

EFFECTS OF LIGHT-EMITTING DIODE (LED 640NM) ON HUMAN GINGIVAL FIBROBLASTS: A COMPARATIVE *IN VITRO* STUDY

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SUMMARY

Purpose. The light-emitting diodes (LEDs) have been applied in oral surgery for tissue stimulation and wound healing. Several Authors have highlighted that fibroblasts subjected to phototherapy have an increased viability, proliferation, bio-modulation of inflammatory cytokines and genes expression. It remains to be determined which are the best irradiation parameters (energy, wavelength, power) for each type of cell in order to obtain the best bio-stimulation.

The aim of this study was to investigate the effects of LED irradiation on primary human gingival fibroblast cells (HGF) on DSP, ELN, HAS1, ELANE, HYAL1, RPL13 genes activation using Real Time PCR. These genes activation is directly connected with elastin protein production and HGF functionality.

Materials and methods. Human gingival tissue biopsies were obtained from three healthy patients during extraction of teeth. The gingival pieces were fragmented with a scalpel and transferred in culture dishes for allow the cells growth. Human gingival fibroblasts at the second passage were seeded on multiple 6-well plates and were stimulated with three different light-emitting diodes (LEDs) fixture. After irradiation, the cells were trypsinized, harvested and lysed for RNA extraction. Genes expression was quantified using Real Time PCR.

Results. We didn't found significant differences in genes activation of HGF of the three different LEDs. The LED irradiation seems to be directly correlated with the elastin and hyaluronoglucosaminidase 1 genes activation that are directly connected with proteins production and HGF functionality.

Conclusions. HGF show an increased deposition of elastin as well as enhanced expression of collagen type I, which is the main protein related to the synthesis and of the collagen-rich matrix.

Key words: light-emitting diode, human gingival fibroblasts, primary fibroblasts cell culture, Real Time Polymerase Chain Reaction.

Introduction

Light therapy has proven to be effective in reducing the damage oral tissue and promotes healing of tissue and is therefore recommended in surgery and dentistry (1, 2). In particular light-emitting diodes (LEDs) have been applied in oral surgery for tissue stimulation and wound healing (3, 4). LED radiation is monochromatic

red-to-near-infrared (NIR) radiation (2). Light in the NIR 600-650 nm and white 400-750 nm range have shown positive effects on cells and tissues. LEDs have many advantages over lasers for use in photo-therapy, in particular in wound healing and for treatment of skin and mucosal ulcers. LEDs are different from low-level laser (LLL) because the LEDs light radiation is not coherent, whereas LLL presents a coherent radiation (2). Concerning the fibroblasts subjected to

phototherapy, several Authors have reported encouraging scientific data, such as enhanced cell viability, proliferation (5, 6), bio-modulation of inflammatory cytokines and genes expression (7, 8). Despite these positive results, it remains to be determined what is the optimal intensity and frequency for each cell type and pathway. In other words, it must be determined which are the best irradiation parameters (energy, wavelength, power) for each type of cell in order to obtain the best bio-stimulation.

In clinical situations with oral erosions or ulcers or in post-surgical phase, light should stimulate the epithelium and connective tissue. It has been demonstrated that LEDs irradiation (600~650 nm, 0.3 J/cm²) and white (400~750 nm, 1.26 J/cm²) are capable of propagating through an epithelial barrier and also to stimulate the underlying fibroblast cells (9).

The aim of this study was to investigate the effects of LEDs on primary human gingival fibroblast cells (HGF) on desmoplakin (DSP), elastin (ELN), hyaluronan synthase 1 (HAS1), elastase (ELANE), hyaluronoglucosaminidase 1 (HYAL1), ribosomal protein L13 (RPL13) gene activation using Real Time PCR. These genes activation is directly connected with proteins production and HGF functionality.

Materials and methods

Primary human gingival fibroblast cell (HGF) culture

Human gingival tissue biopsies were obtained from three healthy patients (68-year-old man, 63-years-old woman and 20-year-old men) during extraction of teeth. The gingival pieces were fragmented with a scalpel and transferred in culture dishes containing Dulbecco's modified Eagle medium (DMEM, Sigma-Aldrich, Inc., St. Louis, Mo) supplemented with 20% foetal calf serum (FBS) and antibiotics, i.e. penicillin 100U/ml and streptomycin 100µg/ml (Sigma Aldrich, Inc.).

