Section A -Research paper

Comparative evaluation of cytotoxicity of endodontic irrigants with and without silver nanoparticles on human periodontal ligament cells: An in vitro study

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ABSTRACT

Introduction: The biolfilms are the main cause of failure of root canal treatment (RCT) due to secondary infection. The main aim of the endodontic therapy is elimination of complex biofilms. During RCT while irrigating various solutions like EDTA, sodium hypochlorite (NaOCl), chlorhexidine (CHX) are applied at various concentrations for the eradication of the smear layer. Special attention has been given on effectiveness of irrigation to remove of smear layer in numerous studies conducted so far. The areas like isthmuses, fins and lateral canals which are relatively untouched by the files are effectively dealt with by irrigation. Antimicrobial nanoparticles have been developed to eliminate the limitations of routinely used antibacterial agents.

Aim : To evaluate and compare the cytotoxicity Of 3% NaOCl, oxytetracycline, 2% CHX and triclosan combined with and without silver nanoparticle as endodontic irrigant on periodontal ligament cells using the Mosmann's Tetrazolium Toxicity(MTT) assay .

Materials and Methods: The roots of premolars extraxted for orthodontic purpose were used to grow periodontal cells (PDL). A water based incubator was used for culture at 37^0C in a humidified atmosphere for 24 hours with 95% air and 5% CO_2. After being divided randomly into nine experimental groups, cells were transplanted to 36-well plates. The Mosmann's Tetrazolium Toxicity assay was used for assessing cytotoxicity of the materials. The proportion of living cells affected how intense the colour was created. Descriptive statistics and repeated measures ANOVA followed by Tukey's Post hoc Test for pairwise comparison were used for statistical analysis.

Results: There was a statistically significant difference in the average number of viable cells between the tested irrigation solution and the control at different time interval (P 0.0001). Addition of silver nanoparticle reduces the cell viability in all the groups. All irrigants with addition of silver nanoparticles at all time intervals did not show any statistically significant difference.

Conclusion: Oxytetracycline and triclosan had the lowest cytotoxicity compared to sodium hypochlorite and Chlorhexidine. Although the addition of silver nanoparticles decreases cell

viability, there was no statistically significant difference between the groups that contained and lacked silver nanoparticles.

Key Words- Irrigants, Cytotoxicity, nanoparticles, viability

INTRODUCTION

Irrigation is an indispensable step for successful outcome of endodintic treatment because fulfills multiple important chemical, mechanical and (micro) biological functions.[1-3] Root canal treatment eliminates bacterial infection from the canals and further prohibits reinfection.[4.5]Irrigating solutions has antibacterial action and destroy bacteria or yeasts on direct contact. Various combinations of irrigation solutions have been precisely used in a sequence for maximum effectiveness of root canal therapy.[6]

Several chemical solutions like saline, chlorhexidine and sodium hypochlorite (NaOCl) are frequently used as endodontic irrigants.[7-10] Each product has unique qualities, the most common and wide spredly used irrigant is NaOCl.[11] Eventhough it is very effective antibacterial agent, NaOCl causes damage to the periradicular tissues on contact. [9,11,12] Therefore, chlorhexidine gluconate (CHX) have been tested as replacement for NaOCl as a potent irrigating solution.[13] However, CHX has always been presented as a liquid, and in some circumstances, its failure to dissolve pulp has proven problematic.[7]The antimicrobial effectiveness of antibiotics like metronidazole, clindamycin and a combination of ciprofloxacin, metronidazole, against root canal bacteria has been explored by a number of studies.[14-20] When used as an endodontic irrigant, Oxytetracycline has been shown to be effective against E. faecalis by Chai et al.[21] and Mittal et al.[22] However, there is not much evidence in the literature regarding cytotoxicity of Oxytetracycline, when used as an endodontic irrigant.

Triclosan is a potent, all-purpose antibacterial agent that is also effective against a wide range of fungi and viruses but cytotoxic effect of triclosan need to be evaluated.[23,24] Nanoparticles have been developed to improvise the properties of irrigants used as antibacterial agents in endodontic therapy. They have larger surface area and higher charge density allow them to contact more closely with surface of bacteria, which increases their antimicrobial activity.[25]

Silver atoms with a diameter of 1 to 100 nm are used to create nanoscale structures known as silver nanoparticles. Silver's toxicity is its most critical feature in biomedical applications. Silver's hazardous effects on human cells only become evident after prolonged exposures.[26,27] Nanotechnology can circumvent this problem by creating smaller silver particles that are less toxic to human cells and more efficient against microbes.[28] Therefore, the present study was designed to compare the cytotoxicity Of 3% NaOCl, 2% CHX, oxytetracycline and triclosan combined with and without silver nanoparticle as endodontic irrigant on periodontal ligament cells using the Mosmann's Tetrazolium Toxicity (MTT) assay .

