Original Article

Comparative Evaluation of Cytotoxicity of Fluoride Varnish as Root Canal Sealer against L929 Mouse Fibroblasts with Conventional Endodontic Sealers

Erfan Hosseini [1], Mahdi Lomee [2], Jamshid Yazdani Charati [3], Abolfazl Hosseinnataj [4], Salma Omidi[5, 6]

[1] Private Practice, Nushahr, Iran
[2]Endodontist, Sari, Iran
[3] Professor, Department of Biostatistics, Health Sciences Research Center, Addiction Institute, Mazandaran University of Medical Sciences, Sari, Iran
[4]Assistant Professor,Department of Biostatistics ,Faculty of health,Mazandaran University of Medical Sciences,Sari, Iran
[5]Assistant Professor, Department of Endodontics, Dental research center, Mazandaran University of Medical Sciences, Sari, Iran
[6] Faculty of Dentistry, Mazandaran University of Medical Sciences, Sari, Iran

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Abstract

Introduction: This study aimed to assess the cytotoxicity of Duraflur fluoride varnish as root canal sealer against L929 mouse fibroblasts in comparison with four commonly used conventional endodontic sealers in the first 48 hours of exposure. **Methods:** In this in vitro, experimental study, L929 mouse fibroblasts were exposed to 1/1, ½, ¼ , and 1/8 concentrations of Duraflur fluoride varnish, AH Plus, Fill Canal, MTA Fillapex, and AH26 sealers. After 48 hours, the methyl thiazolyl tetrazolium (MTT) assay was performed to assess the cytotoxicity of sealers. Cell viability was determined as the percentage of viable cells compared with the control group. The results were analyzed using one-way ANOVA followed by the Tukey's post hoc test for multiple comparisons.. **Results:** MTA Fillapex had the lowest and AH26 had the highest cytotoxicity ($P<0.05$). Fluoride varnish showed high cell viability in 1/8 concentration (91.09%). Its cytotoxicity was close to that of AH Plus with no significant difference ($P=0.49$) but it had higher cytotoxicity than Fill Canal and Fill Apex ($P<0.05$). Fluoride varnish in 1/1 and 1/8 concentrations showed significantly higher cell viability than AH26 ($P<0.001$). **Conclusion:** Fluoride varnish sealer has acceptable biocompatibility comparable to that of conventional sealers. It has lower cytotoxicity than AH26.

Keywords: Cytotoxicity; Endodontic Sealers; Fluoride Varnish; Methyl Thiazolyl Tetrazolium Assay

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Introduction

Complete debridement of the root canal system and its optimal filling with biocompatible root filling materials are imperative for a successful endodontic treatment(1). Gutta-percha is currently the most suitable root canal filling material (2). However, it cannot bond to canal walls. Thus, the gap between the gutta-percha and root canal walls should be filled with endodontic sealers (1-3). However, after completion of root canal treatment, sealers in contact with the periapical tissue may cause tissue irritation and lead to the development of apical periodontitis. Thus, root canal sealers must have optimal biocompatibility and should be well tolerated by the periapical tissue (4).

Several sealer types with advantages and disadvantages and variable physical and biological properties are available in the dental market. Resin-based sealers, zinc-

oxide eugenol (ZOE)-based sealers, calcium hydroxide-based sealers, glass ionomer-based sealers, and silicon-based sealers are among the most common sealer types. AH Plus is currently the gold-standard sealer due to its optimal bonding to dentin and provision of a hermetic seal. Thus, it commonly serves as the standard reference sealer in order to compare with other sealer types (5).

MTA Fillapex is another sealer with the basis of mineral trioxide aggregate, which releases calcium and creates an alkaline environment that enhances hard tissue mineralization. Fill Canal is a ZOE-based sealer, which has long been used in endodontic treatment of teeth (6).

Fluoride varnish has several dental applications and is commonly used as a preventive measure to control dental caries (7, 8). It is also used for the treatment of avulsed teeth with delayed replantation (9), treatment of dentin hypersensitivity(10, 11), and as a root filling material in primary teeth in combination with calcium hydroxide and zinc oxide (12).

Recently, use of fluoride varnish as an endodontic sealer has been recommended owing to its optimal bonding and antibacterial properties (13, 14). Accordingly, Parirokh et al.(1) compared the cytotoxicity of Duraflur fluoride varnish with that of AH26 and AH Plus sealers and found that Duraflur had better compatibility compared with AH26. AH Plus and Duraflur had no significant difference in terms of cell viability.

