Evaluation effect of low level Helium-Neon laser and Iranian propolis extract on Collagen Type I gene expression by human gingival fibroblasts: an *in vitro* study

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Back ground and aim: production of collagen by fibroblast cells is a key component in wound healing. Several studies have shown that low level laser therapy (LLLT) and propolis extract stimulate collagen Type I production. The aim of this study is to evaluation the combined effect of LLL helium neon (632.8 nm) and Iranian propolis extract on collagen Type I gene expression by human gingival fibroblasts (HGF3-PI 53).

Methods and materials: Human gingival fibroblasts after culturing divided into six experimental groups: G1-control group, which received no irradiation and propolis extract, G2-irradiated at 1.5 J/cm², G3-irradiated at 0.15 J/cm², G4-recived extract of propolis, G5- combined extract of propolis and 1.5 J/cm² laser irradiation and G6- combined extract of propolis and 0.15 J/cm² laser irradiation. The experiments were conducted in triplicate. After 24 hour, the total RNA was extracted and cDNA synthesis was performed. Type I collagen mRNA expression was determined with real time PCR.

Results: The obtained results illustrated a statistically significant difference between G3 (0.15 J/cm²) and G1 (control group) in levels of collagen Type I messenger RNA (mRNA) expression ($p < 0.05$). The irradiated cells showed a 1.4 times increase in mRNA expression of the collagen Type I gene. Expression of this gene decreases in other groups that this difference was statistically significant.

Conclusion: LLLT in different dosage and propolis extract may result in decreased or increased collagen type I gene expression. However this effect should be investigated in clinical studies.

Key words: collagen Type I · Gene expression · Low-level laser therapy (LLLT) · propolis extract · Fibroblastic cell

Introduction

Oral mucosal wounds are open lesions in the mouth which are caused by various disorders ¹⁾. These wounds can be the final common manifestation of a

wide range of conditions including traumatic lesions, infectious, vesiculo-bullous, neoplastic and gastrointestinal diseases ²⁾. They may be life threatening by development of infection due to invasion of micro-organisms or contaminants ³⁾. Oral mucosal wounds occur frequently, and the healing of these lesions is important. The wound healing procedure consists of four phases: hemostasis, inflammation, proliferation

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and tissue remodeling⁴⁾. Gingival fibroblasts as a connective tissue cells are responsible for collagen deposition which is a basic element in repair procedure of the tissue injury⁵⁾. These cells are able to produce collagen, elastin, fibronectin, and proteoglycans which play essential roles in gingival connective tissue. In normal tissues, strength, integrity and structure are provided by collagen. When tissues are disrupted due to injury, collagen acts to repair the defect and consequently restore anatomic structure and function. Normal anatomical structure is lost when too much collagen is deposited in the injury place, therefore function is compromised and fibrosis occurs. On the other hand, if an inadequate amount of collagen is deposited, the injury could be considered as weak and may dehiscence⁶⁾.

In recent decades, the use of lasers in physical medicine has become a consolidated practice. In spite of the numerous studies on the application of laser in the biomedical field, it is not easy to justify physical variables as: application technique, dosages, depth, modes and duration of exposure⁷⁾. Low-level lasers therapy (LLLT) as a useful tool has been increasingly used for improving wound healing⁸⁾. LLLT promotes tissue healing by increasing cell proliferation⁹⁾, promotes collagen synthesis and accelerates the formation of granulation tissue^{10, 11)}. Abergel et al confirmed that in fibroblast cultures, He-Ne and Ga-Al-As lasers certainly improve procollagen production without any effect on proliferation¹²⁾.

