INTRODUCTION

Microbial infection is the main aetiological factor of pulpal and periapical diseases. Invasion of the root canal system via microbial irritants leads to infection, necrosis and apical periodontitis (1). Removal of microbial irritants from the root canal system and dentinal tubules and prevention of recontamination are the pertinent to successful root canal treatment (2). This is achieved by several steps of root canal instrumentation procedures (3). However, current mechanical instrumentation techniques alone cannot completely eliminate the bacterial biofilms from the root canal; therefore, irrigants and medicaments are used to disinfect inaccessible areas irritants do not disinfect in between appointments (4).

A number of challenges exist for achieving these goals including; the complexity of root canal system, limitations of intracanal medicaments and irrigants in eliminating the bacteria (5), in addition to complex microbial biofilms which are hard to be removed by the conventional root canal disinfection protocols (6).

Irrigants can act for a short duration while intracanal antiseptic medicaments can act for longer time against microorganisms within the root canal. Despite of their extreme importance, the root
canal irrigants and medicaments still have cytotoxic effects. Therefore, it is essential to develop effective and safe disinfecting strategies in endodontic procedures (7).

The chemical nature of medicaments employed for root canal disinfection are varied; they come from different groups, different chemicals or combinations of drugs. Antibacterial intracanal medication is expected to be effective throughout its application period and to penetrate the dentinal tubules (7, 8).

The most common used intra canal medicament is calcium hydroxide \((\text{Ca(OH}_2\text{)})\), which can destroy the bacterial cell membrane due to its high pH. Many other materials have been introduced as intracanal medicaments such as Chlorhexidine (CHX), triple antibiotic paste (TAP), and silver containing medicaments (8).

Nowadays, the rapid development of nanotechnology helps to develop powerful tools for various biomedical applications. Nanomaterials have gained popularity as antibacterial agents as a result of their broad-spectrum activity and biocompatibility. Nanoparticles in the range of 10 to 100 nm have been known to have bactericidal effects (9).

Irrigant solutions and intracanal medicaments may contact the periapical tissues, either through extrusion from the apex or leaching. Then it should be biocompatible and induce bone healing (10). Despite the wide application of nanoparticles to intracanal medicaments, there is a lack of information concerning their impact on human health and this gap in knowledge regarding the potential cytotoxic effect on human mesenchymal stem cells has to be evaluated (11).

Cytotoxicity is defined as the capacity of a material to impact cellular viability and can be measured at various physiological endpoints such as reduction in cell growth and proliferation, necrosis, apoptosis, or combinations of these aspects. Evaluation of cytotoxic activity of intracanal medicaments is of great importance as it affects the biological and physiological behavior of these cells (12).

Therefore, this study aimed to evaluate the cytotoxicity of newly introduced nano triple antibiotic paste with nano anti-inflammatory drug as intra canal medicaments.

**MATERIALS AND METHODS**

This study was approved by the ethics committee (Faculty of Dentistry, October 6 University, Cairo Egypt) number 4-2020.

Intracanal medicaments used in this study with their specifications, compositions, manufacturers as well as the technique of formation of nanomaterials are listed in Table 1.

**Characterization of particles**

Transmission electron microscope (TEM) (JEOL JEM 2100, Tokyo, Japan) at 200 Kv was used to identify the size and shape of the nanoparticles (Fig. 1a, b). The particle size was found to be in the range of 10 to 40 nm.

The NTAPC particles were white in color, powder in form and when tested for solubility it showed dispersion in water. Scanning electron microscope examination with higher magnifications showed the Nano TAPC particles morphology was characterized by being spherical and flake in shape varying in size with rough surface (Figs. 1c, 1d).

All tested intra canal medicaments were applied to human skin fibroblasts (HSF) 1. Cell viability after 24 and 48 h and 7 days was evaluated using an MTT assay test.

**Cytotoxicity evaluation procedures**

(a) **Cell cultures**

HSF cell line \((2\times10^5)\) were supplied from VACSERA Company, Giza, Egypt. Cells were grown in culture flasks containing Eagle’s minimal essential medium (MEM-E) and supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acid solution, and 100 IU penicillin and 100 units of streptomycin in 0.9% NaCl in a humidified atmosphere of 5% CO\(_2\), 95% air at 37°C until a confluent monolayer was achieved. (All products: Sigma-Aldrich, Corp., St. Louis, MO, USA).

Target cells were maintained in lab for further applications where growth medium was decanted and trypsin (Sigma Aldrich, USA) solution (0.25%) was added for 2-3 min. Trypsin was decanted and cells were incubated until complete disassociation of cells.