Duofluoride XII (FGM, Joinville, SC, Brazil) is a dual fluoride varnish (2.92% fluorine, calcium fluoride + 2.71% fluorine, sodium fluoride, FGM) with proven efficacy for prevention of dental caries (15) and good dentinal adaptability (14). When a new dental material is introduced, its biocompatibility should be determined. Any endodontic sealer must remain compatible with periapical tissues during long-time contact (4).

Considering the novelty of use of fluoride varnish as an endodontic sealer and the significance of finding an ideal sealer to maximize the success rate of endodontic treatment, this study aimed to assess the cytotoxicity of Duofluoride XII (FGM) fluoride varnish in comparison with that of four commonly used conventional sealers namely MTA Fillapex, AH Plus, Fill Canal, and AH26 using the methyl thiazolyl tetrazolium (MTT) assay in the first 48 hours of exposure.

Materials and Methods

In this in vitro, experimental study, L929 mouse fibroblasts with normal proliferation and no fungal or bacterial contamination were obtained from the Pasteur Institute of Iran.

The sample size was determined to be two 96-well plates, and 16 wells were allocated to each sealer.

Preparation of sealers:

Duofluoride XII fluoride varnish (FGM, Joinville, SC, Brazil), AH26 (DeTrey Dentsply, Switzerland), AH Plus (Dentsply, Germany), MTA Fillapex (Angelus, Brazil) and Fill Canal (Technew, Portugal) sealers were evaluated in this study. The powder and liquid of the conventional sealers were mixed according to the manufacturers' instructions such that after mixing, the sealers had to stretch for 1 inch on a glass slab.

Preparation of culture medium:

For the preparation of culture medium, 13.48 g of Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) and 3.7 g of sodium bicarbonate were dissolved in 1 L of distilled water. The pH of the culture medium was adjusted to 7.4 using hydrochloric acid and NaOH. The culture medium was filtered through a filter with 0.2 µ pore size and refrigerated in sterile dishes. Prior to use, 10% fetal bovine serum (FBS; 1 mL per 9 mL of culture medium), 100 U/mL penicillin, and 100 U /mL streptomycin were also added to the culture medium.

Preparation of cells:

L929 mouse fibroblasts were removed from the nitrogen tank and cultured in a 75-cm^2 flask (Nunc, Denmark) containing DMEM supplemented with FBS and antibiotics. The cells were then incubated (Panasonic Healthcare Corporation of north America) at 37°C and 5% CO2. The culture medium was refreshed every 3 days. After the passage of the cells and reaching adequate confluence, the cells were distributed among several flasks. For cell passage, first, the culture medium in the flask was extracted with a sterile pipette and the flask was rinsed with phosphate-buffered saline twice. Next, for a 75-cm^2 flask, 2 mm of trypsin (Merck, Germany) was added to the cells and they were incubated at 37°C for 5 min. Afterward, 2 ml of the culture medium containing 10% FBS was added to the flask to deactivate trypsin. The cells detached from the bottom of the flask were transferred to a 15-mL sterile tube and centrifuged at 2000 rpm for 5 min (Hettich Universal, Germany). The supernatant containing trypsin was discarded and the cell sediment was suspended in a culture medium containing 10% FBS and antibiotic and distributed into two flasks.

Eventually, the cell flasks were incubated at 37°C and 5% CO2.

After detachment of the cells from the bottom of the flask using trypsin, the percentage of viable cells was determined using trypan blue. For this purpose, 20 µL of the suspension was transferred to a Neubauer chamber for cell counting. Number of cells in a large square (comprising of 16 smaller squares) was determined, and the number of viable cells was quantified using the following formula:

Number of cells in 1 ml of the suspension=number of cells in a large square x 10^4

Preparation of sealer extracts:

Fluoride varnish, AH26, AH Plus, MTA Fillapex, and Fill Canal sealers were prepared according to the manufacturers' instructions and transferred into the wells of a 24-well plate (16.2 mm diameter and 2 mm height) before setting. Next, 2.5 mL of the DMEM containing antibiotic (but without FBS) was added to each well. The plate was incubated at 37°C and 5% CO2 for 24 hours. Next, the overlaying culture medium over each sealer was transferred to a test tube, and 10% FBS was also added (0.5 mL per each 5 mL of the sealer extract). This extract was considered as the sealer sample with 1/1 concentration. This sample was serially diluted to obtain culture media with lower concentrations of sealer. DMEM containing antibiotic and 10% FBS was used for dilution to obtain ½, ¼, and 1/8 concentrations of each sealer (16).