MATERIALS AND METHODS

The present study was conducted in the Department Of Conservative Dentistry And Endodontics of a private dental college after approval was duely obtained from the ethical committee. Extracted premolars for orthodontic treatment were used to culture human PDL cells. 96-well plates with Dulbecco's modified Eagle's medium (DMEM) was used to culture PDL fragments. 10% fetal bovine serum (FBS) and antibiotics were added to culture to supplement. In a water-based incubator, cultures were incubated for twenty four hours at thirty seven degree in a humidified environment of 95% air and 5% CO2. 10% DMEM was used as media to cultivate the cell line in culture flask. The culture medium was changed

during the cell culture process every 48 hours, and cells were travelled after a 7 days. After passes, cells had reached a suitable growth for cytotoxicity tests. After that, irrigation fluids were applied to stem cells that had been placed to 36-well plates and randomly separated into 9 experimental groups. Stem cell culture in DMEM was employed as a control group.

GROUP I : Normal Saline
GROUP II : 3% NaOCl
GROUP III : 3% NaOCl + AgNPs
GROUP IV : 2%Chlorhexidine
GROUP V : 2%Chlorhexidine + AgNPs
GROUP VI : Oxytetracycline 50mg/ml
GROUPVII : Oxytetracycline 50mg/ml + AgNPs
GROUPVIII : Triclosan
GROUP IX : Triclosan + AgNPs

The cytotoxicity of the irrigation solutions was appraise using the MTT assay at 1, 5, and 15 minutes of exposure. This solution was filtered and diluted one to ten with DMEM. Plates were incubated for 4 hours at 37 °C with 5% CO2 and 95% humidity after adding 400 L of the diluted MTT solution to each well. After dissolving the MTT crystal, Elisa Reader examined the optical density of the irrigants at 530-680 nm. The intensity of the colour generated was proportional to the percentage of live cells.

STATISTICAL ANALYSIS

The analysis was carried out using the IBM Statistical Package for Social Sciences (Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.), and the data was entered into a Microsoft Excel spreadsheet (Microsoft, USA) for storage. Continuous data was presented by mean and standard deviation (SD) for each group for cell viability. Comparison of means was done using repeated measures ANOVA. Post hoc analysis was done using Tukey's post hoc test. P-value less than 0.05 was considered statistically significant.

RESULTS

The mean percentage of viable cells showed statistically significant difference between control and study groups at 1, 5 and 10 min respectively ($P<0.0001$). Cell viability observed with Oxytetracycline was the highest ($P<0.05$) while Sodium Hypochlorite had the less cell viability in comparison with other irrigants ($P<0.05$) with statistically significant difference at 1, 5 and 10 min. (Table-1)

Table 1: Overall comparison of cell viability in the experimental groups after 1 mins, 5mins and 10 mins of irrigation:

Group	1 min Mean (SD)	5 min Mean (SD)	10 min Mean (SD)
Group 1 (Normal Saline)	99.5 (1.0)	96.25 (1.89)	94.5 (1.91)
Group 2 (3% Na Hypochlorite)	16.5 (1.91)	14.0 (1.63)	10.5 (1.91)
Group 3 (3% Na Hypochlorite +AgNPs)	11.75 (1.7)	11.25 (1.89)	9.5 (1.29)
Group 4 (2% CHX)	22.5 (1.91)	21.5 (1.29)	19.0 (1.41)
Group 5 (2% CHX + AgNPs)	20.25 (4.03)	16.75 (2.21)	16.5 (1.29)

Group 6 (Oxytetracycline)	99.5 (1.91)	98.75 (1.7)	96.75 (0.95)
Group 7 (Oxytetracycline + AgNPs)	96.75 (2.21)	94.25 (2.62)	93.0 (2.58)
Group 8 (Triclosan)	97.75 (1.7)	95.5 (2.08)	91 (1.82)
Group 9 (Triclosan + AgNPs)	92.25 (2.87)	89.0 (2.58)	87 (3.46)
One way Anova F test value	F = 1380.0	F = 1720.0	F =1750.0
P value	p<0.001**	P<0.001**	P<0.001**

The average number of viable cells in all samples changed with time, the average proportion of viable cells declined with greater time of exposure in all the groups and it was satistically significant (Table-2).