Cells were incubated in a humidified atmosphere of 5% CO₂ at 37°C. The medium was changed the next day and twice a week. After 15 days, the pieces of gingival tissue were removed from the culture dishes. Cells were harvested after additional 24h of incubation.

LED irradiation on cells cultures

Human gingival fibroblasts at the second passage were seeded on multiple 6-well plates.

The cells stimulation was performed with three different light-emitting diodes (LEDs) fixture.

The LED 1 (Figure 1) was composed of 12 high-brightness red LEDs, type LR.80, fixed on aluminium base plate with a surface area of 12,5 cm x 10 cm. In this device the LEDs were connected in series and they were willing in two rows of six light 2 cm distant to each other. LEDs were designed to run on direct current low voltage (12V), and 150 mA was the absorbed current.

The LED 2 (Figure 2) was composed of aluminium base plate with a surface area of 9,5 cm x 9 cm on which was fixed a parabolic reflector of 3 cm diameter with inside 3 high-brightness red LEDs, type LR.80. This device was driven by an electronic circuit that allows the production of red light flashes by peaks of current of 700 mA at 5Hz of frequency with a duration of 100 microseconds each.

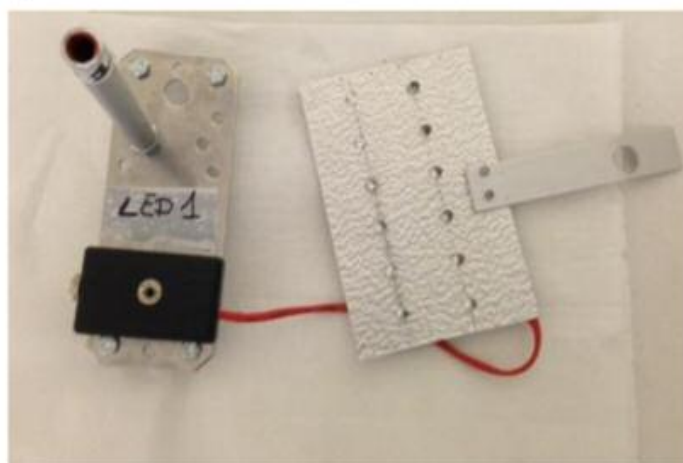
The LED 3 (Figure 3) was composed of 32 RGB LEDs type SMD 5050 (four rows of 9 LED separated 1cm to each other) fixed on aluminium base plate with a surface area of 15 cm x 9 cm. The fixture was setted to emit light at a wavelength of 640 nm. The LEDs were powered with 12 Vdc with an absorbed current of 210 mA.

The LED lamps were placed within 2 cm of the cells and the same cells were exposed to light for 20 minutes once a day for 2 days. A set of untreated cells were used as control.

The cells were maintained in a humidified atmosphere of 5% CO₂ at 37°C for other 24h after LED irradiations.

LED1

a)



b)

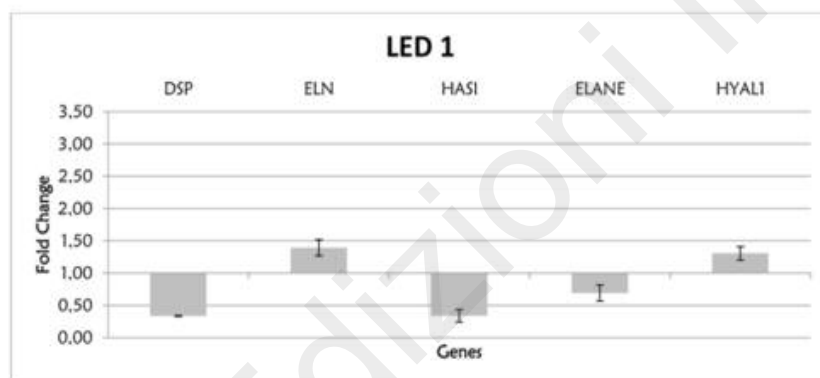


Figure 1

a) LED 1 composed of 12 high-brightness red LEDs, type LR.80. b) Human gingival fibroblast gene expression profile after LED 1 irradiation. SYBR® Green assay. DSP = 0,33; ELN = 1,39; HAS1 = 0,34; ELANE = 0,69; HYAL1 = 1,31; RPL13 = 1,00.

