

## COMPARATIVE EVALUATION OF THE CYTOTOXIC EFFECT OF DIFFERENT INTRACANAL MEDICAMENTS

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### ABSTRACT

**Introduction:** intracanal medicaments have an important role in disinfecting of the pulp space and in endodontic regenerative procedures. These medications come in close contact with the periradicular tissue, thus their degradation products must be biocompatible with the periapical tissue. Otherwise, these medicaments will result in intense inflammatory reaction. **Aim of the study:** The purpose of this in vitro study was to evaluate and compare the cytotoxicity of three intracanal medications; Neem oil, double antibiotic paste (DAP) and calcium hydroxide (Ca(OH)<sub>2</sub>). **Materials and Methods:** Immortalized human gingival fibroblast cells were retrieved from the cell bank and then cultured. Three different intracanal medications (Neem oil, DAP, Ca(OH)<sub>2</sub>) were added to the cultured cells. Cell viability was evaluated using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and Apoptosis/necrosis assays. Statistical analysis was performed using One-way ANOVA and post-hoc Tukey test. **Results:** Ca(OH)<sub>2</sub> recorded the highest significant cytotoxicity followed by Neem oil, then DAP which recorded the lowest significant cytotoxicity. Cytotoxicity of the three intracanal medicaments used were directly proportional to their concentrations **Conclusion:** DAP could be better recommended as intracanal medicament in regenerative endodontic procedures because of its biocompatibility compared to the other tested medications.

### INTRODUCTION

Intracanal medicaments play a complementary role in root canal therapy as they help eliminating the remaining microorganisms in the root canal system that have not been removed by the chemo-mechanical preparation including instrumentation and irrigation processes. They also have an important role in disinfecting of the pulp space in the non-instrumented endodontic regenerative procedures <sup>(1,2)</sup>.

These medications come in close contact with the periradicular tissue, thus their degradation products must be biocompatible with the periapical tissue <sup>(3)</sup>. Otherwise, these medicaments will result in intense inflammatory reaction, mediated by various inflammatory mediators which in turn can induce tissue destruction and interfere with periradicular healing <sup>(4)</sup>.

Biocompatibility of these intracanal medications can be determined by many parameters such as genotoxicity, mutagenicity, carcinogenicity, cytotoxicity or histocompatibility. It is impossible to biologically characterize the materials by using a single test and their properties need to be evaluated both in vitro and in vivo tests in a structured approach<sup>(5,6)</sup>. Cytotoxicity and cell viability assays are used in a broad spectrum in the fields of toxicology and pharmacology. The choice of assay method is important in the assessment of the interaction type<sup>(7)</sup>.

Methyl thiazolyl tetrazolium assay is a colorimetric assay to determine cell viability and cytotoxicity<sup>(8)</sup>. It is based on the ability of mitochondrial succinate dehydrogenases in viable cells to reduce a yellow tetrazolium dye MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) to violet formazan product which is detected spectrophotometrically. The healthy cells exhibit high rates of MTT reduction to formazan while the dead cells fail to do so. Viability in the MTT assay depends on the quantification of formazan at 540 nm which is directly linked with the number of viable cells. High purple color intensity represents higher cell viability while the decrease in purple color intensity implies the reduced cell number and thus cytotoxicity of the given substance<sup>(9)</sup>.

Apoptosis is a programmed mode of cell death that can be triggered by both extrinsic and intrinsic factors<sup>(10)</sup>. The annexin V assay is the most frequently used method to visualize the early-stage apoptosis and to characterize the apoptotic cell populations by flow-cytometry<sup>(11, 12)</sup>. The fluorescent probe annexin V is used for the quantification of apoptosis because it binds to negatively charged phospholipids like phosphatidylserine (PS). In an early stage of apoptosis, the PS molecule translocates from the inner part of the plasma membrane to the outer layer. Because necrotic cells also expose PS due to the loss of mem-

brane integrity, apoptotic cells were distinguished from necrotic cells by propidium iodide (PI) labeling. PI is a vital stain frequently used to identify necrotic cells, based on the loss of membrane integrity. Apoptotic cells maintain their plasma membrane integrity during the major portion of the apoptotic process and become permeable to PI during secondary necrosis<sup>(13)</sup>. In this way vital, apoptotic and dead cells can be distinguished on the basis of double-labeling for annexin V and PI, and analyzed either by flowcytometry or by fluorescence microscopy.

