

Cytotoxicity Evaluation of Combination Irrigant Regimens with MTAD on Two Different Cell Lines

Abstract

Background: Effective management of smear layer ensures adequate clinical success. Use of sodium hypochlorite (NaOCl)/ethylenediaminetetraacetic acid regimen has been the gold standard with limitations. Commercial irrigants incorporate surface modifiers to address these drawbacks. The aim of this study was to evaluate the cytotoxicity of combination regimens on target and nontarget cell lines by trypan blue assay. **Materials and Methods:** Nonsurfactant combination regimen of chlorhexidine (CHX) and NaOCl (2% CHX + 2.5% NaOCl) and surfactant regimens of CHX with cetrимide (CTR) (2% CHX + 0.5% CTR) and CHX with sodium dodecyl sulfate (2% CHX + 1% SDS) were prepared. 0.9% normal saline (NS) and Biopure MTAD (100%) served as control. Cytotoxicity was evaluated on human gingival fibroblast (HGF) and Henrietta Lacks (HeLa) cell lines by trypan blue assay. Thirty microliter of the cell suspension was treated with 20 µl of irrigants. The cell suspension was loaded into Neubauer chamber after 5 min and cell count was performed under inverted microscope and expressed as viability percentage. **Results:** Nonsurfactant combination comprising of 2% CHX + 2.5% NaOCl formed a brownish precipitate while surfactant combination regimens were stable without any precipitate formation. NS and 2% CHX + 0.5% CTR had greater viability scores on both cell lines. Two percent CHX + 1% SDS had better viability on HeLa but were severely cytotoxic on HGF. Two percent CHX + 2.5% NaOCl and MTAD were found to be severely cytotoxic on HeLa with limited viability on HGF. **Conclusion:** The variation in data obtained could be possibly attributed to the difference in the cellular membrane composition and mechanism of action of combination regimens. Experimental surfactant regimen 2% CHX + 0.5% CTR shows lower cytotoxicity than MTAD.

Keywords: Combination regimens, root canal irrigants, surface active agents

Manikandan Ravinathanan, Mithra N. Hegde¹, Veena Shetty², Suchetha Kumari³

Department of Conservative Dentistry and Endodontics, PMS College of Dental Science and Research, Trivandrum, Kerala, Departments of ¹Conservative Dentistry and Endodontics, ²Microbiology and ³Biochemistry, A. B. Shetty Memorial Institute of Dental Sciences, Nitte University, Mangalore, Karnataka, India

Introduction

The goal of endodontic treatment is elimination of microbial infection and subsequent filling of the root canal system facilitating repair of periapical tissues. Mechanical instrumentation results in an amorphous irregular smear layer composed of inorganic and organic material covering the dentin surface.^[1] The microorganisms seated within the dentin tubules are protected by the smear layer; which greatly reduce the success of endodontic therapy.^[2] Numerous irrigants and techniques have thus been recommended for removal of smear layer to achieve proper seal of the root canal filling materials.^[3]

Irrigants must have potent antimicrobial action, be cost-effective and compatible to substrate worked upon.^[4] Although irrigants are widely used in vital and nonvital teeth,

they can be extruded into the periapical tissues in fully mature intact root apices and in wide open apex leading to potential complications.^[5] Thus, cytotoxic potential of the irrigant plays a crucial role to the above factors making the ideal requirements of the irrigant complex. Thus, a definite equilibrium needs to be maintained; resulting in application of more than one ideal irrigant.^[4,6]

Sodium hypochlorite (NaOCl) has been widely used as it fulfills most of the ideal requirements and has a profound effect on the organic component of the smear layer.^[7] Ethylenediaminetetraacetic acid (EDTA) affects the inorganic component of the smear layer and has a lubricating effect in instrumentation.^[8] The combination of higher concentrations of NaOCl (5.25%) and EDTA (17%) although being a potent combination has been shown to display marked reduction in mechanical properties of dentin and erosion of dentinal tubular microstructure resulting in reduced

Address for correspondence:
Dr. Manikandan Ravinathanan,
Department of Conservative
Dentistry and Endodontics,
PMS College of Dental Science
and Research, Vattappara,
Trivandrum, Kerala, India.
E-mail: manikandanravinathanan@gmail.com

Access this article online

Website:

www.contempclindent.org

DOI: 10.4103/ccd.ccd_854_17

Quick Response Code:



How to cite this article: Ravinathanan M, Hegde MN, Shetty V, Kumari S. Cytotoxicity evaluation of combination irrigant regimens with MTAD on two different cell lines. *Contemp Clin Dent* 2018;9:255-9.

