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"ASSESSMENT OF ANTIBACTERIAL ACTIVITY AND CYTOTOXICITY OF MODIFIED NANOBASED ANTIBIOTICS AS ROOT CANAL MEDICAMENTS" (AN IN-VITRO STUDY)

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ABSTRACT

Background: The purpose of this research is to assess the antibacterial activity and cytotoxicity of modified nanobased antibiotics as root canal medicaments in vitro. **Methods**: Depending on the type of intracanal medicine used, the dentinal tubules of 85 root samples that were infected with *Enterococcus faecalis* were randomly allocated to one of 5 groups. A total of 1mg/ml, 0.1mg/ml of [double antibiotic paste (DAP)] or [modified triple antibiotic paste (mTAP)] in regular form, DAP or mTAP loaded nano particles using the [poly lactic-co-glycolic acid (PLGA)] as a nano carrier and PLGA nanoparticles (control) were inserted in the root canal for 1 week. Dentine shavings were obtained after treatment, and colony-forming units were measured. MTT assay was utilized to assess cytocompatibility against human lung fibroblastic cell line [WI-38] after 24 and 48 hours. The data were statistically analyzed ($P < .05$). **Results:** In terms of bacterial eradication, DAP or mTAP loaded nano PLGA performed much better than the regular form. The higher the concentration, the higher the antibacterial effect (1>0.1mg/ml). while it showed no significant difference regarding the antibacterial effect of Nano mTAP. All intracanal medicaments were cytotoxic when in full concentration but when diluted their cytotoxic effect decreased. All the drug loaded nano PLGA showed a higher cytotoxic effect than their regular forms. **Conclusions:** DAP or mTAP loaded nano PLGA are more effective against *Enterococcus faecalis* than their regular counterparts but were more cytotoxic at higher concentrations.

KEYWORDS: Double antibiotic paste, *Enterococcus faecalis*, Nanoparticles, Modified triple antibiotic paste, Cytotoxicity.

INTRODUCTION

The primary cause of pulpal and periapical inflammation and pathosis is microorganisms and their metabolites. A facultative bacteria known as [*Enterococcus faecalis (E. Faecalis)*], which is resistant to a number of intracanal drugs, is commonly found in root canal failures. Ineffective removal of them and their byproducts could lead to lingering discomfort and slowed healing. The application of intracanal medications for disinfecting root canals has been proposed for obtaining for long-term therapeutic success because root canal anatomical complexity and bacterial variety make it difficult for endodontic treatment instruments and techniques to deliver bacteria-free root canals. [Calcium hydroxide (Ca(OH)2)] is a common intra canal medication. The bacterial cell membrane and protein structures are destroyed by Ca(OH)2's high pH. Recent studies have revealed that it has a minimal bactericidal effect on *E. faecalis.*[1] A [triple antibiotic paste (TAP)], comprising primarily of ciprofloxacin, metronidazole, and minocycline, has been suggested for disinfecting root canals.[2] The most significant problem with teeth treated with TAP and minocycline was discoloration. According to current research, TAP is harmful to dental papillae stem cells at high doses, preventing their attachment and proliferation. Therefore, recent research recommends using minimal concentrations of triple antibiotic paste to reduce toxicity.[1] Minocycline should be completely stopped and replaced with [DAP (Double antibiotic paste)], which contains ciprofloxacin and metronidazole, in light of the risks it entails.[3] A substitute for minocycline that can be used in combination with ciprofloxacin and metronidazole is clindamycin. The term modified TAP (mTAP) has been proposed when looking for a feasible antimicrobial alternative to minocycline. In science and technology, nanotechnology has rapidly evolved, leading to an extensive variety of biological applications such as the development of targeted medication delivery systems.[4] Polymers have been used in the delivery of drug research

due to their ability to effectively transport medicine to a specific region, increasing therapeutic efficacy and minimizing side effects. Because of their low toxicity, biodegradability, and biocompatibility, PLGA polymers have attracted great interest for tissue engineering applications and delivery systems in the U.s [Food and Drug Administration (FDA)].[5] As a result, the purpose of this research was to assess the antimicrobial effect and cytotoxicity of modified nano-based antibiotics inside the root canal utilizing PLGA as a nano carrier. The null hypotheses tested were that the nano form of intracanal medication would show no significant differences in terms of antibacterial potential and cytotoxic effect when compared with regular form.

