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Cytotoxic Effect of Different Concentrations of Chitosan and Propolis Nanoparticles on Periodontal Ligament Stem Cells

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Cytotoxic Effect of Different Concentrations of Chitosan and Propolis Nanoparticles on Periodontal Ligament Stem Cells

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Restorative Dentistry
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ABSTRACT

Purpose: The purpose of this study was to assess the cytotoxic effect of different concentrations of Chitosan Nanoparticles (CNPs) and Propolis Nanoparticles (PNPs) on periodontal ligament stem cells using MTT assay. **Materials and Methods:** Immature, impacted third molars were used to isolate stem cells. Cells were seeded in 96-well culture plates. Groups were divided into six experimental groups: Group I: 17% EDTA, Group II: 1% propolis NPs, Group III: 2% propolis NPs, Group IV: 1% chitosan NPs, Group V: 2% chitosan NPs, Group VI: cultured cells not receive any material. Cytotoxic effect of these materials on stem cells was evaluated using MTT assay at 24, 72,120 hours and 14 days. **Results:** In all assay intervals; 2%CNPs group showed the statistically significant highest median value and control group showed the statistically significant lowest median values. After 24 and 72 hours; there was no statistically significant difference between 2% PNPs and 1% CNPs groups followed by 1% PNPs group. Both EDTA and control groups; showed the statistically significantly lowest median values with no statistically significant difference between them. After 120 hours; there was no statistically significant difference between 2% PNPs and 1% CNPs groups in median value followed by 1% PNPs then EDTA groups. After 14 days; there was no statistically significant difference between 1% PNPs, 2% PNPs and 1% CNPs groups in median value followed by EDTA group. **Conclusion**: The ability of 2% Chitosan NPs to promote periodontal ligament stem cell viability makes them a promising irrigating solution. The cell viability of 2% Propolis NPs was comparable to that of 1% Chitosan NPs, which was better than EDTA.

KEYWORDS

Cytotoxity, chitosan nanoparticles, propolis nanoparticles, stem cell.

- Paper extracted from Doctor thesis titled "Evaluation of Cytotoxity on Stem Cell and Pulp Revascularization of Immature Necrotic Dog Teeth with Apical Periodontitis using Nano Natural Material"

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INTRODUCTION

There are challenges in treatment of immature permanent teeth with apical pathosis, not only in root canal debridement and difficult obturation, but also increasing the risk of fracture due to thin dentinal walls. Management of such cases was achieved by custom made obturation, surgery and apexification procedures using Mineral trioxide aggregate [1].

Regenerative endodontic procedure aims to restore the pulp-dentin complex's normal physiologic functions and replace the damaged structures such as dentin, as well as pulp-dentin complex cells with live viable tissues, preferably of the same origin [2]. There are three key elements for successful outcome of regenerative endodontic procedures. First, stem cells which have the ability to proliferate and differentiate. Second, the growth factors which are the extracellular secreted signals.Third, the scaffolds which are three dimensional structures that enhance vascularization and cell organization [3].

Revascularizations of necrotic root canal is achieved by disinfection as presence of bacteria retard tissue growth inside the canal space and prevent stem cells and fibroblasts to adhere or proliferate in infected root canal system, followed by using over instrumentation to establish bleeding into the canal system. [4]. Preservation of stem cells is important in regenerative endodontic treatment. For improving the adhesion of stem cells, a biocompatible irrigant is required as there is a link between the number of adhered stem cells and the irrigant's cytotoxicity. [5].

Irrigant has a central role in cleaning and disinfecting the complex root canal system. Some irrigating solutions dissolve either inorganic or organic tissue in root canal. Sodium hypochlorite (2.5 % NaOCl) is mostly used due to its excellent tissue dissolving and antimicrobial properties. Ethylenediaminetetra acetic acid (17 % EDTA) is a good chelating agent which is used to remove the inorganic part of the smear layer; however, it has little or no antibacterial effect, and prolonged exposures can result in excessive removal of both intratubular and peritubular dentin. So, most researchers have shifted to modern natural substances with fewer side effects, greater antibacterial activity, biocompatibility and nontoxic to the periapical tissues [6].