This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

For reprints contact: reprints@medknow.com

elastic modulus and flexural strength.^[9] Citric acid although similar to EDTA finds limited application.^[10]

Biopure MTAD™ (Dentsply, Tulsa) is a root canal irrigant that contains a mixture of tetracycline isomer, citric acid, and a detergent; originally developed by Torabinejad *et al.* An initial rinse of 1.3% NaOCl is required followed by a final rinse of MTAD to affect the smear layer suggesting it acts as an adjunct to NaOCl rather independently.^[11] Numerous commercial products such as chlorhexidine (CHX) Plus™ (2% CHX with surface modifiers), Chlor-XTRA™ (6%NaOCl with surface modifiers); Vista Dental Products, Racine, W. I and Cetrehexidin™ (0.2% CHX with 0.2% cetrimide [CTR]) Vebas, San Giuliano, Milan, Italy, incorporate surface active agents to the traditional endodontic irrigants.^[12]

Although the antibacterial efficacy and their effect on dentin microhardness of surfactant regimens has been reported,^[12,13] little is known about their cytotoxicity. The role of surfactants may play a key role in modulating the toxicity of the irrigants employed and thus underlying mechanism need to be understood. Thus, the aim of this study was to evaluate the cytotoxicity of combination irrigant regimens with and without surfactants on cell lines by trypan blue assay.

Materials and Methods

Preparation of irrigants and cell line

This *in vitro* study was performed in central research laboratory, A. B. Shetty Memorial Institute of Dental Sciences, NITTE University. Conventional irrigants: 2.5% NaOCl (Prevest Denpro Limited) and 2% CHX (Sigma) were prepared by serial dilution. Surfactants 0.5% CTR, Himedia; 1% sodium dodecyl sulfate (SDS), Merck were prepared by same method. The combination regimens include nonsurfactant (2% CHX + 2.5% NaOCl) and surfactant groups (2% CHX + 0.5% CTR and 2%CHX + 1% SDS). Biopure MTAD (Tulsa Dentsply) and 0.9% normal saline (NS) served as control. Henrietta Lacks (HeLa) and human gingival fibroblast (HGF) cell lines were obtained from Manipal life sciences, Manipal and cultured at 37°C in a humidified atmosphere of 5% CO₂/95% air.

Cell culture and storage

The cells were maintained in a growth medium containing the following constituents: Dulbecco's modified Eagle's medium (Himedia) with 25 mmol/L glucose, 1 mmol/L pyruvate, 4.02 mmol/L L-alanyl-glutamine, and 10% fetal calf serum (Sigma Aldrich). Confluent cells were detached with 0.15% trypsin (Himedia) for 5 min, following which 2 ml of complete medium was added and the cells were centrifuged at 1000 rpm (180 g) for 5 min. Cell suspension was counted using a Neubauer chamber and seeded in 96 well microtiter plates (Himedia) at a density of 1×10^4 cells per well.

Cytotoxicity evaluation

Cytotoxicity was assessed using trypan blue dye. Thirty microliter of the cell suspension was treated with 20 µl of irrigants. Fifty microliter trypan blue dye (0.05%) was added and allowed for 5 min. The cell suspension was loaded into Neubauer chamber and cell count was performed under inverted microscope (Olympus, India). Nonviable cells appear blue stained. At least 200 cells were counted per treatment. Vitality percentage was calculated using the formula: % viability = average number of viable cells/total number of cells × 100.^[14]

Results

The mixture of nonsurfactant irrigant combination (2% CHX + 2.5% NaOCl) resulted in a dark brown precipitate while surfactant combinations (2% CHX + 0.5% CTR and 2% CHX + 1% SDS) were clear and no precipitate was found.

