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*Article*

# PHOTO-BIO-MODULATION IN ORTHODONTICS IN ORDER TO PROMOTE PROLIFERATION AND DIFFERENTIATION OF KERATINOCYTES: AN *IN VITRO* STUDY

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## ABSTRACT

Gingival augmentation techniques proposed in the international literature do not exclude a surgical component, which determines consequent post-surgical discomfort and results are not always predictable. In recent years, the introduction of laser photobiomodulation has led to a less invasive approach, particularly in the treatment of periodontally compromised patients, limiting the surgical phase to seriously compromised cases, with regeneration techniques for the restoration of a correct periodontal tissue anatomy. The aim of this in vitro study is to establish the validity of laser photobiomodulation in order to develop the epithelial keratinized layer of the tissue by stimulating fibroblasts-keratinocytes organotypic cultures and fibroblasts and keratinocytes mono-cultures. We created two groups (test and control), each one composed of 3 fibroblast cultures, 3 keratinocyte cultures and 3 organotypic cultures. We performed laser irradiation of test group at 50 J/cm2 of fluency with one application every 48 h for a total of 5 applications. 48 hours after the last laser application, we investigated the presence and amount of keratins 5 and 8 with citofluorymetric and western blotting analyses. Analyses showed an increase in keratin synthesis in test group cultures, showing a remarkable increase in production of keratin 8 in co-cultures test. Laser photobiomodulation can considerably enhance keratin synthesis when applied with high energy doses and repeated applications to keratinocytes-fibroblasts co-cultures.

**KEYWORDS:** *photobiomodulation, low level laser irradiation, biostimulation, keratinization, keratinocytes, fibroblasts*

## INTRODUCTION

Orthodontic treatment of ectopic dental elements is well documented in international literature (1). Often, when the tooth is repositioned at the end of orthodontic therapy, it is possible to observe a recession of vestibular gingival tissue that surrounds the dental element (2-5). In this case, gingival augmentation techniques, such as coronal advanced flaps

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(CAF) eventually combined with connective tissue grafts (CTG), are required. These techniques require a planning of one or more surgical phases with a resulting post-operative discomfort and results are not always predictable (6, 7).

In recent years, the introduction of laser therapy has led to a less invasive approach in the treatment of the compromised periodontal patient (8-11), confining the surgical component of the treatment only when the anatomy of the periodontal tissues is compromised and there is a need for guided tissue regeneration (12).

Similarly, the same advantages of using laser photobiomodulation may be useful to avoid mucogingival surgery in case of gingival recession of a repositioned ectopic tooth. In an essay we previously published (13), we examined the clinical effects of laser photobiomodulation on alveolar bone remodelling during orthodontic tooth movement and finally on formation of new keratinized gingiva. The results of this study concluded that laser photobiomodulation could promote the formation of attached gingiva around the crown of teeth erupted in oral vestibular mucosa.

Due to these clinical findings, we decided to investigate these results in vitro, applying the same laser photobiomodulation protocol we used in the clinical study to an in vitro reproduced epithelial tissue (14-17), in order to establish laser irradiation potential to increase keratin production and therefore the development of a keratinized layer.

## MATERIALS AND METHODS

We used a kit composed of primary basal fibroblasts-keratinocytes cell lines (Matched Set-Cryopreserved Dermal Fibroblasts and Keratinocytes, Tebu-Bio™). These cells were isolated from human epidermis of a single donor and conserved in two separate vials.

Once in the lab, the cells were unfrozen and placed into their respective culture medium. The keratinocytes were placed in a growth medium (Human Adult Keratinocyte Growth Medium KM-2, Tebu-bio™) composed of MCDB153 and epidermal growth factor (rEGF), insuline, transferrin, bovine serum albumine, ethanolamine, phosphoethanolamine, hydrocortisone, calcium chloride, ephinefrine, bovine pituitary extract (BPE), penicillin, streptomycin and amphotericin B.

Fibroblast cells were placed in a growth medium (Euroclone™) composed of DMEM (4.5 g/L of D-glucose) and 10% fetal bovine serum (FBS), penicillin, streptomycin and amphotericin B. Both fibroblasts and keratinocytes media were changed 24 h after the culture set-up and again at a 48-h interval.

