

CYTOTOXICITY EVALUATION OF DIFFERENT ROOT CANAL SEALERS AT DIFFERENT TIME INTERVALS (AN IN VITRO STUDY)

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ABSTRACT

Aim: to evaluate and compare the cytotoxicity of NeoSEALER Flo, MTApeX and AH Plus in both fresh and set states on human periodontal ligament fibroblasts over a period of one day, one week and one month.

Methods: The sealers were placed in standardized plastic rings to obtain identical sealer specimens. One group of specimens was tested immediately after mixing by preparing its extracts while the rest of the specimens were stored for 24hr at 37C°. Human periodontal ligament fibroblasts were incubated with the extracts of the tested sealers. Cytotoxicity evaluation was done immediately after mixing, and for the extracts of one day, one week and one month according to immersion time. The cytotoxicity of all root canal sealers was determined using the MTT assay. Data were statistically analyzed using repeated measures ANOVA test followed by Bonferroni test and one-way ANOVA test supplemented with Tukey's test ($P < 0.05$).

Results: There was a statistically significant difference in cell viability presented by each tested sealer at different time intervals. Also, there were statistically significant differences in cell viability in all groups within the same time interval except for NeoSEALER Flo and MTApeX at one month evaluation time. NeoSEALER Flo presented the highest cell viability percentages followed by MTApeX and AH Plus respectively at each evaluation time interval.

Conclusions:

- 1- NeoSEALER Flo was the least cytotoxic while AH Plus was the most cytotoxic root canal sealer.
- 2- The increase in cell viability percentages was time-dependent.

KEYWORDS: Bioceramic; Cytotoxicity; MTT assay; Cell viability

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INTRODUCTION

Root canal obturation prevents bacterial infiltration and has been modified from the use of solid filling to gutta-percha cones combined with root canal sealers.^(1,2) Sealers are considered essential materials in the root canal obturation phase by filling spaces where gutta-percha fails to penetrate.⁽³⁾ During the past few years, the use of bioceramic-based sealers was expanded because of their hydroxyapatite formation ability to provide a bond between dentin and filling material. Moreover, they offer efficient antibacterial activity and radiopacity.⁽⁴⁾

From a clinical point of view, almost all root canal sealers are manufactured to be introduced into root canals in a fresh state with flowable consistency. This might possess a risk of material extrusion beyond the apical constriction, leaving the confinement of the root through the apical foramen and escaping into the periapical tissues.^(5,6,7) The sealer-periapical tissue contact is undesirable as it might result in different tissue reactions such as tissue degeneration and delayed wound healing depending on the composition of the extruded sealer. Even if sealers didn't extrude through apical foramen, diffusion of toxic components from these materials into the surrounding environment after their clinical application might have a negative effect on surrounding tissues.⁽⁸⁾ Therefore, endodontic sealers should offer biocompatibility and non-cytotoxicity in both fresh and set states.^(9,10)

NeoSEALER Flo is a recent bioactive bioceramic root canal sealer. As described by the manufacturer, it is characterized by its superior handling properties, promoting hydroxyapatite formation to support the healing process. The manufacturer also claims that, unlike conventional sealers, NeoSEALER Flo offers biocompatibility and antimicrobial properties.⁽¹¹⁾

MTApex root canal sealer is another newly introduced bioceramic root canal sealer. The proprietary gel and tricalcium/dicalcium silicate

powder mixture releases calcium ions. This stimulates bone repair and increases the pH of the canal to help avoid bacterial growth and root resorption as claimed by the manufacturer.⁽¹²⁾

This study was conducted to assess and compare the cytotoxicity of NeoSEALER Flo, MTApex and AH Plus root canal sealers in contact with human periodontal ligament fibroblast cells in both fresh and set states over a period of one day, one week and one month. AH Plus root canal sealer was employed in this study as the standard reference for comparison.

