CYTOTOXICITY OF CHITOSAN NANOPARTICLES AND PLAIN CHITOSAN WITH CHLORHEXIDINE USING FIBROBLAST CELL LINE- AN IN VITRO STUDY

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Abstract

Introduction: Root canal infections are multifactorial and are caused due to a diverse species of organisms present in the oral cavity and the root canal. Sodium hypochlorite has long been the gold standard for irrigation. However, there are several significant disadvantages to using sodium hypochlorite.Chitosan is a natural biopolymer having antimicrobial activity and has all the properties of an ideal irrigant / sodium hypochlorite without its inherent drawbacks.The aim of this study was to evaluate the effect of Sodium hypochlorite, plain chitosan with chlorhexidine, and chitosan nanoparticles with chlorhexidine on the L929 fibroblast cell line.

Materials and Method: The chitosan was obtained and plain chitosan with chlorhexidine, chitosan nanoparticles with chlorhexidine was prepared. For cell viability assay, cells were seeded onto 96-well plates at a concentration of 5×103 cells/well, followed by the addition of test samples of various concentrations prepared in cell culture media. MTT assay was done which measures the reduction of yellow 3-(4,5dimethiazol-2-yl)- 2, 5-diphenhydramine tetrazolium bromide(MTT) by mitochondrial succinate dehydrogenase. The effect of test samples (25-100µg/ml) on cell growth inhibition will be assessed as percentile viability, where vehicle-treated cells will be taken as 100% viable.

Results: Statistical analysis was done using One-way Anova and a post hoc test. $p<0.05$ was considered as significant. $P<0.001$ as compared with negative control. Chitosan nanoparticles with chlorhexidine at different concentrations are $P<0.001$ as compared with sodium hypochlorite.

Conclusion: Chitosan nanoparticles with chlorhexidine had less cytotoxic effect based on its concentration compared to sodium hypochlorite. Hence it can be used as an alternative endodontic irrigant.

Keywords: Endodontic Irrigants, Fibroblast Cells, Root Canal Treatment,Chitosan, Chitosan nanoparticles , Natural Irrigant.

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1. Introduction

Root canal infections are multifactorial and are caused due to a diverse species of organisms present in the oral cavity and the root canal.Necrotic pulp tissue invaded by bacteria causes primary endodontic infections.(1) Endodontic treatment success is dependent on full debridement and disinfection of the root canal space. This is not always totally done since bacteria can be detected in root canals, dentinal tubules, apical ramifications, cementum, or regions of root resorption, restricting tool and irrigant access to root canal systems.(1,2) Irrigants in endodontic therapy must have antibacterial properties, breakdown organic materials in the canal, and rinse out loose debris.(3) Because of its capacity to dissolve organic debris and great antibacterial potential, sodium hypochlorite has long been the gold standard for irrigation. However, there are several significant disadvantages to using sodium hypochlorite, including irritant to periapical tissues, staining of instruments, unpleasant taste, high toxicity, corrosion of instruments, inability to remove smear layer, burning of surrounding tissues, and a decrease in elastic modulus and flexural strength of dentin.(4–6)(7)

Chlorhexidine (CHX) is a broad-spectrum antibacterial agent with significant antimicrobial action but minimal toxicity. Organic tissues, on the other hand, are not dissolved. CHX has been proven in vitro to have long-term antibacterial action in the root canal after being administered as an endodontic irrigant.(8,9) Because of its particular ability to bind to dentin, efficacy as an antibacterial agent, and substantivity in the root canal system, CHX has been proposed as a root canal irrigant.(10–13)

People have become increasingly interested in the advancement of health and medical technologies as their living conditions have improved. Many polymer molecules derived from liver sugar, starch,cellulose,cellulose, chitin, and alginates have found widespread usage in biology, medicine, aesthetics, healthcare, and other disciplines in recent years.(14) (15,16)Chitosan is a deacetylated derivative of chitin, a plentiful natural material with less storage capacity than cellulose.(17–19)Chitosan is a natural alkaline polysaccharide that is renewable and has no toxicity or negative effects. It also has high moisturizing and adsorption capabilities. The Food and Drug Administration (FDA) of the United States has determined that chitosan is safe for use in foods and pharmaceuticals.

