

Cytotoxicity of chlorhexidine and neem extract on cultured human gingival fibroblasts through fluorescence-activated cell sorting analysis : An *in-vitro* study

Umesh Pratap Verma¹, Abhaya Gupta¹, Rakesh Kumar Yadav², Rini Tiwari¹,
Ramesh Sharma³, Anil Kumar Balapure³

Correspondence: Dr. Umesh Pratap Verma
Email: umeshpratapverma@kgmcindia.edu

¹Department of Periodontology, Faculty of Dental Sciences, King George's Medical University, Lucknow, Uttar Pradesh, India,

²Department of Conservative and Endodontics, Faculty of Dental Sciences, King George's Medical University, Lucknow, Uttar Pradesh, India,

³Department of Biochemistry, CSIR-CDRI, Lucknow, Uttar Pradesh, India

ABSTRACT

Objective: To assess the influence of chlorhexidine (CHX), neem vehicle control (NVC), and neem extract (NE) on cultured human gingival fibroblasts (hGFs) using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and fluorescence-activated cell sorting (FACS) analysis. **Materials and Methods:** Fibroblasts were derived from healthy gingival biopsy specimens harvested aseptically. The effects of CHX, NVC, and NE were evaluated on cultured hGFs through FACS and MTT assay. **Results:** MTT assay with hGFs indicated altered morphology with maximum cell death at 10% CHX, while NVC and NE showed similar results at a concentration of 75% and above. On FACS analysis, beyond 1%, CHX adversely affected the cell cycle phase distribution whereas NE exerted a detrimental effect only at 100%. Moreover, both with NVC and NE cells were well differentiated in all the three phases of the cell cycle, with distinction getting lost at 50% to finally causing cell death at 100%. **Conclusions:** CHX beyond 1% concentration exhibited a toxic effect on hGFs at 1, 5, and 15 min time exposure. However, NE did not adversely affect the fibroblasts even up to 50% concentration showing a less toxic effect in comparison with CHX on these cells. The cytoprotective and oral friendly quality of NE emphasize the superiority of NE over CHX.

Key words: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, chlorhexidine, cytotoxicity, fluorescence-activated cell sorting analysis, human gingival fibroblasts, neem extract

INTRODUCTION

Periodontal disease is initiated by microbial plaque. Various mechanical methods and chemical agents are used to prevent the formation and deposition of microbial plaque.^[1] The chemical agents such as

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chlorhexidine (CHX) and povidone iodine have been used in the form of mouth rinse and subgingival irrigating solution.^[2] In addition to chemical agents, some herbal preparations have also been used for the inhibition of plaque-like Sanguinarine^[2] and Neem.^[3] The human gingival fibroblasts (hGFs) are very important cells of periodontal connective tissue that helps in wound healing. The present study focuses on effects of neem and CHX on cultured hGFs through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and fluorescence-activated cell sorting (FACS) analysis.

MATERIALS AND METHODS

This study was conducted at the Department of Periodontics, Faculty of Dental Sciences, King George's Medical University, Lucknow, with permission of the Institutional Ethics Committee in collaboration with CSIR-CDRI.

Preparation of neem extract (NE): The neem leaves and bark were collected from a local area of Lucknow and were shade dried and grounded into fine powder. The samples were then extracted three times with methanol using Polytron Homogen (Kinematica, Pt 6100, Switzerland). The extracts thus obtained were concentrated separately using rotary evaporator (Buchi type) and portioned with hexane by the process of liquid phase extraction to eliminate fats and oils. The obtained methanolic fraction was again portioned with ethyl acetate and water to remove sugars, protein, and carbohydrates. The ethyl acetate fraction thus obtained was concentrated and weighed. The formulations used for this study were prepared using 0.5% ethyl acetate fractions of neem leaves and bark in 15% edible grade ethanol (vehicle control).
#(CSIR-NBRI, Lucknow, India)

Isolation of cell culture: The hGF cell lines were routinely maintained in the Tissue and Cell Culture Laboratory, CSIR-CDRI, Lucknow.