Assessment of cytotoxicity of sealers:

To assess the effect of sealer extracts on cell proliferation, first, the cells attached to the bottom of the flask were detached using trypsin as explained earlier and transferred to the wells of a 96-well plate. Prior to cell transfer to the wells, the percentage of viable cells was determined using the trypan blue test to ensure that the number of viable cells exceeded 90%. The 96-well plate was incubated in the cell culture incubator for 24 hours to allow the transfer of cells to the bottom of the flask. Next, the previous culture medium in each well was extracted and 200 mL of culture medium containing different concentrations of sealer was added. The conventional culture medium was added to the control wells. In each plate, four wells were allocated to each concentration of each sealer. Also, in each plate, nine wells were considered as control wells and were filled with a sealer-free culture medium. Next, the cells were incubated at 37°C and 5% CO2 for 48 hours. The effect of sealers on cells was evaluated using two 96-well plates. The test was repeated four times for each dilution. The methyl thiazolyl tetrazolium (MTT) assay (Sigma, USA) was performed after 48 hours to determine cell proliferation. For the MTT assay, upon completion of exposure time of cells to the sealer extracts, 20 mL of the 5 mg/mL MTT solution was added to each well. Next, the plate containing cells was incubated for 2 hours. Afterward, the overlaying culture medium was removed and 100 µL of dimethyl sulfoxide (Merck, Germany) was added to each well to dissolve the formazan crystals. The optical density of each well was then read at 545 nm wavelength (with 630 nm reference wavelength) using ELISA Reader (Awareness Technology Inc.). The percentage of viable cells was calculated using the formula below:

Percentage of viable cells= Optical density of each well/mean optical density of control wells x 100

Data normality was tested with the Kolomogorov-Smirnow test. Then were analysed using Prism software programs via one-way ANOVA followed by the Tukey's post hoc test for multiple comparisons. The level of significance was set at 0.05.

Results

According to the result of the Kolomogorov-Smirnow test, the data distribution was normal. Therefore, two-way ANOVA was used to evaluate the two factors of sealer and concentration simultaneously. The results were significant for both factors. Therefore, one-way ANOVA was used separately for each factor.

Table I presents the cell viability following exposure to different concentrations of sealers compared with the control group.

Table I. Cell viability following exposure to different concentrations of sealers

Sealer	control	1/8	1/4	1/2	1/1
AH Plus	99.99±1.624^{A}	84.16±5.102^{Aa}	68.70 ±6.989^{Abhk}	27.83±6.087^{Acil}	4.438±1.094^{Adjm}
Fill Canal	100.1±1.626^{B}	90.14±6.431^{C}	83.72±6.198 ^{BDh}	73.73±1.478^{BCl}	38.35±5.976^{BCDj}
MTA Fillapex	99.99±1.628^{E}	95.54±1.342^{g}	91.04±1.977^{EFk}	85.12±1.694^{EFl}	79.54±3.533^{EFm}
fluoride varnish	99.99±1.628^{G}	91.09±4.463^{GHeg}	44.87±3.952^{GH}	9.820±1.422^{GH}	3.038±0.250^{GHf}
AH26	99.99±5.399^{O}	1.085±0.3303^{Oae}	1.402±0.2713^{Ob}	1.176±0.3766 ^{Oc}	0.8593±0.3089 ^{Odf}

Capital letters: significant difference in row. Small letters: significant difference in column

Effect of AH Plus on L929 mouse fibroblasts after 48 hours:

The percentage of cell viability following exposure to different concentrations of AH Plus showed significant differences with the value in the control group (P<0.05). Thus, pairwise comparisons were carried out, which revealed significant differences between 1/2, ¼, and 1/8 concentrations of this sealer (P<0.001).

Effect of Fill Canal on L929 mouse fibroblasts after 48 hours:

The percentage of cell viability following exposure to 1/8 concentration of Fill Canal sealer was not significantly different from that in the control group (P=0.07). However, cell viability following exposure to other concentrations of Fill Canal had significant differences with each other and the control group (P<0.05). Pairwise comparisons revealed significant differences between different concentrations (P<0.05) except for 1/4 versus 1/8, and 1/2 versus 1/4 concentrations (P>0.05).