Triple antibiotic paste (TAP) which is a mixture of metronidazole, ciprofloxacin and minocycline was the first antimicrobial agent to be successfully applied guided in endodontic regeneration<sup>(14)</sup>. Double antibiotic paste (DAP), which is a combination of only metronidazole and ciprofloxacin, has been used successfully in endodontic regeneration and was suggested as a substitute to TAP to overcome the drawbacks of TAP including the discoloration effect of minocycline<sup>(15)</sup>.

However, the most widely used intracanal medicament in endodontic regeneration is calcium hydroxide ( $\text{Ca(OH)}_2$ )<sup>(16)</sup>. The application of  $\text{Ca(OH)}_2$  depends on its ability to neutralize microbial byproducts.  $\text{Ca(OH)}_2$  may result in chronic inflammation and cell necrosis in vivo due to its high pH. In addition,  $\text{Ca(OH)}_2$  is not effective in completely eliminating bacteria from the dentinal tubules<sup>(17)</sup>.

The increasing number of antibiotic-resistant strains and the side effects caused by synthetic drugs<sup>(18)</sup> has encouraged researchers to look for herbal alternatives. The worldwide growth of phytotherapy in preventive and curative programs has stimulated the application of different plant extracts in medical and dental fields. However, the accurate use of plants for therapeutic purposes is based on effectiveness, biocompatibility, and scientific validation.

Neem is a herbal medicament which possesses anti-inflammatory, antibacterial and anti-adherent effects <sup>(19)</sup>. It was recently applied as intracanal medicament with excellent antimicrobial effect<sup>(19)</sup>. Thus, this research aimed to compare the biocompatibility of Neem (as herbal intracanal medicaments), DAP and the commonly used root canal medication Ca(OH)<sub>2</sub> by using both MTT and Apoptosis/necrosis cytotoxic assays. The null hypothesis was that there is no significant differences between the 3 tested medicaments at different concentrations.

## MATERIALS AND METHODS

### Study design and sampling

This study was conducted after the approval of the Research Ethics Committee (number 17/2017), Faculty of Dentistry, Suez Canal University, Ismailia, Egypt.

f: is the effect size= 0.5;  $\alpha$ = 0.05;  $\beta$ = 0.2; Power= 1-  $\beta$  = 0.80

**MTT dye reduction assay sample size:** according to sample size calculations a total sample size of 96 well was used, in which each concentration (M1, M2, M3, M4, M5) of each intracanal medication (A, B, C) was represented by 6 wells and group (d) was represented by 6 wells.

**Apoptosis/necrosis assay sample size:** This procedure was performed in duplicates, where 500  $\mu$ L siliconized tubes were used; each tube consisted of 4 x 10<sup>6</sup> cells at the start of the procedure to compensate for cell loss.

### I. MTT dye reduction assay

#### Specimens preparation and grouping

The intracanal medications used for this study were calcium hydroxide paste (Meta Biomed,

Chungbuk, Korea), double antibiotic paste [1:1 mixture of Metronidazole (50 mg) (Sanofi-Aventis, Gentilly, France) and Ciprofloxacin (50 mg) (Bayer, Leverkusen, Germany) prepared with glycerol (50 mL)], and neem oil (UpNature, Nicosia, Cyprus). The three intracanal medications were initially prepared as a 10 mg/mL stock. For the dose-response curve, the stock solutions were diluted in cell culture medium (DMEM) to achieve a total of five concentrations (0.5, 0.25, 0.125, 0.0625, and 0.00781 mg/mL) (**Table 1**).