MATERIALS AND METHODS

Preparation of medications

- To make 1mg/ml of mTAP, 3 mg of antibiotic powder composed of equal parts of metronidazole (Sanofi-Aventis, Egypt), ciprofloxacin (EPICO, Cairo, Egypt) and clindamycin (Pfizer, Cairo, Egypt) was diluted in 3 ml of deionized water using hot plate & stirrer (MSH-20A, wetige®, Germany) for 30 min then, 0.15gm of Carboxymethyl cellulose (Loba CHIME, India) was dusted slowly and evenly over the mixture while continuously swirling to achieve a uniform gel. The same steps done were repeated to prepare DAP but without clindamycin.
- To prepare 0.1mg/ml gel, 0.3mg of DA(1:1) or mTA (1:1:1) was dissolved in 3 ml of deionized water and the same steps done were repeated.
- The PLGA [nanoparticles (NPs)] were manufactured using the solid-in-oil-in-water (s/o/w) emulsion method.[6] To get a homogeneous PLGA solution, 45 mg of PLGA NPs were dissolved in dichloromethane for 6 hours. To create the solid-in-oil primary emulsion, (double or triple antibiotics) was added to the PLGA solution and sonicated at 55 W for 1 min. To obtain the final solid-in-oil-in-water emulsion, 20 ml of polyvinyl alcohol solution (1% w/v) was sonicated at 55 W for 2 minutes. The resulting nano-sized particles were swirled in the emulsion for 3 hours to allow the solvent to evaporate. To remove the excess solvent, the resulting emulsion was centrifuged at 15,000 g for 15 minutes. The nanoparticles were rinsed three times with deionized distilled water before being resuspended in deionized water. "Table 1" "Fig.1".
- To prepare 10mg/ml gel, 30mg of Drug-loaded nanoparticles(DAP or mTAP) weighed out on a sensitive balance and dissolved in 3 ml of deionized water with stirring by using hot plate & stirrer for 30 min, the solution was then softly and progressively sprinkled with 0.15gm of Carboxymethyl cellulose under 35^{o} temperature with continuous stirring to obtain a homogeneous gel.
- To prepare 1mg/ml gel, 3mg of Drug-loaded nanoparticles (DAP or mTAP) weighed out on a sensitive balance and dissolved in 3 ml of deionized water and the same steps done were repeated. "Fig. 2,3".

Table 1: Representing the physical properties of Nano PLGA.

Appearance (Color):	White.
Appearance (Form):	Gel.
Avg. Size (TEM):Fig(14)	160 ± 20 nm.
Shape (TEM):	Spherical shape.
pH:	5.5±0.5

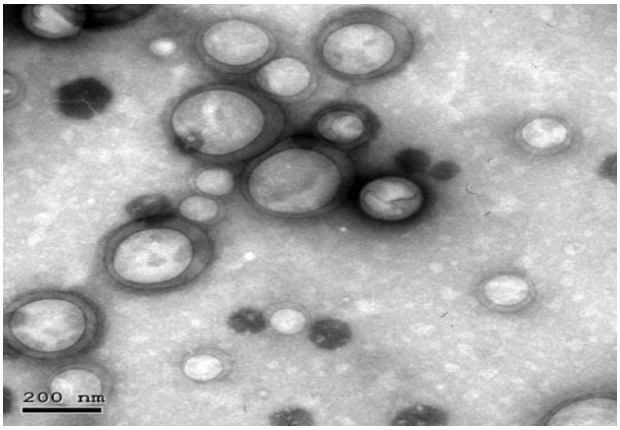

Figure 1: [Transmission electron microscope (TEM)] image of Nano PLGA with scale bar is 200 nm.

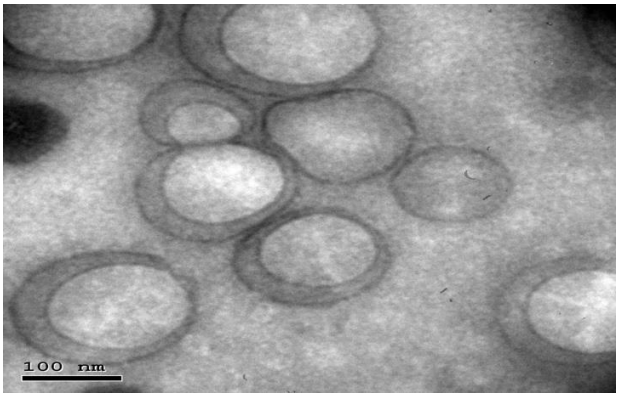

Figure 2: TEM image of Nano PLGA after mTAP loading with scale bar is 100 nm.