RNA Isolation and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

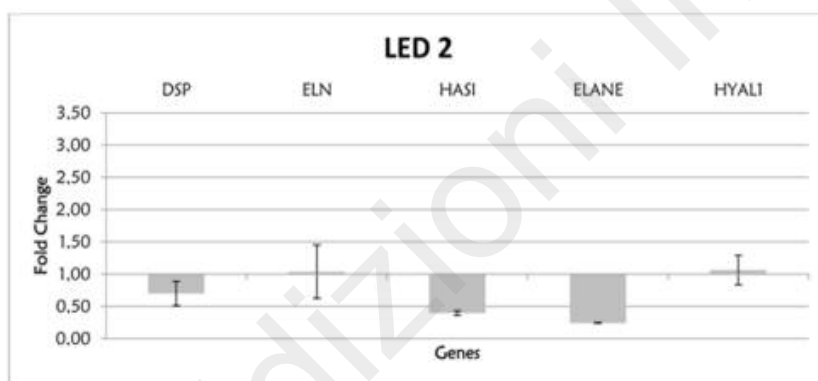
After irradiation, the cells were trypsinized, harvested and lysed for RNA extraction.

The total RNA was isolated using GenElute™ Mammalian Total RNA Miniprep kit (Sigma-Aldrich, Inc., St. Louis, Mo), following the manufacturer's instructions. Then, 2,5 µg of total RNA was used for synthesis cDNA using M-MLV Reverse Transcriptase (Sigma-Aldrich,

Inc., St. Louis, Mo) in according to the manufacturer's instructions.

Real Time Polymerase Chain Reaction (Real Time PCR)

The cDNA was amplified by Real Time PCR. The amplification was performed by using Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA), and the specific assay was designed for the investigated genes.

LED 2**a)****b)****Figure 2**

a) LED 2 composed of aluminium base plate with a surface area of 9,5 cm x 9 cm on which is fix a parabolic reflector of 3 cm diameter with inside 3 high-brightness red LEDs, type LR.80. b) Human gingival fibroblast gene expression profile after LED 2 irradiation. SYBR® Green assay. DSP = 0,70; ELN = 1,04; HAS1 = 0,40; ELANE = 0,24; HYAL1 = 1,06; RPL13 = 1,00.

SYBR assay reactions were performed in a 20 µl volume using the ABI PRISM 7500 (Applied Biosystems). Each reaction contained 10 µl 2X Power SYBR® Green PCR Master Mix (Applied Biosystems), 400nM concentration of each primer and cDNA.

All experiments performed included non-template controls to exclude contamination of reagents. PCR was performed with two biological replicates.

Expression was quantified using Real Time PCR. The gene expression levels were normalized to the expression of the housekeeping gene

Homo sapiens ribosomal protein L13 (RPL13). Quantification was done with the delta/delta calculation method.

Forward and reverse primers for the selected genes were designed using primer express software (Applied Biosystems), and are listed in Table 1.

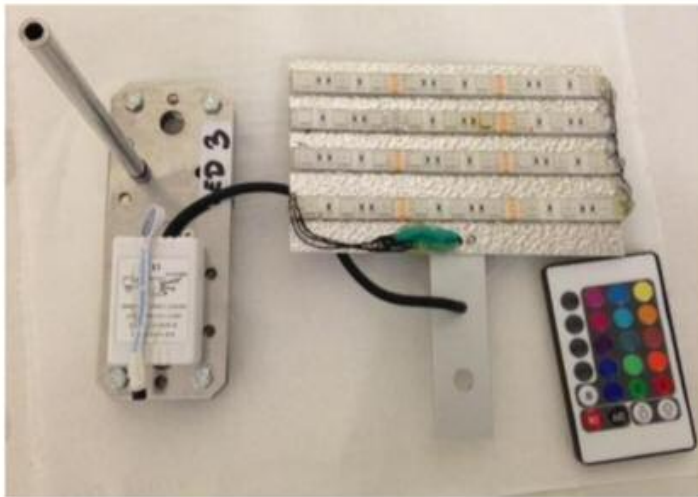


Results

Figures 1, 2, 3 show the effects of the three dif-

LED 3

a)



b)

