Effect of laser on the human gingival fibroblast treated with chlorhexidine

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Abstract: The aim of this study was the using of specific parameters of low level laser treatment (LLLT) to produce bio-stimulatory effects on human gingival fibroblast culture treated with different concentrations of chlorhexidine (CHX) mouth rinse. Fibroblasts were derived from healthy gingival biopsy specimens harvested aseptically and the effect of CHX was evaluated on cultured human gingival fibroblast (HGF) through morphological and biochemical assays. Morphological studies with HGF indicate altered morphology beyond 10% CHX. However, CHX beyond 50% concentration exhibits excessive toxic and damage effect on HGF at 1 minute time exposure. However, the CHX treated cells were irradiated with a diode laser prototype (LASER Table; 630 ± 4 nm; 60 mW) with energy doses of 4 and 5 J/cm^2. Cells were irradiated every 24 h totalizing 3 applications. Twenty-four hours after the last irradiation, cell metabolism was evaluated by the XTT assay and trypan blue assay Data obtained from this study showed that irradiation of fibroblasts with 4 and 5 J/cm^2 resulted in significant increase in cell metabolism and viability compared with the non-irradiated group ($P < 0.05$) in 1, 10, 25, 50% CHX treated cells. On the other hand, HGF treated with 75% and 100% CHX showed non biostimulatory effects post LLLT irradiation. Both energy doses promoted significant increase in the cell number as well as cell metabolism ($P < 0.05$). These results demonstrate that, under the tested conditions, LLLT promoted biostimulation of fibroblasts in vitro.
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1. Introduction

Diseases associated with the periodontium are initiated by microbial plaque which can be controlled or prevented mechanical methods or through chemical means **(Prabhushankar *et al.*, 2010).** 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 subgingival irrigating solution to overcome the drawbacks of mechanical methods **(Verma and Dixit, 2012).**

The ability of Chlorhexidine (CHX) to reduce plaque and gingivitis has been documented in numerous studies. The antibacterial action of CHX and its sustained release over a long period of time makes it a popular anti plaque agent **(Van der Weijden and Slot, 2011).** However, CHX shows some toxic effects on neutrophils, human epithelial cells, gingival fibroblasts, and also causes delay in wound healing. Thus, all chemical preparations have some or the other side effects **(Verma and Dixit, 2012).**

Fibroblasts are responsible for the production of structural proteins, extracellular matrix and are the predominant cellular element in the gingival and periodontal connective tissue **(Gómez-Floreit *et al.*, 2014).** 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 and these investigations have been performed regarding its toxicity on cultured human periodontal gingival (HGF) fibroblasts **(Flemingson *et al.*, 2008).**

However, tissue healing involves an intense activity of diverse cell types, such as epithelial and endothelial cells, as well as fibroblasts which play a key role in this process. In addition to, fibroblasts secrete multiple growth factors during wound reepitelialization and participate actively in the formation of granulation tissue and the synthesis of a complex extracellular matrix after reepitelialization **(Basso *et al.*, 2012).** All these processes directly involve the proliferation and migration capacity to these cells **(Hakkinen *et al.*, 2000).**

The application of lasers as an adjunct to conventional periodontal therapy is becoming more and more prevalent in dental offices as well as the use of

low-level laser therapy (LLLT) has been proposed to promote biostimulation of fibroblasts and accelerate the healing process **(Kreisler *et al.*, 2002).** Previous studies have evaluated the effect of LLLT on the proliferation and migration of human gingival fibroblasts as well as other cellular effects and responses, such as protein production and growth factor expression **(Hakki and Bozkurt, 2012).** Nevertheless, there is a shortage of studies investigating irradiation parameters capable of promoting biostimulatory effects on fibroblasts in order to establish an ideal irradiation protocol for these cells **(Peplow *et al.*, 2010).**

Therefore, the aim of this study was to determine the most adequate energy doses using specific parameters of LLLT to produce biostimulatory effects on human gingival fibroblast cultures treated with different concentrations of Chlorhexidine (CHX) *in vitro* model.