Several researches in the past have focused on the use of natural medicines for healing Oral mucosal wounds. Therefore, some studies have evaluated the injury healing treated by propolis^{13, 14)}. Propolis is a balsamic-resinous material with viscous consistency which is made by bees (*Apis mellifera*). One of its chemical components is flavonoid. It plays three main roles as antioxidants, antimicrobial agents, and modulators of the immune system¹⁵⁾. Some components of propolis may play significant bio modulatory function on the dynamics of fibroblastic activity and collagen synthesis as well^{16, 17)}. Albuquerque-Junior et al demonstrated that the integration of Brazilian red propolis into collagen-based films could improve injury healing, because of modulating the dynamics of the inflammatory evolution and collagen deposition process¹⁸⁾.

Ghaibi et al evaluated the combined effect of laser and oral administration of Iranian propolis extract on skin wound healing in male rats and showed that oral administration of propolis or low power laser radiation can increase the wound healing rate¹⁹⁾. In the

past studies about collagen synthesis, the exact effect of propolis is still not fully clarified and optimal parameters of LLLT are still not defined. Therefore, the aim of our study was to evaluate single or combined effects of LLL helium neon (he-ne) and Iranian propolis extract on collagen type I gene expression of human gingival fibroblasts (HGF3-PI 53). Cell culture investigations facilitate an experimental environment in which the effect of LLLT and propolis extract on gingival fibroblasts can be investigated by eliminating many parameters that may interfere with clinical trials.

Materials and Methods

Fibroblast cell culturing

Human gingival fibroblasts (HGF3-PI 53 NCBI code C502) have been provided by Pasteur Institute, Iran. Culture process was performed according to the cell culture protocol of American Type Culture Collection (ATCC). The cells were cultured in RPMI (Gibco, USA) supplemented with 10% fetal bovine serum (FBS). In addition, this medium was supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Culture media condition was changed every two days through the test procedure. Overlying liquid culture media was removed with micropipettes and 5 ml fresh culture media added to each 25 cm² flask. Cell passage was performed. Cells initially were washed with phosphate buffered saline (PBS) followed by adding of 1 ml trypsin solution (0.25 %) for 3 min. Afterward, FBS (5 ml) was added to neutralize the trypsin. The cells were removed by centrifugation at 1200 rpm. The cells were re-suspended in fresh culture medium and the cell suspension was transferred into three flasks. HGF3-PI 53 cells were grouped in three 6-well plates at an initial density of 5×10^6 cells/well and incubated for 24 hr at 37°C under 5% CO₂ atmosphere prior to exposure to laser irradiation and extract of propolis.

Propolis extraction process

Collected Propolis samples from different locations in Iran were used in this study. Hand-collected propolis was dried out in the dark before it's processing. The samples were ground mechanically and bottled in 10 g portions. The portions of 10 g were put into flasks, and 100 g of 70% ethanol (w/v, POCH S.A., Poland) was added. Propolis was subjected to 14 days of extraction in order to obtain ethanol extract of propolis (EEP). The flask was located in laboratory shaker in a dark, closed bottle for two weeks in ordinary temperature of

laboratory. After that time, the extract was cooled in 3°C for 24 hours to precipitate all unsolvable substances. Rough particles were removed from the propolis extract, filtered using filter paper (Whatman no. 4, UK). The obtained filtrate was evaporated, by means of rotary vacuum evaporator (Rotavapor R-215, BUCHI Labortechnik AG, Switzerland), in 45°C. In this path, a viscous substance having brown color was achieved, which was later dissolved in ethanol in order to receive 2 µg/µL of the working concentration. Our experiment has been started with high concentration of propolis extract (70 µg/µL), then the concentration of propolis extract has been decreased. Through this procedure, we reached to appropriate concentration 2µg/µL. In this concentration, decrease in cell viability was not seen under light microscope while it was added into culture media. Finally, 1µL of this extract admixed with 1cc of media culture (RPMI 1640 + FBS 10%) of wells and cells incubated for 24 hours.

Low level laser irradiation

Laser irradiation was provided by means of a 632.8 nm helium neon (he-ne) laser and a maximal output power of 500 mW. The wells were irradiated from 10cm distance using a laser beam. The output laser beam was directed toward the center of the wells over a 9.6 cm² zone during medium culture. Required irradiation time was 60 sec. The cells were irradiated 24 h after seeding and The Gene expression was assessed 24 h after irradiation.