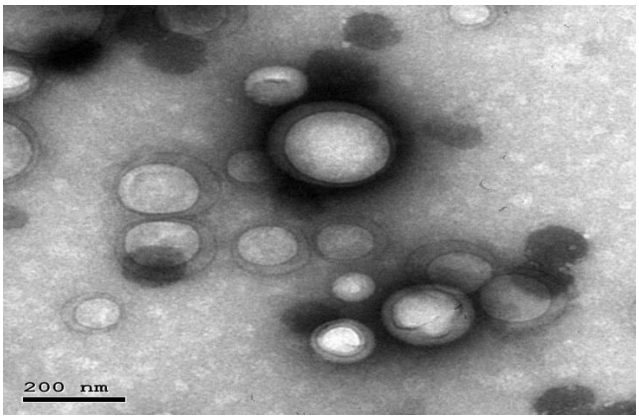

Figure 3: TEM image of Nano PLGA after DAP loading with scale bar is 200 nm.

Specimens selection and preparation

Eighty-five extracted single-rooted mandibular premolars were obtained from an oral and maxillofacial surgery clinic due to orthodontic or periodontal disease. The crown underneath the CEJ and the apical section of the root were cut with a diamond disc for slow speed handpiece under coolant. This procedure produced 6 mm radicular dentine cylinders from the middle third of the root. Gates Glidden drill No.3 was used to standardize the internal size of the root canal space (Dentsply Maillefer, Ballaigues-Switzerland). Smear layer was eliminated by washing the specimens in an ultrasonic bath (Vector 55, Jeltraft, Jelenko) for 5 minutes with 17% EDTA, then rinsed with 5% NaOCl for 5 minutes, and finally, distilled water for 10 minutes. All specimens were sealed externally using 2 layers of nail polish before being autoclaved at 121^{o}C for thirty minutes.

Sample Classification

The study involves 85 samples. Eighty-four samples were randomly assigned to one of five test groups and two groups as control (n=14). The following antimicrobial agents were represented by the five test groups:

Group 1: DAP
Group 2: mTAP
Group 3: DAP loaded nano PLGA
Group 4: TAP loaded nano PLGA
Group 5: Nano PLGA

Each group was then divided into two groups based on antibiotic concentrations (1,0.1mg/ml of DAP, TAP, and drug loaded nano PLGA) and (9,0.9mg/ml of nano PLGA) (n=7). The positive control group was utilized throughout the experiment to verify bacterial viability. To ensure process sterility, the group with a negative control was used. The last sample was utilized as a biofilm development indicator.

Biofilm formation

A clinical strain of *E. faecalis* from the lab of microbiology was utilized for forming biofilms, and the bacterial strain was injected in [Brain Heart Infusion Broth (BHIB)], cultured at 37 °C for 24 hours, and the level of turbidity was corrected to the No. 1 MacFarland standard. The specimens from the test and the positive control groups were infected by injecting 1 ml of the microbial suspension into each root canal with a sterile micropipette. All samples were incubated at 37°C in sealed tubes. Every 72 hours, this technique was carried out with a 24-hour pure culture being created and adapted to the MacFarland turbidity standard No. 1. To ensure the sterility of the processes, the negative control samples were soaked in sterile BHI broth and refilled with sterile saline every 72 hours. For 21 days, the teeth were kept in an environment that was humid at 37°C.

Confirmation of Biofilm Formation

After 21 days, SEM was used to analyze the growth of bacterial biofilms on root canal dentin. A randomly

chosen specimen was picked, and longitudinal grooves were made over its whole length. After immersing it in the bacterial suspension, it was separated into two halves with a hammer and chisel, as described by (Daood U et al., 2021).[7] For fixation, every half was soaked in 2.5% glutaraldehyde (pH 7.4) for 1 hour at 4°C, rinsed with [phosphate buffer saline (PBS)] for 15 minutes, and then postfixed in 1% (wt/vol) osmium tetroxide for 30 minutes. They were then washed with PBS before being dehydrated in an increasing ethanol series and dried using a critical point dryer. The sample has been mounted and sputtered with a 200-layer gold-palladium coating. The sample was analyzed using a Quanta FEG 250 microscope 20 KV at various magnifications (3000-24000x). SEM clearly demonstrated *E. Faecalis* deposition and biofilm growth on the root canal walls "Fig. 4".