2. Material and Methods

1. **Chlorhexidine (CHX):**

CHX was procured from 0.2% concentration of CHX available commercially was regarded as 100%. The 1%, 10%, 25%, 50%, 75 and 100% dilutions of CHX with medium Dulbecco's modified Eagle's medium (DMEM, Sigma, Linz, Austria) were used for treating the human gingival fibroblasts (HGF) cells.

2. Isolation and culture of Human Gingival Fibroblast (HGF):

HGF were grown from biopsies obtained during oral surgery from volunteers in the age group ranging from 16-24 years, with a clinically healthy gingival. HGF were cultured and maintained in plastic culture flasks (NUNC) according to the technique described by **(Khadra *et al.*, 2005).** The cells were cultured in DMEM (Sigma, Linz, Austria), supplemented with 5% fetal calf serum (FCS) and penicillin / streptomycin solution. The cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cultures were supplied with fresh medium every other day. Only cells between the 4th and 8th passages were used in experimental procedures. After cell culture, the cells were counted and plated on sterile 24 well acrylic plates ($3x10^4$ cells/cm^2) using Plain DMEM supplemented with 10% fetal bovine serum (FBS) for 48 h. The cells following exposure to various concentrations of CHX solution for 1 min. **(Mariotti and Rumpf, 1999)** were washed twice with DMEM, followed by the addition of fresh medium and cultured for the next 48 h at 37°C in a humidified 5% CO2 incubator.

3. LLLT on Fibroblast Culture:

The LLLT device used in this study was a red diode laser prototype (LASER Table; 630 ± 4 nm wavelength, 60 m W maximum power output), which was specifically designed to provide a uniform irradiation of each well (2 cm^2) in which cultured cells are seeded. The power loss through the acrylic plate was calculated using a power meter (Coherent LM-2 VIS High-Sensitivity Optical Sensor, USA), which was placed inside the culture plate. After this measure, the power loss of the plate was determined as 5%. After that, the power of diode laser was checked and standardized. Therefore, a final power of 0.057 W reached the cultured cells. For the evaluation of cell metabolism, the radiation originated from the LASER Table was delivered on the base of each 24-well plate with energy doses of 4 and 5 J/cm^2. The laser light reached the cells on the bottom of each well with a final power of 0.057W because of the loss of optical power in each well due to the interposition of the acrylic plate. The cells were irradiated every 24 h totalizing 3 applications during 3 consecutive days. The cells assigned to control groups received the same treatment as that of the experimental groups. The 24-well plates containing the control cells were maintained at the LASER Table for the same irradiation times used in the respective irradiated groups. Twenty-four hours after the last irradiation, the metabolic activity of the cells was evaluated using the XTT assay.

4. XTT Assay:

Twenty-four hours after the last irradiation, the viability and proliferation of fibroblasts were determined by an XTT assay (Cell Proliferation Kit produced by Bio. Ind. Ltd) as described by **(Chae *et al.*, 2007).** It was evaluated 24h post LLLT treatment and the optical (OD) of untreated (control) cell was considered to be 100%. The relative viability of GF cells was calculated by the formula: (OD of irradiated sample / OD of treated cells) x 100.

5. Viable Cell Counting (Trypan blue assay):

Trypan blue assay was used to evaluate the number of viable cells in the culture after LLLT application. This test provides a direct assessment of the total number of viable cells in the samples as the trypan blue dye can penetrate only porous, permeable membranes of lethally damaged (dead) cells, which is clearly detectable under optical microscopy according to **(Basso *et al.*, 2012).** Cell counting was performed in the experimental and control groups 24 h after the last irradiation. The number of viable cells was calculated by deducting the number of nonviable cells from the number of total cells **(Basso *et al.*, 2012)**. The number of cells obtained in the counting corresponded to n × 10^4 cells / milliliter of suspension.

