.

Results:

The number of cells and the mean percentage of viable, apoptotic and necrotic cells for Epiphany SE and Resilon are shown in Table 1 and 2.

Table 1. The percentage of viable, apoptotic, and necrotic cells for Epiphany SE and Resilon in cytotoxicity testing.

Epiphany SE	Hours	Live Cell number (%)	Apoptotic cell number (%)	Necrotic cells (%)	P<
		$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	
	0	89.61 ± 1.07A,a	7.38 ± 0.40B,f	2.88 ± 0.26C,e	0.001
	0.50	82.33 ± 1.66A,a	8.66 ± 0.60B,f	8.05 ± 1.25B,e	0.001
	1	69.47 ± 5.50A,b	12.41 ± 0.04B,e	16.80 ± 2.09B,c,d	0.001
	3	58.93 ± 2.36A,c	24.69 ± 1.76B,c	14.66 ± 2.22C,d	0.001
	6	48.19 ± 1.63A,d	28.49 ± 0.33B,b	21.13 ± 1.32C,c,d	0.001
	12	43.47 ± 0.51A,d,e	33.55 ± 1.55B,a	22.19 ± 1.73C,c	0.001
	18	36.88 ± 2.41B,e	19.63 ± 0.58C,d	43.74 ± 1.70A,b	0.001
	24	27.11 ± 3.54B,f	17.66 ± 0.99B,d	56.08 ± 3.92A,a	0.001
	P Value	0.001	0.001	0.001	
Resilon	**Hours**	**Live Cell number (%)**	**Apoptotic cell number (%)**	**Necrotic cells (%)**	**P<**
		$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	
	0	90.72 ± 1.04A,a	5.95 ± 0.79B,e	3.04 ± 0.44C,e	0.001
	0.50	86.28 ± 1.75A,ab	7.25 ± 0.59B,e	5.77 ± 0.59B,ed	0.001
	1	80.97 ± 2.44A,b	11.28 ± 1.75B,de	8.72 ± 1.11B,d	0.001
	3	73,99 ± 2.80A,c	14.47 ± 2.48B,d	12.94 ± 2.88B,c	0.001
	6	65.11 ± 3.21A,d	20.91 ± 1.87B,c	14.58 ± 1.35B,b,c	0.001
	12	55.61 ± 1.11A,e	26.63 ± 1.56B,b	17.08 ± 1.13C,a,b	0.001
	18	48.46 ± 2.25A,f	33.08 ± 1.83B,a	18.86 ± 0.62C,a	0.001
	24	41.36 ± 1.66A,g	37.16 ± 2.44A,a	20.36 ± 0.51B,a	0.001
	P Value	0.001	0.001	0.001	

*A, B, C: No statistical difference was seen between the row with the same letter. (P > 0.05)

a,b,c,d,e,f,g: No statistical difference was seen between the column with the same letter. (P > 0.05)

Table 2. The mean number of viable, apoptotic, and necrotic cells for Epiphany SE and Resilon in cytotoxicity testing.

Epiphany Se	Hours	Live Cell number	Apoptotic cell number	Necrotic cells	P<
		$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	
	0	178.55 ± 1.78A,a	13.66 ± 1.20B,d	6.83 ± 1.52C,f	0.001
	0.50	165.10 ± 3.04A,a	17.23 ± 1.20B,d	17.49 ± 1.33B,ef	0.001
	1	130.77 ± 1.88A,b	23.72 ± 1.19B,d	33.27 ± 4.47B,cde	0.001
	3	117.94 ± 4.60A,bc	49.16 ± 3.37B,b	30.44 ± 4.71C,de	0.001
	6	96.38 ± 3.27A,cd	58.88 ± 1.66B,b	49.99 ± 3.80B,c	0.001
	12	86.94 ± 1.02A,d	77.55 ± 5.89B,b	44.11 ± 3.20B,cd	0.002
	18	74.44 ± 5.47A,de	35.94 ± 2.25B,c	78.49 ± 11.99A,b	0.014
	24	54.72 ± 6.27B,e	35.33 ± 1.98B,c	112.16 ± 7.83A,a	0.002
	P Value	0.001	0.001	0.001	
Resilon	**Hours**	**Live Cell number**	**Apoptotic cell number**	**Necrotic cells**	**P<**
		$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	
	0	181.00 ± 3.58A,a	10.67 ± 2.35B,e	6.09 ± 1.54C,e	0.001
	0.50	172.55 ± 6.07A,ab	14.50 ± 2.05B,e	11.55 ± 2.05B,de	0.001
	1	160.94 ± 7.34A,db	22.55 ± 6.08B,de	17.39 ± 3.80B,d	0.001
	3	148.00 ± 9.71A,dc	28.94 ± 8.60B,dc	25.89 ± 9.83B,c	0.001
	6	130.22 ± 11.14A,d	39.92 ± 7.89B,c	29.16 ± 4.67B,bc	0.001
	12	111.22 ± 3.87A,e	53.50 ± 5.49B,b	34.16 ± 3.93C,ab	0.001
	18	96.88 ± 7.80A,f	66.16 ± 6.35B,a	37.72 ± 2.17C,a	0.001
	24	82.72 ± 5.77A,g	74.33 ± 8.48A,a	40.16 ± 2.12B,a	0.001
	P Value	0.001	0.001	0.001	