The suspended cells were counted by hemocytometer and distributed in 96 well tissue culture plates as 100μl of culture medium with cell density so 30⁴ \(\text{cells/cm}^2\) (TPPSwiss). Plates were incubated for 24-48 h at 37°C in a 5% CO\(_2\) atmosphere to allow the cells attachment.

(b) **Sample size calculation**

Using ANOVA test, assuming an effect size F ratio of 0.5, type I error of 0.05 and power of 0.8, a total sample size of 30 specimens (10 per group) was required to test the difference between the three groups. The sample size was calculated using G Power software version 3.1.9.2.

A total of 30 samples were used, 10 for each intracanal medicament shown in (Table 1).

(c) **Extraction procedure**

Tested materials were collected aseptically under Laminar Air Flow (LAF) and suspended in MEM-E medium to obtain 10 mg/ml of each sample for 7 days. Each nano extract media was then collected using Millex-GS sterile filter with pore size 0.22 μm while 0.45 μm pore size filters were used for collection of the extract media of regular particle size samples.

(d) **Determination of cytotoxic medicament concentrations**

Tested medicaments were two-fold serially diluted in sterile test tubes. Several dilutions of the extraction media were obtained using MEM-E Medium to achieve a total of three concentrations (10, 0.625 and 0.07 mg/mL) to determine the concentration in which the cell will survive.

(e) **Cytotoxicity test**

1) **MTT assay**

    Cell viability was evaluated by performing the MTT colorimetric assay. MTT 0.5 mg/ cm\(^2\) was added to the pre-treated cells as 50 μL/cm\(^2\), tissue culture plates were incubated at
The treated flasks showed cytological changes and detached cells. The detached cells were collected by cold centrifugation. The pelleted cells were fixed with methanol then prepared for flow cytometry processing.

2) Flow cytometry analysis

Flow cytometric DNA analysis was done using the fluorescence-activated cell sorting [FACSCAN] flow cytometer (14).

The remaining parts of the cell pellet of the treated and control samples were transferred into a falcon tube and the cell culture medium was removed and replaced by the buffer solution of the cell cycle test. The samples were again centrifuged for 5 minutes at 300 rpm at room temperature [20 to 25°C]. The supernatant was aspirated leaving approximately 50 μL of the residual buffer in the tubes to avoid disturbing the pellet at room temperature. 125 μL of solution ‘A’ containing trypsin was added to digest the cell membrane for 10 minutes. 125 μL of solution ‘B’ contacting trypsin in-

| TABLE 1. Material specification, composition and manufacturer |
|---------------------------------|-----------------|-----------------|-----------------|
| Material                        | Specification   | Composition      | Manufacturer     |
| Metapex (Control group)         | Non-setting     | Calcium hydroxide | META BIOMED Co,  |
|                                 | calcium         | and iodoform     | Chungbuk, Korea.|
|                                 | hydroxide       |                 |                  |
| Triple antibiotic paste (TAP)   | Mixture of      | Using commercially available tablets | Flagyl 500 mg:  |
|                                 | ciprofloxacin,  | of Ciprofloxacin (Ciprofloxacin 500 mg),  | (Sanofi Aventis, |
|                                 | metronidazole   | Metronidazole (Flagyl 500 mg) and | Cairo, Egypt)   |
|                                 | and minocycline | Minocycline (Minocin 50 mg). Following | (Amriya pharm,  |
|                                 |                 | the removal of the enteric coating of the | Alexandria, Egypt) |
|                                 |                 | tablets, the contents were ground using  | Minocin 50 mg:   |
|                                 |                 | a mortar and pestle and mixed in an equal | (Sedico, Giza, Egypt) |
|                                 |                 | amounts by weight (1:1:1) in a mixing |                  |
|                                 |                 | pad (100 mg of each) and then will be |                  |
|                                 |                 | dissolved in 100 mL of sterile water to |                  |
|                                 |                 | prepare 1 mg/mL solution of TAP in a |                  |
|                                 |                 | creamy consistency |                  |
| Catafast                         | 50 mg NSAIIDs   | Every sachet contains 50 mg diclofenac  | NOVARTIS PHARMA S.A.E., Cairo, Egypt |
|                                 | granules for   | potassium, Potassium hydrogen carbonate, | NanoTech Center (Cairo, Egypt). |
|                                 | oral solution  | mannitol; aspartame, saccharin sodium, |                  |
|                                 | (Diclofenac     | glyceryl dibehenate, mint flavor, anise flavor. |                  |
|                                 | potassium)     |                  |                  |
| Nano Triple antibiotic paste (TAP) | Mixture of nano form of ciprofloxacin, | Nano TAPC consists of TAPC: Ciprofloxacin (Ciprofloxacin 500 mg), Metronidazole (Flagyl 500 mg) and Minocycline (Minocin 50 mg), mixed in equal amounts by weight (1:1:1) 100 mg of each, together with the 50 mg Catafast granules. This mixture was transferred into nano structure material using Top-down technique through the combination of various processes including fine grinding, sonication homogenisation and ultrafiltration to prevent agglomeration. Ball milling was used to produce nanomaterials by mechanical attrition in which kinetic energy from a grinding medium was transferred to material undergoing reduction. Nanomaterials were put back together with compaction and consolidation in an industrial scale process to form materials with enhanced properties (13). |                  |