Chitosan, on the other hand, is insoluble in water and most organic solvents, limiting its uses in a variety of sectors. Chitosan derivatives are created by chemically modifying chitosan-reactive functional groups. The OH and NH2 active groups on the chitosan molecule are susceptible to chemical reactions in this case.(20) Chemical modification can not only improve the physical and chemical characteristics of chitosan, but also preserve its distinctive qualities and broaden the application range of chitosan derivatives. Modified chitosan derivatives have improved biocompatibility, bioactivity, biodegradability, and non-toxicity while retaining the original bactericidal, antibacterial, anticancer, and antiviral pharmacological effects, such as the ability to induce erythrocyte aggregation, promote platelet activation, and activate complement systems other than chitosan.(21,22)

Chitosan derivatives are currently frequently utilised in medical materials and biomedicine. Chitosan derivatives have been created as nanostructures, including nanoparticles, hydrogels, microspheres, and micelles, as nanotechnology has advanced. Chitosan derivatives can be employed as drug carriers, adjuvants, and vaccine delivery carriers.(22) As a result, chitosan derivatives and their nanomaterials may be widely employed and increased in terms of chitosan application sectors.(23,24)There is a paucity of research exists on the cytotoxicity of chitosan nanoparticles on fibroblast cell lines so, the aim of this study is to evaluate the cytotoxicity effect of Sodium hypochlorite, plain chitosan with chlorhexidine, and chitosan nanoparticles with chlorhexidine on the L929 fibroblast cell line.

2. Materials and Methods

Synthesis and optimization of chitosan nanoparticles with chlorhexidine

500 mg of chitosan was dissolved in 50 ml of 1% acetic acid solution and agitated for 25 minutes at room temperature at 1000 rpm until the solution turned clear. The resultant solution was sonicated before being titrated with NaOH or HCL solution calibrated to pH5 and filtered through 0.2 mesh. 5 mL of nano-magnetic solution was added to 75 mL of deionized water and sonicated for 10 minutes for the coating step. The solution of chitosan was then added and sonicated for 5 minutes. The resulting solution was clear. To 50 ml of the produced nano chitosan solution, 50 ml of 2% chlorhexidine was added. The resultant solution was sonicated for 10 minutes till clear.

Synthesis of plain chitosan nanoparticles with chlorhexidine

500 mg of chitosan was dissolved in 50 ml of 1% acetic acid solution and agitated for 25 minutes at room temperature at 1000 rpm until the solution turned clear. The resultant solution was sonicated before being titrated with NaOH or HCL solution calibrated to pH5 and filtered through 0.2µ mesh. To 50 mL of the produced chitosan solution, 50 mL of

2% Chlorhexidiene was added. The resultant solution was sonicated for 10 minutes till clear.

Chemicals

The materials used for MTT test were 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 10% fetal bovine serum (FBS), 100 units/ml of fungizone, dimethyl sulfoxide (DMSO), human fibroblast cell lines (primary culture), Eagle's minimum essential medium (EMEM), kanamycin, and phosphate-buffered saline.

Maintenance of Cell Lines

NCCS Pune provided L929 fibroblast cell lines for this study. The L929 cells were cultivated in a humidified atmosphere at 37°C in a humidified CO2 (5%) chamber and 95 percent air in the cell growth DMEM medium with 10% foetal bovine serum, L–glutamine, 1% penicillin (100 U/ml), and streptomycin (100 g/ml). 0.25 percent EDTA Trypsin was used to separate the cells. Trypsin was neutralised using DMEM containing 10% FBS and PSGF, and cells were physically separated using a pipette. There were 96-well polycarbonate culture plates with 200 l of media in each well. The plates were then incubated for 24 hours at 37°C in a humidified environment containing 5% CO2 and 95% air to allow the cells to adhere to the plates.