Nanotechnology is a new approach in dentistry. One nanometer is equal to one billionth of a meter. Nanomaterials have different properties from those of the same material in mass scale [7]. Chitosan is a natural biopolymer isolated from shellfish, shrimp and crab. Chitosan is produced by deacetylation of chitin; the most widely used natural biopolymer after cellulose. Many reports have shown that chitosan have good antimicrobial effect against Gram-positive and Gram-negative bacteria, biocompatibility, nontoxicity, chelating potential, has the ability to elevates dentin resistance to collagenase degradation and improve dentin surface properties. Chitosan and chitosan NPs applications include tissue engineering, a coating applied to implants to aid in bone regeneration, enamel caries remineralization, an antibacterial agent for the prevention of oral diseases, wound healing, and a variety of other applications [8,9].

Chitosan nanoparticles have chitosan properties as well as nanoparticle characteristics such as small size, surface and interface effect. It was reported that chitosan nanoparticles enhance the differentiation of the stem cells of apical papilla in regenerative endodontics by providing a controlled release of transforming growth factor-β [10]. It was reported that chitosan NPs promote healing by altering the inflammatory response of macrophages and also could inactivate biofilm [11]. Nano-sized chitosan are anticipated to be more effective in penetrating and disrupting bacterial cell membranes because of their polycationic/polyanionic nature with their charge density and high surface area. So, Nano-chitosan is expected to be more effective against a variety of organisms [12].

Propolis (also known as bee glue) is a viscous natural organic substance derived from beeswax. Propolis has a high bactericidal activity. Therefore,

it was used as a natural antimicrobial agent [13]. Propolis contains phenolic acid and flavonoids which have antibacterial, antiviral and antifungal properties. Biocompatibility of propolis is the outcome of its anti-inflammatory propertie. The chemical composition of propolis is primarily influenced by plant source, the collection time and location. This encouraged using it in dentistry for many treatments as root canal disinfection, a storage media for avulsed teeth, direct and indirect pulp capping, caries prevention and accelerating surgical wound healing [14].

It was reported that propolis can break the bacterial resistance produced by certain bacterial strains as E faecalis and S aureus. It was also reported that ethanolic propolis extract induced dentin bridge formation and bone regeneration when propolis comes in contact with exposed pulpal tissues [15].

Global trends toward natural products have been the stimulus for further investigation of chitosan NPs and propolis NPs properties. So this study was carried out to assess the cytotoxicity of Nano chitosan and Nano propolis on periodontal ligament stem cells.

MATERIAL AND METHODS

This study was been approved by Research Ethic Committee Faculty of dental medicine Al -Azhar University code (REC-EN-21-01).

Prepration of chitosan Nanoparticles:

The chitosan powder (CS) was dissolved in 1% acetic acid at room temperature with magnetic stirring (a device consists of a rotating magnet or a stationary electromagnet that creates a rotating magnetic field. This device is used to make immerse in a liquid or mixing a solution). After that, a solution of sodium tripoly-phosphate (TPP) was added to the chitosan solution in the ratio 2.5: 1(v/v) (chitosan: TPP) [16]. The tripoly-phosphate solution was added drop-by-drop to the mixture using a Polytron homogenizer at 5,000 rpm. After stirring, the CS-TPP solutions were centrifuged at 13,000 rpm for 4 minutes to collect nanoparticles. The supernatant was discarded, and the nanoparticles were thoroughly rinsed with distilled water. The zone of opalescent suspension was visible with the formation of the nanoparticles. Chitosan NPs were prepared at two different concentrations: 1% and 2%.

Preparation of Propolis Nanoparticles:

Propolis was filtered using filter papers after being placed in a flask with 500 ml of 80% ethanol and placed on a hot plate and stirred. To isolate pure propolis particles, the filtered solution was mixed 1:10 with distilled water [17]. To obtain propolis NPs, the suspension was placed in an ultrasonic bath for 20-30 minutes. After that, propolis NPs in the colloid state was obtained. Propolis NPs were prepared in two concentrations 1% and 2%.

Cell culture:

Periodontal ligament stem cells were isolated from impacted immature third molars of a healthy 18-25 year-old patients who informed about taking stem cells from their third molars and signed a written informed consent. The teeth were rinsed with phosphate buffered saline (PBS) solution right after extraction and were stored in this sterile solution. Cell line was grown using 450 ml of Roswell Park Memorial Institute (RPMI)-160 culture media with 50 ml of 10% Foetal Bovine Serum (FBS), 5 ml of 2mm glutamine and 5 ml of antibiotics (0.1 mg/ml streptomycin with100 U penicillin) were added. Culture media and supplements were prepared at biochemistry and molecular biology branch of medicine faculty in Cairo University. To ensure sterility, aseptic conditions in a culture hood were followed.