Effect of MTA Fillapex on L929 mouse fibroblasts after 48 hours:

The percentage of cell viability following exposure to 1/8 concentration of MTA Fillapex was not significantly different from that in the control group (P=0.07). However, cell viability following exposure to other concentrations of Fill Canal had significant differences with each other and the control group (P<0.05). Pairwise comparisons revealed significant differences between different concentrations (P<0.05) except for 1/4 versus 1/8 concentrations (P=0.06).

Effect of fluoride varnish on L929 mouse fibroblasts after 48 hours:

The percentage of cell viability following exposure to different concentrations of fluoride varnish showed significant differences with the value in the control group (P<0.05). Pairwise comparisons revealed significant differences between all concentrations (P<0.05).

Effect of AH26 on L929 mouse fibroblasts after 48 hours:

The percentage of cell viability following exposure to different concentrations of AH26 showed significant differences with the value in the control group (P<0.05). Pairwise comparisons revealed no significant difference between the concentrations (P>0.05).

Comparison of cytotoxicity of sealers:

In general, the cytotoxicity of fluoride varnish and AH Plus was close with no significant difference (P=0.49). Both sealers were toxic in 1/1, 1/2 and 1/4 concentrations but their cytotoxicity decreased in 1/8 concentration.

Comparison of cytotoxicity of fluoride varnish sealer with that of Fill Canal and MTA Fillapex revealed that fluoride varnish had significantly higher cytotoxicity than both of them (P<0.05). The difference in cytotoxicity of fluoride varnish and MTA Fillapex was greater than the difference between fluoride varnish and Fill Canal. An interesting finding was that fluoride varnish showed high cell viability in 1/8 concentration (91.09%). Fluoride varnish in 1/1 and 1/8 concentrations showed significantly higher cell viability than AH26 (P<0.001).

Comparison of AH Plus with AH26 showed that AH Plus in all concentrations had higher cell viability than AH26 (P<0.001). Furthermore, the comparison of AH Plus with Fill Canal and MTA Fillapex showed that the cell

viability of AH Plus was significantly lower (P<0.05) than that of the aforementioned two sealers. The difference in this respect between fluoride varnish and AH Plus was not significant (P>0.05).

Comparison of cytotoxicity of Fill Canal and MTA Fillapex revealed no significant difference (P=0.06). However, among all, MTA Fillapex had the lowest cytotoxicity and highest cell viability. The difference of MTA Fillapex in this respect with AH Plus, AH26 and fluoride varnish was significant (P<0.05). MTA Fillapex was the only sealer that had higher cell viability in 1/8 concentration than fluoride varnish (95.54%). Figure 1 compares the cell viability of sealers evaluated in this study.