Cytotoxicity test by MTT assay

The EMEM, Kanamycin, 1% pen strep, 10% FBS, and 100 units/ml of fungizone 100 µl was added as a control media to the 96 well microtiter plates. The microplates are filled with 100 µl of fibroblast cells with a density of 3×10^3 in EMEM, kanamycin, 1% pen strep, 10% FBS, and 100 units/ml of fungizone as negative control. The cells were permitted to adhere for 24 hours, and the growth medium using micropipette and the monolayer of cells was washed twice with MEM without FBS to remove dead cells and excess FBS.

1ml of medium (without FBS) containing different dilution of chitosan nanoparticles and plain chitosan (25-100µg/ml) were added in respective wells; 20 µl of MTT (5 mg/ml in PBS) were added to each well, and the cells incubated for a further 6‑7 hrs in 5% CO_2 incubator. After removal of the medium, 1ml of DMSO was added to each well and the positive control (Sodium hypochlorite) was tested. The supernatant was removed and 50 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The plates were placed on a shaker for 15 min and the absorbance was read on an enzyme-linked immunosorbent assay (ELISA) reader at 570 nm. Each experiment was carried out in triplicate and the half maximal inhibitory concentration (IC_{50}) of the test samples as the percentage survival of the cells was calculated.

Statistical Analysis

The results were presented as mean±SD. One-way analysis of variance (ANOVA) and the post hoc least-significant difference test were used to establish statistical significance. $P<0.05$ was seen as substantial. (See Table2)

	Control	10µg/ml	20µg/ml	30µg/ml	50µg/ml	100µg/ml	200µg/ml
Group I (plain chitosan with chlorhexidine)	100%	90.8%	85.8%	84.9%	81.7%	77.9%	74.9%
Group II (nano chitosan with chlorhexidine)	100%	98.2%	96.1%	95.6%	95.13%	94.99%	94.02%
Group III (Sodium Hypochlorite)	100%	93.9%	81%	77.3%	57.8%	26%	21%

Table 1- Cell survival rate at different concentration of irrigants

Group I-	Mean	100	90.09684	86.29644	84.23886	81.97853	79.06579	76.27181
	SE	0	3.454782	4.712032	5.296399	4.636402	5.391979	5.366517
	p-Value		0.033993	0.032633	0.031903	0.020668	0.020469	0.016602
Group II-	Mean	100	94.57806	93.65801	90.1865	90.46056	89.51445	87.20678
	SE	0	3.516533	2.318845	3.722606	5.322874	4.606787	5.630882
	p-Value		0.087015	0.03694	0.037676	0.070616	0.048557	0.048562

Table II- Values are expressed as Mean±SD (n=3); *P<0.001, as compared with Negative control. ^{a}P<0.001, as compared with NaOCl.