Before proceeding with the set-up of the experimental groups, cell cultures were cultivated until they achieved at least 70-80% of confluence. In order to obtain valid organotypic co-cultures that could reproduce the epithelial tissue, we tested various growth media, changing the concentration of the two types of media used for the fibroblast and the keratinocyte cells. Finally, we found that a 1:1 mixture of the two growth media (with a final 5% of FBS) permitted an ideal coexistence and survival of the two cell populations in the same cultures.


**Fig. 1.** *100x photography of organotypic fibroblast cultures.*

We were then able to create organotypic fibroblast-keratinocytes co-cultures by positioning keratinocytes cells on top of fibroblast cultures with the 1:1 mixture growth medium (Fig. 1). We prepared two identical experimental groups (test and control) dividing the cultures in 12-well transparent plastic multi-wells. Each well had a 1 cm diameter. At the time of the experimental group set-up, fibroblast cells were seeded at a density of 50000 cells per well, meanwhile keratinocyte cells were seeded at a density of 100000 cells per well, both for monolayer cultures and for organotypic co-cultures.

For each monolayer culture and for each co-culture we used the same culture medium used to set-up the organotypic co-cultures. Each experimental group was composed of 3 fibroblast cells cultures, 3 keratinocytes cells cultures and 3 fibroblast-keratinocyte cells co-cultures.

**Fig. 2.** *Laser biostimulation of test group cultures.*

Twenty-four h after the group set-up, we placed the cultures of the test group under sterile extractor fan and we irradiated each culture with a 980-nm continuous wave diode laser (Wiser Doctor Smile, Lambda™) every 48 h for 10 days. Laser energy was delivered through a particular hand-piece (Onda Piana, Lambda™) that keeps energy properties unaltered in a variable distance from 0 to 100 cm, and delivers the same amount of energy in every point in a 1 $cm^2$ area. The hand-piece was perpendicularly stabilized by means of a mechanical arm at a distance of 1 cm from the culture surface, and the laser device was set with the following parameters: output power 1 Watt, exposure time 50 s, energy density 50 $J/cm^2$ (Fig. 2). During the irradiation phase of this work, we changed the culture medium of both groups every 3 days.

Forty-eight h after the last irradiation, we collected data from the cultures of both groups by using flow cytofluorimetry (FACS) and western blotting. Using the analyses we estimated presence and quantity of keratin 5 and 8 using monoclonal antibodies reactive to cytokeratins 5 and 8 (KRT 5/8, Antibodies-online™). FACS analysis data was statistically processed with t-student analysis (95% confidence level).

## RESULTS

Data obtained with FACS analysis show a low positivity to keratin 5-8 of fibroblast cultures of both test and control groups (1.5% and 0.5%, respectively), as expected. Both keratinocytes and organotypic cultures showed a statistically significantly difference of keratin 5-8 expression between test and control group ($p=0.0007$ and $p<0.0001$, respectively) in favor of the control group, while on the other hand, it showed an increase of positive cells in the irradiated group in both keratinocytes (84.2% in the test group and 82.9% in the control group) and organotypic cultures (6.1% in test group and 4.7% in control group) (Fig. 3).

Data obtained with Western blotting analysis showed a decrease in keratin 5 expression of irradiated keratinocytes cultures, but an increase in keratin 8 expression of the same cultures. This analysis also showed a small increase of keratin 5 expression of the organotypic cultures of the irradiated group, and a high increase of keratin 8 expression of the same cultures (Fig. 4, 5).

