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Research Article

DEVELOPMENT OF A HUMAN GINGIVAL FIBROBLAST (HGF) CELL LINE FOR THE EVALUATION OF A NOVEL MOUTHWASH FROM *AZADIRACHTA INDICA* VIS-À-VIS CHLORHEXIDINE

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ABSTRACT

Chemical plaque inhibitors are toxic when used for longer duration. However, herbal preparations are less toxic as compared to chemical mouthwashes and shows excellent results. Owing to these reasons, Neem and Sanguinarine have been used for the inhibition of plaque and gingivitis. The present study attempts to assess the influence of Chlorhexidine (CHX) and Neem Extract (NE) on Cultured Human Gingival Fibroblasts (hGF). Fibroblasts were derived from healthy gingival biopsy specimens harvested aseptically. The effects of CHX and NE were evaluated on cultured hGF through morphological and biochemical assays. Morphological studies with hGF indicate altered morphology beyond 1% CHX. However, NE shows similar results at higher concentrations. Cytotoxicity and Antioxidant analysis of NE displays remarkable safety as compared with CHX with less than 32% cytotoxicity even at 100% conc. CHX beyond 1% concentration exhibits toxic effect on hGF at 1 minute time exposure. However, NE does not adversely affect the fibroblasts even up to 50% concentration showing less toxic effect in comparison with CHX on these cells. The cytoprotective, oral friendly quality of NE emphasize the superiority of NE over CHX.

Keywords: Chlorhexidine, Neem Extract, Human Gingival Fibroblast, Cytotoxicity

INTRODUCTION

Diseases associated with the periodontium are initiated by microbial plaque which can be controlled or prevented mechanical methods or through chemical means[1]. Mechanical methods like brushing, flossing and tooth-picks, etc. have advantages and their own limitations. Chemical agents like Chlorhexidine Gluconate, essential oils, Povidone-iodine, antibiotics, chelating agents, enzymes, oxygenating agents, metal salt and Quaternary ammonium compounds have been used in the form of mouth rinse and sub gingival irrigating solution[2] to overcome the drawbacks of mechanical methods.

The ability of Chlorhexidine (CHX) to reduce plaque and gingivitis has been documented in numerous studies[3]. The antibacterial action of CHX and its sustained release over a long period of time makes it a popular anti plaque agent. However, CHX shows some toxic effects on neutrophils[4], human epithelial cells[5], gingival fibroblasts[6], and also causes delay in wound healing[7]. Thus, all chemical preparations have some or the other side effects. Owing to these limitations, some herbal preparations have been also used for such purposes. Most commonly used plant preparations are Sanguinarine[2] and Neem[8], etc. In the recent literature, Neem has been shown to be effective in reducing plaque and gingivitis[9]. There are no studies available regarding the toxic effect of Neem when used locally in the oral cavity for a long duration.

Fibroblasts are responsible for the production of structural proteins, extracellular matrix and are the predominant cellular element in the gingival and periodontal connective tissue. Thus, any toxic effects on these cells have important implications in periodontal wound healing. Though numerous studies have evaluated the toxicity of Chlorhexidine on fibroblasts by various methods, but to date no investigations have been performed regarding its toxicity on cultured human periodontal gingival (hGF) fibroblasts.

The present study has been undertaken with the aim of evaluating the cyto-static/proliferative/toxic action of Chlorhexidine (CHX), and Neem Extract (NE), as mouthwashes on hGF through cytomorphological, biochemical techniques such as Sulforhodamine B (SRB) & DPPH assay.

MATERIAL AND METHODS

Seven volunteers in the age group ranging from 20-28 years, with a clinically healthy gingiva (with appropriate institutional approvals) were selected for harvesting gingival biopsies. The effect of mouthwashes, Chlorhexidine (CHX) and Neem Extract (NE) on Cultured Human Gingival Fibroblast cells (hGF) was tested.

CHX was procured from Global dent aids Pvt. Ltd, New Delhi. All the reagents used for tissue culture purposes were procured from Sigma Chemicals Co. St. Louis, MO (USA) Sterile plastic ware, flasks, multi-welled tissue culture plates, filterware etc. were obtained from Corning, Boston, MA (USA).

Preparation of Neem Extract (NE)

The Neem extract was prepared from the fresh soft twigs of Neem, extracted with 10% w/v of chilled 0.15% KCl by grinding with a pestle and mortar. The extract was centrifuged at 1000 x g for 5 minute at 4ºC to rid of the debris and finally sterilized by filtering through 45µm syringe filter.