Experimental design for CHX, neem vehicle control (NVC), and NE exposure to gingival fibroblasts cultures: CHX mouthwash (0.2%)^{*} (ICPA, Ankleshwar, Gujarat, India), NVC, and NE were regarded as 100% solutions and were diluted to 0.1%, 1.0%, 10%, 25%, 50%, and 75% for experimental purposes. The 100% solution/extract with the help of NaOH (1N) was first adjusted to pH 7.4. This was treated as stock solution and diluted as per requirement in Dulbecco's Modified Eagle Medium

(DMEM) (pH 7.4), pH 7.4. The pH of all solutions was ascertained to be 7.4 before use.

From a freshly trypsinized flask, 5×10^4 cells were plated in 12-welled tissue culture plates and incubated for 24 h at 37°C in a humidified CO₂ incubator. Subsequently, the cells were exposed with different concentrations of CHX mouthwash, NVC, and NE for 1 min, 5 min, and 15 min at 37°C. Finally, the cells were incubated at 37°C in a CO₂ incubator for 48 h. The cells were visualized under phase contrast microscope with 35 mm camera at $\times 100$ and $\times 200$ magnifications using Kodak Color $\times 400$ ASA photo film.

MTT assay: In the freshly prepared culture plates after incubation for 24 h at 37°C, 20 μ l of MTT (5 μ g/ml) was added per well and then the cultures were incubated further for 4–6 h.

Thereafter, the media was aspirated and the resultant formed crystals were dissolved in 200 μ l Dimethyl sulfoxide (DMSO). The plate was read at 550 nm through ELISA plate reader.^[4]

FACS analysis: Logarithmically growing gingival fibroblast cells were harvested with 0.05% trypsin-EDTA solution. 0.2×10^6 cells/well were plated in 6-welled plates for 24 h in DMEM. Ligands were added at 1%–100% doses for 5 min and cells harvested by trypsinization were washed with chilled phosphate-buffered saline (PBS) and centrifuged at 100 rpm for 10 min at 4°C. Cells were permeabilized with 70% chilled ethanol for 30 min at 4°C. The pellet was resuspended in PBS containing propidium iodide (PI) (40 μ g/ml) and analyzed by flow cytometry for cell cycle study.^[5]

Mean, standard deviation, and standard error were calculated as per the standard formulae and the significance of changes was tested using paired “*t*” test.^[6]

RESULTS

The cell cultures were exposed to CHX mouthwash, NVC, and NE at a concentration of 0.1%, 1.0%, 10%,

Table 1: The change in pH of chlorhexidine mouthwash, neem vehicle control, and neem extract after rinsing for 20-25 s

Mouthwash/extract	Prerinsing pH	Postrinsing pH
CHX	5.0	6.9
NVC	6.5	7.4
NE	6.8	7.1

CHX: Chlorhexidine, NVC: Neem vehicle control, NE: Neem extract

25%, 50%, 75%, and 100% for 1 min, 5 min, and 15 min duration for MTT and FACS analysis.

Table 1 depicts the pH of CHX, NVC, and NE pre- and postrinsing for 20–25 s. Tables 2–7 show optical density of viable hGF using MTT assay in the presence of the above solutions. FACS analysis for changes in the membrane permeability and cell cycle in hGF for 5 minute” needs deletion.

In CHX-treated groups, the majority of the cells were found to be in “G₀/G₁” phase with very few cells in “S” phase and G₂/M phase of cell cycle at 1% concentration of CHX. However, at 25% concentration, the cells plateau into single continuous indistinct cycle, denoting the cytotoxicity of the mouthwash to die out at 50% and 100% concentration.