Table 2: Pairwise comparison of cell viability in the experimental groups without additions of Silver Nanoparticle after 1 mins of irrigation using Tukey's post hoc test

Without Addition of Silver Nanoparticles				
Group	**Comparison Group**	**1 min**	**5 min**	**10 min**
Group 1 (Normal Saline)vs	Group 2 (3% Na Hypochlorite)	p<0.001**	p<0.001**	p<0.001**
	Group 4 (2% CHX)	p<0.001**	p<0.001**	p<0.001**
	Group 6 (Oxytetracycline)	p =0.941	p =0.307	p =0.796
	Group 8 (Triclosan)	p =0.997	p =0.815	p =0.280
Group 2 (3% Na Hypochlorite)vs	Group 4 (2% CHX)	p =0.023*	p =0.001*	p<0.001**
	Group 6 (Oxytetracycline)	p<0.001**	p<0.001**	p<0.001**
	Group 8 (Triclosan)	p<0.001**	p<0.001**	p<0.001**
Group 4 (2% CHX) vs	Group 6 (Oxytetracycline)	p<0.001**	p<0.001**	p<0.001**
	Group 8 (Triclosan)	p<0.001**	p<0.001**	p<0.001**
Group 6 (Oxytetracycline)vs	Group 8 (Triclosan)	p =1.000	p =0.993	p =0.009*

On overall comparison of cell viability in the experimental groups after 10 mins using One way Anova F test, there was found to be highly statistical significant difference (p<0.001) among nine groups. (Table2,3).

Table 3: Pairwise comparison of cell viability in the experimental groups with additions of Silver Nanoparticle after 1 mins, 5 mins and 10 mins of irrigation using Tukey's post hoc test

Addition of Silver Nanoparticles				
Group	**Comparison Group**	**1 min**	**5 min**	**10 min**
Group 1	Group 3	p<0.001**	p<0.001**	p<0.001**

(Normal Saline) vs	(3% Na Hypochlorite + AgNPs)			
	Group 5 (2% CHX + AgNPs)	p<0.001**	p<0.001**	p<0.001**
	Group 7 ytetracycline+AgNPs)	P =0.743	P =0.892	P =0.974
	Group 9 (Triclosan + AgNPs)	P =0.003*	P =0.001*	p<0.001**
Group 3 (3% Na Hypochlorite + AgNPs) vs	Group 5 (2% CHX + AgNPs)	p<0.001**	p =0.017*	P=0.001*
	Group 7 (Oxytetracycline + AgNPs)	p<0.001**	p<0.001**	X p<0.001**
	Group 9 (Triclosan + AgNPs)	p<0.001**	p<0.001**	p<0.001**
Group 5 (2% CHX + AgNPs)vs	Group 7 (Oxytetracycline+ AgNPs)	p<0.001**	p<0.001**	p<0.001**
	Group 9 (Triclosan + AgNPs)	p<0.001**	p<0.001**	p<0.001**
Group 7 ytetracycline+AgNPs) vs	Group 9 (Triclosan + AgNPs)	p =0.169	p =0.026*	P =0.006*

Addition of silver nanoparticle reduces the viable cells in all the tested irrigation solution. No statistically significant difference (p>0.05) was observed between both group with and without silver nanoparticles at all time intervals (1 min, 5 min, 10 min) (Table-4).

Table 4: Intergroup comparison of cell viability in the experimental groups without and with additions of Silver Nanoparticle after 1 mins, 5 mins and 10 mins of irrigation using Tukey's post hoc test

	1 min	**5 min**	**10 min**
Group 2 (3% Na Hypochlorite) vs Group 3 (3% Na Hypochlorite + AgNPs)	p =0.125	p =0.612	p=0.998
Group 4 (2% CHX) vs Group 5 (2% CHX + AgNPs)	p =0.892	p =0.057	p =0.695
Group 6 (Oxytetracycline) Vs Group 7 (Oxytetracycline + AgNPs)	p =0.125	p<0.001**	p=0.607
Group 8 (Triclosan) Vs	p<0.001**	p<0.001**	p=0.148

Group 9 (Triclosan + AgNPs)			

$p>0.05$ – no significant difference $*p<0.05$ – significant $**p<0.001$ – highly significant

DISCUSSION

The primary objectives of endodontic treatment is the elimination of pulpal and dentinal debris from the root canal system. This can be achieved by using an irrigant or mixture of irrigants both during and after the instrumentation of the canal system. Various traditional and newer chemical agents like Acids (Tannic acids, Citric acids), normal saline, hydrogen peroxide, Ethylene Diamine Tetracetic Acid (EDTA), sodium hypochlorite, Chlorhexidine are used.[29,30]

Irrigation is integral part of endodontic therapy. The irrigation solution ease the removal of bacteria, pulp fragments, and dentinal shavings from the canal through a flushing procedure. In apical third packing and extrusion of debris beyond apex can be avoided with irrigation. Some irrigating fluids can disintegrate both pulp tissue and dentine. Additionally, irrigating solutions have antibacterial properties. [31] Nevertheless, some irrigating substances potentially to be cytotoxic and if they get into the periapical tissues, they can be rather painful. The present range of irrigants cannot be termed as perfect. The successful outcome of treatment is hinged on using the different components in the rightful order.