Isolation and culture of Human Gingival Fibroblast (hGF)

Fibroblasts used in these experiments were derived from aseptically obtained gingival biopsy specimens. The tissue explants were harvested with the help of a BP knife blade No. 15 and transferred in chilled Dulbecco's Modified Eagle's Medium (DMEM), containing 20% fetal Bovine serum (FBS) and 10 mM HEPES, Penicillin (100 U/ml), Streptomycin (200 µg/ml) and Gentamycin (50 µg/ml) at pH 7.4. The explants were transported immediately to the Tissue Culture Laboratory CDRI in minimal time on ice.

In the laboratory, the biopsy specimens were transferred to a dish and washed with fresh, chilled medium (DMEM) thrice. The explants were then cut with an iris forceps into smaller cubes approximately 1 mm^3 (~1-2 mg) in a petri-dish containing medium with 20% FBS in a laminar flow to ensure sterility. Subsequently, they were transferred into a T-25 tissue culture flask with adequate medium for complete immersion of tissue explants. The flasks were incubated in a humidified CO_2 incubator at 37 °C with 5% CO_2 and left undisturbed. By Day 5, a mixed population of epithelial and fibroblast cells emerged in and around the explants as examined by Nikon™ ECLIPSE Ti Phase Contrast Microscope. By Day 15, the epithelial cells perished while the fibroblasts with typical oblong, flattened, spindle shaped, and densely packed morphology were retained. The medium in the flask was replaced with fresh medium as and when required. Complete monolayer was evidenced by Day 21. DMEM containing 10% FBS was employed to subculture the monolayer into fresh flasks following trypsination as reported previously[10].

Growth & Viability

A confluent flask of hGF was trypsinized to obtain a single cell suspension. 0.05 x 10^6 cells were plated onto a 24 well plate with 500 µl DMEM supplemented with penicillin (100U /ml), streptomycin (200µg /ml) and FBS 10% and incubated at 37^0 C in a humidified 5% CO_2 incubator. After 24 hours of culture, the cells were treated with CHX & NE. 0.2% concentration of CHX available commercially was regarded as 100%. 1%, 10%, 25%, 50% and 100% dilutions of CHX and NE with medium were used for treating the cells. The cells following exposure to various concentrations of solution for 1 min, were washed thrice with DMEM, followed by the addition of fresh medium and cultured for the next 48 h at 37^0 C in a humidified 5% CO_2 incubator. At the end of the experiment, the cells were photographed by Nikon™ ECLIPSE Ti Phase Contrast Microscope.

Sulforhodamine-B Assay

At the end of the culture period, Sulforhodamine-B (SRB) assay was performed[11, 12]. Briefly, the cells were fixed with chilled 10% Tetrachloroacetic acid (TCA), washed thrice with distilled water and air dried. 0.4% SRB solution in 1% acetic acid was added into each well and incubated for 30 min at room temperature. After incubation, the cells were washed thrice with 1% acetic acid solution and air dried. Finally, the SRB bound to the cellular protein was extracted with 10 mM Tris (pH 10.5) and optical density read at 560 nm by Spectrophptometer (SpectraMax M2: Molecular Devices).

DPPH Assay

DPPH assay was performed according to Singh *et al* [13]. Briefly, DPPH was dissolved in methanol (500 µM) to obtain the stable free radical DPPH•. NE & CHX at various doses (1.0 -100%) was diluted in the ratio 1:1 with the DPPH• solution in a 96-well microtiter plate and the absorbance of the reaction mixture was measured within 30 min at 520 nm. Fresh DPPH• solution was prepared daily. NE and CHX were tested in triplicate.

RESULTS

Derivation of Human Gingival Fibroblast (hGF)

The explants were left undisturbed for up to 5 days to allow their attachment to the substratum and observed under Nikon Phase Contrast Microscope for evaluating the emergence of cells, if any. A stream of fibroblast was found emerging in and around the explants which formed a complete monolayer by 15 days. From time to time, the medium in the flask was replaced with fresh medium. Once the complete monolayer had developed in the culture flask, it was sub cultured by trypsination. The fibroblasts displaying typical flattened tapering morphology were densely packed and firmly attached to the substratum.

Cellular Morphology

The hGF were exposed for 1 min to CHX and NE each, individually at conc. ranging from 1%-100% and photographed at 100X magnification (Fig 1). In the untreated control cells, the hGF were found to be evenly distributed with flattened morphology In case of CHX, the cells begin to demonstrate adverse effects beyond 1% whose activity increased proportionally to the conc. of the mouthwash employed. However, with NE, the cells tolerated the mouth rinses reasonably well until 75% conc. At & beyond 75%, the hGF started getting adversely affected morphologically.