**Table 1:** Variables of the study and levels of investigation for MTT

Variable	Level	Referred to
Type of intracanal medication	A	<b>Group (A), Neem</b> Cells were grown in medium conditioned by neem oil.
	B	<b>Group (B), DAP</b> Cells were grown in medium conditioned by double antibiotic paste
	C	<b>Group (C), Ca(OH)<sub>2</sub></b> Cells were grown in medium conditioned by calcium hydroxide.
	D	<b>Group (D), Control</b> Cells were grown in fresh medium
Concentration (M)	M <sub>1</sub>	Concentration of 0.5
	M <sub>2</sub>	Concentration of 0.25
	M <sub>3</sub>	Concentration of 0.125
	M <sub>4</sub>	Concentration of 0.0625
	M <sub>5</sub>	Concentration of 0.00781

### Cell culture:

Immortalized human gingival fibroblast cells were retrieved from the cell bank of Egyptian Company for Production of Vaccines (Vacsera). Cells were cultured as adherent monolayers in plastic tissue culture dishes in complete growth medium: Dulbecco's Modified Eagle Medium

(DMEM high-glucose) (Merck, Darmstadt, Germany) supplemented with 10% fetal bovine serum (Sigma, Seelze, Germany), 2 ML-glutamine (Sigma, Seelze, Germany), penicillin G 100 mg/mL (Sigma, Seelze, Germany), streptomycin 100 µg/mL (Sigma, Seelze, Germany) and 1% Fungizone (Sigma, Seelze, Germany). Cells were preserved in an incubator (Thermo Fisher Scientific, Waltham, Massachusetts, USA) at 37°C in humidified 95% air and 5% CO<sub>2</sub> for 1 h. Cell culture medium was refreshed each 2 or 3 days until the cells reached 80% confluence for optimal cell harvesting, and then cells were detached with 0.5% (w/v) trypsin-EDTA for subcultures. Growing cells were diluted in fresh medium and seeded into 96-well plates (1.0 × 10<sup>4</sup> cells/well). After incubation for 24 h, the medium was aspirated from all wells and replaced blindly with 100 µL/well of serial dilutions of the used intracanal medicaments (0.05, 0.025, 0.125, 0.0625 and 0.00781 mg/mL), or control medium. Cells were incubated for another 24 h (six wells/ five serial dilutions/ three intracanal medicaments). All steps were carried out under aseptic conditions (vertical cleanroom workbench BIO-CL).

### MTT dye reduction assay

MTT dye [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Abcam, USA) was prepared as 0.5 mg/mL in ice-cold phosphate-buffered saline (PBS) at 37°C just before use. To determine cell viability, a total of 10 µL MTT dye was added to each well and the plate was incubated at 37°C in air containing 5% CO<sub>2</sub> and at 95% relative humidity for 2–12 h to allow mitochondrial succinate dehydrogenases in viable cells to reduce intracellular soluble yellow tetrazolium dye MTT to insoluble violet formazan dye (**Fig. 1**). Formazan was solubilized before recording absorbance readings by adding 100 µL dimethyl sulfoxide (DMSO) detergent reagent to each well to avoid formazan

accumulation as an insoluble precipitate inside cells as well as being deposited near the cell surface and in the culture medium<sup>(20)</sup>. After 10 minutes at room temperature to ensure that all crystals were dissolved, optical densities of each plate were read with microplate reader at 550–600 nm. All assays were performed in triplicates to ensure reproducibility. The absorption value obtained with the untreated fibroblasts as a control was deemed to indicate 100% viability. The percentage of viable cells was determined using the following formula:

$$\text{Percentage of Viable Cells} = (A/B) \times 100$$

Where A = viable cells in the experimental well and B = viable cells in the control well. More than 90% cell viability considered as noncytotoxic, 60% to 90% as slightly cytotoxic, 30% - 59% as moderately cytotoxic, and less than 30% cell viability considered as highly cytotoxic<sup>(21)</sup>.

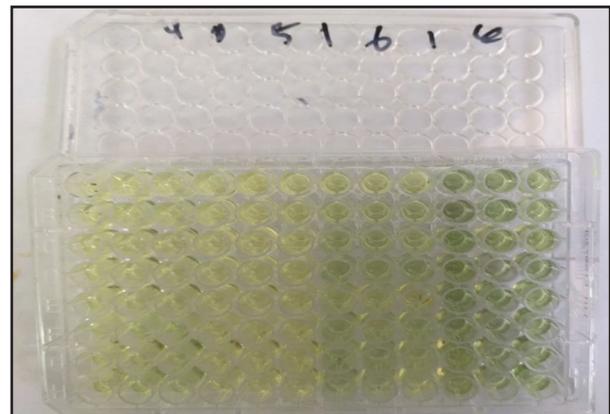


Fig. (1) A photograph showing reduction of intracellular soluble yellow tetrazolium dye MTT to insoluble violet formazan dye.

