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# Evaluation of the Antimicrobial Effect of Pre-Synthesized Novel Antibiotic Electrospun Nanofibers as an Intracanal Delivery Strategy for Regenerative Endodontics: A Randomized Clinical Trial

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## Abstract

**AIM:** The aim of this study is to evaluate the antimicrobial effect of pre-synthesized novel antibiotic loaded electrospun nanofibers and compare it with conventional triple antibiotic paste when used in patients with immature necrotic teeth.

**METHODS:** Antibiotic loaded nanofibers were fabricated by electrospinning**.** Thirty-four patients with immature necrotic teeth were included in the study. In the first visit, access cavity preparation was performed to obtain the first bacteriological sample (S1). The canals were thoroughly irrigated using sodium hypochlorite 1.5% and a second sampling was performed (S2). Patients were randomly divided into two groups according to the intracanal medicament used: Modified triple antibiotic paste (MTAP) loaded electrospun nanofibers or MTAP paste. At the second appointment, the third samples (S3) were taken. The intracanal bacterial count was determined using the spread plate culture technique. Scanning electron microscopy (SEM) was used to examine the morphology of the fabricated MTAP loaded electrospun nanofibers.

**RESULTS:** Both MTAP nanofibers and MTAP paste resulted in significant reduction of bacterial count after the irrigation step. MTAP nanofibers resulted in significantly higher percent reduction of bacterial count ($p < 0.05$).

**CONCLUSIONS:** It was concluded that electrospinning technology can be used to fabricate antibiotic containing nanofibers which can results in enhanced disinfection in regenerative endodontic procedures.

## Introduction

In the era of regenerative medicine, proper microbial control is mandatory for success of regenerative endodontic procedures (REPs), as regeneration and repair can never occur in presence of a persistent infection [1]. REPs for immature teeth comprise minimal-to-no mechanical instrumentation. That is why they count on the chemical disinfection step and application of intracanal medicaments to achieve proper disinfection. The previous studies showed that the majority of failed regenerative cases were attributed to the presence of a persistent root canal infection [2], [3].

Despite the fact that triple antibiotic paste (TAP) is used in an increasingly number of clinical cases since its introduction by Hoshino [4], still the majority of studies on the antimicrobial effects of the material are *in vitro* studies carried out under a very controlled environment [5]. Tooth discoloration was mainly attributed to minocycline as a component of the TAP [6]. Minocycline binds to the calcium ions in dentin by chelation, and induces a color change [7]. Minocycline is believed to undesirably affect angiogenesis by reducing vascular endothelial growth factor secretion, which suppresses the neovasculogenesis of endothelial cells [5], [8]. Thus, a number of suggestions were proposed including sealing the coronal dentinal tubules with bonding agent as well as eliminating minocycline using only double antibiotic paste [9]. Replacement of minocycline with cefaclor, amoxicillin, or clindamycin was also proposed. On the other hand, clindamycin is less cytotoxic, exerts a remarkable post-antibiotic activity and even more, can induce a proangiogenic effect similar to vascular endothelial growth factor [10], [11].

Moreover, the toxicity of TAP on stem cells in regenerative endodontics is well established [5], [6]. Regeneration of the pulp dentin complex involves both

human dental pulp stem cells (HDPSCs) and stem cells of the apical papilla (SCAP) [12], [13], [14], maintaining the viability of both types of cells is crucial for success. Concerns about the deleterious toxic effects on stem cells have led the way for research for more biocompatible means for delivery of the antibiotic [15].

Electrospinning, which refers to “electrostatic spinning,” is an intervention that relies on application of high voltage electric force to draw charged threads from polymer solutions to form nanofiber, where the fiber’s diameter ranges from few to hundreds of nanometers [16]. Electrospinning technology was used to manufacture drug loaded polymer-based nanofibers containing minimal effective minute concentrations for drug delivery strategies. Electrospun nanofibers containing antibiotic have proven to be a promising approach to achieve decontamination of the root canal *in vitro* [17], [18], [19], [20], [21], [22], [23], [24].