Cell Growth and viability

SRB assay

In order to corroborate and confirm the results obtained from microscopical evaluation of hGF, SRB assays were performed. These assays are the benchmarks for ascertaining the cytostatic/proliferative/toxic effect of any drug or ligand such as CHX and NE. For this purpose, the effect of CHX and NE for 1 minute on hGF exposure was underkatken using SRB assay (Fig 2 & Table 1). We chose concentrations ranging from 1 – 100% of ligand, incubated with the hGF for 1 min and compared the results with the untreated control. Results in (Fig 2 & Table 1) clearly demonstrate dose-dependent, steady state, and cytotoxic kinetics induced upon hGF by 1 minute exposure of CHX. CHX shows dose-dependent (1-100%) inhibition while NE gradually increases inhibition at 10% (31.92%) followed by the attainment of steady state at subsequent concentrations. These observations are in synchrony with dose of the morphological studies. Expectedly, NE displays remarkable safety as compared with the synthetic CHX. The results therefore suggest and justify the use of Neem for oral hygiene over CHX mouthwash.

DPPH based antioxidant quantification of NE and CHX

Based on quenching formula {Q = 100 (A_0 – Ac) / A_0} the data was calculated[13] and presented in Fig. 3 & Table 2. CHX and NE were microtitrated with 500 µM DPPH. Reduction of DPPH through CHX started beyond 1% whose activity increased proportionally with the conc. of CHX employed. However, with NE, the cells demonstrated higher reduction from 1-25% beyond that saturation was attained.

DISCUSSION

Antiseptic mouth rinses play an important role in daily dental care, and their use is strongly encouraged throughout the world, particularly for the prevention and treatment of periodontal diseases. Chlorhexidine (CHX) is widely used as an adjunct therapy in the treatment of various periodontal diseases due to its antiseptic activity with a broad range of antimicrobial activity[14]. Neem has attracted the widest attention globally for a myriad of reasons and is used to maintain the oral hygiene[15]. It has been shown to be effective in reducing plaque and gingivitis [9]. Wolinsky *et al* [16] have reported the inhibitory effects of aqueous extract derived from the bark containing Neem stick on bacterial aggregation, growth adhesion to hydroxyapatite and production of insoluble material which may regulate plaque formation. Several workers have also proven the efficacy of Neem as an antibacterial, anti-inflammatory agent etc. However, to our knowledge no systematic study is available regarding its cytotoxicity on human Gingival Fibroblast (hGF). The present study has been undertaken for this reason. The efficacy of Neem Extract (NE) has been compared with Chlorhexidine (CHX) on cultured hGF derived from periodontal gingival biopsy of healthy volunteers. In addition, the antioxidant potential of NE and CHX has also been determined, which plays an important role in cytotoxicity.

The cultured hGF fibroblast cells were exposed to the above mouthwashes range from concentration of 1-100% of 1 minute. Generally, gargling for 1 min is recommended so we decided 1 min as time exposure for our study to titrate the exposure at various concentrations similar to Mariotti *et al* [17]. The cytotoxicity data compared with untreated control obtained from SRB assay clearly indicates that CHX dose-dependently induces cytotoxicity initiating at 1% (43.10%). However, NE did not show any dose-dependent cytotoxicity and maintained the level of cell death to about 31% even at 100% concentration. This clearly indicates that the CHX is more toxic at much lower concentrations as compared to NE. The cytotoxic effect of CHX has been well documented by several workers in detail. In addition, the cytopathic effect of CHX on human fibroblasts and HeLa cells has been demonstrated by Goldschmidt *et al* [6]. According to Schiott [18-22] *et al*., two years daily mouth wash of CHX in human patients showed no systemic effects and no oral difference from the control. However, Bassetti and Kallenberger describe that the use of CHX delays the process of wound healing[7].

Morphological studies also indicate that hGF gingival fibroblast upon exposure to CHX and NE at different doses for 1 minute display specific effects. 1% CHX and 50% NE displayed minimal number of cells with altered morphology (Fig. 1).This possibly indicates no cytotoxicity. However, CHX at 100% concentration showed cell lysis depicting high toxicity over NE (100%). Similar response has also been observed with CHX by Goldschmidt [6], suggesting that high concentration of toxic substances somehow fixes the cells to the surface of petri-plate. In another study where gingival fibroblasts were exposed to 0.12% of CHX, the cells rounded up and detached from the substratum within few hours[1].