### II. Apoptosis/necrosis assay

This procedure was performed in 500 µL siliconized tubes; each tube consisted of 4 × 10<sup>6</sup> cells at the start of the procedure to compensate for cell loss.

### Cell Preparation

After incubation and detaching of fibroblast with 0.25% trypsin-EDTA, cells were distributed on the used siliconized tubes. Samples were centrifuged at 335x g for 10 minutes and supernatant fluid was poured, then suspended in 2 mL 1x PBS (no calcium, no magnesium). Centrifugation and suspension were repeated three times, first suspension was in PBS and the following two times were in 1 mL 1x Annexin V binding buffer (BioLegend, California, USA). The three different intracanal medicaments were added to the tubes and incubated at 37°C in humidified 95% air and 5% CO<sub>2</sub> for 24 h.

### Application of Annexin V/Propidium Iodide Stain

Annexin V (5 µL) (BioLegend, California, USA) was added to each tube, and then tubes were incubated in the dark for 15 minutes at room temperature. 100 µL of 1x Annexin V binding buffer was added to each reaction tube. 4 µL of propidium iodide (PI) (BioLegend, California, USA) that has been diluted (1:10 in 1x Annexin V binding buffer) was added to the reaction tube. Tubes were then incubated in the dark for 15 minutes at room temperature. 500 µL 1x Annexin V binding buffer was added to wash the cells. Samples were centrifuged at 335x g for 10 minutes and the supernatant was decanted. Cells were resuspended in 500 µL 1x Annexin V binding buffer and 500 µL 2% formaldehyde to create a 1% formaldehyde (fixative) solution. Thereafter, samples were fixed on ice for 10 minutes, stored overnight at 4°C in the dark, then 1 mL 1x PBS was added to each tube, and mixed by flicking. Centrifugation at 425xg for 8 minutes with pouring of supernatant two times. This was repeated two more times. Finally, tubes were incubated for 15min at 37°C.

Samples were analyzed on a fluorescence-activated cell sorter (FACS) flow cytometer (Beckman Coulter Diagnostics, Indiana, USA).

This software allows assessing specific populations, individualized by gates according to size (FSC), granularity (SSC), and fluorescence (FL) parameters. Early apoptotic cells stained positively with annexin V, whereas late apoptotic cells stained positively with annexin V and PI. Percentages of viable cells, apoptotic cells and necrotic cell populations were determined as described by *Vermes et al.*,<sup>(22)</sup>. The experiment was done in duplicates.

## RESULTS

### I. MTT-dye reduction assay

#### A. Comparison between different concentrations in each intracanal medication (Intragroup analysis)

There was a statistically significant difference between all concentrations in all the three experimental groups. Cytotoxicity of the three medicaments was directly proportional with the concentrations as the percentage of viable cells increase with decreasing the concentrations (**Fig. 2**).

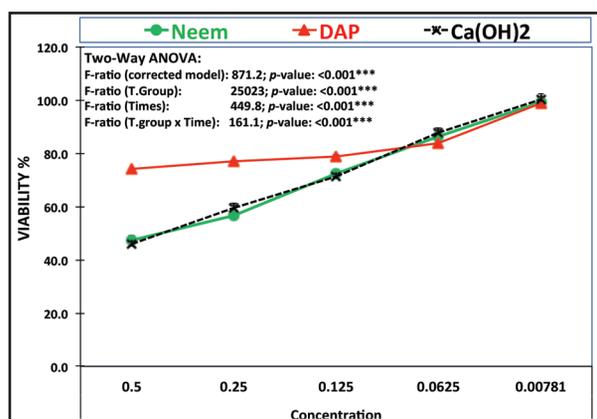


Fig. (2) Line curve showing comparison between the change in viability (%) (Y-axis) under the effect of increasing concentration (X-axis).