Six experimental groups have been considered for the evaluation of laser's and propolis extracts bio modulation performance. These groups named as: G1-control group, which received no irradiation and propolis extract; G2-irradiated at 1.5 J/cm²; G3-irradiated at 0.15 J/cm²; G4-recived extract of propolis; G5-combined extract of propolis and 1.5 J/cm² laser irradiation and G6- combined extract of propolis and 0.15 J/cm² laser irradiation. It is worthy to note that, we performed all steps of this research in three times.

Real-time PCR Primer Design

Total RNA was extracted by the Gene Jet RNA Purification Kit (Thermo Scientific, USA) after 24 hours of incubation and cDNA synthesis was performed using Revert Aid First Strand cDNA Synthesis kit (Thermo Scientific, USA). The cDNA products were used for standard Real time Polymerase Chain Reaction (PCR). Real time PCR reactions were performed by Maxima™ SYBR/ROX qPCR Master Mix (Thermo Scientific, USA) and monitored in Rotor-gene Q real-time analyzer (Corbet, Australia). The effect of expres-

sion of collagen type I was investigated by Real time PCR (data not shown) and alteration of the level of expression was assessed with RT-PCR in triplicate and followed by prediction of the average threshold cycle which was normalized with GAPDH. The fold change of each target gene was estimated using $\Delta\Delta CT$ method. The primers were designed by primer 3 programs, and complete cDNA sequences obtained from the NIH Gene Bank Entrez program. The sequence of primers used is specified in **Table 1**.

Statistical analysis

Statistical analyses were performed in accordance with the Pfaffl method ²⁰⁾ and capability of the Res2009 software application ²¹⁾, taking into consideration the standard deviation values and the "pair wise fixed reallocation randomization test". A significance of $p < 0.05$ has assumed.

Results

The obtained results illustrated a statistically significant difference between G3 (0.15 J/cm²) and G1 (control group) in levels of collagen type I messenger RNA (mRNA) expression ($p < 0.05$). The irradiated cells showed a 1.4 times increase in mRNA expression of the collagen type I gene. Expression of this gene decreases in other groups compared to control group, that this difference was statistically significant ($p < 0.05$). Difference in other groups were statistically significant ($p < 0.05$) when compared with each other except between (G2, G4), (G2, G5) and (G4, G5) groups (**Fig. 1**).

Discussion

In dentistry, LLLT has been widely studied to accelerate the healing process, especially in the treatment of ulcerative oral mucosal lesions ^{22, 23)}. One possible reason might be explained by augmenting mitotic activity ²⁴⁻²⁶⁾, as well as by changes in collagen synthe-

Table 1: Primer sequences used to analyze the gene expression of irradiated cells

Gene	Primer sequences
COLA1	F: 5'-GTGCGATGACGTGATCTGTGA-3' R: 5'-CGGTGGTTTCTTGGTCGGT-3'

sis^{24, 25, 27–29}). In this study, a low-power He-Ne laser (632.8 nm) was used to stimulate cultured human gingival fibroblasts. This laser was chosen because it is known that this wavelength could be optimal for wound healing³⁰. In the current study, most parameters related to the laser irradiation are constant (e.g., wavelength, power output, irradiated area, exposure times), except the energy densities. The laser wavelength was 632.8 nm with a power output of 5mW and the area covered by the light was of 9.6 cm². Consequently, we have demonstrated that differences in energy density have different effects on cell growth. Our results corroborate with theories stating that low power laser can stimulate cell proliferation, but only within combination of exposition parameters, in a narrow energy, and power density bands³¹. This shows that preventing effect on collagen gene expression achieved when using 1.5 J/cm². There is a controversy on the effect of laser irradiation on collagen metabolism. Some of past studies have shown LLLT had no effect on procollagen and collagen synthesis^{11, 32}.