Optical density (OD) representing the% residual living cells was determined according to the following equation:

Viability %=(OD test 1×100)
OD control

Number of residual living cells=(OD of treated cells/OD of untreated cells) x Number of negative control cells (1×10⁴ cells/0.1 cm³). The concentration induced 50% viability/ death of treated cells; [inhibitory concentration (IC50)], was calculated. The tissue culture (TC) flasks were treated with the IC50 value. The treated flasks showed cytological changes and detached cells. The detached cells were collected by cold centrifugation. The pelleted cells were fixed with methanol then prepared for flow cytometry processing.
hibitor was added for another 10 minutes to stop the action of trypsin in solution ‘A’. The tubes were wrapped with tin foil to protect them from light and 125 μL of solution ‘C’ was added and the tubes were kept in ice bath for at least ten minutes till running in the flow cytometer within a maximum 2-3 hours.

- **Annexin V stain**
  Annexin V Apoptosis Detection Kit is based on the observation of initiated apoptosis. Cells translocate the membrane phosphatidylinerine [PS] from the inner face of the plasma membrane to the cell surface. Once it reached the cell surface, PS can be easily detected by staining with a fluorescent conjugate [Annexin V]; a protein that has a high affinity for PS. The one-step staining procedure takes only 10 minutes. Detection can be analyzed by flow cytometry. The kit can differentiate between apoptosis and necrosis when performing both Annexin V and Propidium Iodide [PI] staining.

- **Annexin V-FITC assay protocol**
  Incubation of cells with Annexin V-FITC:
  1. Apoptosis was induced by test medications using flowcytometry.
  2. Cells were collected by centrifugation and desired cell count was optimized as 2×10^5/ml using hemocytometer.
  3. Cells were re-suspended in 500 μl of 1X Binding Buffer.
  4. Annexin V-FITC and 5 μl PI 50 mg/ml of were added.
  5. Stained cells were incubated at room temperature for 5 min in the dark.
• Flow cytometry analysis
   Apoptotic profile traced using Annexin –V and PI stains using flow cytometry, [Ex=488 nm; Em=530 nm] using FITC signal detector [usually FL1] and PI staining by the phycerythrin emission signal detector [usually FL2].

Statistical analysis
Statistical analysis Data presented as mean and standard deviation (SD) when appropriate. Data Explored for Normality using D’Agostino-Pearson test. The significance level was set at P≤0.05 (α=0.05). Two-Way ANOVA test was used to compare between tested materials at different concentrations and times. Statistical analysis was performed with IBM® SPSS® (SPSS Inc., IBM Corporation, NY, USA) Statistics Version 25 for Windows.

RESULTS

1) MTT assay
(a) Inhibitory concentration (IC50 values)
The inhibitory concentration of TAPC, Nano TAPC and Ca(OH)₂ were calculated from the regression curve of log concentration versus the cell viability (%). The estimated IC 50 values for the tested intracanal medicaments after 24 hours were 6.147, 4.430 and 2.028 mg/ml respectively (Fig. 2).

(b) Percentage of viability values
The effect of different intracanal medicaments on percentage of viability of fibroblast cells value are listed in Tables 2-4, and Figs. 3-5.

2) Flow cytometry test results
(a) Apopotic effect evaluation of intra canal medicaments
The mean values of apoptotic and necrotic cells in cultures assessed using flow cytometry analysis for different intracanal medicaments were listed in Table 5 and Figs. 6a-c.

(b) The effect of particle size (TAPC vs. Nano TAPC)
Comparison of flow cytometry test results (Mean values± SDs) between regular and nano scale forms for TAPC experimental group were explained in Table 6 and Fig. 7.

DISCUSSION
Intra-canal medicaments are usually applied between visits, and have several advantages. They act by inhibiting proliferation of bacteria and minimizing ingress of pathogens in between visits through a leaking temporary restoration (15).

