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Cytotoxicity of Chitosan Nanoparticles_Incorporated with Different Endodontic Irrigation Solutions (An in Vitro Study)

Sarah Flaah Ali (1) *
Fanar T.A. Al-jadwaa (2)
Suhad Mwafaq Hamdoon (3)

(1,2) Department of Conservative Dentistry, College of Dentistry, University of Mosul, Mosul, Iraq
(3) Department of Dental Basic Sciences, College of Dentistry, University of Mosul, Mosul, Iraq

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***Corresponding Author:**
Email:
sarah.22dep16@student.uomosul.edu.iq
Department of Conservative Dentistry, College of Dentistry, University of Mosul, Mosul, Iraq

Abstract

Aim: The investigation was done to evaluate the cytotoxicity of two concentrations of chitosan-nanoparticles (0.2% ,0.5%) _incorporated with sodium hypochlorite (5.25%) and chlorhexidine (2%) at different time periods. Methods: For evaluating cytotoxicity, human periodontal fibroblast cell lines were exposed to different endodontic irrigation solutions. Cytotoxicity was assessed immediately, after 24 hours, after 72 hours and after 168 hours, using Methyl Thiazol Tetrazolium (MTT) assay. Result: According to the result of this study, sodium hypochlorite 5.25% was the most cytotoxic solution (lower cell viability) followed by chlorhexidine 2%, nano-chitosan 0.5% while nano-chitosan 0.2% was the lowest cytotoxic irrigation solution (higher cell viability). After mixing of sodium hypochlorite and chlorhexidine with nanoparticles we noticed that incorporating the nanoparticles lowers the cytotoxicity of these irrigation solutions. Conclusion: This research demonstrates that chitosan nanoparticles can improve cytotoxicity of 5.25% sodium hypochlorite and 2% chlorhexidine when they mixed together.

Introduction:

Irrigations play an essential role in clearing the root canal walls from debris and smear layer as well as their antibacterial properties, which improve the adherence and penetration of endodontic sealer and obturation material. In endodontic therapy, the most frequently used irrigation solutions are Chlorhexidine (CHX),sodium hypochlorite (NaOCl) and ethylene diamine tetra-acetic acid (EDTA). The field of endodontics is constantly searching for substitute irrigants with superior antibacterial activity and fewer adverse effects because each of these irrigants has drawbacks and adverse consequences, including cytotoxicity, allergic responses, and dentin erosion [1]. Consequently, investigators are studying more effective irrigation solutions and disinfection methods. New irrigation agents that are less irritating to periapical tissues and more effective at disinfecting have been the subject of numerous studies. Numerous natural materials, including herbal solution, propolis, chitin, and antimicrobial nanoparticles have been explored in these studies. These substitutions are thought to have less toxicity, less irritation, and antibacterial activity that is comparable to that of NaOCl [2]. Because of the drawbacks of conventional irrigation solutions, the use of irrigation solutions based on nanoparticles become more frequently used. The utilization of nanotechnology to treat oral conditions with the goal of enhancing oral health is known as "nano dentistry" [3]. Nanoparticles are present in various size and forms. Nanoparticles range in size from 1 to 100 nm. They have numerous features including their tiny diameters and increased chemical activity. Because of their biocidal, anti-adhesive and transport characteristics, nanoparticles can be used in a variety of infection control strategies particularly in the complex environment of an oral cavity. When compared to their conventional equivalents, the most effective features of nanoparticles in antibacterial action are their larger surface areas and higher concentrations at the target region [4].

Chitosan nanoparticles, are a cationic polymer that comes from natural sources like shrimp and crab shells [5], a naturally occurring polysaccharide that is non-toxic and renewable. Additionally, it has strong adsorption and moisturizing properties. Its safe profile, biodegradability, and biocompatibility have led to its utilization in various biological and pharmacological fields [6]. Chitosan has been confirmed to be safe for use in foods and medications by FDA (Food and Drug Administration of the United States) [7].

Hypothesis

The following hypotheses to be tested in the current study:

1.The null hypothesis (H0) states lack of difference in cytotoxicity that caused by adding nanoparticles to tested irrigation solutions.

2.The alternative hypothesis (H1) states that there is difference in cytotoxicity caused by adding nanoparticles to tested irrigation solutions.

Materials and Methods

Chitosan Nanoparticles Preparation

Nano-chitosan powder (purity more than 95.7%, particle size ≤ 80 nm) were purchased from SHAANXI SANGHERB BIO-TECH INC. The 0.2% and 0.5% nano-chitosan irrigation will be produced by dissolving 0.2 and 0.5 gm of the chitosan nanoparticles powder, respectively, in 100 ml of distilled water and 1% acetic acid, stirred for two hours using magnetic stirring machine at room temperature(±23°C) until a crystalline homogenous solution ,PH =4 , measured by a digital PH meter [8].