6. Statistical Analysis:

Data from XTT, Trypan blue were an analyzed by student t- test and $P < 0.05$ was considered as significant

3. Results

Cellular Morphology:

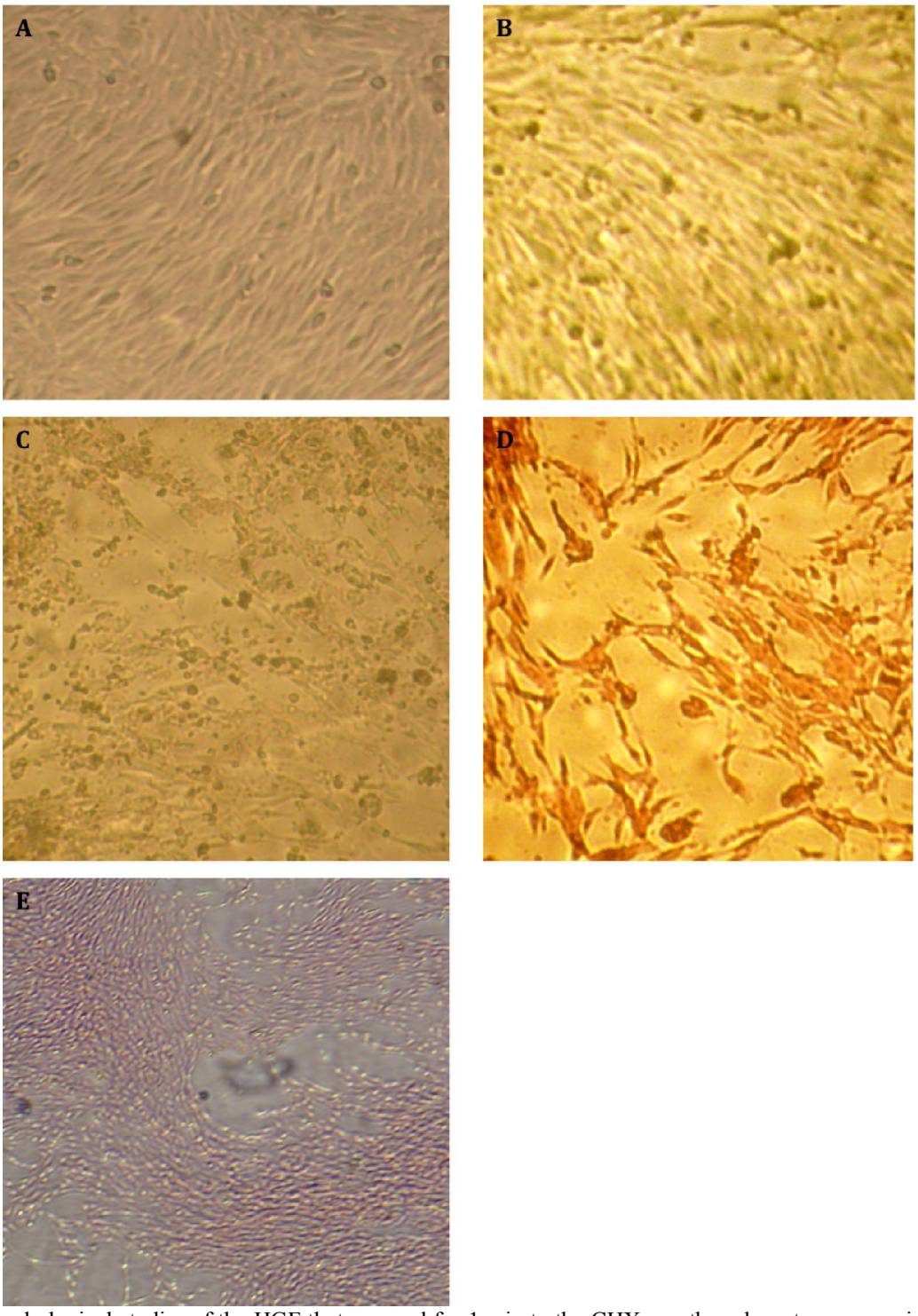

Fig.1: Morphological studies of the HGF that exposed for 1 min to the CHX mouthwashes at conc. ranging from 1-100% and photographed after 24h. In the untreated control cells, the HGF were found to be evenly distributed with flattened morphology (A). Dose-dependent adversity initiates beyond 10% of CHX (B). However, 50% conc., the HGF started getting adversely affected morphologically (C). At 100% conc. HGF showed cell lysis (D). LLLT irradiation showed observable cell proliferation effects on 50% CHX treated cells (E) (100X).