Calcium hydroxide as an intracanal medicament, has been successfully used in endodontic treatment for decades. It can stimulate the formation of calcified tissue, to induce antibacterial properties and to disinfect root canals after one month of application in 97% of treated cases (16).

Another combination of antibiotics, called TAP was introduced especially for the regeneration and revascularization protocol and the treatment of open apex teeth with necrotic pulp. This material has also shown other applications in endodontics. Several studies have reported the antibacterial efficacy of TAP containing ciprofloxacin, metronidazole, and doxycycline against the pathogens commonly found inside the root canal.
Figure 3. Line graph showing the mean values of % viability (± SD) of all groups at different time intervals for (Conc. 10mg/ml)

Figure 4. Line graph showing the mean values of % viability (±SD) of all groups at different time intervals for (Conc. 0.65 mg/ml)

Figure 5. Line graph showing the mean values of % viability (±SD) of all groups at different time intervals for (Conc. 0.07 mg/ml)

TABLE 5. Percentage of apoptotic and necrotic cells in cultures assessed by using flow cytometry analysis for different intracanal medicaments

| Variables | Early apoptosis | Late apoptosis | Necrosis |
|-----------|----------------|---------------|---------|
| TAPC | 2.35±0.070 | 3.17±0.098 | 1.61±0.104 |
| Nano TAPC | 7.23±0.104 | 3.65±0.128 | 2.14±0.087 |
| Ca(OH)₂ | 5.91±0.268 | 13.26±0.854 | 3.28±0.072 |
| P value | <0.001* | <0.001* | <0.001* |

*: Significant from control group (P<0.05), ns; non-significant from control group

Figure 6. (a–c) The dot plots are representative of control and human fibroblast cells exposed to various intra canal medicaments, representing the populations of viable cells [bottom left: annexin V–, propidium iodide (PI–)], early apoptotic (bottom right: annexin V+, PI–), late apoptotic (top right: annexin V+, PI+), and necrotic (top left: PI+) cells. The x-axis is the fluorescence intensity of annexin-V; the y-axis is the fluorescence intensity of PI
system including Enterococcus faecalis. Because of complexity of the root canal infection, a combination of antibiotics may be needed to address the diverse flora encountered in root canal infections (17).

Salem-Milani et al. (18) was the first to comparatively evaluate anti-bacterial efficiency of ibuprofen, diclofenac and Ca(OH)₂ using the agar diffusion test, the authors revealed the anti-bacterial properties of NSAIDs (ibuprofen, diclofenac) against E. faecalis; whereas Ca(OH)₂ failed at the same. The exact mechanism of antibacterial activity of diclofenac and ibuprofen remains unclarified. Studies have postulated the following mechanism(s) of action: inhibition of bacterial DNA synthesis, impairment of membrane activity, anti-plasmid activity, alteration in genes encoding transport/binding proteins as well as down-regulation of efflux pumps, reduced quorum sensing-mediated motility leading to reduced biofilm (19).

Diclofenac potassium anti-inflammatory drug was proved to be efficient in reducing post-operative pain when it was added to triple antibiotic paste as intracanal medication, its antiinflammatory effect was comparable to that of calcium hydroxide when used in asymptomatic teeth with necrotic pulps (20).

Recently, nanotechnology has become increasingly utilized for dental applications because of their great broad-spectrum antibacterial effectiveness (21). This study aimed to evaluate the cytotoxic effects of TAP and TAP with nano-particles. Evaluation of the intracanal medicaments cytotoxicity is highly important, as it may affect the biological and physiological behavior of the cells. Hence, the concentration of the medicaments are important for effective treatment (22).

Three different concentrations (10, 0.625 and 0.07mg/ml) of the intracanal medicaments were used at three different time intervals (24hr., 48hr. and 7 days) to assess cytotoxicity. The higher the concentration and the longer the contact time, the stronger the cytotoxic effect (23).

MTT was used for the evaluation of the cytotoxicity as it considered simple, rapid and reliable test, MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a water-soluble tetrazolium salt, which converted to an insoluble purple formazan by cleavage of the tetrazolium ring by succinate dehydrogenase (LDH) within the mitochondria. The formazan product is impermeable to the cell membranes and therefore it accumulates in healthy cells (24).