In NVC-treated group, at 1% concentration, it causes damage to the cell membrane and 25%–50% concentration shows a decrease in the number of cells in G₀/G₁ phase, but at 100% concentration, the cells do not show much distinction between various phases despite remaining viable. Briefly, the cells very well tolerated the vehicle control up to 50% concentration beyond which the toxicity was seen up to 100% concentration.

In NE-treated group, at 1% and 25% concentration, very mild effect was observed on the cell membrane

with cells dying at a concentration of 50% and 100% [Figure 1].

DISCUSSION

In the present study, cytotoxicity of different concentrations of NE was assessed on freshly developed hGF cell line and compared with CHX as control using MTT assay. Further cell membrane permeability, DNA perturbations, and effects on different phases of cell cycle on mouthwash exposure were analyzed by FACS analysis. MTT assay is a cytotoxicity assay that measures the mitochondrial activity of live cells. Damour *et al.*^[7] have also observed the cytotoxic effect on cultured human fibroblasts using MTT assay.

The cytotoxicity assay shows that 10% concentration of CHX is responsible for maximum cell death as indicated in Table 2 whereas NVC showed the same result at 100% concentration [Table 3]. NE showed maximum cytotoxicity on hGF at concentration of 75% as indicated in Table 4. From the above data, it is evident that the CHX is more cytotoxic as compared to NVC and NE, which is evaluated further in next parameter.

The cytotoxic effect of CHX on cultured human periodontal ligament and hGF by Chang *et al.*^[8] and Rajabalian *et al.*^[9] is in agreement with the findings

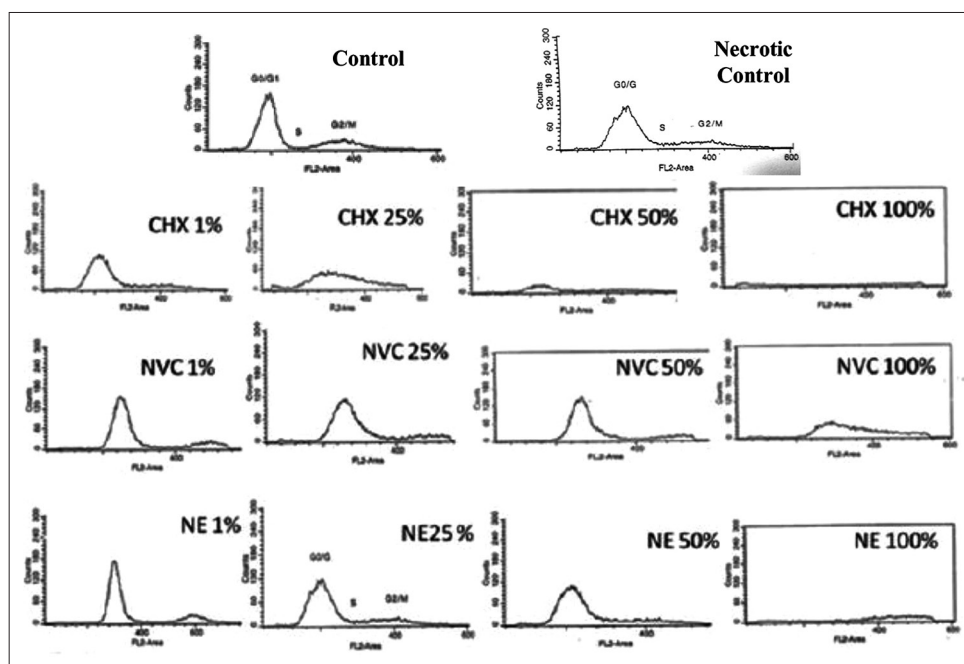


Figure 1: Flow cytometry analysis for cell cycle studies on human gingival fibroblasts versus the ligands was conducted. After exposing the cells with different ligands at 1%, 25%, 50%, and 100% doses for 1 min, the cells were analyzed by fluorescence-activated cell sorting for cell cycle. Control depict cells in all the three phases