**B. Comparison between three intracanal medications at different concentrations (Inter-group analysis) the level of significance was set at <0.05.**

In the current study, at concentrations 0.5, 0.25 and 0.125 mg/mL, DAP recorded the highest significant of viable cell recording 74%, 77% and 79% for each concentration respectively. At lower concentration 0.0625 mg/mL, the three medicaments (DAP, Ca(OH)<sub>2</sub>, Neem) were slightly cytotoxic. However, at concentration 0.00781 mg/mL there was no significant difference between the three intracanal medications, all three medicaments were

biocompatible and were considered non cytotoxic with viable cells concentration > 90% (Fig. 3).

**Apoptosis/necrosis assay**

Regarding the healthy apoptotic cells, DAP showed the statistically significant highest mean value followed by Ca(OH)<sub>2</sub> then Neem oil. On the other hand, the early and late apoptotic cells, Neem oil exhibited the statistically significant highest apoptotic rate and necrosis, followed by Ca(OH)<sub>2</sub>. Meanwhile DAP exhibited the statistically significant lowest apoptotic rate (Fig. 4, Fig. 5).

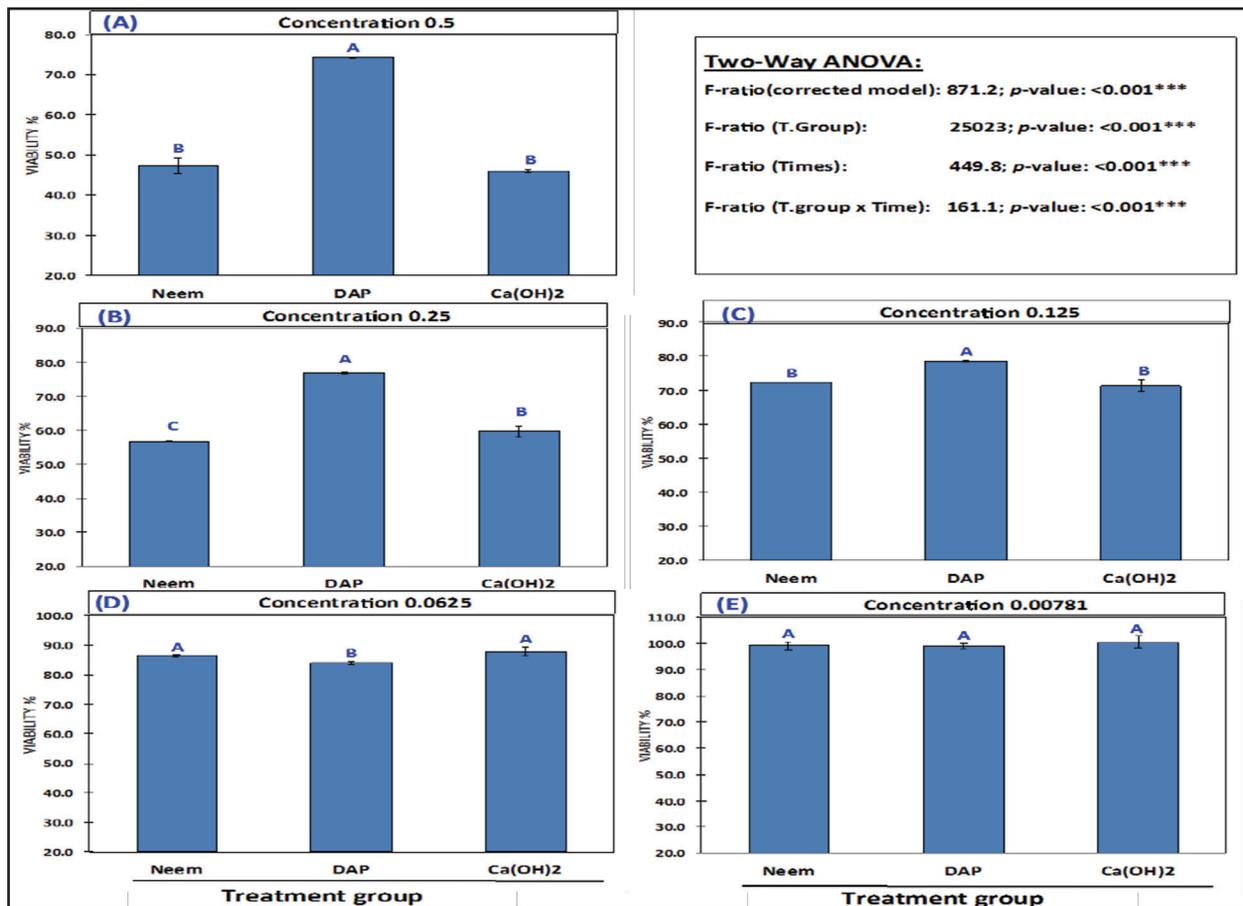


Fig. (3) Bar chart showing viability % at different treatment groups (Neem, DAP, and Ca(OH)<sub>2</sub>) tested at all concentration (0.5, 0.25, 0.125, 0.0625, 0.00781 mg/mL).