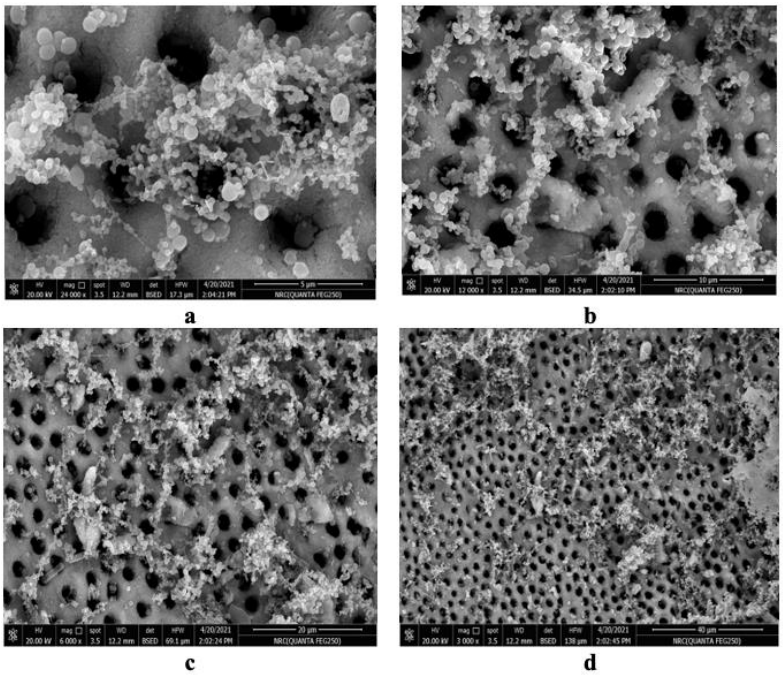

Figure 4: A photographs showing presence of E. Faecalis verified by SEM with different magnifications. a: at magnification 24000 x, b: at magnification 12000 x, c: at magnification 6000 x, d: at magnification 3000 x.

Application of medication and antibacterial evaluation

After the incubation period, the intracanal medicaments were applied to each sample according to the classification of each group using a sterile plastic syringe of gauge 19-G, the samples were then sealed at the ends with paraffin wax to mimic the clinical state and maintained for one week at 37°C. Following the scheduled time, 20 mL of sterile saline used to irrigate the root canals to wash out the intracanal medication, gentle scrubbing of root canal walls with size 40 H file was done and collected using two sterile paper points size 50 inserted into a test tube having 1.0 ml of saline. Each paper point was vortexed for 30 seconds to ensure homogeneity. In saline, serial 10-fold dilutions (1:10, 1:100, and 1:1000) were prepared, and 0.1 mm out of each dilution was smeared on Brain heart infusion agar plates before incubating at 37 °C for 48 hours. All procedures were conducted inside a laminar flow chamber. [Colony forming units (CFU)] were counted per 1 ml. To calculate the total CFU/ml of sample, visible colonies/plates were multiplied by the corresponding dilution factor and by 10.

Cytotoxicity

Human lung fibroblastic cell line [WI-38] was treated with the examined intra canal medications. Cell viability after 24 and 48 hours was evaluated using MTT assay.

Process of extraction

The medications were obtained aseptically under Laminar Air Flow and placed in MEM-E medium to achieve 10 mg/ml of each sample for 24, 48 hours. Control samples having only medium were incubated in the same manner. Millex-GS sterile filters (Millipore-Burlington, USA) were used to collect each extraction medium. In sterile test tubes, medications were serially diluted twice. Using MEM-E Medium, several dilutions of the extraction media were achieved, resulting in a total of twelve concentrations (10,5,2.5,1.25-0.07mg/ml), etc.

Cytotoxicity Test

The MTT colorimetric method was used to evaluate cell vitality. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was a water-soluble tetrazolium salt that was transformed to an insoluble purple formazan by succinate dehydrogenase within the mitochondria by tetrazolium ring cleavage. Because the formazan product is impermeable to cell membranes, it

accumulates in healthy cells. MTT 0.5 mg/ml was applied to the pre-treated cells as 50 µl/well, and the tissue plates for culture were placed in incubators at 37°C for 4 hours. After decanting the MTT stain, cells that were stained were rinsed twice with PBS. [Di Methyl Sulfoxide (DMSO)] was used to dissolve the formed MTT Formazan complex crystals. The optical density of dissolved crystals was determined at 570 nm using a [enzyme-linked immunosorbent assay (ELISA)] plate reader (Biotek-ELx-800-Vermont, USA) after every measurement was collected three times. The vitality % and drug concentration were shown opposite one another. The [Optical density (OD)] reflecting the percentage of residual alive cells was calculated using the following equation:

$$\text{VIABILITY \%} = \frac{\text{OD TEST 1 X 100}}{\text{OD CONTROL}}$$

Number of residual living cells = (OD of treated cells/ OD of untreated cells) x Number of negative control cells (104 cells/0.1ml).

Statistical analysis

To determine any statistical difference between groups, the findings were evaluated using one-way analysis of variance (ANOVA) and Tukey Honestly Significant Difference (HSD) tests in the Minitab 19 programme (version 19, Minitab, LLC, USA). The level of significance in the current study was set at $P \leq 0.05$.