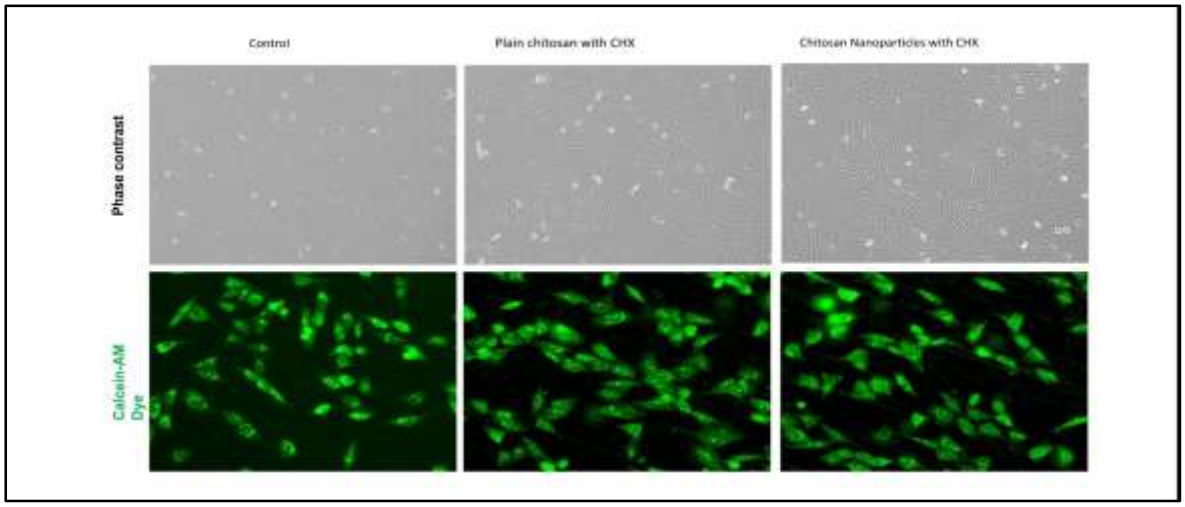

Fig 1: Cells were treated with Group 1 and Group 2 for 24 h along with the control group. Images were obtained using an inverted Phase contrast (10x) and Fluorescence microscope (20x).