In this study, we fabricated electrospun nanofibers loaded with a modified triple antibiotic paste containing metronidazole, ciprofloxacin, and clindamycin and evaluated its antimicrobial effect**.** To the best of our knowledge, no previous clinical studies investigated the effect of antibiotic electrospun nanofibers in patients with necrotic immature teeth undergoing regenerative endodontic treatment.

## Materials and Methods

### *Study design*

The study was designed as a prospective, parallel, blinded, and randomized clinical trial with 1:1 allocation ratio. The protocol of this study was registered in the national institute of health clinical trials registry [NCT03690960]. The protocol of this study was reviewed and approved by the Institutional review board and ethics committee (IRBs/ECs) in the Faculty of Dentistry - Cairo University. All participants provided signed informed consent. Participants were recruited from the endodontic clinic’s out patients at Faculty of dentistry, Cairo University, Cairo governorate. Patients were treated in accordance with the Helsinki Declaration.

Sample size calculation was performed, and a total of 34 patients (divided into two groups of 17 patients each) were included in the study, which set the power of the study at 80%. The Type I error probability related to this test was 0.05. A computer software was used to generate a random sequence. Opaque envelopes containing folded numbered papers were prepared to be dragged by the patients. The patients did not know their treatment group. The laboratory technician and statistician were also blinded to the study groups.

### *Patient selection*

The inclusion criteria included healthy patients between 9 and 25 years old with non-vital, asymptomatic single-rooted tooth with immature root. Exclusion criteria were patients with allergy to any of the used antibiotics, patients taking antibiotics in the past 3 months. Teeth with vital pulp, previously initiated treatment or teeth that cannot be isolated properly were also excluded from the study.

### *Fabrication of antibiotic containing nanofibers*

Polyvinyl alcohol (PVA) (Oxford laboratories, India) was dissolved in sterile saline solution at 7% weight/volume ratio to prepare the polymer solution which was then stirred using a magnetic stirrer (WiseStir, Germany) at 100°C for 2 h [16]. After which 15% citric acid powder (Adwic - El Nasr, Egypt) was added to the solution. Afterward, three antibiotics powder of metronidazole, ciprofloxacin, and clindamycin were added. Equal proportions (335 mg of each drug) were added to the polymer solution. The antibiotic concentration was 30 wt.% relative to the polymer weight [17]. After 1 h of stirring, plastic syringes with a metallic tip needle were loaded with the solution and the solution was electrospun using an electrospinning system (NanoEbers LLC, Egypt). The processing parameters applied were; a flow rate of 0.4 mL/h, the distance between the needle tip and the collecting mandrel was 15-cm, and electrical voltage of 20 kV. The collected nanofibers membrane was heat treated on a glass watch in a dry heat oven for 10 min at 120°C. The nanofibers membrane was then cut into pieces with a final MTAP concentration of 0.1 mg/ml. Finally, the nanofibers were vacuum dried and exposed to UV irradiation for sterilization [11], [23].

### *Regenerative endodontic procedures*

#### *The first appointment*

Medical and dental history were obtained from all patients participating in this research. After confirmation of the diagnosis, teeth were anaesthetized and isolated. The crowns and surrounding structures were disinfected with 30% $H_2O_2$ and 2.5% sodium hypochlorite for 30s after which 5% sodium thiosulfate was used. Sterility was confirmed by swabbing a sample from the crown surface. A sterile round bur was used for preparing the access cavity with sterile saline solution. The first bacteriological samples (S1) were taken by inserting three successive sterile paper points in the canal for 1 min each. Determination of working length was done using apex locator and confirmed using radiograph (Soredex, Digora, USA). Light mechanical preparation was done using stainless

steel manual K-files (MANI, INC., Japan) and the canals were thoroughly irrigated using 20 ml of sodium hypochlorite 1.5% followed by 5 ml of 0.5% sodium thiosulfate. The final wash was done using saline and a second sampling were performed (S2) samples were then immediately placed in sterile ready to fill vials containing the transport media for culturing. All samples were transported to the microbiology laboratory within 1 h [25], [26].