Table 2: Effect of chlorhexidine on human gingival fibroblasts using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

Concentration (%)	1 min	5 min	15 min
0.1	0.856±0.032	0.729±0.028	0.793±0.040
1.0	0.771±0.057	0.754±0.038	0.705±0.038
10	0.323±0.004	0.011±0.005	0.013±0.003
25	0.098±0.003	0.019±0.002	0.015±0.004
50	0.110±0.007	0.013±0.004	0.015±0.002
75	0.108±0.006	0.018±0.008	0.023±0.006
100	0.095±0.001	0.018±0.006	0.034±0.008

Table 3: Effect of neem vehicle control on human gingival fibroblasts using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

Concentration (%)	1 min	5 min	15 min
0.1	0.760±0.093	0.743±0.085	0.884±0.073
1.0	0.869±0.099	0.897±0.120	0.815±0.003
10	0.739±0.048	0.810±0.091	0.850±0.115
25	0.800±0.035	0.960±0.280	0.578±0.414
50	0.814±0.068	0.864±0.102	0.852±0.004
75	0.682±0.237	0.554±0.121	0.520±0.005
100	0.093±0.012	0.107±0.001	0.093±0.005

Table 4: Effect of neem extract on human gingival fibroblasts using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

Concentration (%)	1 min	5 min	15 min
0.1	0.863±0.037	0.757±0.052	0.923±0.041
1.0	0.789±0.079	0.877±0.041	0.926±0.035
10	0.778±0.011	0.614±0.052	0.518±0.029
25	0.715±0.031	0.520±0.030	0.473±0.016
50	0.572±0.030	0.486±0.049	0.408±0.012
75	0.261±0.030	0.028±0.000	0.023±0.005
100	0.013±0.002	0.015±0.001	0.007±0.003

Table 5: Treatment time comparison of chlorhexidine treatment to fibroblasts at different concentrations using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

Concentration (%)	<i>t</i>		
	1 min versus 5 min	1 min versus 15 min	5 min versus 15 min
0.1	3.186*	1.403	1.287
1.0	0.242	0.949	0.905
10	48.016***	63.123***	0.237
25	23.383***	15.977***	0.875
50	11.838***	13.231***	0.279
75	9.522**	10.439***	0.553
100	12.694***	7.141**	1.530

P*<0.05 (significant); *P*<0.01 (highly significant); ****P*<0.001 (very highly significant)

of the present study. Treatment time comparison of the response of fibroblasts to different concentrations of CHX (Table.5),NVC(Table.6) and NE(Table.7) has been done utilizing two parametrs i.e concentration (%) of the different agents and different time periods combinations like 1minute Vs 5 minute, 1 minute Vs 15 minute and 5 minute Vs 15 minute revealing critically useful information.

Based on the aforesaid discussion on the cytotoxicity assay and analysis of the three mouthwashes in question, i.e., CHX, NVC, and NE, it was decided to employ FACS parameters to the exposed fibroblasts. The time period of exposure was narrowed down to 5 min, and the concentrations evaluated were 1%, 25%, 50%, and 100% since 1 min is the lower limit and 15 min is the upper limit of exposure in our studies.

As explained earlier, the flow data initially essentially contains two controls, one being the untreated fibroblasts (normal control) and the second being the necrotic control where fibroblasts necrosed on freezing-thawing in liquid nitrogen. This is the standard protocol since necrosis causes damage to the plasma membrane thereby allowing the assay dye Propidium iodide (PI) to penetrate the cell and bind to DNA. In Figure.1 the FL area provide a measure of fluorescence uptake with peaks denoting different phases of cell cycle. There is a dose dependent increase in PI fluorescent probe uptake from 1% to 50% CHX concentration with decline seen at 100% of CHX concentration..