RESULTS

1-Antibacterial effect

a. The impact of concentration on the antibacterial effect of several intracanal medicaments "Table 2-3".

For regular intracanal medications

Concentration showed statistically significant effect on antibacterial effect of both DAP and mTAP intracanal medications.

Table 2: Showing The impact of concentration on the antibacterial effect of several regular intracanal medicaments.

Groups	Regular DAP 0.1mg	Regular DAP 1mg	Regular mTAP 0.1mg	Regular mTAP 1mg
CFU/ml	$145*10^3 \pm 28.4*10^{3\ a}$	$8.5*10^3 \pm 1.643*10^{3\ b}$	$70*10^3 \pm 6.57*10^{3\ a}$	$20*10^3 \pm 0.632*10^{3\ b}$
P-Value	<0.001		<0.001	

Means that don't share same letter are significantly different.
$P \leq 0.05$ is considered Significant.

For Nano intracanal medications

Concentration showed statistically significant effect on antibacterial effect of both Nano PLGA and DAP loaded nano PLGA intracanal medications while it showed no statistically significant effect on antibacterial effect of Nano mTAP.

Table 3: Showing The impact of concentration on the antibacterial effect of several Nano intracanal medicaments.

Groups	Nano PLGA 0.9mg	Nano PLGA 9mg	DAP loaded nano PLGA 0.1mg	DAP loaded nano PLGA 1mg	mTAP loaded nano PLGA 0.1mg	mTAP loaded nano PLGA 1mg
CFU/ml	$55.5*10^3 \pm 26.8*10^{3\ a}$	$211.167*10^3 \pm 0.983*10^{3\ b}$	$30.5*10^3 \pm 4.93*10^{3\ a}$	$4*10^3 \pm 3.29*10^{3\ b}$	$4.5*10^3 \pm 2.74*10^{3\ a}$	$7*10^3 \pm 1.095*10^{3\ a}$
P-Value	<0.001		<0.001		0.083	

Means that don't share same letter are significantly different.
$P \leq 0.05$ is considered Significant.

b. The impact of particle size (regular vs. nano) on the antibacterial effect of several intracanal medicaments "Table 4".

Particle size showed statistically significant effect on antibacterial effect of all tested intracanal medications at different concentrations.

Table 4: Showing The impact of particle size (regular vs. nano) on the antibacterial effect of several intracanal medicaments.

Group / Concentration		DAP		mTAP	
		0.1mg	1mg	0.1mg	1mg
CFU/ml	Regular	$145*10^3 \pm 28.4*10^{3\ a}$	$8.5*10^3 \pm 1.643*10^{3\ a}$	$70*10^3 \pm 6.57*10^{3\ a}$	$20*10^3 \pm 0.632*10^{3\ a}$
	Nano	$30.5*10^3 \pm 4.93*10^{3\ b}$	$4*10^3 \pm 3.29*10^{3\ b}$	$4.5*10^3 \pm 2.74*10^{3\ b}$	$7*10^3 \pm 1.095*10^{3\ b}$
P-Value		<0.001	0.020	<0.001	<0.001

Means that don't share same letter are significantly different in the same column.
$P \leq 0.05$ is considered Significant.

2-Cytotoxicity

2.a Percentage of vitality values

2.a.1 The impact of several intracanal medications on the percent of fibroblast cells vitality

Values "Table 5-6".

At 1mg concentration

- At 24-hour interval, there is a significant statistical difference among all the tested groups with **Nano PLGA** showed the highest cytotoxicity (13.677 ±1.379) while **Regular mTAP** showed the lowest cytotoxicity (34.310 ±1.358).
- At 48-hour interval **Nano PLGA** showed the highest cytotoxicity (9.415 ±1.716) while **Regular mTAP showed** the lowest cytotoxicity (31.396 ±0.928).
- At 48-hour interval **Nano PLGA** showed a statistically significant difference compared to other groups except **mTAP loaded nano PLGA**. **DAP loaded nano PLGA** showed statistically significant difference compared to other tested groups except for **Regular DAP. Regular mTAP** showed statistically significant difference with all tested groups.

Table 5: Cytotoxicity test results measured by % of viable cells of all groups at different time intervals.

Groups	Nano PLGA	DAP loaded nano PLGA	mTAP loaded nano PLGA	Regular DAP	Regular mTAP	P-Value
24 h	13.677 ±1.379 [a]	22.11 ±2.71 [d]	17.502 ±1.002 [c]	29.612±1.691 [b]	34.310 ±1.358 [e]	<0.001
48 h	9.415 ±1.716 [a]	24.67 ±2.16 [b]	13.180 ±1.608 [a]	20.42 ±3.88 [b]	31.396 ±0.928 [c]	<0.001

Means that don't share same letter are significantly different in the same raw.
P ≤ 0.05 is considered Significant.