The patients were randomly allocated into two groups according to the type of intracanal medicament**:**

- Intervention group: MTAP nanofibers (prefabricated electrospun nanofibers loaded with metronidazole, ciprofloxacin, and clindamycin) were placed into the root canals using hand pluggers (Sedradent solutions, Egypt).
- Comparator group: Equal proportions of antibiotic powders of metronidazole, ciprofloxacin, and clindamycin were combined with saline to make a homogeneous MTAP paste with a creamy consistency and a concentration of 1 g/ml. The mixture was injected into the canal using a syringe that was 2 mm shorter than the working length. Excess antibiotic was removed from the access cavity to a point just below the cementoenamel junction.

*The second appointment*

The second visit was scheduled after 2–3 weeks, the nanofiber or MTAP paste were removed with 5 ml of sterile saline solution and the third samples (S3) were taken in the same previously described manner. Irrigation was done with 17% Ethylenediaminetetraacetic acid (EDTA) and the final wash was done using saline. To induce bleeding into the canal, a manual K-file was inserted beyond the working length. The bleeding was permitted to reach a depth of 3 mm below the cemento-enamel junction. The blood column was left for 3 min to allow for formation of a blood clot followed by a placement of a CollaCote membrane (Zimmer Dental, Carlsbad, CA). Then a 3-mm barrier of mineral trioxide aggregate (Cerkamed, Poland) was placed to reach a level of 2–3 mm beyond the cementoenamel junction. The tooth was restored with glass-ionomer and composite resin [26].

***Intracanal bacterial count assessment***

The intracanal bacterial count was determined using the spread plate culture technique. Samples in sterile ready to fill vials were dispersed for 60 s with vortex and the solution was serially diluted. For aerobic bacterial culture, brain heart infusion agar plates (Oxoid microbiology, LTD, England) were inoculated with 50 μl of these diluted samples and cultured under aseptic conditions, then incubated at 37°C for 48 h. For anaerobic bacterial culture, agar plates were inoculated with the other 50 μl of diluted samples under aseptic conditions, and placed in an anaerobic sealed jar with GasPak (Anaerogen (AN0035), Oxoid microbiology, England) and anaerobic indicator (BR0055B, Oxoid microbiology, England) followed by incubation for 48 h at 37°C. The number of colonies forming units per millimeter of each dilution (CFUs) on the agar plate was eventually counted [25], [26], [27].

***Evaluation of the MTAP loaded electrospun nanofiber***

SEM (SEM Model Quanta 250 FEG, FEI company, Netherlands) with an Image-J software was used to observe the morphology of the developed nanofibers. An ion sputter (Hitachi Ion Sputter MC1000, Japan) was used to coat the samples with gold particles for imaging [11].

***Statistical analysis***

Statistical analysis was performed with IBM SPSS Statistics (IBM Corporation, Version 25, NY, USA), CFU/ml counts were converted into LOG 10. Parametric data were analyzed using independent t-test for comparisons between two groups and multi-way ANOVA followed by Tukey *post hoc* test for multiple group comparisons. The significance level was set at $p \leq 0.05$ within all tests.

## Results

A total of 34 patients met the inclusion criteria and were enrolled in the study. Patients were randomized into two groups of 17 patients each. Thirty-four patients were included in the analysis. The mean age in the intervention group (MTAP loaded electrospun nanofibers) was 15.94 ± 5.88 years, while for the comparator group (MTAP paste), the mean age was 17.00 ± 3.61 years with no statistically significant difference between groups ($p = 0.532$). For the gender, the intervention group (MTAP loaded electrospun nanofibers) consisted of ten males and seven females while the comparator group (MTAP paste) consisted of eight males and nine females with no statistically significant difference ($p = 0.49$).