Cells were cultured in 75cm2 flasks at 37°C CO2 incubator. Every day, cells were examined under a microscope (Leica, USA) to ensure that they were healthy and growing as expected. The cells were mostly attached to the flask's bottom, elongated in shape. Culture medium was clear pinky orange

in colour that indicated absence of contamination. The culture medium was changed every 2-3 days (to avoid the media to turn yellow as consequence of the acidic pH generated by cell metabolic activity and exhausted of nutrients as it could lead to cell death) during the cell culture process, and the cells were passaged after one week.

Cells had reached confluence after four passages. As the cells were in the phase of growth and reached approximately 80% confluent (80% of surface of flask covered by cell monolayer) they required to be sub-cultured (If cells became over confluent, they would begin to die and might not be recoverable).

To sub-culture, the fresh culture medium was warmed for at least 30 minutes in a 37°C water bath or incubator. Media were poured from flask into waste pot. Enough trypsin EDTA was added to cover the cells at the bottom of the flask, e.g., 5 ml for a 75 cm2 flask. To ensure trypsin contact with all cells, the flask was gently rolled. The flask was put into a 37°C incubator. The flask was filled with culture media as soon as the cells were detached (the FBS in this media inactivated the trypsin).

Cells were sub-cultured when reaching 80% confluent. Cell culture groups divided into:

Group I: Cells were cultured in 17% EDTA **Group II**: Cells were cultured in 1% propolis NPs .**Group III**: Cells were cultured in 2% propolis NPs. **Group IV**: Cells were cultured in 1% chitosan NPs .**Group V**: Cells were cultured in 2% chitosan NPs. **Group VI**: Cultured cells not receive any material were used as a control group. Cells of all groups cultured for 24, 72,120 hours and 14 days.

MTT cell proliferation assay:

Numerous in-vitro assays aimed at quantifying a cell population's response to external factors are built on cell viability and proliferation measurements. The (TACSTM TREVIGEN® 8405 Hegerman Ct. Gaithersburg) provided MTT Reagent that was ready to use. The MTT assay (The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) is a colorimetric assay for determining the metabolic activity of cells.

The number of viable cells present can be determined by using oxido-reductase enzymes under specific conditions, and these enzymes are capable of reducing the tetrazolium dye. The number of living cells in culture is directly proportional to the absorbance.

To allow for microplate adherence, cells (1x103) were placed in a 96 well microplate 24 hours before the MTT assay. The cells were given 50 μL of serum-free media and 10 μL of MTT solution in each well. The plate was incubated for 3 hours at 37°C.After incubation, each well received 100 μL of detergent solution (MTT formazan).The plate was wrapped in foil and shaken for 15 minutes on an orbital shaker before being incubated overnight at 37°C. An Enzyme-Linked Immuno-Sorbent Assay (ELISA) plate reader was used to measure colour absorbance at (optical density) OD=590 nm (Stat Fax 2200, Awareness Technologies, Florida, USA). An Enzyme-Linked Immuno-Sorbent Assay (ELISA) plate reader was used to measure colour absorbance at OD=590 nm (Stat Fax 2200, Awareness Technologies, Florida, USA). For each sample, duplicate readings were taken and averaged. The amount of absorption was proportional to the number of cells. In all of the groups studied, colour absorbance was plotted at 590 versus cell proliferation.

Statistical Analysis

The distribution of numerical data was checked for normality, and normality tests (Kolmogorov-Smirnov and Shapiro-Wilk tests) were used. The data from the MTT assay had a non-normal (non-parametric) distribution. The median, range, mean, and standard deviation (SD) values were used to present the data. To compare the groups, the Kruskal-Wallis test was used. Friedman's test was used to study the changes by time within each group. When Kruskal-Wallis or Friedman's tests were significant, Dunn's test was used for

pair-wise comparisons. The significance level was set at $P \leq 0.05$. Statistical analysis was performed with (IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp).

RESULTS

1. Comparison between the groups. (Table 1)

After 24 as well as 72 hours; there was a statistically significant difference between the tested groups. Pair-wise comparisons between the groups revealed that 2%Chitosan NPs group showed the statistically significantly highest median value. There was no statistically significant difference between 2% Propolis NPs and 1% Chitosan NPs groups; both showed statistically significantly lower median values than 2% Chitosan NPs group followed by 1% Propolis NPs group. There was no statistically significant difference between EDTA and control groups; both showed the statistically significantly lowest median values.