At 0.1mg concentration

- At 24 hours interval **Regular DAP** showed the highest cytotoxicity (99.07 ±4.10) while **Regular mTAP** showed the lowest cytotoxicity (104.01 ±4.56).
- At 48 hours interval **Nano PLGA** showed the highest cytotoxicity (98.77 ±2.53) while **DAP loaded nano PLGA** showed the lowest cytotoxicity (100.99 ±2.33).
- There is no difference of statistical significance between all of the tested groups at both 24-hour and 48-hour intervals.

Table 6: Cytotoxicity test results measured by % of viable cells of all groups at different time intervals.

Groups	Nano PLGA	DAP loaded nano PLGA	mTAP loaded nano PLGA	Regular DAP	Regular mTAP	P-Value
24 h	100.60 ±3.41 [a]	103.83 ±3.56 [a]	100.633 ±0.35 [a]	99.07 ±4.10 [a]	104.01 ±4.56 [a]	0.241
48 h	98.77 ±2.53 [a]	100.99 ±2.33 [a]	100.38 ±2.42 [a]	99.49 ±2.20 [a]	100.28 ±4.10 [a]	0.820

Means that don't share same letter are significantly different in the same raw.
P ≤ 0.05 is considered Significant.

2.a.2 **The impact of observation time on percentage of vitality of different intra canal medicaments "Table 7-8".**

At 1mg concentration

Observation time showed statistically significant effect on medication cytotoxicity of all groups except for **DAP loaded nano PLGA** and **Regular mTAP** groups which revealed no statistically significant impact on it.

Table 7: Cytotoxicity test results measured by % of viable cells of all groups at different time intervals.

Groups	Nano PLGA	DAP loaded nano PLGA	mTAP loaded nano PLGA	Regular DAP	Regular mTAP
24 h	13.677 ±1.379 [a]	22.11 ±2.71 [a]	17.502 ±1.002 [a]	29.612 ±1.691 [a]	34.310 ±1.358 [a]
48 h	9.415 ±1.716 [b]	24.67 ±2.16 [a]	13.180 ±1.608 [b]	20.42 ±3.88 [b]	31.396 ±0.928 [a]
P-Value	0.008	0.190	0.004	0.005	0.269

Means that don't share same letter are significantly different in the same column.
P ≤ 0.05 is considered Significant.

At 0.1mg concentration

Observation time revealed no statistically significant impact on medication cytotoxicity of all groups.

Table 8: Cytotoxicity test results measured by % of viable cells of all groups at different time intervals.

Groups	Nano PLGA	DAP loaded nano PLGA	mTAP loaded nano PLGA	Regular DAP	Regular mTAP
24 h	100.60 ±3.41 [a]	103.83 ±3.56 [a]	100.633 ±0.35 [a]	99.07 ±4.10 [a]	104.01 ±4.56 [a]
48 h	98.77 ±2.53 [a]	100.99 ±2.33 [a]	100.38 ±2.42 [a]	99.49 ±2.20 [a]	100.28 ±4.10 [a]
P-value	0.422	0.230	0.845	0.863	0.269

Means that don't share same letter are significantly different in the same column.
P ≤ 0.05 is considered Significant.

2.a.3 The impact of particle size (regular vs. nano) on percentage of vitality of different intra canal medicaments "Table 9-10".

At 1mg concentration

Particle size showed statistically significant effect on medication cytotoxicity of all groups except for DAP group at 48-h interval.

Table 9: The viability% of fibroblast cells of regular and nano scales of intracanal medicaments.

Time \ Group		DAP		mTAP	
		24 h	48 h	24 h	48 h
Particle size	Regular	29.612 ±1.691 [b]	20.42 ±3.88 [a]	34.310 ±1.358 [a]	31.396 ±0.928 [a]
	Nano	22.11 ±2.71 [a]	24.67 ±2.16 [a]	17.502 ±1.002 [b]	13.180 ±1.608 [b]
P-Value		0.003	0.104	<0.001	<0.001

Means that don't share same letter are significantly different in the same column.
P ≤ 0.05 is considered Significant.

At 0.1mg concentration

Particle size showed no statistically significant effect on medication cytotoxicity of all groups.

Table 10: The viability% of fibroblast cells of regular and nano scales of intracanal medicaments.