***Bacterial count***

Comparison of bacterial count between the study groups (Table 1 and Figure 1) showed that, for aerobic bacteria at S1, there was no statistically

**Table 1: Descriptive statistics and test of significance comparing the bacterial count (log10) between the study groups**

| Bacterial count | Aerobic, mean ± SD | | | | Anaerobic, mean ± SD | | | |
|---|---|---|---|---|---|---|---|---|
| | S1 | S2 | S3 | *P* | S1 | S2 | S3 | p |
| MTAP nanofiber | 6.35 ± 0.35 | 5.27 ± 0.8 | 0.72 ± 1.61 | <0.001* | 6.17 ± 0.42 | 5.02 ± 0.71 | 0.24 ± 0.97 | <0.001* |
| MTAP paste | 6.23 ± 0.33 | 5.05 ± 1.9 | 2.6 ± 2.5 | <0.001* | 6.19 ± 0.37 | 4.04 ± 2.7 | 2.18 ± 2.4 | <0.001* |
| p | 0.3 (NS) | 0.6 (NS) | 0.015* | | 0.8 (NS) | 0.16 (NS) | 0.004* | |

*Significant. Significance level P ≤ 0.05. NS: Non-significant, SD: Standard deviation, MTAP: Modified triple antibiotic paste, S1: First bacteriological sample, S2: Second sampling, S3: Third samples.

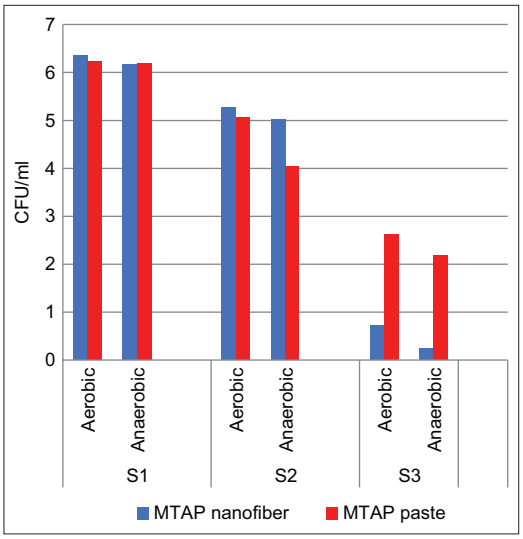


*Figure 1: Bar chart illustrating mean value of bacterial count (log 10) in the study groups*

significant difference between bacterial count in both groups at the start of the treatment, (p = 0.3). At S2, there was no statistically significant difference between bacterial count in both groups, (p = 0.6). At S3, there was a statistically significant higher mean value of bacterial count recorded in the MTAP paste group compared to MTAP loaded electrospun nanofibers group, (p = 0.015). Within each group, there was a statistically significant difference between bacterial count at S1, S2, and S3 (p < 0.001).

For anaerobic bacteria, at S1, there was no statistically significant difference between bacterial count in both groups at the start of the treatment after access cavity preparation, (p = 0.8). At S2, there was no statistically significant difference between bacterial count in both groups, (p = 0.16). At S3, there was a statistically significant higher mean value of bacterial count recorded in the MTAP paste group compared to MTAP loaded electrospun nanofibers group, (p = 0.004). Within each group, there was a statistically significant difference between bacterial count at S1, S2, and S3 (p < 0.001).

### *Percent reduction (%) of the bacterial count*

Comparison of the percent reduction of bacterial count between the study groups (Table 2 and Figure 2) showed that, for aerobic Bacteria from S1 to S2, there was no statistically significant difference between both groups, (p = 0.8). From S2 to S3, there was a statistically significant higher mean percent of reduction in MTAP loaded electrospun nanofibers group compared to MTAP paste group, (p = 0.002). From S1 to S3, there was a statistically significant higher percent of reduction in the MTAP loaded electrospun nanofibers group to MTAP paste group (p = 0.015).