MATERIALS AND METHODS

Specimen and extract preparation:

The experiment was performed using three endodontic sealers listed in **Table (1)**. The sealers were mixed in accordance with the manufacturers' instructions, under aseptic conditions then applied into standardized plastic rings (5 mm in diameter, 5 mm in height) to obtain identical sealer specimens with similar volumes. One group of specimens (freshly mixed sealers) was tested immediately after mixing by preparing its extracts while the rest of the specimens (set specimens) were placed in a humidified 5% CO₂ (pH regulator), 95% air atmosphere for 24hr at 37C°.⁽¹³⁾

Extracts of the tested sealers were prepared in 24-well plates by immersing them in Dulbecco's Modified Eagle's Medium (DMEM) cell culture using the surface area-to-volume ratio of approximately 150mm² /ml between the surface of the specimens and the volume of medium then incubated in the dark at 37C ° for one day, one week and one month.

Human periodontal ligament fibroblasts:

Human periodontal ligament fibroblasts (HPDLF) (Cat. No. ABC-TC3750, Accegen Biotechnology, New Jersey, USA) were used in this study. Cells were routinely cultivated in DMEM

TABLE (1): Endodontic sealers used in this study.

Sealer name	Manufacturer name	Composition
NeoSEALER Flo	Avalon Biomed	Tantalite, tricalcium silicate, calcium aluminate, dicalcium silicate, tricalcium aluminate, calcium sulfate.
MTApex	Ultradent	Tricalcium silicate, tricalcium aluminate, tantalum oxide, water-based gel.
AH Plus	Dentsply	Epoxy resin, zirconium oxide, iron oxide, calcium tungstate, silicone oil, aerosol.

(Dulbecco's Modified Eagle's Medium) (Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS) (Merck Life Science, Darmstadt, Germany), 100 $\mu\text{g}/\text{mL}$ penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin. The culture was incubated at 37 °C, 5% CO₂, and 95% humidity. After achieving the confluent growth and maturity needed for experimentation, the cells were detached by trypsinization and plated in 96-cluster well culture plates at a concentration of 1×10^4 cells/well. Each well contained 100 μl of cell suspension and incubated for 24hr at 37°C under 5% CO₂.

Cells exposure to extracts:

Cells were then exposed to the extracts of the tested sealers (200 μL) in each well (6 wells for each group of each sealer). Cells incubated in DMEM alone without sealer extracts served as control. Culture plates containing cells were incubated at 37 °C, 5% CO₂, and 95% humidity for 24hr. Cytotoxicity testing was done immediately after mixing (for the extract of fresh mix), and for the extract of one day, one week and one month according to immersion time.

Cell survival was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After 24hr, the extracts containing materials were removed, and 1 mL of MTT solution at a concentration of 0.5 mg/ mL of medium was added to the plates and then incubated for 2hr in the dark at 37 C°, 5% CO₂ and 95% humidity. The fluid was then aspirated from the culture and

200 μL of dimethyl sulfoxide was poured into each well to lyse the cells and elute their intracellular formazan salt. Then, the plates were stirred at room temperature for 10 min. to dissolve the formazan crystals. ⁽¹³⁾ The presence of cellular viability was examined by the development of purple color due to the formation of formazan crystals. The absorbance (i.e., optical density) of the purple formazan-stained dimethyl sulfoxide was measured at 570 nm using ELISA plate reader (ELX-800, Biotek-USA).

Cell viability was calculated using the following formula ^(14, 15): (Test sample absorbance / Control sample absorbance) \times 100. The viability of HPDLF was used to assess the cytotoxicity of root canal sealers. Cytotoxicity responses were rated as severe ($\leq 30\%$), moderate (30%-60%), mild (60%-90%) or non-cytotoxic ($\geq 90\%$).

For microscopic evaluation, detached and adhered cells were collected and re-suspended in phosphate buffered saline (PBS). A part (50 μL) was dispensed on the clean ethanol washed glass slide, air dried and fixed using methanol as a preparatory step for cytological examination.