From 1% to 5.25 percent of sodium hypochlorite is utilized as irrigant. There will be hazardous consequences as the concentration rises. According to a study by Siquerra, using them at different doses had no effect on their antibacterial effects. When 3% NaOCl was used as the irrigation solution in this investigation, the cytotoxicity was significantly higher than with other irrigation solutions.[11]

Another irrigating solution used in study is Chlorhexidine which is an efficient antiseptic and used to control plaque accumulation in the oral cavity, 0.1 to 0.2% aqueous solutions are advised for the same. Similar to other endodontic disinfection agents, chlorhexidine's efficacy depends on pH and suffers greatly when organic matter is present. Microbes are eliminated by chlorhexidine, but organic debris and biofilm is not eliminated. For the greatest antibacterial impact at the completion of chemomechanical preparation, 2% chlorhexidine is typically preferred. [29] The ability of chlorhexidine to bind to negatively charged oral surfaces (such as teeth, Its efficacy depends on its gradual release from these retention sites (mucous membrane, pellicle, and restorations), which sustained antibacterial property for longer duration. This method is known as substantivity. These properties have been linked to tetracycline and chlorhexidine. [32]

According to research by Zambon JJ et al[24], triclosan is a broad-spectrum antibacterial drug that has action against microbes, fungi and viruses. It does not harm human cells because people lack the EACPR enzyme. Only a modest dose of the very effective inhibitor triclosan is required for strong antibiotic activity. [24.33 Studies has proven the antibiotic action of triclosan as root canal irrigant but much literature is not available on cytotoxicity of triclosan.[33] Hence in this study we evaluated the cytotoxicity of triclosan with other commonly used irrigants.

Numerous research on the tetracyclines' antibacterial potency have been done. Ex-vivo investigation by Rakesh et al., the antibacterial effectiveness of injectable oxytetracycline as irrigant was assessed to overcome issue of antibiotic insolubility in water. [22] E. faecalis was more effectively restrained from growing by oxytetracyline than by the other groups. Chai et al. examined the antibacterial efficiency of antibacterials and calcium hydroxide against biofilm in an in vitro model and discovered that oxytetracycline, Ca(OH), and erythromycin

were 100% effective. [21] Despite the fact that oxytetracycline has antibacterial properties, its cytotoxicity on tooth tissue needs to be assessed. [34]

It has been suggested that nanotechnology will improve oral healthcare. Silver nanoparticles (AgNP) are microscopic particles with at least one dimension less than 100nm.In Endodontics[35], Nanosilver gutta percha improved anti-bacterial efficacy of gutta percha.[25] In nano particle induced toxicity, non-specific oxidative damage is the primary major concern. According to several earlier investigations, the primary causes of their cytotoxicity may include oxidative stress, mitochondrial malfunction, DNA damage, and cytokine production.[29]

Human periodontal ligament cells may be grown rather easily, and, like other moderately differentiated cell lines, their growth properties and sensitivity to cytotoxins can range significantly from culture to culture.[36] We employed the MTT test to assess the cytotoxicity of the irrigants as well. Our findings showed that human periodontal ligament cells were time dependently cytotoxic to NaOCl, CHX, oxytetracycline, and triclosan solutions.The percentage of viable cells after exposure to tested irrigants in ascending order was Sodium Hypochlorite, Chlorhexidine, Triclosan and Oxytetracycline. According to the study's findings, NaOCl had the highest level of cytotoxicity, followed by Triclosan, CHX, and oxytetracycline. Sodium hypochlorite leads to formation of hypochlorous acid. Hypochlorous acid is an oxidizing agent, It acts as solvent and on contact with organic tissue it causes release of chlorine. [37] However, Chlorine causes emission of free radicals leading to increase in reactive oxygen species (ROS). A high pH of NaOCL also triggers the release of hydroxyl ions. This leads to damage of the mitochondria and in turn causes change in the integrity of the cytoplasmic membrane. This causes formation of transition pore in the mitochondrial membranes. As a result, oxidative phosphorylation fails and (ATP) formation is reduced causing ROS formation. This oxidative stress in cells causes peroxidation of lipids, impairment of protein synthesis and damage to DNA. Cumulatively oxidative stress along with reduction of ATP formation results in death of cell. [37] (99-37). Our observations are in consensus with findings of Koulaouzidou et al [38] and Serper et al [39] which shows higher cytotoxicity of NaOCl as compared to CHX and EDTA. Heggers JP et al. on investigating tissue toxicity of sodium hypochlorite at different concentrations and at varying time intervals found that higher concentration and prolonged exposure increases the cytotoxicity. [40]