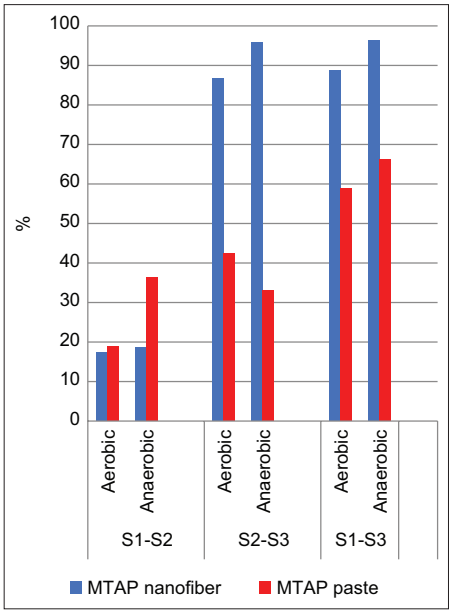


*Figure 2: Bar chart illustrating the percent reduction (%) of bacterial count (log 10) in the study groups*

For anaerobic Bacteria, from S1 to S2, there was no statistically significant difference between both groups, (p = 0.1). From S2 to S3, there was a statistically significant higher percent of bacterial reduction in MTAP loaded electrospun nanofibers group compared to MTAP paste group, (p < 0.001). From S1 to S3, there was a statistically significant higher percent of bacterial reduction in the MTAP loaded electrospun nanofibers group compared to MTAP paste group, (p = 0.005).

### *Scanning electron microscope imaging (SEM) of the fabricated nanofibers*

SEM imaging confirmed the ability to fabricate antibiotic containing nanofibrous structures with relatively uniform diameters in the nano-submicron scale. The fiber diameter of the antibiotic containing loaded electrospun nanofibers ranged between 400 nm–600 nm. At 500× magnification the SEM images showed a dense, compact, nonwoven nanofibrous mesh with even architecture. At 2500× magnification, a nanofibrous 3D network consisting of well-defined randomly oriented loaded electrospun nanofibers with interconnected pores was observed. At 6000× magnification, The SEM images showed

**Table 2: Descriptive statistics and test of significance comparing the percent reduction (%) of bacterial count (log10) between the study groups**

| Reduction (%) | Aerobic, mean ± SD | | | Anaerobic, mean ± SD | | |
|---|---|---|---|---|---|---|
| | S1-S2 (%) | S2-S3 (%) | S1-S3 (%) | S1-S2 (%) | S2-S3 (%) | S2-S3 (%) |
| MTAP nanofiber | 17.25 ± 10.1 | 86.58 ± 30.18 | 88.72 ± 25.14 | 18.6 ± 9.6 | 95.8 ± 17.3 | 96.3 ± 15.4 |
| MTAP paste | 18.94 ± 31.6 | 42.4 ± 44.6–100 | 58.8 ± 40.5 | 36.27 ± 42.75 | 32.95 ± 39.85 | 66.22 ± 37.60 |
| p | 0.8 (NS) | 0.002* | 0.015* | 0.1 (NS) | <0.001* | 0.005* |

*Significant. Significance level P ≤ 0.05. NS: Non-significant, SD: Standard deviation, S1: First bacteriological sample, S2: Second sampling, S3: Third samples.

that the loaded electrospun nanofibers have a smooth, uniform surface morphology and structure without any beads along the fiber. Moreover, at higher magnification 10000 X, noticeable branching of the fibers was noted along with larger interfiber spaces (Figure 3).