The viability scores on HeLa were 2% CHX + 2.5% NaOCl (11.8%), 2% CHX + 0.5% CTR (72%), 2% CHX + 1% SDS (65%), 0.9% NS (93.5%), and MTAD (0%) as seen in Figure 1. On HGF, the following values were obtained: 2% CHX + 2.5% NaOCl (33.75%), 2% CHX + 0.5% CTR (63.75%), 2% CHX + 1% SDS (28.75%), 0.9% NS (66.25%), and MTAD (32.5%) as depicted in Figure 2.

Discussion

This *in vitro* study evaluated the cytotoxicity of experimental combination regimens (with and without surfactant) with commercially available surfactant irrigant MTAD. We utilized continuous (HeLa) and primary (HGF) cell lines to evaluate the effects by trypan blue assay. To the best of our knowledge, this approach has not been evaluated, thus highlighting its significance.

In vitro cell culture studies can reveal a great amount of information regarding the cytotoxicity of materials used and might indicate the effects observed *in vivo*.^[15] The use of two different cell lines comprising of target (HGF) and nontarget (HeLa) in nature helps us to better understand

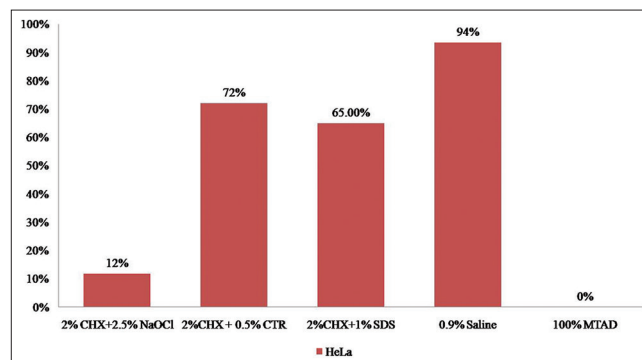


Figure 1: Vitality percentage of combination irrigant regimens on Henrietta Lacks

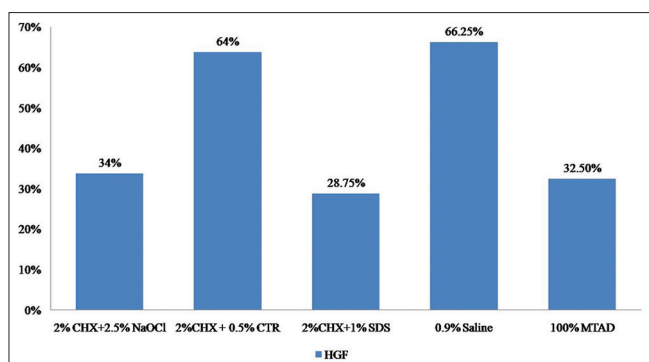


Figure 2: Vitality percentage of combination irrigant regimens on human gingival fibroblast

the underlying mechanism while other parameters are kept constant.^[16,17] The trypan blue exclusion test is a qualitative and quantitative method used to indicate cytotoxicity, where dead cells take up the blue stain of the dye, whereas the live cells have yellow nuclei.^[18]

Two percent CHX was chosen as the primary irrigant of choice based on our previous study results.^[19] 2.5% NaOCl had similar effect of 5% NaOCl to form precipitate with 2% CHX and caused reduced antibacterial efficacy.^[20] Surfactants 0.5% CTR and 1% SDS had antibacterial efficacy even when used alone or in combinations with CHX even at lower concentrations.^[21] A contact time of 5 min was chosen for the irrigants with the cell lines because MTAD (as per manufacturer's instruction) requires the above time as a final rinse in clinical endodontics.^[22]