The observance of florescence at 100% CHX concentration as shown in Figure 1 is possibly due to the fixing of cells at the highest concentration and PI binding to the DNA-like material. Thus, it can be concluded that even 1% CHX exposure to the fibroblasts adversely affects the plasma membrane which increases proportionally until 50% concentration.

The results of fibroblasts membrane damage on exposure to CHX as previously discussed were carried downstream to the adverse changes in the cell cycle where 1% exposure showed adverse effects in all the phases as compared to normal control. Phases G₀/G₁, S, and G₂/M show the toxic effects with reduced distribution of cells in each. The cell cycles were progressively affected until 100% CHX concentration.

The PI uptake at 1% and 25% of NVC exposure to fibroblasts was found to be similar to that observed in the ecrotic control rather than normal control [Figure 1]. At 50% NVC, the PI uptake declined

Table 6: Treatment time comparison of neem vehicle control treatment to fibroblasts at different concentrations using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

Concentration (%)	<i>t</i>		
	1 min versus 5 min	1 min versus 15 min	5 min versus 15 min
0.1	0.234	1.819	2.180
1.0	0.315	0.889	1.136
10	1.188	1.535	0.472
25	0.979	0.923	1.320
50	0.700	0.835	0.185
75	2.793*	4.932**	0.438
100	1.710	0.086	2.561

P*<0.05 (significant); *P*<0.01 (highly significant)**Table 7: Treatment time comparison of neem extract treatment to fibroblasts at different concentrations using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay**

Concentration (%)	<i>t</i>		
	1 min versus 5 min	1 min versus 15 min	5 min versus 15 min
0.1	1.666	1.083	2.519
1.0	0.992	1.599	0.919
10	3.102*	8.340**	1.607
25	4.518*	6.956**	1.376
50	1.483	4.097**	1.533
75	7.961**	8.014**	0.989
100	1.131	1.268	2.135

P*<0.05 (significant); *P*<0.01 (highly significant)

drastically to rise again at 100% concentration to maximal level, indicating maximum membrane changes. The results at 100% NVC exposure are akin to those witnessed with necrotic control.

Despite the fact that the plasma membrane architecture of fibroblasts changes with NVC exposure, the cells remain evenly distributed in the different phases of the cell cycle from 1% to 50 % concentration of NVC. It is interesting to note the relationship between the fluorescence versus cell cycle changes. At 100% NVC concentration, the phases, i.e., G_0/G_1 to G_2/M , including the S phase become indistinguishable.

Comparison with CHX clearly indicate that even 1% concentration of CHX exposure can bring about changes in normal control making them look more like necrotic control. Further, it was inferred that upto 75% NVC was better tolerated than similar

concentration of CHX as a mouth rinse. The most important mouth rinse evaluated through FACS in the present study, i.e., NE, revealed most interesting data when compared to the results obtained with CHX and NVC. At 1% NE exposure, although the PI uptake was slightly of a higher order, but the cell cycle data revealed that cells were more sharply distinct in all 3 phases of cell cycle than the normal control. This clearly suggested some “unknown factor” that might reside in the NE, causing the cells to withstand the slight perturbations in the fluorescence, but yet providing more crisper and distinct phases over the normal control. The results on NE are in complete disagreement over the necrotic control, suggesting its utility. As the concentration of NE is raised from 25% to 100%, the PI uptake does not alter much too adverse status at 50% and 100% concentration, respectively.

CONCLUSIONS

NE, thus, exhibits the best results as compared to CHX and NVC since the last two show membrane damage even at 1% concentration. Conversely, the NE shows the cells undamaged in terms of fluorescence and improvement in cell cycle stages even over the normal control. The fact that membrane integrity is unaffected up to 50% exposure to NE, a crucial feature, makes us choose it as the best among the three mouth rinses tested.

The *in vitro* studies conducted here would greatly help extrapolate the situation under intact *in vivo* conditions that exist in a human where fibroblast interaction with other cellular components including the vasculature is crucial.

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Conflicts of interest

There are no conflicts of interest.

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