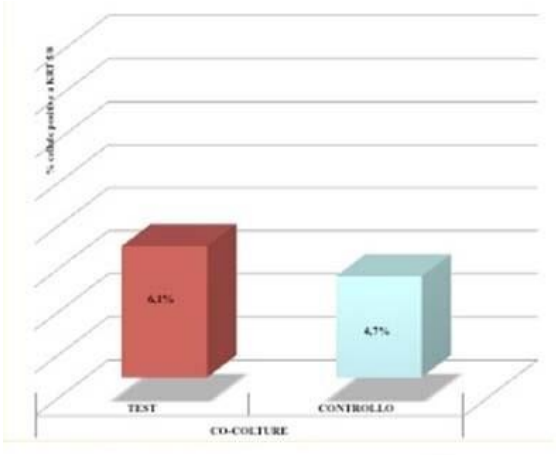


**Fig. 3.** *Example of FACS analysis in test group organotypic cultures.*

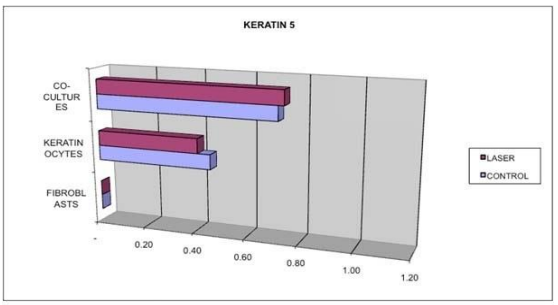


**Fig. 4.** *Western blotting analysis of keratin-5 expression.*

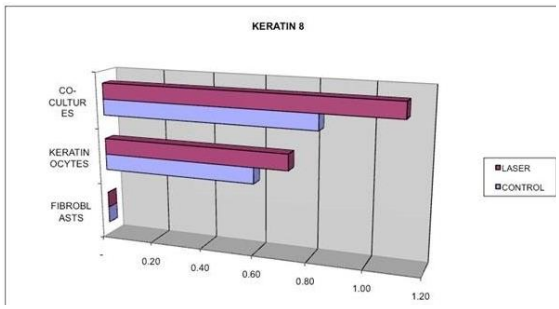


**Fig. 5.** *Western blotting analysis of keratin-8 expression.*

## DISCUSSION

Studies that examine laser photobiomodulation applied to cell cultures are widely present in international literature (18-26). The energy density used in these studies is on the average 5 $J/cm^2$. It has been observed that a higher energy value density applied to cell cultures may lead to an inhibition in terms of cell vitality and proliferation, leading ultimately to cell death. However, we decided to use a relatively high energy density (50 $J/cm^2$) with repeated applications (every 48 h for 10 days).

The base hypothesis involves a sufficiently strong stimulus to push cells to increase the production of keratins, similar to the phenomenon of epidermal keratosis following repeated and constant traumatic events which lead to the formation of palmar or plantar callus. The possible periodontal clinical application is represented by the chance to increase the volume of the gingival keratinized layer around dental elements and/or implants.

The international literature is rich in studies that have evaluated laser photobiomodulation on cell cultures (27-31). These studies evaluate the effects of laser photobiomodulation in terms of vitality, proliferation, adhesion, migration, growth

factors and protein synthesis with an application range of laser energy density of 0.12 to 20 J/cm$^2$. A literature review did not however enlighten to the effects of laser photobiomodulation on keratin production by either fibroblasts and/or keratinocytes. Despite using significantly higher energy density values than those suggested in international literature, we managed to maintain cell survival in all of our specimens. This was possible using a particular hand-piece assembled to the diode laser that lead to an optimal energy absorption of the cell cultures avoiding inhibitory or apoptotic effects that are often described when using an energy density higher than 20 J/cm$^2$.

We had an increase of the keratin-positive cell rate in test group culture compared to non-biostimulated cultures, both for keratinocyte and organotypic cultures while there was a decrease of the total positivity to keratin in the test group specimens. We could conclude that laser photobiomodulation caused a reduction of the number of cells expressing keratin, leading therefore to a minor total expression of keratin as observed through FACS analyses, but an increase of keratin-positive cells in relation with the total population of the test group specimens. Therefore, laser photobiomodulation was not able to obtain an increase of the total amount of keratin in the treated cultures, but stimulated a higher number of cells to synthesize keratin, compared to non-treated group.

The results of Western blotting analyses lead mainly to two conclusions: a decrease of keratin-5 expression was observed in the keratinocyte test group, a small increase of both keratin-8 expression of keratinocyte and keratin 5 in the organotypic cultures in the test group and a great increase of keratin-8 expression in the organotypic cultures in the test group. We can conclude that the difference in keratin expression of the two group specimens (i.e. a decrease of total expression in the test group) as shown by FACS analyses, may be explained by the fact that the keratinocytes used in this study are able to produce less keratin 5 than keratin 8 and that on the other hand, had a strong increase following laser photobiomodulation as shown by Western blotting analysis.

## CONCLUSIONS

In conclusion, the increase of both keratin-5 and -8 in treated organotypic cultures suggest that a synergy between laser photobiomodulation and the mesenchymal compartment represented by fibroblasts is crucial to stimulate a higher synthesis of keratin in the test group organotypic cultures.

*Author Contributions*

G.C. designed the research study. G.C. performed the research. P.C. and G.C. wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

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*Conflict of Interest*

The authors declare no conflict of interest.

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