Sample Grouping

Experimental groups were divided into nine groups as follow:

Group I: Nano-chitosan(NC) 0.2%.
Group II: Nano-chitosan(NC) 0.5%.
Group III: NaOCl **5.25%.**
Group IV: CHX 2%.
Group V: Nano-chitosan(NC) 0.2%+ NaOCl 5.25%.
Group VI: Nano-chitosan(NC) 0.5%+ NaOCl 5.25%.

Group VII: Nano-chitosan(NC) 0.2%+ CHX 2%.

Group VIII: Nano-chitosan(NC) 0.5%+ CHX 2%.

Group IX: Control (cells + culture media).

MTT Cytotoxicity Assay

The Methyl Thiazol Tetrazolium assay was used to measure the metabolic function of cells. The tetrazolium dye MTT, which is chemically 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, can be reduced by NAD(P)H-dependent cellular oxido-reductase enzymes to its purple insoluble formazan [9].The MTT assay was conducted as an indirect screening test to determine the cytotoxic effects of the tested irrigation solutions in comparison to the control group on the isolated fibroblast-like cells.

Cell Culture

At the University of Malaysia, Department of Natural Products Research and Drug Discovery, the cytotoxicity of endodontic irrigation was assessed on human periodontal fibroblasts. The cells were cultivated in 96-well polystyrene plates that contain Dulbecco's modified Eagle's medium (DMEM), enriched with 10% fetal bovine serum (FBS) , 250 µg/ml gentamycin sulfate, 5 µg/ml amphotericin B, and were incubated for 24h at 37°C in water based incubator in a humidified atmosphere(95% air and 5% CO2) [10].

0.25% trypsin was mployed to separate confluent cell and aliquots of the splitted cells were then subcultured. For the experimental procedures, cells from passages 7th through 14th were utilized [11].

Procedure of MTT assay

The used MTT assay Kit is composed of the following:

1. MTT solution: 3-(4,5-dimethylthiazol-2yl) 2,5diphenyl tetrazolium bromide (MW= 414) (1mL x 10vials).
2. Solution for solubilization (50 mL x 2 bottle).

According to following protocol, MTT assay was used to assess the cells vitality according to their response to the tested irrigation solutions (at immediate (0h), 24h, 72h, and 168h) as following [12,13]:

1. In a 96-well plate, the cells (1 x 10^5cells mL^{-1}) were cultivated until each well had 200 µL of full culture media. After gently stirring the plates and covering them with sterile parafilm, they were incubated for twenty-four hours at 37 °C with 5% CO2.
2. Following cell incubation, 200 µl of culture media containing the tested solutions for each immersion time period were added after the culture media was eliminated. The responsiveness of the cells was tested using cells received treatment with 10% ethanol as a positive control and negative control (cells + medium only). For every group, three repetitions, or triplicates, were carried out. After that, the plates were incubated for 24 hrs at 37°C with 5% CO2.
3. Each well received 10 µL of MTT solution upon exposure to the tested solutions. For an additional four hours, the plates were incubated at 37 °C with 5% CO2.
4. Following carefully removing the medium, each well received 100 µL of dimethyl sulfoxide solubilization solution, which then incubated for five minutes.
5. Lastly, an enzyme-linked immunosorbent assay (ELISA) reader employed to assess absorbency (also known as optical density) at a wavelength of 575 nm. The data was then statistically analyzed to determine whether the material was cytotoxic or not.

Statistical Analysis

It has been completed by "SPSS software" (SPSS version 20, IBM, USA). Descriptive statistics of cell viability have been represented as mean and standard deviation.

Results

The mean and standard deviation of vitality of fibroblast-like cell treated with tested irrigation solutions at different time periods (0h, 24h, 72h, 168h) are presented in Table (1). Table (1) showed that 5.25% NaOCl was the most cytotoxic solution (lower cell viability)(43.788) followed by 2% CHX (74.614), 0.5% NC (89.660) while 0.2% NC was the lowest cytotoxic irrigation solution (higher cell viability)

(91.473). After mixing of NaOCl and CHX with nanoparticles we noticed that this mixing lower the cytotoxicity of NaOCl and CHX.

Discussion

The main goal of endodontic therapy is pulp tissue removal. The system of root canal is cleaned of bacteria and microbial toxins by endodontic irrigation. The periapical and dentinal tissues may come into contact with irrigation solutions, among other adjacent tissues. In the contact region, irrigants that are not biocompatible may promote the growth of cells, adhesion, and disintegration of the enzyme system [14].

It is crucial to evaluate the material's biocompatibility that used in root canal therapy since biocompatibility is a prerequisite for using any type of endodontic irrigation solution in clinical practice [15].