The majority of in-vitro studies have illustrated that the use of LLLT in a cell culture of fibroblasts

and epithelial cells enhances cell proliferation and gene expression of collagen type I^{10, 33–35}.

Pourzarandian et al showed that the low-level Er:YAG laser irradiation stimulates proliferation of cultured gingival fibroblasts. They showed that, the stimulative action on gingival fibroblasts proliferation through the production of PGE2 via the expression of

COX-2^{36, 37}.

Based on the study of Martignago et al, the laser irradiation (λ 904 nm) with a dose of 2 J/cm² increases the expression of collagen genes type I alpha 1 (COL1 α 1) in the fibroblast cells of mice (L929) cultivated in vitro³⁸. The study of Frozanfar et al.³⁹, shows that the influence of LLLT (λ 810 nm) on the expression of the COL1 gene has been evaluated, after three laser applications at 4 J/cm² in a culture of human gingival fibroblast. It was found that multiple applications of laser irradiation caused a stimulatory effect on the levels of COL1 mRNA. The irradiation of fibroblast cells derived from the Achilles tendon of pigs, by LLLT (λ 820 and 635 nm) in doses of 1, 2, and 3 J/cm², had caused increase in the expression of mRNA of the COL1 gene 24 h after a single exposure to irradiation with all doses employed⁴⁰. An experimental study on gingival fibroblast culture showed that irradiation with diode laser increases the production of the corresponding mRNA for the synthesis of collagen type I⁴¹. An experimental test by Chellini et al. using Nd:YAG laser for LLLT (1.5 J/cm²), showed that fibroblasts' (cell line derived from mice NIH/3T3 fibroblasts) irradiation LLLT resulted in significant production of collagen type I⁴². There are significant differences between other studies and current research work including wavelength and cell types, frequency, energy, optic fiber diameter, time, and distance of irradiation. Therefore a direct comparison cannot be implied herein. Our results confirm that the data found in the cell cultures

Fig. 1: Relative expression of the collagen type I gene; L: laser, P: propolis

of human fibroblasts, after laser applications (λ 632.8nm) at a dose of 0.15 J/cm² in the gingival fibroblast cells of human, a bio modulation effect increasing the expression of the COL1 gene was observed. In addition, we observed decrease in the expression of the COL1 gene for a dose of 1.5 J/cm². This result suggests an inhibitory effect on the expression of this gene by the dose used. Hakki and Bozkurt⁴³⁾ illustrated that irradiation of human gingival fibroblast at a wavelength of 904 nm (6 J/cm²) seriously reduced the expression of collagen type I in the periodontal pocket application when compared to the no irradiated control, bio stimulation, and infected pocket groups. Moreover, studies by Houred et al.⁴⁴⁾ established a down regulation of COL1 α 1, COL1 α 2, COL3 α 1, COL5 α 1, and COL5 α 2 in response to irradiation of fibroblast cells as 660 nm and diode laser at an effect of 5 J/cm². We showed it selectively restrained collagen products in fibroblast culture which recommend that this laser modality may be useful for the cure of fibrosis condition as keloids and hypertrophic scar, scleroderma and oral submucose fibrosis.