Our study results revealed that nonsurfactant combination regimen comprising of 2% CHX + 2.5% NaOCl was highly cytotoxic on HeLa and HGF. The possible explanation could be due to the interaction between the irrigants resulting in precipitate formation. The precipitate which possibly contains para chloroaniline is cytotoxic, and thus, these irrigant combinations should never be attempted in clinical endodontics.^[23]

The surfactant combination comprising of 2% CHX + 0.5% CTR had higher viability scores next to NS. CHX and CTR are cationic in nature and thus possibly could not have interacted resulting in higher viability on both cell lines. The cationic environment of the molecules encourages linking with anionic phospholipid bilayer on the cell membrane and is capable of altering the cytoplasmic membrane integrity. Inactivation of the enzymes of cytoplasmic membrane brings serious consequences such as protein denaturation and cell death.^[24]

On the contrary, 2% CHX + 1% SDS had better viability on HeLa but were found to be highly cytotoxic on HGF. CHX being cationic and SDS being anionic interact with each other resulting in charge neutralization and thus may have accounted for limited permeability of SDS across the cellular membrane on HeLa.^[25] The possible explanation

for lower viability on HGF may be attributed to tertiary structure unfolding in the submicellar and chain expansion in the micellar range of SDS concentrations resulting in perturbation of protein structure. SDS can solubilize proteins, leading to rapid influx of cationic CHX across the cellular membrane causing damage to cytoplasmic contents and cell death.^[26]

MTAD contains 0.5% polysorbate (tween 80); a nonionic emulsifier, viscous, water-soluble yellow liquid. Lower concentration of citric acid (4.25%) present in the irrigant could have contributed to cytotoxicity of the irrigant as well.^[27] Tetracycline analogs (doxycycline in MTAD) have been shown to inhibit matrix metalloproteinases and to induce apoptosis in several cancer cell types. They were found to be cytotoxic and also cause DNA damage as well; thus resulting in nonviability of HeLa cells and limited viability on HGF.^[28] However, the underlying mechanism may be presumed principally due to the acidic nature of MTAD (pH 4) that could have caused cellular lysis in addition to the above factors.^[29]

Our study results are in accordance with Onçağ *et al.* who stated 2% CHX and cetrexidin (0.2% CHX + 0.2% CTR) had lower toxicity than 5.25% NaOCl.^[30] Estrela *et al.* used cetylpyridinium chloride (CPC) in concentrations of 0.1% and 0.2% as an endodontic irrigant for management of infected canals.^[31] Dong *et al.* reported that SDS is cytotoxic when used alone.^[32] On the contrary, Barbosa *et al.* reported that a combination of calcium hydroxide and SDS can be used as an endodontic irrigant.^[33] Fiume *et al.* reported that SDS in products intended for prolonged contact with the skin, concentrations should not exceed 1%.^[34]

Marins *et al.* found MTAD to be genotoxic on murine fibroblast cell and did not cause cell death. The lower concentrations of MTAD used (in contrast to manufacturers instruction) could have accounted for higher cell viability scores.^[35] Zhang *et al.* stated that MTAD was not cytotoxic when assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method.^[36] As stated by Eisenbrand *et al.*, single cell comet assays which are an integral part of cytotoxicity are not recommended on samples showing more than 30% cytotoxicity and thus were not performed.^[37]

The results of this study confirm that cell membrane was the main target of the irrigants employed, with differences in their mechanism of action. Variability among the two cell lines may be attributed due to the differences in the structure, metabolism, and composition of the cell membrane. The use of target tissue may simulate close replica to *in vivo* clinical situations.