After 120 hours; there was a statistically significant difference between the groups. Pair-wise comparisons between the groups revealed that Chitosan 2% NPs group showed the statistically significantly highest median value. There was no statistically significant difference between 2% Propolis NPs and 1% Chitosan NPs groups; both showed statistically significantly lower median values than 2% Chitosan NPs group followed by 1% Propolis NPs group then EDTA group. Control group showed the statistically significantly lowest median value.

After 14 days; there was a statistically significant difference between the groups. Pair-wise comparisons between the groups revealed that 2% Chitosan NPs group showed the statistically significantly highest median value. There was no statistically significant difference between 1% Propolis NPs, 2% Propolis NPs and 1% Chitosan NPs groups; all showed statistically significantly lower median values than 2% Chitosan NPs group followed by EDTA group. Control group showed the statistically significantly lowest median value.

Table (1) Descriptive statistics and results of Kruskal-Wallis test for comparison between MTT assays in the six groups.

Group	**24 hours** (n = 6)		**72 hours** (n = 6)		**120 hours** (n = 6)		**14 days** (n = 6)	
	Mean (SD)	**Median (Range)**	**Mean (SD)**	**Median (Range)**	**Mean (SD)**	**Median (Range)**	**Mean (SD)**	**Median (Range)**
EDTA	0.076 (0.043)	0.072 (0.028-0.136) ^{D}	0.171 (0.038)	0.175 (0.129-0.209) ^{D}	0.455 (0.046)	0.431 (0.418-0.522) ^{D}	1.441 (0.376)	1.36 (0.966-1.95) ^{C}
Propolis NPs 1%	0.351 (0.102)	0.354 (0.202-0.478) ^{C}	0.581 (0.104)	0.576 (0.411-0.732) ^{C}	1.175 (0.054)	1.152 (1.121-1.252) ^{C}	2.1 (0.388)	2.22 (1.42-2.46) ^{B}
Propolis NPs 2%	0.407 (0.08)	0.449 (0.293-0.481) ^{B}	0.764 (0.066)	0.762 (0.671-0.849) ^{B}	1.608 (0.116)	1.602 (1.488-1.795) ^{B}	2.327 (0.16)	2.355 (2.12-2.52) ^{B}
Chitosan NPs 1%	0.473 (0.093)	0.477 (0.326-0.598) ^{B}	0.792 (0.165)	0.798 (0.559-0.986) ^{B}	1.705 (0.241)	1.781 (1.264-1.893) ^{B}	2.493 (0.354)	2.521 (2.08-2.9) ^{B}
Chitosan NPs 2%	0.956 (0.288)	0.899 (0.719-1.464) ^{A}	2.198 (0.195)	2.212 (1.892-2.427) ^{A}	3.24 (0.079)	3.285 (3.129-3.298) ^{A}	3.42 (0.443)	3.443 (2.7-3.936) ^{A}
Control	0.045 (0.041)	0.034 (0.002-0.094) ^{D}	0.151 (0.024)	0.147 (0.127-0.193) ^{D}	0.309 (0.115)	0.293 (0.157-0.474) ^{E}	0.598 (0.154)	0.581 (0.373-0.811) ^{D}
P-value	<0.001*		<0.001*		<0.001*		<0.001*	
Effect size (Eta Squared)	0.849		0.869		0.931		0.826	

**: Significant at $P \leq 0.05$, Different superscripts in the same column indicate statistically significant difference between groups according to Dunn's test*

2. Changes by time within each group. (Table 2, Fig. 1)

As regards EDTA,1% Propolis NPs, 2% Propolis NPs, 1% Chitosan NPs as well as control groups; there was a statistically significant increase in MTT assay values from 24 to 72, 72 to 120 hours as well as from 120 hours to 14 days.

While for 2% Chitosan NPs group; there was a statistically significant increase in MTT assay values from 24 to 72 as well as 72 to 120 hours followed by non-statistically significant change from 120 hours to 14 days.