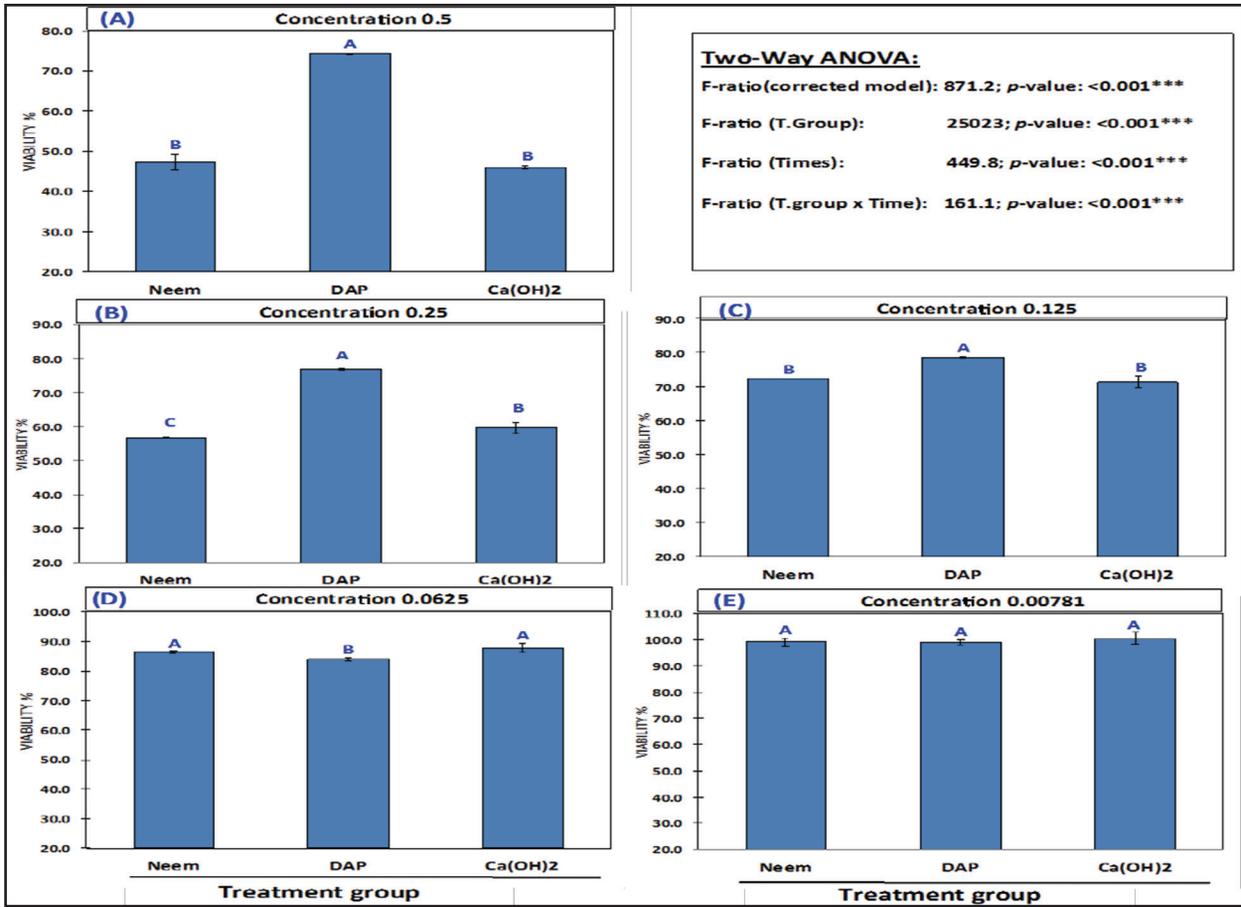


Fig. (4) Bar chart showing comparison between the mean change values of the apoptotic levels of the three intracanal medications.