Cytotoxicity was measured using both MTT assay and flowcytometry because single cytotoxicity assay is not considered to be sufficient to evaluate or predict all aspects of the material’s cytotoxicity as using a colorimetric assay to analyze a given compound do not know give sufficient indication of cytotoxicity. Flowcytometry is a stable and reliable indicator of the real cell viability, this is based on its capacity to exclude dye in the living cells. The Annexin V Propidium iodide (PI) protocol was used, Annexin V can assess if cells are viable, apoptotic, or necrotic through differences in plasma membrane integrity and permeability while PI does not stain live or early apoptotic cells due to presence of an intact plasma membrane (25).

Fibroblasts were used to assess cytotoxicity of intracanal medicaments, as they are more behaviour representative of in-vivo cells. Fibroblasts act as vital immune-regulatory cells that involved in local inflammatory and immune responses. They produce cytokines, chemokines, growth factors and other biologically active molecules, and their function may be modulated by the microenvironment (26). The results of the present study showed that the cytotoxicity was affected by the concentration and particle size of the utilized medicaments.

Calcium hydroxide showed the highest level of cytotoxicity amongst all the studied groups, this may be explained by the release of the reactive hydroxyl ions and the high pH which may cause cell necrosis and apoptosis as previously proved by Selis et al. (11) who stated that Ca(OH)₂ had cytotoxic potential against fibroblast cells.

Triple antibiotic paste showed less cytotoxic effect than calcium hydroxide. The lower cytotoxicity of TAPC may be attributed to the inhibition of cell collagenase and matrix metalloproteinase, also doxycycline increases collagen formation and angiogenesis in the process of wound healing. Hosseini et al. (27) compared the cytotoxic effect of TAP and Ca(OH)₂ against fibroblast cells and also concluded that Ca(OH)₂ had more cytotoxic effect than that of the triple antibiotic paste.

The Nano TAPC showed more cytotoxic effect on human skin fibroblast cells than its corresponding regular TAPC. This might be explained by decrease in the particle size from macroscopic to nano scale that lead to changes in physicochemical proper-

| Variables     | Early apoptosis | Late apoptosis | Necrosis |
|---------------|----------------|---------------|----------|
| TAP           | 2.350.070*     | 3.170.098*    | 1.610.104* |
| Nano TAPC     | 7.230.104b     | 3.650.128b    | 2.140.087b |
| P value       | <0.001*        | 0.006*        | 0.003*   |

*: Significant (P<0.05), ns: non-significant (P>0.05)
Different small letters mean statistical significance in column

Figure 7. Bar chart showing flowcytometry early, late apoptosis and necrosis values of TAPC and Nano TAPC

The Nano TAPC showed more cytotoxic effect on human skin fibroblast cells than its corresponding regular TAPC. This might be explained by decrease in the particle size from macroscopic to nano scale that lead to changes in physicochemical proper-
ties of the materials and resulted in particles with smaller size that may elicit different biological responses including toxicity (16, 28). Also may be from disruption of the mitochondrial respiratory chain by the nano particles leading to production of reactive oxygen species (ROS) and interruption of adenosine triphosphate (ATP) synthesis, which in turn caused DNA damage (28). Samie et al. found slight increase in lactic acid dehydrogenase leakage which is cell damage indicator that was observed with nanoparticles, indicating that these particles interfere with cell metabolism (29). The higher cytotoxic effect of the nano TAPC intracanal medicaments could also be attributed to the ability of nanoparticles to bind to the negatively charged part of the cell membrane, causing disturbance in its functions such as permeability and respiration, causing leaking of the cytoplasmic content and eventually rupture of the cell. As a result, the nanoparticles will infiltrate inside the cytoplasmic content and interact with sulphur and phosphorus containing proteins such as DNA and RNA, causing further damage to the cell (30).

CONCLUSION
The cytotoxic effect of Nano TAPC was lower than that of calcium hydroxide and higher than that of TAPC. Although it has the highest apoptotic effect when compared to TAPC and calcium hydroxide but still there is no statistically significant difference between them.

Disclosures
Conflict of interest: The author declares that there is no conflict of interest related to this research.

Ethics Committee Approval: This was approved by the local ethics committee number 4/2020 Faculty of Dentistry, University of October 6.

Peer-review: Externally peer-reviewed.

Financial Disclosure: There is no financial involvement with any commercial organization in the subject or materials discussed in this manuscript.

Authorship contributions: Concept – Y.N.; Design – S.A.H.; Supervision – Y.N.; Funding - Y.N., H.S., S.A.H.; Materials - Y.N., H.S., S.A.H.; Data collection &/or processing – Y.N.; Analysis and/or interpretation – Y.N., S.A.H.; Literature search – H.S.; Writing – Y.N., H.S.; Critical Review – Y.N., H.S., S.A.H.

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