Table (2) Descriptive statistics and results of Friedman's test for comparison between MTT assay at different time periods in each group:

Group	**24 hours** (n = 6)		**72 hours** (n = 6)		**120 hours** (n = 6)		**14 days** (n = 6)		*P*-value	*Effect size (w)*
	Mean (SD)	**Median (Range)**	**Mean (SD)**	**Median (Range)**	**Mean (SD)**	**Median (Range)**	**Mean (SD)**	**Median (Range)**		
17% EDTA	0.076 (0.043)	0.072 (0.028-0.136)^{D}	0.171 (0.038)	0.175 (0.129-0.209)^{C}	0.455 (0.046)	0.431 (0.418-0.522)^{B}	1.441 (0.376)	1.36 (0.966-1.95)^{A}	<0.001*	1
Propolis NPs 1%	0.351 (0.102)	0.354 (0.202-0.478)^{D}	0.581 (0.104)	0.576 (0.411-0.732)^{C}	1.175 (0.054)	1.152 (1.121-1.252)^{B}	2.1 (0.388)	2.22 (1.42-2.46)^{A}	<0.001*	1
Propolis NPs 2%	0.407 (0.08)	0.449 (0.293-0.481)^{D}	0.764 (0.066)	0.762 (0.671-0.849)^{C}	1.608 (0.116)	1.602 (1.488-1.795)^{B}	2.327 (0.16)	2.355 (2.12-2.52)^{A}	<0.001*	1
ChitosanNPs 1%	0.473 (0.093)	0.477 (0.326-0.598)^{D}	0.792 (0.165)	0.798 (0.559-0.986)^{C}	1.705 (0.241)	1.781 (1.264-1.893)^{B}	2.493 (0.354)	2.521 (2.08-2.9)^{A}	<0.001*	1
Chitosan NPs 2%	0.956 (0.288)	0.899 (0.19-1.464)^{C}	2.198 (0.195)	2.212 (1.892-2.427)^{B}	3.24 (0.079)	3.285 (3.129-3.298)^{A}	3.42 (0.443)	3.443 (2.7-3.936)^{A}	0.001*	0.911
Control	0.045 (0.041)	0.034 (0.002-0.094)^{D}	0.151 (0.024)	0.147 (0.127-0.193)^{C}	0.309 (0.115)	0.293 (0.17-0.474)^{B}	0.598 (0.154)	0.581 (0.373-0.811^{A}	<0.001*	1

**: Significant at P ≤ 0.05, Different superscripts in the same row indicate statistically significant change by time according to Dunn's test*

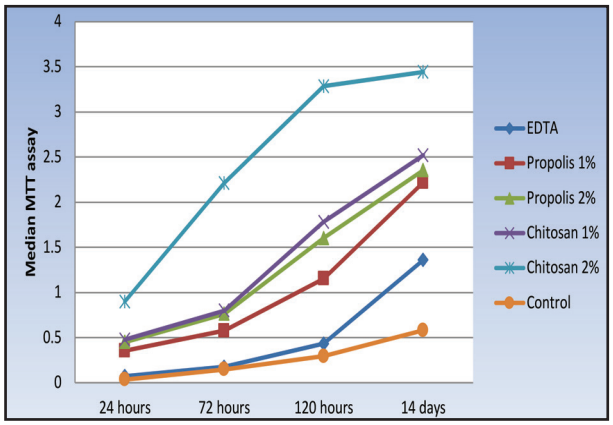

Figure (1) Line chart representing median values for MTT assay at different time periods within each group

DISCUSSION

Cytotoxic effect of any given substance is evaluated by cell viability assay as it measures the behaviour of cell cultures against tested substance. To simulate the effect of the irrigation immediately after contact with the cells and the effect of medication over time, different times were used.

There are several factors which influence the number of viable cells, such as the ability of substance to disperse within the medium, nutritional reserve, the cells adaptation to the medium, and the difference in each substance's cytotoxic effect. The non-cytotoxic effect of test solutions boosts the cells' ability to self-renew [18].

Stem cells were taken from the immature third molar which exhibits a high capacity for differentiation into mineralizing and actively migrating odontoblasts. The third molar is frequently the last tooth to erupt and normally extracted at an early stage of development thus contains a significant population of stem cells [19].

The dentin matrix trapped many important active growth factors during dentinogenesis. Some of these growth factors are known to have important role on the proliferation and differentiation of MSCs such as vascular endothelial growth factor and transforming growth factor-beta 1. It was reported that 17% EDTA enhance new dentin formation, as it promotes the differentiation of dental pulp stem cells (DPSCs) into odontoblast-like cells [20].

EthyleneDiamineTetraacetic Acid (EDTA) is known to remove the smear layer. In addition to provide disinfection, release growth factors from dentin[21], however, treatment with EDTA in a longer period could be recommended to enhance the release of growth factors from the dentin although it may induce a reduction of microhardness and erosion of root canal dentin [22].