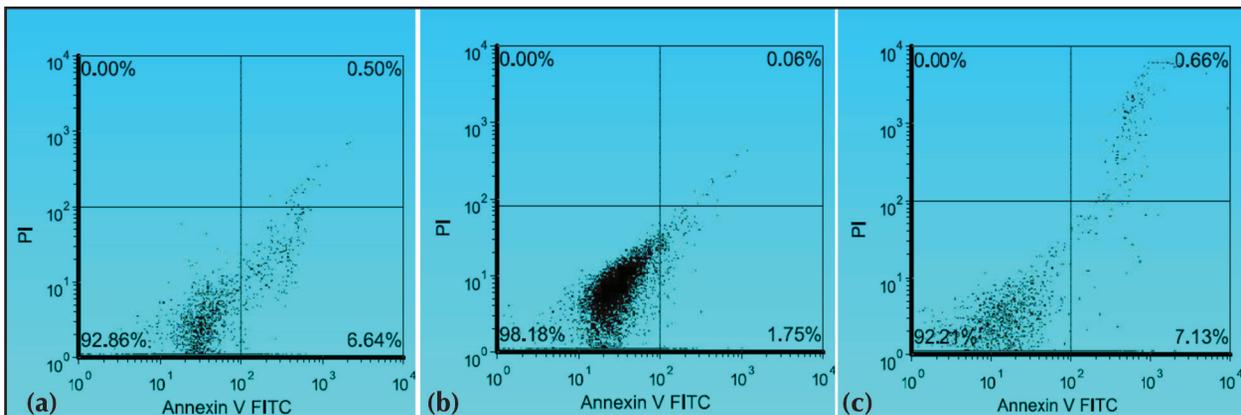


Fig. (5) Contour diagram of FITC-Annexin V/PI flow cytometry of HGFC after exposing to three different intracanal medications (a) Ca(OH)<sub>2</sub>, (b) DAP, (c) Neem Oil. The lower left quadrants of each panels show the viable cells, which exclude PI and are negative for FITC-Annexin V binding. The upper right quadrants contain the non-viable, necrotic cells, positive for FITC-Annexin V binding and for PI uptake. The lower right quadrants represent the apoptotic cells, FITC-Annexin V positive and PI negative demonstrating cytoplasmic membrane integrity.

## DISCUSSION

A wide variety of intracanal medications is available. The most common is  $(\text{Ca}(\text{OH})_2)$  and its combination with other materials such as iodoform. Antibiotics also have gained popularity especially with the need of a broad spectrum antimicrobial agent for disinfection in regenerative endodontics which relies mainly on the action of irrigants and intracanal medicaments. These medications are proved to be effective antibacterial agents under laboratory conditions, but their efficacies in clinical use are unpredictable and exhibit a degree of toxicity and allergic reactions that may cause tissue injury. Recent trend toward using medication extracted from natural plants has increased rapidly to overcome the drawbacks of the commercially available intracanal medicaments<sup>(23)</sup>.

Neem has been proven to be a powerful antimicrobial agent by inhibiting the increase of microorganisms which cause infectious diseases. It is also characterized by its anti-adherence activity as it alters bacterial adhesion and ability of the microorganisms to colonize<sup>(24)</sup>.

Intracanal medications must be biocompatible because they come into direct contact with periapical tissues during endodontic treatment either by extrusion through the apex or leaching of the fine particles overtime<sup>(5)</sup>, otherwise these medications can result in high levels of inflammation, which can induce tissue destruction<sup>(26)</sup>. Therefore, these drugs should be able to induce repair of the injured area without interfering with osteogenesis and cementogenesis<sup>(26)</sup>.

MTT is one of the most commonly applied colorimetric assays for evaluation of cell viability and cytotoxicity of several drugs at different concentrations<sup>(7)</sup>. However, the sensitivity of an MTT assay is lower than that of fluorescent or

luminescent assays, as MTT reduction assay is a marker reflecting viable cell metabolism and not specifically cell proliferation<sup>(27)</sup>. Therefore, Annexin V apoptosis/necrosis assay has been added to this study as it allows easy and sensitive analysis of the changes in the structure of cell membrane with the application of fluorescence methods. In this way vital, apoptotic and dead cells can be distinguished and analyzed by flow-cytometry microscopy<sup>(28)</sup>.