Statistical analysis:

The data were analyzed using the Statistical Package for the Social Sciences (IBM.SPSS) software, version 26. Descriptive analysis included the cell viability percentage in terms of mean and standard deviation. After using Shapiro-Wilk test for normality, repeated measures ANOVA test followed by Bonferroni post hoc test were used to compare

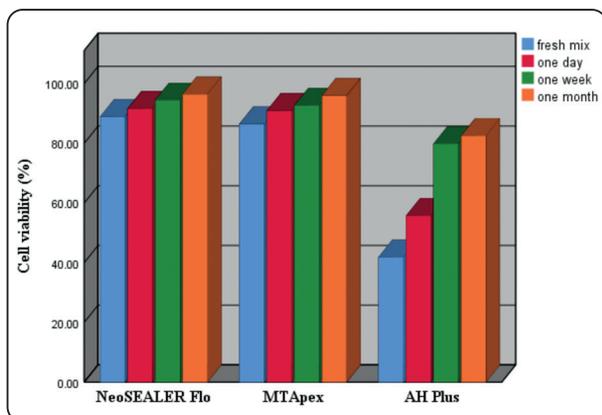


Fig. (1): Clustered bar chart showing the percentage of cell viability in all groups at both fresh and set states “different time intervals”.

the mean percentage of cell viability between different time intervals in the same group. The one-way ANOVA test supplemented with Tukey’s post hoc test were used to compare the mean percentage of cell viability between all groups at the same time interval. The level of significance was set at ($P < 0.05$).

RESULTS

The obtained results are shown in **Figure (1)**. There was a statistically significant difference in cell viability presented by each tested sealer at different time intervals. Also, there were statistically significant differences in cell viability in all groups within the same evaluation time except for NeoSEALER Flo and MTApex at one month evaluation time ($P > 0.05$).

NeoSEALER Flo presented the significantly highest cell viability followed by MTApex and AH Plus respectively at each evaluation time interval which was in line with microscopic findings of cell viability **Figure (2)**. Regarding the fresh mix state, both NeoSEALER Flo and MTApex showed mild cytotoxicity while AH Plus was moderately cytotoxic. The cytotoxicity of all tested sealers noticeably decreased over time. At one-month evaluation time, both NeoSEALER Flo and MTApex were non-cytotoxic while AH Plus showed mild cytotoxicity as shown in **Table (2)**.

TABLE (2): Comparison of mean percentage of cell viability for all tested sealers at different time intervals.

Sealer name	Metric	Fresh mix (0 day)	1 day	1 week	1 month
NeoSEALER Flo	Mean (%)	88.91 ^{1*/a**}	91.67 ^{2/a}	94.48 ^{3/a}	96.53 ^{4/a}
	Std. deviation	0.74	0.82	0.85	0.72
	N***	7	7	7	7
MTApex	Mean (%)	86.42 ^{1/b}	90.95 ^{2/b}	92.71 ^{3/b}	95.98 ^{4/a}
	Std. deviation	0.64	0.68	0.49	0.77
	N	7	7	7	7
AH Plus	Mean (%)	41.91 ^{1/c}	55.73 ^{2/c}	79.89 ^{3/c}	82.51 ^{4/b}
	Std. deviation	0.81	0.71	0.46	0.77
	N	7	7	7	7

* Numbers (horizontal analysis) indicate the mean percentage of cell viability presented by each root canal sealer at different time intervals.

** Letters (vertical analysis) indicate the mean percentage of cell viability presented by different root canal sealers within the same time interval. Means sharing the same number or the same letter indicate no statistically significant differences.