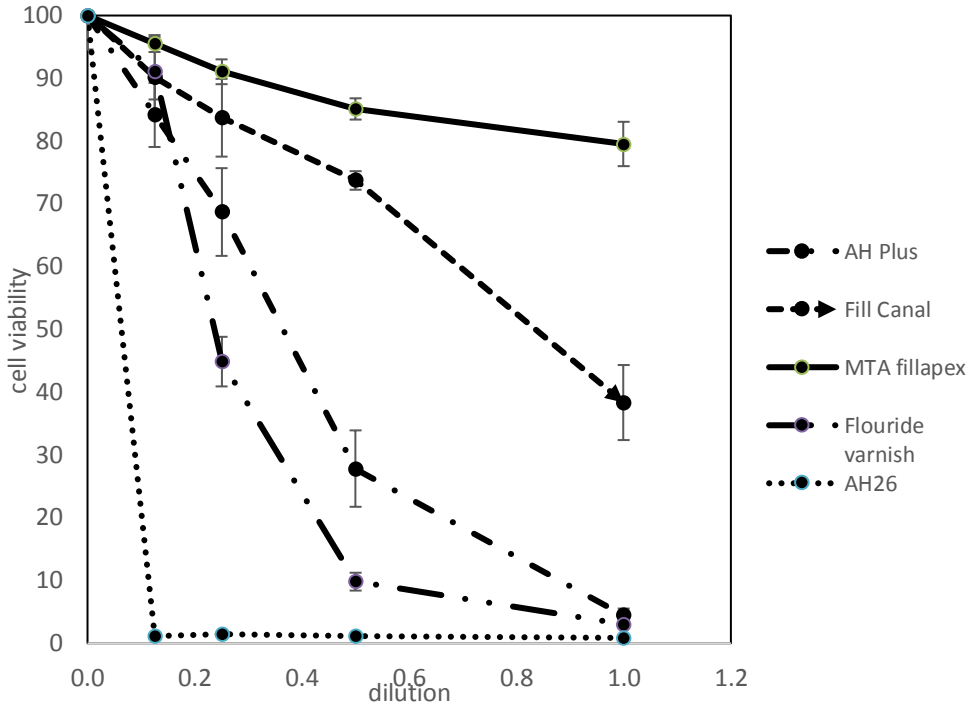

Figure1: comparison of cell viability of different sealer

Discussion

Evidence shows that root filling materials cause periapical irritation when leaked into the periradicular tissue. Thus, the main question is the severity of tissue irritation caused by different root filling materials (17). This study assessed the cytotoxicity of Duofluoride XII fluoride varnish as root canal sealer against L929 mouse fibroblasts in comparison with four commonly used conventional endodontic sealers in the first 48 hours of exposure. Duofluoride XII (FGM, Joinville, SC, Brazil) is a dual fluoride varnish (2.92% fluorine, calcium fluoride + 2.71% fluorine, sodium fluoride, FGM) that in previous studies, its anti-decay properties and dentinal adaptability were confirmed. However, its biocompatibility has not been yet compared with conventional sealers. L929 mouse fibroblasts were used in this study due to their easy accessibility and culture, yielding reproducible results. This cell line is commonly used for cytotoxicity testing (18-24). MTT assay was used for the assessment of cytotoxicity since it is the standard test for this purpose (18-24).

In assessment of cytotoxicity of endodontic materials, some researchers directly exposed the cells to materials such as Ashraf et al, and Bracket et al. (19, 20). However, in some other studies, the sealer extract was mixed with the culture medium to assess its effects on cells (16, 25, 26). Direct placement of sealer in cell culture plate may cause physical injury to the cells and increase the risk of microbial contamination of cell culture plate. Thus, the second method (use of sealer extract) was employed in our study.

In the clinical setting, the sealer is applied to the root canal system immediately after preparation. However, evidence shows that freshly mixed, unset sealer has maximum cytotoxicity for the periapical tissue in case of exposure (16, 26).

A novel material for clinical use should always be evaluated by biocompatibility tests before introducing to the market (1) Since this was the first study that evaluated the cytotoxicity of Duofluoride XII, as a sealer different dilutions of the sealer extract were prepared and used similar to the previous study (4). It is not possible to clinically determine the concentration of the substance to texture, due to the lack of an accurate estimate of concentration of the sealers in the setting and non-setting state to the cells. It is usually examined the effect of sealers at different concentrations of the face Concentrations that can cause cell damage To be specified (27).