The main focuses of past researches that evaluated the effect of propolis extract on collagen synthesis were often on animal models. Increase in collagen deposition in wounds treated with ethanolic extract of propolis has been observed in some of them⁴⁵⁻⁴⁸⁾. Kilicoglu et al.⁴⁹⁾ showed better performance of fibroblast proliferation, activation and synthesis capabilities in the presence of propolis. Oliveira et al.⁴⁵⁾ found a similar view that propolis speeds up the healing procedure both through its anti-inflammatory effect, and by direct action on fibroblast proliferation. Günay et al.⁵⁰⁾ used propolis gel to evaluate its result on fibroplasia in post tooth extraction wounds. They illustrated that it had no significant effect on fibroblast expansion. In vitro studies assessed the effect of propolis on cytotoxicity and proliferation of fibroblast cells that included its high or mild cytotoxicity^{51, 52)}, or no effect⁵³⁾, and the proliferative action of this product⁵⁴⁾, depending on several factors as concentration, exposure time, and the in vitro /in vivo conditions. The effect of different alcoholic propolis solution was evaluated on cultures of fibroblast. Results illustrated that lower concentration of propolis (1%, 2% or 4%) were more efficient and not toxic to the fibroblasts⁵⁵⁾. In the study of Ann Jacob et al, Malaysian propolis showed maximum proliferation of fibroblast at 500 µg/mL with no significant difference (i.e. $p > 0.05$) compared to control group. Brazilian red propolis showed a slight increase in proliferation at 10 and 100 µg/mL concentrations, respectively with no significant

difference ($p > 0.05$) compared to control, while concentrations above these had inhibitory effects⁵⁶⁾. An opposing observation on the effect of Tubi-bee propolis on glioblastoma and normal fibroblast cell lines has been reported by Borges et al.⁵⁷⁾. They concluded that propolis exerted a strong inhibition on the proliferation of both cell lines. Funari et al.⁵¹⁾ observed that concentration of 31.25 µg/mL of propolis was contaminated to mouse fibroblasts where it caused a 50 % decrease in cell viability. In the study of Małgorzata, propolis at a concentration of 100 µg/mL decreased cell growth 27 % when it was compared with the controlling case⁵⁸⁾. In the present study, we observed decrease in expression of collagen type I gene of fibroblasts that were exposed with propolis extract, probably due to toxic effect of propolis on fibroblast cells. As a result, combined effect of propolis extract and laser irradiation have decreased expression of collagen type I gene. This finding does not exclude the possibility of other changes in the collagen metabolism. It could also provide an important indication that high energy LLLT treatment could not ensure the best clinical stimulation effects. That is to say, suitably determined laser density might cause optimal treatment effects in gingival tissue healing. Nevertheless, the real advantages of in vivo studies needs further resolve. This study also shows that the mRNA expressions of collagen type I increase irrespective of the energy density. Hence, some latent effects may be seen on fibroblasts after different treatments of LLL.

While this study addresses gene expressions under low level laser irradiation (LLLI) and propolis extract in vitro, there are some limitations to the study which could be considered in future studies. First, our study design involved a 24 h result after LLLI. It is not obvious that what the accumulated effect of longer durations on gene expressions is. Our study focused on the gene expressions of collagen type I, however many components are required for the gingival tissue to undergo healing processes. Other components of this matrix may respond differently to LLLI and propolis extract. Finally, though animal models have been helpful, particularly with regard to the gingiva, any suggestion that the results derived from an in vitro animal model would necessarily apply to an in vivo human gingiva should be treated with caution. In summary, the production of mRNA increases under LLLI. mRNA expressions by varying the dosage of LLLI highlights the fact that effective irradiation energy may reach maximal effects in connective tissue healing. The optimal LLLI dose for mRNA expressions of collagen type I in this study was found to be 0.15 J/cm². It is

hoped that a deep understanding of the dosage effects of gene expressions is achieved while applying LLLI will lead to greater insight into both the injury and rehabilitation of the gingival tissue.

Conclusion

Based on the results of the present study, it has been illustrated that LLLT within the parameters presented is capable of stimulating the expression of the collagen type I genes in a culture of the human gingival fibro-

last cells. Those cells irradiated with 0.15 J/cm² showed increase in gene expression of the COL type I gene, when those irradiated at 1.5 J/cm² or received propolis extract or combined laser (0.15 J/cm² or 1.5 J/cm²) and propolis extract showed decrease in expression of this gene. It is worth to note that the gene expression of collagen has just been evaluated in this paper and more researches based on western blot or ELISA results for detection of collagen protein are suggested.

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