It may be concluded that CHX beyond 1% concentration exhibits toxic effect on periodontal gingival fibroblasts at 1 minute time

exposure. However, NE does not adversely affect the fibroblasts even up to 75 % concentration showing less toxic effect in comparison with CHX on these cells. These results corroborate well with the results of DPPH assay (Fig 3 & Table 2). CHX showing negligible antioxidant activity leads to enhanced cytotoxicity. Contrarily, Neem showing dose-dependent activity protects the cells from death i.e. showing cyto-protective effect. This therefore suggests that CHX at 1% only and NE until 75% possessing antioxidant property is well tolerated by hGF hinting at their relative safety which may be of relevance in the *in vivo* situations.

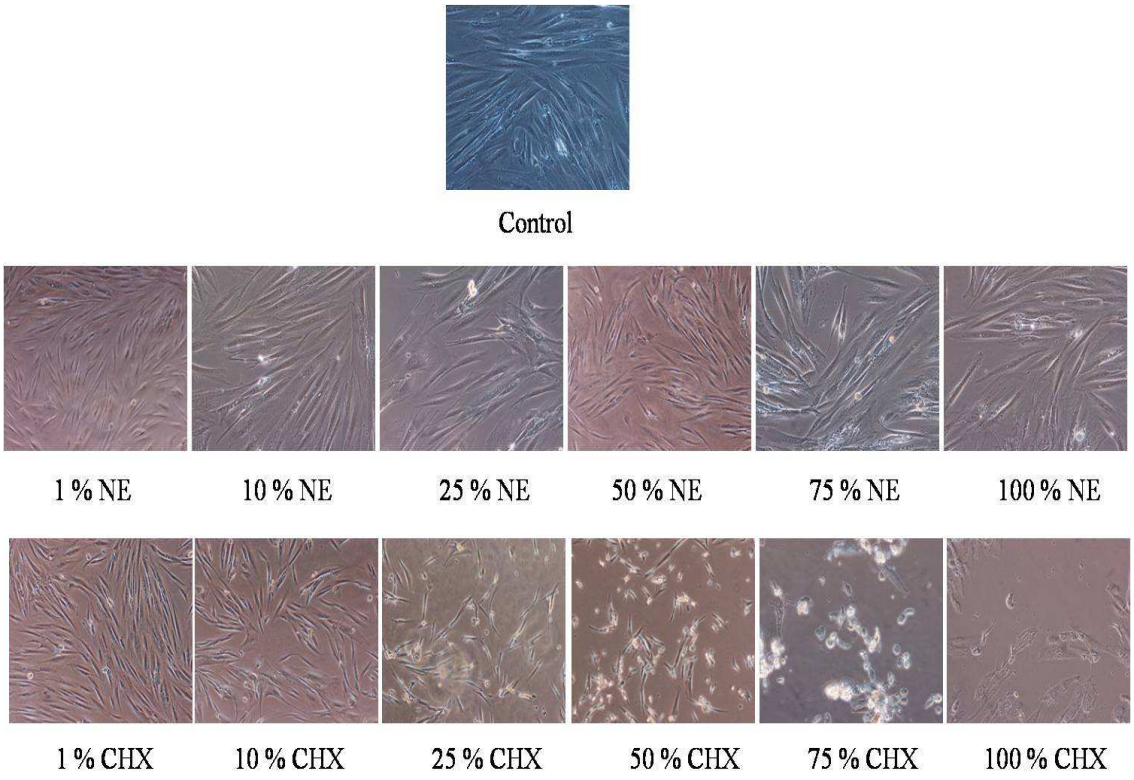

Fig. 1: For morphological studies the hGF were exposed for 1 min to the mouthwashes eg. CHX, and NE each, individually at conc. ranging from 1-100% and photographed after 24h at 100X magnification. In the untreated control cells, the hGF were found to be evenly distributed with flattened morphology. Dose-dependent adversity initiates beyond 1% of CHX. However, with NE, the cells tolerated the mouth rinses reasonably well until 50% conc. of each. At and beyond 50%, the hGF started getting adversely affected morphologically.