In the present study, the cytotoxicity of fluoride varnish and AH Plus sealers was close to each other with no significant difference such that they were both toxic in 1/1, ½, and ¼ concentrations at 48 hours. Their cytotoxicity decreased in 1/8 concentration and the percentage of cell viability increased. However, AH26 was toxic in 1/1 to 1/8 concentrations at 48 hours, which is due to its higher cytotoxicity, which had significant differences with all other sealers. However, it should be noted that this effect can be attributed to testing under in vitro conditions. The cytotoxicity of AH26 is attributed to its epoxy bis-phenol resin content as well as formaldehyde release during and after setting (28). Fluoride varnish sealer had lower cytotoxicity than AH26 in our study. Our results were similar to those of Parirokh et al. (1) with the difference that we performed the MTT assay at 48 hours and assessed 5 sealers; whereas, Parirokh et al. (1) assessed AH Plus, AH26 and fluoride varnish sealers. The MTT assay showed that all three sealers in ½, ¼ and 1/8 concentrations had lower cell viability compared with the control group. AH Plus showed significantly higher cell viability than AH26. Also, similar to our study, the cell viability of AH Plus sealer in ½ and ¼ concentrations was higher than that of fluoride varnish. Fluoride varnish showed higher cell viability than AH26. The results of both studies indicate that fluoride varnish can be used as a suitable sealer with lower cytotoxicity than AH26 (1) but in both articles the cytotoxicity of fluoride varnish was higher than that of AH plus sealer. The possible mechanisms of fluoride toxicity are (a) As the fluoride comes in contact with moisture this results in the formation of hydrofluoric acid and this acid formation results in burning of tissues due to low pH. (b) Cellular poisoning results due to inhibition of enzymes required for the physiological functioning of cells. (c) Fluoride is one of the most reactive elements that attacks oxygen and disrupt the metabolism resulting in the production of hydrogen peroxide as a product. In addition, fluoride results in excessive production of free radicles that disrupt the antioxidant formation (27). Huang et al. (25) reported results similar to ours. They showed that the cytotoxicity of AH26 and AH Plus was higher than that of a ZOE-based sealer (Canals) at 1, 2, and 3 days. Similarly, in our study, comparison of AH26 and AH Plus with Fill Canal ZOE-based sealer showed a significant difference in cytotoxicity, indicating higher cytotoxicity of AH26 and AH Plus sealers. Al Anezi et al. (21) reported results similar to our findings such that the cytotoxicity of AH26 sealer was found to be higher than that of MTA sealer. Bin et al. (16) assessed the cytotoxicity of MTA Fillapex and compared it with AH Plus. The MTT assay results revealed cell viability over 50% for all concentrations of MTA Fillapex, which was in agreement with our results. In the present study, the highest concentration of MTA Fillapex i.e. 1/1 showed 79.54% cell viability. Silva et al. (29) assessed the cytotoxicity of MTA Fillapex and reported that the cytotoxicity of MTA Fillapex was higher than that of AH Plus, which was different from our findings. This difference can be due to the time of assessment since they assessed the cytotoxicity of sealers after 168 hours while we performed the MTT assay after 48 hours and as we know, the cytotoxicity of resin-based sealers decreases over time (29). Badole et al. (2) compared the cytotoxicity of four sealers and similar to our study, reported that AH26 had maximum cytotoxicity in the first 24 hours. Javidi et al. (4) assessed the cytotoxicity of NZOE and compared it with AH26 and Pulpdent using the MTT assay. In line with our findings, they indicated that AH26 was more toxic than other sealers. Jafari et al. (30) compared the cytotoxicity of MTA Fillapex, AH26, and Apatite root canal sealer using the MTT assay and reported results in accordance with our findings. They demonstrated that after 72 hours, MTA Fillapex had lower cytotoxicity than AH26 sealer but this difference was no longer significant after 7 days. De Toledo Leonardo et al. (31) compared the cytotoxicity of five sealers such as Fill Canal and AH26 and reported that Fill Canal had the lowest cytotoxicity. In our study, Fill Canal and MTA Fillapex showed minimum cytotoxicity as well. Silva et al. (32) compared the cytotoxicity of five sealers such as MTA Fillapex and AH Plus. They did not observe any significant difference in cytotoxicity of these two sealers after 2 days. However, after 3 days, MTA Fillapex was more toxic than all other sealers, which was different from our findings. This difference can be due to the fact that they did not assess different concentrations of sealers while we tested the cytotoxicity of four different concentrations of sealers. Jagtap et al. (33) assessed the cytotoxic effects of MTA Fillapex, Apexit Plus calcium hydroxide-based sealer, AH Plus and Tubli Seal ZOE-based sealer on human periodontal ligament fibroblasts using the MTT assay. They showed that MTA Fillapex had maximum cytotoxicity at 2 weeks followed by Tubli Seal and Apexit Plus. AH Plus showed minimum cytotoxicity.

Most in vitro studies on cytotoxicity of sealers have reported that the cytotoxicity of epoxy resin-based sealers (AH Plus and AH 26) is high especially early after mixing. The same is true for ZOE-based sealers. However, the cytotoxicity of AH26 is often lower (16, 26, 34, 35).

Future studies are required to assess the cytotoxicity of fluoride varnish sealer at different time points after mixing and setting of sealer. Also, other properties of fluoride varnish sealer such as its sealing ability, solubility and setting time should be investigated in further studies.

Conclusion

Fluoride varnish has acceptable biocompatibility comparable to that of commonly used sealers. Application of its lower concentrations, with lower cytotoxicity, can be considered as an alternative to conventional sealers. Fluoride varnish had optimal cell viability compared to that of AH Plus gold standard sealer, and had lower cytotoxicity than AH26. Thus, it seems to be a promising alternative sealer for use in the clinical setting.

Conflict of Interest

The authors declare no conflict of interest.

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Corresponding Author
Salma Omidi
Department of Endodontics, Faculty of Dentistry, Mazandaran University of Medical Sciences, Sari, Iran
Tell: 09111534687
Email: salmaomidi@yahoo.com