In spite of all efforts to prevent it, endodontic irrigation will be pushed into tissues present in periapical region during root canal therapy. It is well recognized that endodontic irrigation are not constrained for use in root canals, they can also reach the periapical tissues (through the apical foramen) which are made up of cement, periodental ligament and the alveolar bone [16]. In order to reduce future hazards for the patient and the physician, biochemical studies measuring the hereditary and harmful effects of endodontic irrigants on the interacting tissues are essential [17].

One of common colorimetric technique to determine cell vitality, proliferation, activation is the MTT assay. It is sensitive, qualitative and extremely reliable [18]. It depends on capacity of living cell to alter the soluble tetrazolium salt into a blue formazan end product via mitochondrial dehydrogenase enzymes. Because the MTT assay is sensitive, we employed it to assess the cytotoxicity of dental material. The benefits of this approach include its ease of use, rapidity, repeatability and lack of a need for radioisotopes [19].

The initial point of contact where irrigation solution are unrestrained throughout the root canal, is the periodontal ligament because of its anatomical proximity to the root apex . Usual cell in this area that come into touch with irrigation solution is fibroblast. A human periodontal fibroblast cell type therefore employed in this investigation [2○].

NaOCl concentrations ranging from 0.5–5.25% have been available, with higher concentrations having greater antibacterial activity but also higher toxicity . Cellular cytoplasmic membrane integrity is compromised by NaOCl toxicity, moreover high pH (hydroxyl ion action) which causes persistent enzymatic suppression. CHX is used widely in periodontal therapy because of its substantivity and broad spectrum antibacterial effect. For these same reasons, it is also utilized as endodontic irrigant and intracanal medicatment in endodontic therapy . CHX also exhibit a cytotoxic effect on fibroblast cells but it lower than the cytotoxicity of NaOCl [21].

Natural material is frequently utilized as substitutes for root canal irrigation solution. Consequently, the application of natural chitosan has grown in popularity. [22]. Chitosan, a cationic biopolymer, has aroused researchers interest in the recent years due to its low toxicity and biocompatibility [23].

Irrigation solution cytotoxicity is dose- and duration-dependent, according to Aydin et al. Due to its decreased toxicity, chitosan may make an improved irrigation solution over sodium hypochlorite [15].

In this study, we measure cytotoxicity at different time periods to know if the cytotoxic effect of the irrigation solutions increase or decrease with time. 5.25% NaOCl has the lower cell viability (higher cytotoxicity) (43.788) than other irrigation solutions. The cell viability of fibroblast decrease with time (more toxicity after 168 hrs). Also, 2% CHX result in low cell viability (high cytotoxicity) (74.614) but it lower than NaOCl which is the most cytotoxic solution among the other. This finding is in agreement with researches by Yasuda et al. and Mollashahi et al. who demonstrated that CHX is not more toxic than NaOCl . Chitosan result in highest cell viability (lower cytotoxicity) (0.2% NC= 91.473,

0.5% NC = 89.660) so it is better irrigation solution than NaOCl and CHX because it is the most less toxic among the other irrigation solutions [24,25].

The cytotoxicity of chitosan on different cells was the focus for many investigations. According to Chellat et al., chitosan had no effect on fibroblast cells, presumably because to methodological variations in cell line and concentration [26]. In the current investigation,0.2% NC had less cytotoxicity than 0.5%. Incorporating chitosan nanoparticles of different concentrations with 5.25% NaOCl and 2% CHX significantly reduced cytotoxicity ($P < 0.05$).

Conclusion

According to my study, mixture of nanoparticles with NaOCl and CHX had lower toxicity than when they used alone without mixing. The combination of NC with NaOCl and CHX can be considered as a novel irrigant that requires additional investigation for replacing the endodontic irrigant.

Table (1): MTT cell viability results (%) in response to the tested irrigation solutions at the different time periods (i.e. immediate (0 h), 24 h, 72 h and 168 h).

Sample grouping	0 h		24 h		72 h		168 h	
	mean	SD	mean	SD	mean	SD	mean	SD
G I	97.569	0.834	94.637	0.594	93.865	0.530	91.473	1.718
G II	97.569	0.834	93.75	0.530	92.708	0.759	89.660	1.506
G III	97.569	0.834	75.887	2.0884	50.841	1.626	43.788	1.487
G IV	97.569	0.834	86.767	3.505	84.105	1.465	74.614	1.182
G V	97.569	0.834	91.744	0.658	87.075	0.812	80.401	2.039
G VI	97.569	0.834	87.731	1.712	81.404	0.998	76.774	0.928
G VII	97.569	0.834	94.945	0.467	92.019	0.582	91.357	0.522
G VIII	97.568	0.834	91.820	1.159	85.995	1.219	81.597	1.219
G IX	98.86	0.205	98.86	0.205	98.86	0.205	98.86	0.205

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