When evaluating cytotoxic effect of different intracanal medicaments, cell type is an important factor for consideration. Human gingival fibroblasts were used in this experiment due to their quick and easy growth<sup>(29)</sup>, in addition to their availability in periapical tissues and the periodontal ligament, which are areas susceptible to the effects of intracanal medications and their degradation products. Fibroblasts are also the largest producers of collagen tissue and, therefore, actively participate in periapical tissue repair process<sup>(30)</sup>.

In MTT dye reduction assay, the intergroup analysis showed statistically significant difference between the tested groups  $\text{Ca}(\text{OH})_2$  and Neem oil at concentrations (0.5 and 0.25 mg/mL) with obvious toxicity to the fibroblasts. For DAP, the four concentrations (0.5, 0.25, 0.125 and 0.0625 mg/mL) were slightly cytotoxic to fibroblasts. However, at concentration 0.00781 mg/mL there was no significant difference between the three intracanal medications, where all materials were biocompatible.

These findings were consistent with previous studies<sup>(31,32)</sup> which evaluated the effect of different concentrations of DAP on the survival rate of human dental pulp stem cells (DPSC) using LDH assay and found that the three lowest tested concentrations of DAP 0.5, 0.25 and 0.125 mg/mL were non-toxic to DPSC. Same findings affirmed by **Kim et al.**,<sup>(23)</sup> who evaluated the dentin treated with two concentrations

of DAP (0.5 and 1 mg/mL) and EDTA regarding the proliferation of DPSCs and found that there was no significant differences in the proliferation of DPSCs with both concentration regardless of the use of EDTA.

Regarding, the reaction of  $\text{Ca(OH)}_2$  to fibroblast, it showed moderate cytotoxicity at concentration 0.5 mg/mL. While at concentrations 0.25, 0.125 mg/ mL  $\text{Ca(OH)}_2$  displayed slight cytotoxicity. This could be referred to the direct relation between the pH and the concentration of the drug, as the pH decreases when using lower concentration, consequently, cytotoxicity of  $\text{Ca(OH)}_2$  decreased by using decreased concentration<sup>(33)</sup>.

Neem oil displayed moderate cytotoxicity at concentration 0.5% and remained at same level of cytotoxicity at concentration 0.25 mg/mL. This could be explained by the Neem formula used in this study design, which was 100% pure cold pressed and it is well known that its effect is concentration dependent<sup>(34, 35)</sup>, so the side effects increase when the concentration increase. In addition, **Franco et al.**,<sup>(36)</sup> reported that when using Neem oil in medical and dental conditions or in connective tissue disorders it should be diluted with water because the concentration is a crucial point.

The MTT results were parallel to the Apoptosis/necrosis assay, where DAP group showed the statistically significant highest mean value of healthy apoptotic cells ( $98.213 \pm 0.27$ ) followed by  $\text{Ca(OH)}_2$  then Neem oil. These results might be attributed to the metronidazole present in the formula of DAP that has no cytotoxic effect on stem cells<sup>(18, 37)</sup>. Also, might be due to glycerin involved in DAP formula which is considered biocompatible<sup>(38, 39)</sup>.

Regarding the early and late apoptotic cells, Neem oil exhibited the highest apoptotic rate compared with other medicaments. These findings

could be explained by the antitumor nature of Neem<sup>(40)</sup> that induces apoptosis and leads to cell mortality<sup>(41)</sup>. It has been demonstrated that Neem alters cell cycle and prompts apoptosis in various carcinoma via both extrinsic and intrinsic apoptotic pathways<sup>(42)</sup>. We rejected the null hypothesis as there was significant difference between the cytotoxicity of the three tested intracanal medications ( $\text{Ca(OH)}_2$ , DAP, Neem oil) ( $P$ -value  $< 0.001$ ).

## CONCLUSION

In terms of MTT assay; cytotoxicity of the three medicaments was directly proportional with the concentrations as the percentage of viable cells increases with decreasing the concentration.

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