Conclusion

Experimental surfactant combination regimen (2% CHX + 0.5% CTR) appears to be promising alternative

irrigant being least cytotoxic, cost-effective, and readily prepared *in vitro* than MTAD or other commercial surfactant irrigants (nonavailable in India). Further studies on tooth models are required to confirm the results obtained.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

References

- McComb D, Smith DC. A preliminary scanning electron microscopic study of root canals after endodontic procedures. *J Endod* 1975;1:238-42.
- Torabinejad M, Handysides R, Khademi AA, Bakland LK. Clinical implications of the smear layer in endodontics: A review. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2002;94:658-66.
- Orstavik D, Haapasalo M. Disinfection by endodontic irrigants and dressings of experimentally infected dentinal tubules. *Endod Dent Traumatol* 1990;6:142-9.
- Zehnder M. Root canal irrigants. *J Endod* 2006;32:389-98.
- Ehrich DG, Brian JD Jr., Walker WA. Sodium hypochlorite accident: Inadvertent injection into the maxillary sinus. *J Endod* 1993;19:180-2.
- Visalli G, Baluce B, La Maestra S, Micale RT, Cingano L, De Flora S, *et al.* Genotoxic damage in the oral mucosa cells of subjects carrying restorative dental fillings. *Arch Toxicol* 2013;87:179-87.
- Grandini S, Balleri P, Ferrari M. Evaluation of glyde file prep in combination with sodium hypochlorite as a root canal irrigant. *J Endod* 2002;28:300-3.
- Aktener BO, Bilkay U. Smear layer removal with different concentrations of EDTA-ethylenediamine mixtures. *J Endod* 1993;19:228-31.
- Marending M, Luder HU, Brunner TJ, Knecht S, Stark WJ, Zehnder M, *et al.* Effect of sodium hypochlorite on human root dentine – mechanical, chemical and structural evaluation. *Int Endod J* 2007;40:786-93.
- Eldeniz AU, Erdemir A, Belli S. Effect of EDTA and citric acid solutions on the microhardness and the roughness of human root canal dentin. *J Endod* 2005;31:107-10.
- Torabinejad M, Khademi AA, Babagoli J, Cho Y, Johnson WB, Bozhilov K, *et al.* A new solution for the removal of the smear layer. *J Endod* 2003;29:170-5.
- Aslantas EE, Buzoglu HD, Altundasar E, Serper A. Effect of EDTA, sodium hypochlorite, and chlorhexidine gluconate with or without surface modifiers on dentin microhardness. *J Endod* 2014;40:876-9.
- Jungbluth H, Peters C, Peters O, Sener B, Zehnder M. Physicochemical and pulp tissue dissolution properties of some household bleach brands compared with a dental sodium hypochlorite solution. *J Endod* 2012;38:372-5.
- McKelvey-Martin VJ, Green MH, Schmezer P, Pool-Zobel BL, De Méo MP, Collins A, *et al.* The single cell gel electrophoresis assay (comet assay): A European review. *Mutat Res* 1993;288:47-63.
- Geurtsen W. Substances released from dental resin composites and glass ionomer cements. *Eur J Oral Sci* 1998;106:687-95.
- Merdad K, Pascon AE, Kulkarni G, Santerre P, Friedman S. Short-term cytotoxicity assessment of components of the epiphany resin-percha obturating system by indirect and direct contact Millipore filter assays. *J Endod* 2007;33:24-7.
- Nishikiori R, Nomura Y, Sawajiri M, Masuki K, Hirata I, Okazaki M, *et al.* Influence of chlorine dioxide on cell death and cell cycle of human gingival fibroblasts. *J Dent* 2008;36:993-8.
- Hartmann A, Agurell E, Beevers C, Brendler-Schwaab S, Burlinson B, Clay P, *et al.* Recommendations for conducting the *in vivo* alkaline comet assay 4th international comet assay workshop. *Mutagenesis* 2003;18:45-51.