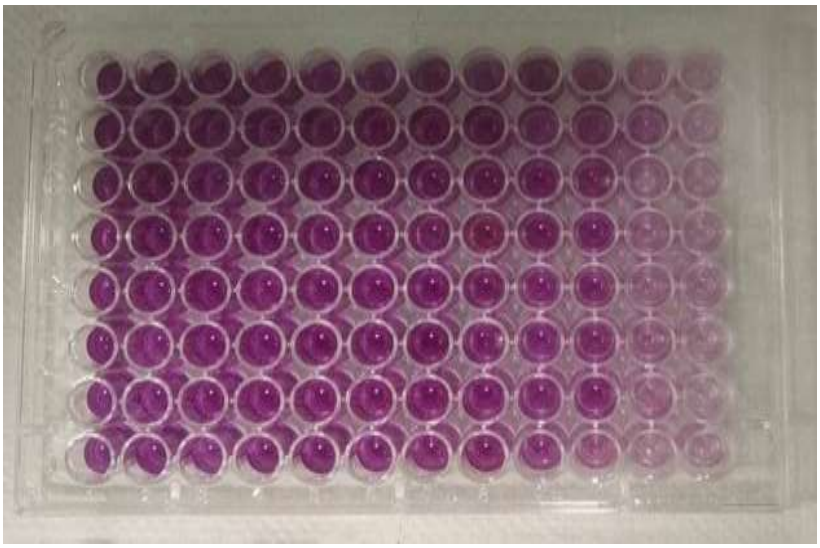

Fig 3: MTT Assay

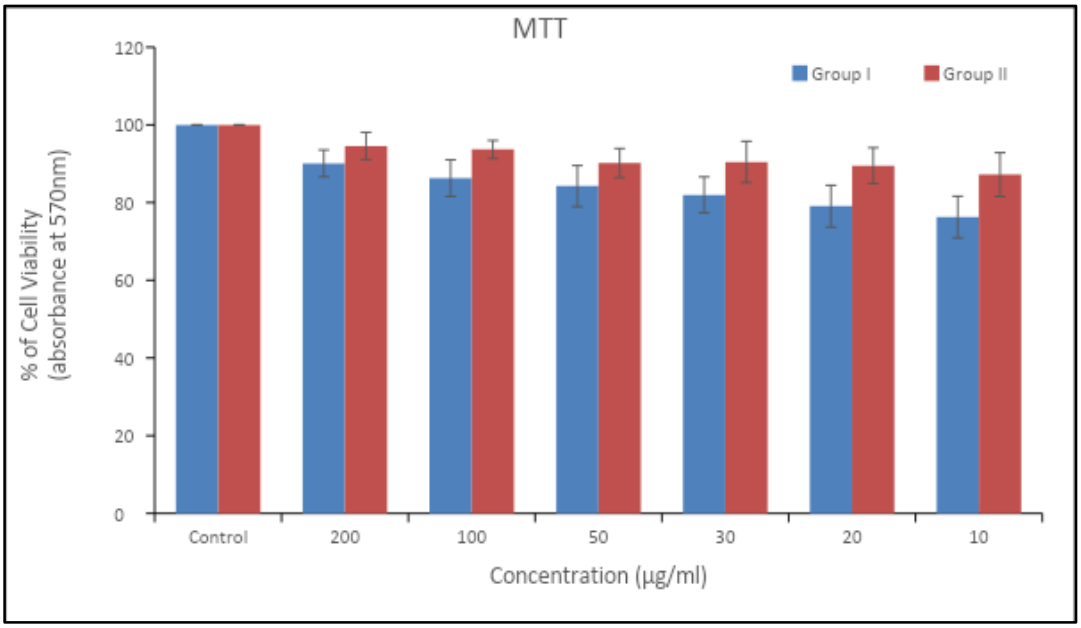

Fig 3: The cell viability of NIH-3T3 cells were treated with different concentration of Group I- plain chitosan with chlorhexidine & II- nano chitosan with chlorhexidine (10, 20, 30, 50, 100 and 200 µg/ml) for 24 h, and cell viability was evaluated by MTT assay. Data are shown as means ± SD (n = 3).* compared with the control group, $p < 0.001$.

3. Results

Cytotoxicity in cell culture is typically expressed as LC50. The LC50 of chitosan nanoparticles with chlorhexidine is <100ug/ml. P<0.001 as compared with negative control. Chitosan nanoparticles with chlorhexidine at different concentrations are P<0.003 as compared with sodium hypochlorite.

4. Discussion

The present in-vitro study evaluated the cytotoxicity of nano chitosan with chlorhexidine and chitosan with chlorhexidine on the L929 fibroblast cell line. The results showed that the nano chitosan with chlorhexidine and chitosan with chlorhexidine at 30µg/ml concentration showed about 95.6% and 84.8% cell viability and even at 100µg/ml showed cell viability of about % and 94.9 % cell viability, Which is less toxic to L929 fibroblasts cells when compared to sodium hypochlorite which was 26% at 100µg/ml.

The goal of endodontic therapy for teeth with pulpal and periapical pathosis is to rid the root canal system of germs. Chemomechanical methods have little effect on microorganisms in the dentinal tubules. There has been no significant difference in antibacterial efficacy between NaOCl concentrations ranging from 0.5 percent to 5% in clinical and laboratory trials. (25–27) in the root canal (canal wall samples).(28)

The antimicrobial activity of NaOCl was essentially identical to that of a mixture of 2% chlorhexidine and 2% chitosan.Chlorhexidine digluconate (CHX) has been proposed as a root canal irrigant because of its particular ability to attach to dentin and its efficacy as an antibacterial agent against E. coli. faecalis in the root canal system and its substantivity.(29,30)

In cell culture, cytotoxicity is usually represented as LC50, which signifies that a certain concentration of a substance is deadly to 50% of the cells. Furthermore, the LC50, or the dose of a medication that kills half of the examined cells in culture, is the most frequent approach to define cytotoxicity in cell culture. (31) Our team has extensive knowledge and research experience that has translate into high quality publications(32–41)

The cytotoxic effects of plain chitosan with chlorhexidine and nano chitosan with chlorhexidine at concentrations of 10g/ml, 20g/ml, 30g/ml, 50g/ml, 100g/ml, and 200g/ml on human gingival fibroblast were investigated using the MTT assay in comparison to positive and negative controls on human gingival fibroblast. Plain chitosan with chlorhexidine and nano chitosan with chlorhexidine had no harmful impact, according to the findings. Plain chitosan with chlorhexidine and nano chitosan with chlorhexidine were shown to be non-cytotoxic and capable of killing E. faecalis, indicating that it might be examined further as a root canal irrigation material. Further research will be conducted using appropriate experimental models to study its biomedical applications with a thorough methodology.

5. Conclusion

Chitosan nanoparticles with chlorhexidine had less cytotoxic effect based on its concentration compared

to sodium hypochlorite. Hence it can be used as an alternative endodontic irrigant.

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