Time \ Group		DAP		mTAP	
		24 h	48 h	24 h	48 h
Particle size	Regular	99.07 ±4.10 [a]	99.49 ±2.20 [a]	104.01 ±4.56 [a]	100.28 ±4.10 [a]
	Nano	103.83 ±3.56 [a]	100.99 ±2.33 [a]	100.633 ±0.35 [a]	100.38 ±2.42 [a]
P-Value		0.130	0.385	0.190	0.966

Means that don't share same letter are significantly different in the same column.
P ≤ 0.05 is considered Significant.

DISCUSSION

The purpose of root canal treatment is to reduce the microbial load inside the root canal to promote healing and boost success rates. Therefore, antibacterial agents have been developed aiming to increase their activity against bacterial biofilms.[8] Because of its broad-spectrum antibacterial capabilities, nanotechnology is rapidly being used in medical and dental applications. As a good carrier, nanomaterials can enhance and support traditional antibiotics. They can also help to target antibiotics to an infection site, reducing systemic side effects.[9]

Therefore, this study aimed to compare different concentrations of two forms (regular & drug loaded nano PLGA) of two materials: Double antibiotic paste (DAP), Clindamycin modified triple antibiotic paste (mTAP) regarding their anti bacterial potential and cytotoxicity.

Enterococcus faecalis was chosen for our study because it can withstand extreme conditions.[8] In this study, a three-week-old Enterococcus feacalis biofilm was used to ensure a mature bacterial biofilm onto root canal dentin, which is more resistant to disinfectants.[1] Antibiotics could be one of the current therapeutic strategies indicated for eradicating *E. faecalis* from the root canal system. In this study, the DAP and mTAP were utilized instead of the TAP since minocycline was excluded in DAP[10] or substituted by clindamycin in mTAP.[11]

The two concentrations used in this study, 0.1 and 1 mg/mL, were in accordance with the AAE2015 guideline

for regenerative endodontic treatment.[12] The antibacterial effect of an intracanal medicament depends on whether it reaches the inaccessible area of the root canal. This can be achieved by adding carboxymethyl cellulose to the medicaments to create gel form, which is widely used in pharmaceutical and nutritional industries due to its biocompatible and biodegradable characteristics.[13] Poly lactic-co-glycolic acid (PLGA) polymer was utilized as a drug carrier in this study. PLGA is biocompatible, biodegradable and most importantly is FDA approved polymer. When utilised in biomedical applications, PLGA as a nanocarrier is thought to have low systemic toxicity.[5,14] The current study's findings antibacterial potential of the tested intracanal medicaments showed that all tested intracanal medications with different concentrations were effective against *E. faecalis* except nano PLGA. However, the higher concentration of 1 mg/mL was significantly higher than the lower concentration of 0.1mg/mL for both regular DAP, mTAP, and DA loaded nano PLGA, while it showed no significant difference of mTA loaded nano PLGA. These results came in agreement with a study (Asnaashari M et al., 2019)[11] who concluded that 1mg/ml of (MTAP) expunged the bacteria completely. Furthermore, it was in agreement with (Mclntrye PW et al., 2019)[15] who concluded that hydrogels containing 1mg/ml of DAP, had a significant direct antibacterial effect. However, these results contradict the findings of (Latham J et al., 2016)[16] who assumed that Ca(OH)2 or 1,0.1mg/mL concentrations of either TAP or DAP did not effectively disinfect the canal. Hense results lead us to reject the first null hypothesis since, for the particle size, the drug-loaded nano PLGA demonstrated a stronger antibacterial impact than their regular form at both concentrations. This may be because of their small particle size, which is believed to be better because it has a larger surface area, allowing for greater drug release over time. Loss of membrane permeability and improper membrane function have been linked to electrostatic interactions among positively charged NPs with negatively charged bacterial cells, as well as an accumulation of additional NPs on the bacteria's cell membrane. This causes major bacterial cell activities like respiration, transport of nutrients, and energy transduction to be disrupted, which ultimately leads in bacterial cell death.[17] This is similar to the findings of (Karczewski A et al., 2018)[18], who concluded that CLIN-m triple antibiotic nanofibers displayed significant antibacterial activity against all bacteria and could be an effective substitute for minocycline-based antibiotic pastes.