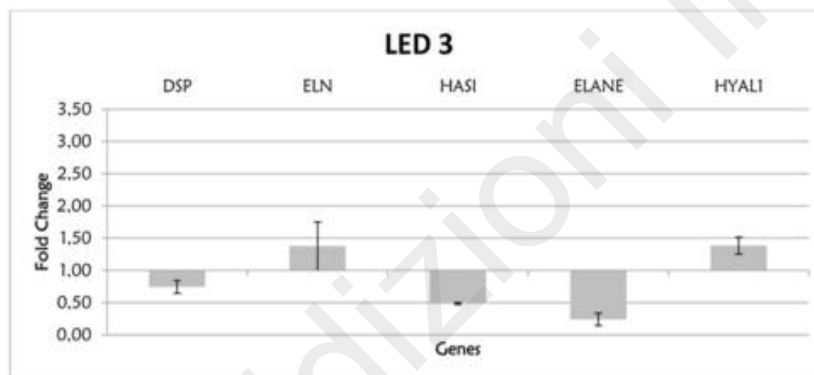


Figure 3

a) LED 3 composed of 32 RGB LEDs type SMD 5050. b) Human gingival fibroblast gene expression profile after LED 3 irradiation. SYBR® Green assay. DSP = 0,74; ELN = 1,38; HAS1 = 0,48; ELANE = 0,24; HYAL1 = 1,38; RPL13 = 1,00.

ferent LEDs irradiation on the different genes (HAS1, HYAL1, ELN, ELANE, and DSP) involved in growth, health and elasticity cells. Specifically, the LED irradiation seems to be directly correlated with the elastin (ELN) and hyaluronoglucosaminidase 1 (HYAL1) genes activation.

Discussion

Despite the promising cell-biostimulatory re-

sults obtained with phototherapy, such as modulation of tissue inflammation and stimulation of fibroblast cell metabolism, information about the effects of red LEDs on the HGF metabolism is poor (10). Therefore, the aim of this study was to evaluate the effects of three different LEDs irradiation on HGF.

The use of light therapy for HGF bio-stimulation is an interesting adjuvant treatment for healing during surgical procedures in operative dentistry. Based on the analysis of positive data provided by previous studies, researchers have been encouraged to develop more investigations to es-

Table 1 - Primers sequences for SYBR® Green assay.

Gene symbol	Gene name	Primer sequence (5' → 3')
DSP	<i>Homo sapiens</i> desmoplakin	F - ATGACCTGAGGAGAGGACGAA R - AGGCTCTCTCTTCTGTACCAC
ELN	<i>Homo sapiens</i> elastin	F - CTAATACGGTGCTGCTGGC R - CATGGGATGGGGTTACAAAG
HAS1	<i>Homo sapiens</i> hyaluronan synthase 1	F - CTCGGAGATTCGGTGGACTA R - CGCTGATGCAGGATACACAG
ELANE	<i>Homo sapiens</i> elastase, neutrophil expressed	F - CTACGACCCCGTAAACTTGCT R - CCTCACGAGAGTGCAGACGTT
HYAL1	<i>Homo sapiens</i> hyaluronoglucosaminidase 1	F - ACAGATGTATGTGCAACACCG R - AAGGGCCCCAGTGTAGTGTC
RPL13	<i>Homo sapiens</i> ribosomal protein L13	F - ATTCACAAGAAGGGAGACAG R - GAAATTCTTCTTCTCCTCAGTG

Footnotes: F= forward; R= reverse

establish the most beneficial physical parameters of LEDs or laser irradiation on HGF (11).