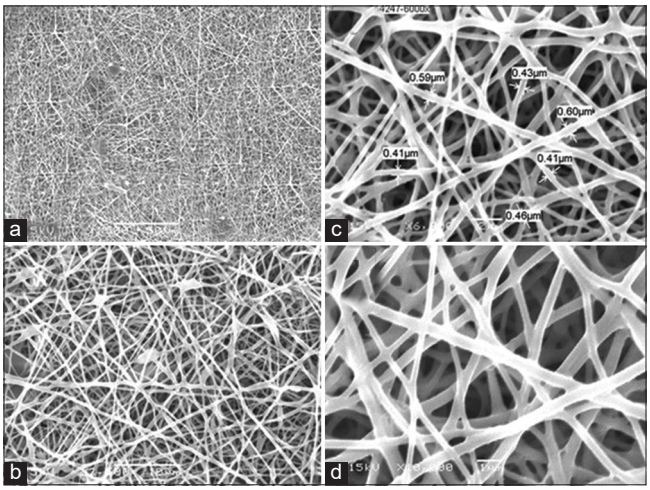


*Figure 3: SEM photograph of the MTAP coated electrospun nanofibers at different magnifications; 500× (a), 2500× (b), 6000× (c) and 10000× (d)*

## Discussion

Regenerative endodontic procedures for the management of necrotic immature teeth have been widely investigated and applied. Due to the fact that necrotic immature teeth have thin dentinal walls that mandates the use of minimal instrumentation, disinfection relies mainly on chemical methods [3], [28].

In our study, electrospinning technology was used to fabricate the modified triple antibiotic loaded nanofibers. PVA is an exceptional polymer with admirable properties as biocompatibility, inertness and safety. PVA is FDA approved for clinical application in humans and is widely used in a number of pharmaceutical products [23]. Another interesting feature, the polymer is readily soluble in water which eliminated the need to use to toxic solvents. The polymer (PVA) was dissolved at a concentration of 7% W/V in the solvent to ensure production of smooth bead free nanofibers in the nanoscale. At a lower concentration the stretching force was weak resulting in fragmentation of the fiber jet into droplets, while increasing the polymer's concentration results in larger diameter nanofibers [29], [30]. In our study, the parameters used to obtain a continuous steady jet for the PVA/antibiotic solutions were 20 kv voltage, a flow rate of 0.4 ml/h, and a 15 cm needle to collector distance.

Various methods were implemented to incorporate antibiotics into nanofibers via electrospinning including blending, coaxial electrospinning, encapsulation, and attachment. In our study, blending was used before the electrospinning process to actively incorporate the antibiotic within the polymer solution. Blending electrospinning is a widely used form of electrospinning being a simple and versatile method as long as proper solubility of the antibiotic in the polymer solution is achieved [31]. The antibiotic mixture was added to the polymer solution at a 25–35 Wt.%, this concentration was previously applied successfully in numerous studies [11], [17], [18].

In our study, a modified antibiotic combination was used, where minocycline was replaced with clindamycin. Minocycline results in severe adverse effects that counterbalance the regenerative process as a result of inhibition of angiogenesis and toxic effects on stem cells [12], [13]. In addition, minocycline causes tooth discoloration because it binds to calcium ions by chelation and form insoluble complexes [9]. In our study, a two-visit revascularization protocol was followed. A recent systematic review concluded that the success rate of single visit REPs was much lower than the multiple visit success rate for REPs [32]. Root canals were irrigated using 1.5% sodium hypochlorite as it was proved that it has lower toxicity to stem cells in the apical tissues compared to full concentration 5.25% sodium hypochlorite [33].

For the comparator group, antibiotics were mixed with saline to form a slurry thick mix, the same 1 gm/ml concentration was used before and showed successful results [25], [26], [27]. For regenerative endodontic procedures, it is now recommended to use intracanal antibiotics at concentrations of 0.1–1 mg/ml to maintain stem cell viability [33]. However, this low concentration always results in a liquid mixture difficult to apply inside the root canal [26]. The use of MTAP in the form of nanofibers allowed for the use of the minimum yet effective recommended antibiotic concentration in the intervention group.