Chitosan is an organic natural biopolymer derived from chitin. The source of chitin is the shells of shrimps and crabs. Chitosan is known to be a biodegradable non-toxic biomaterial[23]. Biocompatible material must not interact with the body system or promotes rejection such as hypersensitivity or inflammatory response and allow cell attachment and proliferation. Chitosan NPs can be used as a vehicle to deliver biomolecules due to molecular structural similarity with extracellular matrix, their antibacterial/antibiofilm properties and distinct cell uptake mechanisms [24, 25]. Nanochitosan was prepared in this study using ionotropic gelation method because it is simple and do not use organic solvents or apply high shear force [26].

Egyptian propolis has anti-inflammatory properties due to caffeic acid and flavonoids present in Propolis. Arachidonic acid's lipoxygenase pathway is inhibited by flavonoids. As a result, they play a crucial role in lowering the inflammatory response[27]. Flavonoids are topoisomerase inhibitors. Topoisomerase is an enzyme that plays important role in chromosome transcription, replication and has the ability to regulate the superhelical area of chromosomal DNA. Therefore, propolis does not cause DNA damage [28]. Because propolis has a potential cytotoxic effect on various cancer cell lines, it was necessary to investigate its cytotoxicity in stem cells. [29]

The MTT assay was chosen for this study because it is a standardised method for determining the effect on cell viability, safe, simple, easily repeatable, rapid, minimally toxic, and superior for determining the activity of mitochondrial dehydrogenase in living cells. It measures mitochondrial dehydrogenase activity, depending on the water soluble methylthiazol tetrazolium being converted to an insoluble purple formazan where the amount of formazan crystals formed is proportional to the activity of the living cells as these crystals are impermeable to cell membranes [30]. Tetrazolium salt reduction is now widely recognised as a reliable method for determining cell proliferation.

In the current study, in all assay intervals (24, 72,120 hours and 14 days); 2% Chitosan NPs showed the statistically significant highest mean value .This could be attributed to the fact that, Chitosan NPs release endogenous matrix components, such as transforming growth factor-β which have the ability to promote stem cell differentiation and proliferation [31].

In the current study, 2% chitosan NPs is not toxic to periodontal ligament stem cells. This finding was consistent with previous studies [20, 27, 32] which showed that in a short time interval, chitosan NPs were less cytotoxic and induced fewer apoptotic changes in DPSCs than propolis NPs. The results of the present study were in disagreement with a recent study, which reported that Chitosan nanoparticles in concentrations of 0.2 mg/mL, 0.5 mg/mL, 1 mg/mL, and 2 mg/mL possess a cytotoxic effect on the normal hDPCs in dose-dependent manners [12]. This could be attributed to different method of prepration of chitosan NPs.

Regarding to 24 as well as 72 hours; there was no statistically significant difference between 2% Propolis NPs and 1% Chitosan NPs groups, as propolis induces the synthesis of collagen by dental pulp cells. Propolis also has the ability to stimulate the production of transforming growth factor TGF-β1 which is important for the differentiation of odontoblasts. Zinc present in propolis promotes cell differentiation and proliferation [32].

In our study, 2% propolis NPs is not toxic to periodontal ligament stem cells. This finding was consistent with previous studies [20,31]. Which showed that propolis NPs have proliferating effect on pulpal stem cells DPSCs.

Since different concentrations of propolis were used. 1% Propolis NPs showed statistically significantly lower median value. This could attribute to increased propolis concentration resulted in increased cell proliferation and vitality. As a result, propolis' biocompatibility was established. [33].

Regarding cytotoxity of Propolis NPs and Chitosan NPs on viability of periodontal ligament stem cells over 24, 72, 120 hours and 14 days revealed that viability of stem cells increased by time. This effect was dose and time dependent. Therefore, a better outcome was associated with higher concentrations and longer exposure [34].

CONCLUSION

The ability of 2% Chitosan NPs to promote periodontal ligament stem cell viability makes them a promising irrigating solution. The cell viability of 2% Propolis NPs was comparable to that of 1% Chitosan NPs, which was better than EDTA.

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RECOMMENDATIONS: Further studies are recommended to investigate the clinical outcome of chitosan and propolis nanoparticles on regenerative endodontics.

CONFLICT OF INTEREST: None declared.

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