Current studies have assessed LEDs energy densities varying from 0.3 J/ cm² and 1.26 J/ cm² LED wavelength. Some Authors (9, 10) recommend power values from 1 to 500 mW and energy densities from 0.04 to 50 J/cm² for the irradiation of cells in culture with low-intensity therapy parameters. The red parameters of this study were based the indications of previous Authors who have studied biomodulation of HGF subjected to LEDs therapy (12, 13). In the present study, it was shown that red LED irradiation can increase the viability and number of HGF as well as elastin and hyaluronoglucosaminidase 1 genes activation.

Several studies have assessed the viability of HGF exposed to phototherapy (9-13). Some Authors (10) showed that viability of HGF was enhanced after exposure to one irradiation session with red LED. Therefore, in the present study we decided to evaluate the responses of HGF exposed to irradiation of three different LEDs irradiation.

As determined for cell viability, the number of viable HGF in the present study was also enhanced after irradiation with red LED at the chosen parameters. The ELN and HYAL1 genes ex-

pression and bio-stimulation seem to be related to cytochrome C oxidase activation, which enhances levels in the respiratory chain and adenosine triphosphate (ATP), and these biochemical changes led to macroscopic effects such as increased cell proliferation (6). Analysis of these data confirms that LED therapy may increase the viability and stimulate the proliferation of HGF *in vitro*.

Analysis of these data corroborates previous studies in which HGF (10,11) were subjected to phototherapy. In general, these cells presented increased deposition of elastin and other proteins as well as enhanced expression of hyaluronoglucosaminidase 1, which is the main protein related to the synthesis and of the collagen-rich matrix not only under phototherapy but also with biostimulation substances (14-18). The molecular pathways that cause increased production of these specific proteins have not been described thus far. However, this macro effect of phototherapy on HGF suggests that light can be an interesting adjuvant treatment to up-regulate the deposition of collagen-rich matrix by HGF during healing phase. In HGF different energy doses can be the ideal parameter depending on the desired outcome. Overall, *in vitro* light therapy with red LEDs bio-stimulated all HGF func-

tions assessed in the present study. Therefore, the scientific data obtained in the present study can drive future laboratory investigations or even *in vivo* studies in animal models to establish irradiation parameters for optimal and friendly clinical phototherapy procedures in HGF tissue regeneration. In recent year medical treatments have successfully used the LEDS in different treatments (healing-resistant wounds and ulcers e.g., chronic diabetic ulcers; in pain management, and in spinal cord and nervous system injuries) when other methods had limited success. Typical applications for LEDS irradiation are ulcerations, including decubitus ulcers and wound healing in the diabetic foot disorders. The LEDS may trigger vasomotor reflexes and enhance the effectivity of the respiratory chain. The LEDS stimulate the blood flow that can help in healing infections and other tissue disorders by accelerating supply with nutrients and increased clearance of metabolites and metabolic waste products. It may also attract cells of the immune system, such as neutrophils and other leukocytes as well as macrophages, because the effectiveness to induce cell migration by light is well established.

However, LED is still not a part of mainstream medicine and dentistry. We have highlighted some important recent developments in dentistry and in studies of cellular and molecular mechanisms behind the clinical findings. Future studies should focus on the efficacy of LED in oral surgery and implant dentistry, in particular in the treatment of peri-implantitis. Tooth replacement with implants is a well-known technique used worldwide in the last forty years (19-64). Peri-implantitis can happen with high frequencies in patients affected by periodontal diseases (37, 38, 40, 65-102).

In fact, even if the main factor for survival rate of implants is the quality of bone of receiving sites, the bacteria of peri-implantitis may be the main cause of failure of implants (70-73, 77, 84, 85, 103-105). So another field of application of LED could be peri-implantitis treatment by phototherapy.

Conclusion

In conclusion, there is no difference in genes activation of the three different LEDS. HGF presented increased deposition of elastin as well as enhanced expression of collagen type I, which is the main protein related to the synthesis and of the collagen-rich matrix.

The effect of phototherapy on HGF suggests that light can be an interesting adjuvant treatment to up-regulate the deposition of collagen-rich matrix by HGF during healing phase. Therefore, the scientific data obtained in the present study can drive future laboratory investigations or even *in vivo* studies in animal models to establish irradiation parameters for optimal and friendly clinical phototherapy procedures in HGF tissue regeneration.

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