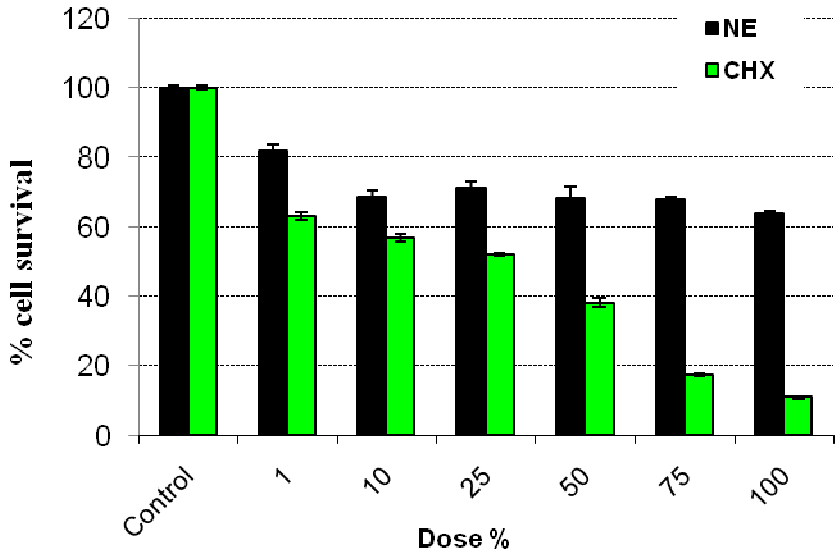

Fig. 2: Cytotoxicity evaluation of Chlorhexidine (CHX) and Neem Extract (NE) in hGF Primary cells using SRB Assay. 0.05×10^6 cells were cultured for 48 h in DMEM supplemented with 10% Fetal bovine serum (FBS) and subsequently exposed with various doses of CHX and NE for 24 h. Percentage survival was determined as per the formula (Absorbance of drug treated cells / Absorbance of Control cells)×100 and compared with Control, untreated cells regarded as 100%. Data shown are the mean±S.E. of one of the three similar experiments each performed in triplicate.

Table 1: Effect of Neem Extract (NE) and Chlorhexidine (CHX) in various concentration after one minute exposure on Human Gingival Fibroblast (hGF) using SRB Assay

% Conc. Of Solution	NE (% cell survival/ cytotoxicity)	CHX (% cell survival/ cytotoxicity)
Control	100	100
1	81.66 / 18.35	56.90 / 43.10
10	68.09 / 31.92	63.12 / 36.88
25	63.98 / 36.02	52.09 / 47.91
50	68.91 / 31.10	38.01 / 61.99
75	71.26 / 28.75	17.17 / 82.84
100	68.36 / 31.62	11.03 / 88.98

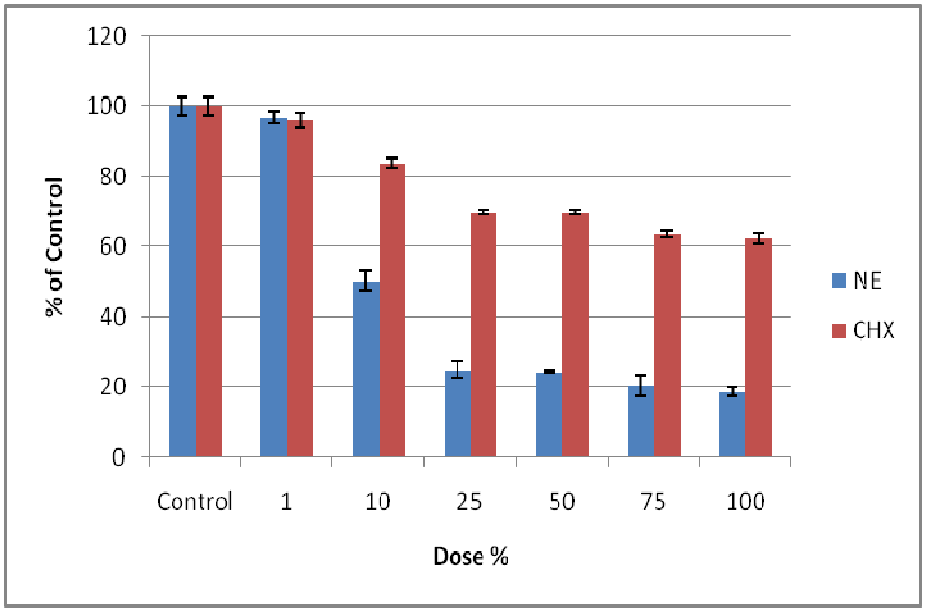

Fig. 3: Free-radical scavenging activity of NE & CHX is measured by using the DPPH assay: Results are mean ± SD of one of the three similar experiments each performed in triplicate.

Table 2: Scavanging activity of Neem Extract (NE) & Chlorhexidine (CHX) in various concentration using DPPH Assay

% Conc. Of Solution	% Scaveanging of 500µM DPPH by NE	% Scaveanging of 500µM DPPH by CHX
1	96.87	96.03
10	50.26	83.74
25	24.95	69.83
50	24.36	69.83
75	20.53	63.65
100	18.62	62.40

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