Although culture technique comes with limitations, numerous studies have shown that success of treatment is directly related to decrease in number of microorganisms [34], [35]. To decrease the odds of false positive results, teeth were isolated with rubber

dam, the tooth, and the rubber dam were disinfected and sterility control samples were collected, after which a sterile bur was used to access the pulp chamber [36].

In our study, SEM was used to examine the fabricated MTAP loaded electrospun nanofibers' morphology as the nanofibers are non-conductive [37], sputter coater was used before scanning to deposit a thin nanolayer of gold ions to ensure obtaining images with the highest resolution [38]. Our results showed that successful electrospinning of PVA polymer solution containing antibiotic for local drug delivery is possible. The antibiotic containing nanofibers were continuous and even. No bead formation was detected under scanning electron microscopy. The fiber diameter ranged between 400 nm and 600 nm; smaller diameter nanofibers are known to provide more support cell proliferation compared to larger diameters approaching 1000 nm [39]. Moreover, noticeable branching of the nanofibers was noticed along with large inter-fiber spaces which can be of great value in regenerative procedures [39].

Overall, bacterial levels were significantly decreased with each phase of the treatment. At S1, after access cavity preparation, all teeth sampled were positive for bacteria. No statistically significant difference between groups was found indicating similar participants at the start of the trial. The mean counts were close to other studies in immature teeth [25], [26], [40]. However, it was higher than the previous studies performed on teeth with mature roots. This may be credited to the fact that sampling in a tooth with a very large canal space is much easier and more conductive to higher bacterial growth [25].

At S2, after irrigation with sodium hypochlorite, all cases showed a significant reduction in the aerobic, anaerobic, and total bacterial count. This agrees with other studies results [25], [26], [40]**.** However, in the present study, the majority of teeth still continued to be positive for bacterial analysis after the 1.5% sodium hypochlorite irrigation this comes in disagreement with Nagata *et al*. [26]. This disagreement in the antimicrobial efficacy after the irrigation protocol may be attributed to the much higher concentration of sodium hypochlorite (6%) used in their study**.** However, the toxicity of such high concentration to DPSC and SCAP is now well-established and this concentration is no more recommended [12], [33].

After intracanal medicament placement at S3, there was a further significant reduction in the CFU count for both groups. This further reduction comes in agreement with the previous studies [25], [26], [27]. Our results showed a significant higher bacterial reduction after intracanal medicament placement in both groups compared to the bacterial reduction after the irrigation step. This comes in agreement with the results of the study by de-jesus-soares [25]. The anatomy of the root canals of immature teeth might have enhanced the effectiveness of the used medicaments, along with the minimal instrumentation implemented and lower concentration of irrigants, contributed to the more pronounced effect of the intracanal medicament in regenerative endodontics [6].

Comparing both study groups, regarding the bacterial count no statistically significant difference was found between groups at S1 and S2. However, a statistically significant higher number of bacterial cells was found in the MTAP paste group at S3. A statistically significant higher mean value of percent reduction was recorded in the MTAP loaded electrospun nanofibers group compared to the MTAP paste group from S2 to S3 and from S1 to S3. This significant difference may be accredited to the sustained release nature of the MTAP loaded electrospun nanofibers as a drug delivery method. The previous studies showed that nanofibrous scaffolds containing antibiotic mixtures exhibited a consistent and sustained antimicrobial effect of for a period of 21 day inside the root canals [11], [18].

## Conclusions

From the findings of our study, it could be concluded that electrospinning technology is a successful method for fabrication of MTAP loaded electrospun nanofibers. Substitution of minocycline by clindamycin in the formula of triple antibiotic paste resulted in production of smaller diameter nanofibers, a potent antimicrobial effect while avoiding the undesirable tooth discoloration. Along with the ease of placement of nanofibers inside the root canals, sustained drug release and proposed nontoxic effect on stem cells, MTAP loaded electrospun nanofibers had a statistically significant higher antimicrobial effect in our study compared to MTAP paste.

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