The mTAP in regular and with loaded nano PLGA form at 0.1mg/ml showed more effectiveness in eliminating the bacteria than 0.1 mg/ml of DAP in both forms, which might be related to the combined spectrum of antimicrobial activity and synergistic or additive actions of antibiotics "ciprofloxacin, metronidazole, and clindamycin" found in mTAP. When administered as a topical root canal treatment, the combination of these three antibiotics overcomes bacterial resistance and achieves stronger antimicrobial efficacy.[19] As was reported by (Karczewski A et al., 2018)[18] that the antimicrobial activity of CLIN-m triple antibiotic nanofibers and aliquots was higher against Aa and Ef than the CLIN alone. This result also came in agreement with (Latham J et al., 2016)[16] who suggested that TAP at 10 mg/mL was more efficient than DAP and Ultracal Ca(OH)2 in removing bacteria from the root canal system. Conversely, (Chamorro-Petronacci CM et al., 2022)[20] found that the combination of ciprofloxacin and metronidazole had the most effective effects against *E. faecalis* than TAP in ordered mesoporous silica at different concentrations this may be due to using ordered mesoporous silica as loading material and higher concentrations of antibiotics.

The cytotoxic evaluation was essential since it could impact the biology and physiologic behaviour of the cells. As a result, the characteristics and concentrations of the medication are critical for successful therapeutic outcomes. Cytotoxicity results were shown to be concentration-dependent. This is in agreement with (Ruparel NB et al., 2012)[21] who examined TAP and DAP with various concentrations on the survival of SCAPs and concluded that higher concentrations have detrimental effects on SCAP survival compared to lower concentrations. Also, that was in agreement with (Sabrah AH et al., 2015)[22] who evaluated the cytotoxic effect of several TAP and (DAP) dilutions (0.125, 0.25, 0.5, 1, and 10 mg/ml) on DPSCs, and found that except for 0.125 mg/ml, all antibiotic dilutions severely decreased the viability of DPSC.

Conversely, (Latham J et al., 2016)[16] claimed that indirect cytotoxicity is caused by insufficient disinfection of the root canal system by utilising lower concentrations like 0.1mg/ml.

Furthermore, the cytotoxicity was directly proportional to the duration of treatment in high concentrations only except for DA loaded nano PLGA and regular mTAP. Which came in agreement with (Chuensombate S et al., 2013)[23] who exposed the cells to either TAP or every single antibiotic component of TAP using different concentrations for 1, 3, 5, and 7 days, they found that the cytotoxicity increased in a concentration- and time-dependent manner. The nano-scale intracanal medicaments had a more cytotoxic effect than their corresponding regular scale at higher concentrations, so these results reject our second null hypothesis. This could be attributed to a change in particle size from macroscopic to nanoscale, which results in alteration in the physical and chemical characteristics of the substances and smaller particles, that could induce various biological reactions, including toxicity. It is additionally possible that the nanoparticles disrupted the respiratory chain of mitochondria, resulting in the generation of [reactive oxygen species (ROS)] and a cessation of [adenosine triphosphate(ATP)] production,

leading to DNA damage.[24,25] Samiei et al., 2018[26] discovered that when nanoparticles were present, there was a small rise in lactic acid dehydrogenase leaking, which is a cell damage sign, showing that these particles impair the metabolism of cell. This came in agreement with (Karczewski A et al., 2018)[18] who discovered a substantial reduction in human DPSC viability following exposure to aliquots obtained from CLIN and CLIN-m nanofibers when compared to PDS and the control groups. This came in disagreement with (Pankajakshan D et al., 2016)[27] who discovered that DPSC adhesion and spreading on triple antibiotic-containing nanofibers treated dentin were better than TAP using qualitative representative CLSM images. However at low concentrations observation time and particle size showed no statistically significant effect on medication cytotoxicity of all groups, therefor these results support our second null hypothesis. This came in agreement with (Yadlapati M et al., 2014)[28] who evaluated TAP, DAP, minocycline, and calcium hydroxide cytotoxicity and influence on pro-inflammatory cytokine mRNA expression levels in human periodontal ligament (HPDL) fibroblasts. They discovered no significant differences in cytokine pro-inflammatory gene expression levels following 24-h or 48-h exposure to any of the materials, with the exception of IL6, which had considerably greater mRNA levels with the 24-h TAP, which could be related to the minocycline component contained in TAP. Regular mTAP showed the lowest cytotoxicity of all tested groups in high concentration at both intervals. The suppression of cell collagenase and matrix metalloproteinase may account for mTAP's lowered cytotoxicity.[29] Moreover clindamycin additionally showed proangiogenic activity in vitro studies.[18]

CONCLUSIONS

It is possible to conclude from the current investigation that nano intracanal medicaments are more effective against *Enterococcus faecalis* than their regular counterparts. The regular forms of intracanal medicaments have a less cytotoxic effect on fibroblast cells. Higher concentrations of 1 mg/ml are more effective against *Enterococcus faecalis* than lower concentrations of 0.1mg/ml but were more cytotoxic. None of the represented antibacterial agents was able to completely disinfect the root canal.

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