The results of Yu-Chao Chang et al[41] are not in agreement with our study, in which they observed that CHX is more cytotoxic than NaOCl. In present study triclosan shows lesser cytotoxicity than NaOCl and CHX. Our findings are similar to Babich H et al.[42],who observed cytotoxicity of triclosan is lesser than CHX and sanguinarine chloride towards gingival epithelial cells.

In comparison to other tested irrigants, CHX was determined to be the second most cytotoxic according to the findings of our study. Cytotoxicity of CHX is attributed to increased permeabililty of plasma membrane by binding to it and permitting the leakage of lysosomal enzymes. Our results are in consesnsus with in vitro studies conducted by Mollashahi et al and Yasuda et al. where in they suggested that CHX is less cytotoxic than EDTA and NaOCl. [43,44] As well as Babich et al. also found in their study that CHX has cytotoxic effect more according to time of exposure. In vivo evaluation of CHX reported moderate inflammation after 2 days foreign body granuloma formation 2 weeks later.[42]

On the contrary, Trevino EG et al showed that CHX had the highest toxicity to the apical papilla stem cells compared to NaOCl and EDTA. [45] In present study triclosan shows lesser cytotoxicity than NaOCl and CHX. Although studies have been done to assess in vivo toxicology and pharmacology of triclosan, the studies on its effect at cellular level remains scarce. Cytotoxic effect of triclosan could be due to, exposure of triclosan disrupts membrane

potential, preventing mitochondrial swelling suppression of ADP stimulated respiration. Triclosan directly inhibits complex II activity, further leading to hypercondensation and degradation of chromatin, which causes cell death.Our results are in consensus with Babich H et al.,who reported cytotoxicity of triclosan is lesser than CHX and sanguinarine chloride towards gingival epithelial cells. [42]

Oxytetracycline was shown in the study to be less cytotoxic than the other examined irrigants. Oxytetracycline probably exerted cytotoxic effect by inhibiting various enzymes which are zinc-dependent within the family of matrix metalloproteinase (MMP), which includes collagenases, gelatinases, and stromelysins.The inhibitory activity of the MMPs is by the virtue of their ability to chelate divalent cations in the catalytic domain. This conclusion is consistent with findings independently reported by Zhenxing Chi et al., Boleas S et al., and Ferreira CS et al., which showed that oxytetracycline has a higher biocompatibility. [46] Nevertheless, they are in contrast with a study reported by H. Spielmann et al which displayed higher cytotoxicity of oxytetacycline. [47]

In present study results shows that addition of AgNPs with other irigants increases the cytotoxicity of irrigation solution, but no statistical significant difference ($p>0.05$) was notice. In endodontic literature there is no single study cited regarding evaluation of cytotoxicity of endodontic irrigants combined with silver nanoparticle on human periodontal ligament cells. However Hackenberg *et al*.[48] conducted a study on AgNPs as irrigant where they found that cytotoxicity of AgNPs increases with time on human mesenchymal stem cells.

There are certain limitations of this study. This study is confined to cultured HPDFLC only. Thus it does not take into aaccount the dynamics present in the root canal like defense mechanism of biofilm and immune response of the person. The cytotoxicity of irrigants was predicated on the exposure dose, exposure time, and medium composition. [38,39,41]. This study estimated cytotoxicity absolutely at cellular level, therefore generalization of the results to vivo studies cannot be done.

CONCLUSION

It can be concluded that Oxytettracycline and triclosan had the lowest cytotoxicity when compared to sodium hypochlorite and Chlorhexidine. Different approaches for assessing cytotoxicity of materials have different outcomes. Therefore, it is advised to apply a variety of techniques to improve the precision and dependability of outcomes. Our results cannot be directly applied to in vivo investigations since cytotoxicity estimation in our in vitro investigation is purely cellular. Therefore, further *in vivo* studies are required for assessing the biocompatibility of these materials. Thus, further studies investigating new root canal irrigants on animal models and eventually humans should be done to assess in vivo cytotoxicity and biocompatibility.

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