*A, B, C: No statistical difference was seen between the row with the same letter. (P > 0.05)
a,b,c,d,e,f,g: No statistical difference was seen between the column with the same letter. (P > 0.05)

Resilon and Epiphany induced apoptosis in vero cells, which was not observed between 0 and 6 hours (Figure 1a, Figure 1b).

In the Resilon groups, apoptotic cells increased between 6 and 24 hours, while necrotic cells were detected at 3 hours. Significant differences were observed at 12 hours and later compared to the time point before 12 hours ($p < 0.05$). Morphological changes indicative of apoptosis were observed in 5.95% to 11.27% of cells treated with Resilon at 1 hour post inoculation (POI). At 6 hours POI, the percentage of treated cells increased to 20.91%. Viable vero cells decreased regularly between 0 and 24 hours, with significant differences observed compared to baseline times ($p < 0.05$). Apoptotic cells increased from 0.5 to 12 hours, but decreased at 18 and 24 hours. In contrast, necrotic cells increased at each stage of the study period, with a statistically significant increase at 18 and 24 hours ($p < 0.05$). The proportion of apoptotic cells rose from 26.23% at 12 hours to 33.08% at 18 hours and 37.36% at 24 hours (Figure 1c, 1d, and 1e).

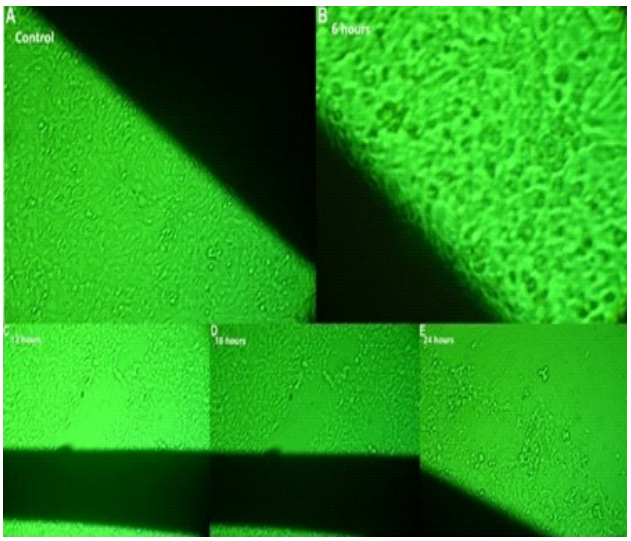

Fig 1a-1e: Images of viable, apoptotic, and necrotic cells at a) Control group b) 6 hours c) 12 hours, d) 18 hours, e) 24 hours

When Resilon was evaluated for necrotic cells, it was less than for apoptotic cells and was found to be 3.04%. The rates of necrotic cells at the end of 0,5, 1, 3, 6, 12, 18 and 24 hours were 5.77%, 8.71%, 12.94%, 14.58%, 17.08, 18.96% and 20.05% in target hours, respectively.

In the Epiphany SE groups, the number of cells that was 7.38% at 0 hours reached 28.49% at the end of 6 hours. The rates of apoptotic cells were 24.69%, 33.55%, 19.63% and 17.66% at the end of 3, 12, 18 and 24 hours respectively. Epiphany SE caused apoptotic changes on the cells, the tendency to increase at 3 and 12 hours was clear. A trend towards a decrease in the number of apoptotic cells was observed from the end of the 18th hour. When Epiphany was evaluated for necrotic cells, it was lower than for apoptotic cells and was determined to be 2.88%. The rates of necrotic cells were determined at the end of 0,5, 1, 3, 6, 12, 18 and 24 hours to be 8.05%, 16.80%, 14.66%, 21.13%, 22.19%, 43.74% and 56.08% in target hours, respectively.

Discussion

The success of endodontic treatment depends on the complete filling of the root canals. However, root canal paste and/or root canal filling material may overflow from the root canals and contact with the periapical tissues. This may cause inflammation in the periapical tissue. The biocompatibility of root canal filling materials plays an important role in root canal therapy. It is well known that root canal filling materials can cause local and systemic adverse effects due to the release of extractable monomers and other organic and inorganic components[16]. Direct contact of these obturation materials with the periradicular tissues can cause cellular necrosis and delayed periradicular tissue healing. Numerous investigations employing diverse testing approaches and materials have been conducted to assess biocompatibility. Primary methodologies in these studies involve cytotoxicity assessments on cellular or tissue cultures, along with implantation into subcutaneous connective tissue or bone within experimental animals[17]. The dental field needs to acknowledge that the significance of biocompatibility is on par with the consideration of physical and chemical properties when choosing materials for root canal obturation. Currently, cell culture systems are used to study the toxicology of various biological materials.

