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

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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. The chemical agents such as...
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, Gujrat, 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 ×100 and ×200 magnifications using Kodak Color ×400 ASA photo film.

MTT assay: In the freshly prepared culture plates after incubation for 24 h at 37°C, 20 µl of MTT (5 µg/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 µl 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 × 10⁶ 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 µg/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%,

| 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

Table 1: The change in pH of chlorhexidine mouthwash, neem vehicle control, and neem extract after rinsing for 20-25 s
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| 0.844±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 (%) | 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)

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.
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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 (%) | 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 (%) | 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., G₀/G₁ to G₂/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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