The HGF were exposed for 1 min to CHX at conc. ranging from 1% -100% (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 10% whose activity increased proportionally to the conc. of the mouthwash employed.

Analysis of Cell Metabolism (XTT Assay):

Comparisons of the fibroblasts' viability/ proliferation data from the XTT assay showed the mean OD value of the control group (0.97), the highest among all groups, to be significantly greater (p<0.05) than that of any of the four treated groups with 25%, 50%, 75% and 100% CHX. However, the other two groups (1% and 10%) were not statistically significant compared to control group (Table 1). Data from treated human gingival fibroblast cultures (XTT assay) after LLLT, according to the energy dose are presented in Table 2. Irradiation of the fibroblast cultures with doses of 4 J/cm² and 5 J/cm² resulted in 23% and 18% increases in cell metabolism, respectively, differing significantly from the control group (P < 0.05). At the both dosages applied, HGF was significantly higher in the irradiated groups treated with 1, 10, 25, and 50% CHX than in the treated non irradiated groups. Conversely, the cells treated with 75% and 100% CHX and irradiated presented the significant lowest metabolic rate compared with the non irradiated treated group (8% and 19%) decrease, respectively in case of 4 J/cm², and (12% and 19%) decrease in case of 5 J/cm², P < 0.05). Regarding the energy dose, no statistically significant difference between the irradiated groups with the both doses (4 and 5 J/cm²) was observed (P > 0.05).

Table 1: Mean OD human gingival fibroblasts proliferation detected by the XTT assay according to the CHX concentration used

Group	*CHX Conc.*	*Mean OD*
Control	-	0.97[a]*
Gr.1	1%	0.95[a]
Gr.2	10%	0.88[a]
Gr.3	25%	0.69
Gr.4	50%	0.38
Gr.5	75%	0.35
Gr.6	100%	0.19

*Same letters indicate no statistically significant difference (P > 0.05).

Viable Cell Counting (Trypan Blue Assay):

The number of viable cells (%) after LLLT application, according to the energy dose, is presented in Table 3. Comparison among the energy doses revealed that irradiation of the CHX treated human gingival fibroblast cultures with 4 J/cm^2 and 5 J/cm^2 increased the number of viable cells and differing significantly from the treated non-irradiated (P < 0.05), but without statistically significant difference between each other (P > 0.05). Conversely, the cells treated with 75% and 100% CHX and irradiated presented the decreased the number of viable cells without statistically significant difference from non-irradiated cultures.

Table 2: Proliferation of HGF cultures treated with CHX detected by the XTT assay according to the energy dose used in the low-level laser therapy.

CHX treated groups	***XTT% Energy dose (J/cm2)***	
	4	5
Control	123	118
1%	119	117
10%	118	113
25%	114	111
50%	109	108
75%	92	88
100%	81	79

Table 3: Number of viable cells (%) detected by the trypan blue assay in CHX treated HGF cultures, according to the energy doses used in the low-level laser therapy.

CHX treated groups	***Number of viable cells (%) Energy dose (J/cm2)***	
	4	5
Control	148	151
1%	137	133
10%	142	139
25%	144	139
50%	122	130
75%	98	95
100%	95	92

4. 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 **(Verma and Dixit, 2012).**

The cytotoxic effect of CHX has been well documented by several researchers in detail. In addition, the cytopathic effect of CHX on human fibroblasts and HeLa cells has been demonstrated by **(Goldschmidt *et al.*, 1977)**. According to the previous studies, two years daily mouth wash of CHX in human patients showed no systemic effects and no oral difference from the control. However, some studies

were described that the use of CHX delays the process of wound healing **(Verma and Dixit, 2012).**