Cell culture is a rapid and relatively inexpensive method of assessing the cytotoxicity of dental materials. Cell culture studies are the first step in assessing the response of cells to root canal obturation materials[17]. Biological responses to these materials may include apoptosis and necrosis. In this study, the cytotoxic effect of the polymers contained in the restorative materials Resilon and Epiphany SE was investigated in Vero cell cultures and their apoptotic and necrotic changes in vitro and morphological effects were assessed.

Apoptosis, the most important cytotoxic change, is the death of cells associated with various chemical substances, toxic substances and infectious agents. Necrosis can occur due to cell damage caused by various traumatic events.

In this study, the healthy cell count was 90.72% - 73.99% between 0 and 3 hours after administration of Resilon. In the first 3 hours, the cell count indicates a high healthy population, but in the next 6 hours there is a decrease in the healthy rate. In the first 3 hours, the rates of both apoptotic and necrotic cells are low. This may be physiologically normal. At the end of 3 hours, the apoptotic and necrotic cell rates were 14.47% and 12.94% respectively. And then an increase begins. The apoptotic effect of Resilon was found to be 37.16% after 24 hours, and this increased at a higher rate than the rate of increase in necrotic cells, which rose to 20.35%. This situation indicates that Resilon has a cytotoxic effect, albeit a small one. The role of apoptosis is greater than that of necrosis in the cytotoxic effect of Resilon.

Baraba et al. documented that Epiphany sealer triggered cell death by provoking substantial apoptosis within 24 hours. The primer and thinning resin components of resin-based root canal sealers, as well as their combinations, exhibited cytotoxicity and induced apoptosis. However, the sealants did not exhibit a significant impact on the viability of human leukocytes[18]. Eldeniz et al. discovered that Epiphany demonstrated high cytotoxicity towards both human gingival fibroblasts and L929 cells, resulting in less than 30% viable cells after a four-hour treatment. The authors attribute this cytotoxicity to the combination of Epiphany's dual-cure methacrylate resin sealer and a formulation containing bisphenol A glycidyl methacrylate (BisGMA), ethoxylated BisGMA, urethane dimethacrylate (UDMA), and a hydrophilic blend of difunctional methacrylates. Additionally, the presence of residual monomers in both fresh and aged samples might contribute to the observed cytotoxicity of this sealer[19]. The results of these studies are consistent with the results of the present study. The tests with Epiphany SE showed that the increase started at hour 3 and peaked at hour 24. Epiphany SE caused a reduction in the amount of healthy live cells in the 0 -3 hour period more than Resilon, and the healthy live cell ratio was found to be 90.72 - 58.93%. At the end of the third hour, the apoptotic and necrotic cell ratios were found to be 24.69% and 14.66% respectively. These ratios were higher than the effects of Resilon during the same period. At the end of the 24th hour, the necrotic cell ratio was 56.08% and the apoptotic cell ratio was 17.66%. According to these data, Epiphany SE had a greater cytotoxic effect than Resilon, and the cytotoxic changes occurred as necrosis rather than apoptosis. This suggests that Epiphany SE may induce inflammation when in contact with tissue. This higher cytotoxic effect of Resilon was expected, mainly due to the presence of polyester and barium sulphate in the Resilon tips. In addition, the cytotoxic effect of Epiphany SE is related to the mixture of HEMA, BISGMA, EBPADMA and acid methacrylate resins. In addition, Resende et al. showed that the solubility of Epiphany was higher than that considered acceptable, allowing the release of toxic substances. The solubility of Epiphany were greater than values considered acceptable, with higher amounts of calcium ion release. Epiphany SE revealed more organized, compacted, and homogeneous polymers in a reduced resin matrix when compared with the other groups.[20]. The high cytotoxicity of both unpolymerised resin-based sealants could be due to leaching of filler particles[21] or release of unreacted monomers[22]. These cytotoxic reactions may explain the results obtained in the present study.

Although this morphological study demonstrates the cytotoxic effects of Resilon and Epiphany SE, causing necrosis of fibroblastic cells, no studies to date have investigated the molecular changes that occur in the periradicular tissues of these materials. Molecular and long-term clinical studies in patients are needed to evaluate the in vivo responses when Resilon and Epiphany SE are extruded into the periradicular tissue. The optimal material for root canal treatment should not be harmful to the periapical tissues. It is important to note that these materials should be non-toxic to promote successful root canal treatment. In this histological study, the cytotoxic effect of Epiphany and Resilon should be taken into account and care should be taken not to over obturation the root canals.

Conclusion

It is inferred that necrosis of fibroblastic cells is caused by the cytotoxic effects of both Resilon and Epiphany SE. Hence, it can cause problems with root canal over obturation.

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