- Ravinanthanan M, Hegde MN. Comparative evaluation of antimicrobial efficacy of routine endodontic irrigants with surfactants against MTAD on *Enterococcus faecalis*- An *in vitro* microbiological study. *Int J Res Rev Pharm Appl Sci* 2012;2:55-64.
- Basrani BR, Manek S, Sodhi RN, Fillery E, Manzur A. Interaction between sodium hypochlorite and chlorhexidine gluconate. *J Endod* 2007;33:966-9.
- Ravinanthanan M, Hegde MN, Shetty V, Kumari S. Antimicrobial efficacy of endodontic irrigants and combination surfactant regimens on *Enterococcus faecalis*. An *in vitro* microbiological study. *Int J Curr Acad Res* 2017;6:3740-6.
- Newberry BM, Shabahang S, Johnson N, Aprecio RM, Torabinejad M. The antimicrobial effect of biopure MTAD on eight strains of *Enterococcus faecalis*: An *in vitro* investigation. *J Endod* 2007;33:1352-4.
- Luddin N, Ahmed HM. The antibacterial activity of sodium hypochlorite and chlorhexidine against *Enterococcus faecalis*: A review on agar diffusion and direct contact methods. *J Conserv Dent* 2013;16:9-16.
- Davies A, Bentley M, Field BS. Comparison of the action of vantocil, cetrime and chlorhexidine on *Escherichia coli* and its spheroplasts and the protoplasts of gram positive bacteria. *J Appl Bacteriol* 1968;31:448-61.
- Barkvoll P, Rølla G, Svendsen K. Interaction between chlorhexidine digluconate and sodium lauryl sulfate *in vivo*. *J Clin Periodontol* 1989;16:593-5.
- Bhuyan AK. On the mechanism of SDS-induced protein denaturation. *Biopolymers* 2010;93:186-99.
- Navarro-Escobar E, González-Rodríguez MP, Ferrer-Luque CM. Cytotoxic effects of two acid solutions and 2.5% sodium hypochlorite used in endodontic therapy. *Med Oral Patol Oral Cir Bucal* 2010;15:e90-4.
- Song H, Fares M, Maguire KR, Sidén A, Potáková Z. Cytotoxic effects of tetracycline analogues (doxycycline, minocycline and COL-3) in acute myeloid leukemia HL-60 cells. *PLoS One* 2014;9:e114457.
- Ravinanthanan M, Hegde MN. Evaluation of critical concentration and pH of endodontic irrigants to eliminate *Enterococcus faecalis* – An *in vitro* study. *J Indian Acad Dent Spec* 2011;2:36-42.
- Onçağ O, Hoşgör M, Hilmioğlu S, Zekioğlu O, Eronat C, Burhanoglu D, *et al.* Comparison of antibacterial and toxic effects of various root canal irrigants. *Int Endod J* 2003;36:423-32.
- Estrela C, Sousa-Neto MD, Alves DR, Alencar AH, Santos TO, Pécora JD, *et al.* A preliminary study of the antibacterial potential of cetylpyridinium chloride in root canals infected by *E. faecalis*. *Braz Dent J* 2012;23:645-53.
- Dong L, Witkowski CM, Craig MM, Greenwade MM, Joseph KL. Cytotoxicity effects of different surfactant molecules conjugated to carbon nanotubes on human astrocytoma cells. *Nanoscale Res Lett* 2009;4:1517-23.
- Barbosa SV, Barroso CM, Ruiz PA. Cytotoxicity of endodontic irrigants containing calcium hydroxide and sodium lauryl

- sulphate on fibroblasts derived from mouse L929 cell line. *Braz Dent J* 2009;20:118-21.
34. Fiume M, Bergfeld WF, Belsito DV, Klaassen CD, Marks JG Jr, Shank RC, *et al.* Final report on the safety assessment of sodium cetearyl sulfate and related alkyl sulfates as used in cosmetics. *Int J Toxicol* 2010;29:115S-32S.
35. Marins JS, Sassone LM, Fidel SR, Ribeiro DA. *In vitro* genotoxicity and cytotoxicity in murine fibroblasts exposed to EDTA, NaOCl, MTAD and citric acid. *Braz Dent J* 2012;23:527-33.
36. Zhang WU, Torabinejad M, Li Y. Evaluation of cytotoxicity of MTAD using the MTT-Tetrazolium method. *J Endod* 2003;29:654-7.
37. Eisenbrand G, Pool-Zobel B, Baker V, Balls M, Blaauboer BJ, Boobis A, *et al.* Methods of *in vitro* toxicology. *Food Chem Toxicol* 2002;40:193-236.