In this study, the morphological changes in CHX treated cultures indicate that human gingival fibroblast upon exposure to CHX at different doses for 1 minute display specific effects with altered morphology at 25, 50, and 75% concentration (Fig. 1). However, CHX at 100% concentration showed cell lysis. Similar response has also been observed with CHX by **(Goldschmidt et al. 1977)** 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 **(Prabhushankar *et al.*, 2010).**

In addition to, the present results were corroborated well with the results of XTT assay (Table 2). CHX treated cultures showing negligible proliferation activity in case of 75% and 100% concentration. Contrarily, from 1% to 10% showing dose-dependent cells proliferation protects the cells from death i.e. showing cyto-protective effect. This therefore suggests that CHX at 1% to 10% only possessing relative safety which may be of relevance in the in vivo situations.

On the other hand, LLLT has been widely investigated and indicated for accelerating the healing process, especially in the treatment of ulcerative oral mucosa lesions **(Chor *et al.*, 2004; Abramoff *et al.*, 2008).** Nevertheless, current research involving irradiation of cell cultures has not yet established the irradiation patterns specific for the different cell lines. In the present study, the metabolic activity of human gingival fibroblast cultures treated with CHX in different concentrations and determined with XTT assay after LLLT with different energy doses was evaluated to determine the adequate doses to produce biostimulatory effects on these cells in vitro.

The results were showed that both 4 and 5 J/cm^2 doses increased cell metabolism significantly compared to 1, 10, 25, 50% CHX treated non irradiated cells. Therefore, these two doses were effective irradiation doses selected to produce biostimulatory effects on HGF cells. These were agree with **(Damante *et al.*, 2009; Basso *et al.*, 2012)** who observed the increase of cell proliferation after irradiation of gingival fibroblasts using a similar laser prototype to the one used in the present study. In the same way as in the present study, the XTT results also served as guide for subsequent experiments that evaluated the expression of growth factors by cultured fibroblasts.

On the other hand, no biostimulatory effects of LLLT were observed on HGF cells treated with 75 and 100% CHX concentrations either with the 4 and 5 J/cm^2 doses. However, a significant increase in the number of viable cells that presented normal morphological characteristics (Trypan blue assay) was observed after LLLT using doses of 4 and 5 J/cm^2 on treated cells. These results confirm those of previous laboratory investigations in which LLLT increased the proliferation of gingival fibroblasts **(AlGhamdi *et al.*, 2012; Kreisler *et al.*, 2002)** also reported increase of fibroblast cell culture in vitro after direct and consecutive low level laser irradiations. The mechanism by which LLLT can promote biostimulation and induce proliferation of different cell types remains a controversial subject **(AlGhamdi *et al.*, 2012).**

Fibroblast cell proliferation are essential events for tissue healing and are directly related with its success so, the effect of LLLT on the capacity of gingival fibroblast proliferation, using two energy doses capable of increasing cell metabolism was evaluated in this study and it was demonstrated that LLLT using 630 nm laser source was able to increase the proliferation capacity and viability of fibroblasts that partially damaged with CHX treatment and the results revealed no significant difference between the energy doses. These results are in accordance with those of previous investigations **(Basso *et al.*, 2012).**

In addition, the bio stimulation of human gingival fibroblast cultures by LLLT with consequent increase in the number of viable cells demonstrates the efficacy of specific laser parameters and irradiation technique on the healing process. As well as, the obtained results are supportive to those of previous in vivo studies in which acceleration of the healing process was observed after LLLT **(Chor *et al.*, 2004; Abramoff *et al.*, 2008).**

In conclusion, the findings of the present study demonstrated that CHX beyond 10% concentration exhibits toxic effect on periodontal gingival fibroblasts at 1 minute time exposure. However, 75 % and 100% concentration showing excessive toxic effects in comparison with the other CHX concentrations on these cells. As well as, the preset laser parameters in combination with the sequential irradiation technique caused biostimulation, proliferation and viability of CHX treated human gingival fibroblast cultures. These encouraging laboratory outcomes should guide forthcoming studies involving tissue irradiation with laser and its effects on in vivo tissue healing.

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