***N represents number of specimens.

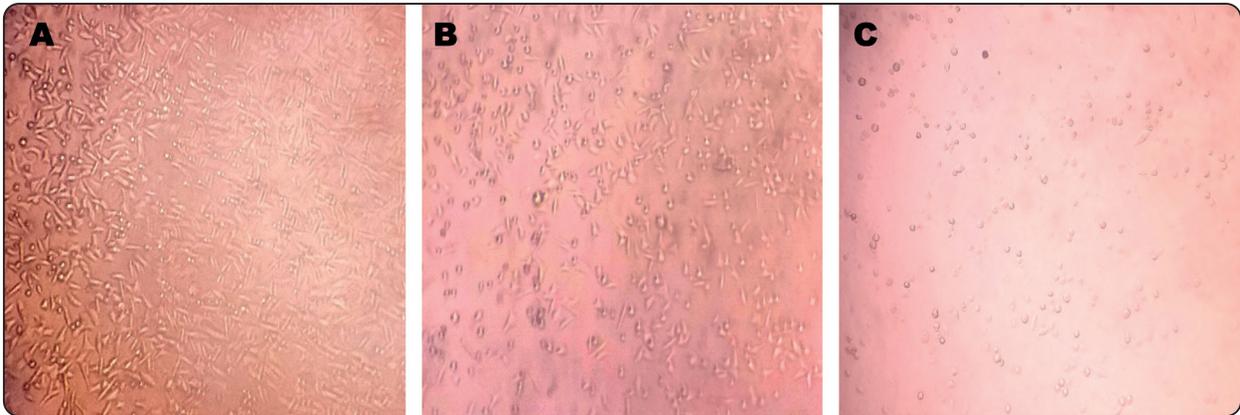


Fig. (2): Micrographs showing cell viability in all groups (fresh mix state) (10X magnification) A: NeoSEALER Flo B: MTApex C: AH Plus. In NeoSEALER Flo: cells showing optimum confluence and spindle-shaped fibroblastic morphology. In MTApex: cells showing relatively less confluence. Contrary to AH Plus: poor confluence and more rounded morphology denoting degeneration.

DISCUSSION

Various types of cell lines have been employed in the cytotoxic evaluation of endodontic sealers. However, fibroblast cells were specially chosen in this study because these cells are considered the most prominent type of cells in the periodontal ligament ⁽¹⁶⁾ and play an eminent role in the function and regeneration of periodontal connective tissues. Moreover, the human periodontal ligament fibroblast cell line represents an accurate imitation of the clinical situation. This cell line imitates the real clinical scenario where the sealers might contact these cells. This possible contact between root canal sealers and periapical tissue which might happen accidentally necessitates the use of biocompatible products ^(17, 18, 19).

It is possible that toxic constituents of root canal sealers can be leached out into the surrounding environment in both fresh and set states. Therefore, in this study, a cytotoxicity experiment was carried out to evaluate the cytotoxic potential of NeoSEALER Flo, MTApex and AH plus. The extracts from the previously mentioned sealers were prepared as per ISO-10993-5 guidelines ⁽²⁰⁾.

MTT assay is a colorimetric assay for quantitative measurement of metabolically active cells based

on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water-soluble tetrazolium salt (MTT) into dark blue formazan crystals. This MTT assay measurement is explained by the inability of dead or necrotic cells to release the colored formazan. Therefore, this assay can be used to accurately differentiate between viable and dead cells. This method of measurement is characterized by its simplicity and precision.

This experiment was designed to evaluate the cytotoxic effect of the previously mentioned root canal sealers on human periodontal ligament fibroblasts (HPDLF) directly after mixing (fresh mix) and also after the complete setting of root canal sealers. Moreover, it is crucial to evaluate the set sealers over an extended period of time after setting. Therefore, the evaluation of cytotoxicity was carried out on one day, one week and one month.

Regarding the results of this study, all tested sealers expressed higher cytotoxicity values in the fresh mix specimen groups. However, NeoSEALER Flo showed lower cytotoxicity values in comparison with MTApex and AH Plus. AH Plus was the most cytotoxic sealer on HPDLF in this study. This could be attributed to the bioceramic nature of NeoSEALER Flo and MTApex. Bioceramic

sealers are well known for their osteoconductive manner and high alkalinity which provides an antibacterial effect and continued release of calcium ions. ⁽²¹⁾ Moreover, the cytotoxic effect of AH Plus might be attributed to the minimal formaldehyde release as a product of the hexamethylenetetramine decomposition. ^(22,23) Epoxy resin sealers such as AH 26 and AH Plus release their maximum amount of formaldehyde in the first days followed by gradual declination. ⁽²⁴⁾ This could explain the initial higher cytotoxicity of AH Plus in the fresh mix group.

Interestingly, the percentages of cell viability presented by all tested sealers increased over time which is in agreement with Silva et al. ⁽²⁵⁾ It is worth mentioning that the experimental steps conducted in an in vitro cytotoxicity assay represent the preliminary stage of biocompatibility analysis of a material. This type of methodology is possibly affected by several factors, most importantly, the type of cell line used.

CONCLUSIONS

Based on the findings of this study,

- 1- In terms of cytotoxicity, NeoSEALER Flo was the least cytotoxic while AH Plus was the most cytotoxic root canal sealer.
- 2- The increase in cell viability percentages presented by all tested sealers was time-dependent.

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