

## ***In vitro* effect of 470 nm LED (Light Emitting Diode) in keloid fibroblasts<sup>1</sup>**

Efeito *in vitro* do LED (*Light Emitting Diode*) de 470 nm em fibroblastos de quelóide

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### ABSTRACT

**Purpose:** To quantify keloid fibroblasts after irradiation with 470nm blue LED, *in vitro*. **Methods:** Fibroblasts from keloid and adjacent skin have been obtained from 6 patients. Cells have been cultivated and maintained in DMEM culture medium. In Petri dishes, they were irradiated with energy doses of 6J, 12J and 18J. After 24 h, counting was done by the average of the triplicates for each sample. **Results:** There were no significant differences in the number of irradiated keloid fibroblasts at the studied doses ( $p=0.261$ ). In adjacent skin fibroblasts, differences were observed ( $p=0.025$ ) concerning the doses of 18 J and 6 J ( $p=0.03$ ). **Conclusions:** There was a reduction in the number of adjacent skin fibroblasts irradiated with 470nm blue LED at the energy dose of 18 J compared to the ones irradiated at the energy dose of 6 J. There were no changes in keloid fibroblasts counting at any of the doses applied, 24 h after irradiation.

**Key words:** Fibroblasts. Keloid. Phototherapy. Lasers. Skin. Laser Therapy, Low-Level.

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### RESUMO

**Objetivo:** Quantificar fibroblastos de quelóide após irradiação com LED azul de 470nm, *in vitro*. **Métodos:** Foram obtidos fibroblastos de quelóide e pele adjacente, de seis pacientes. As células foram cultivadas e mantidas em meio de cultura DMEM. Em placas de Petri, receberam irradiação com doses de energia de 6J, 12J e 18J. Após 24 horas a contagem foi feita pela média da triplicata para cada amostra. **Resultados:** Não houve diferença na quantidade de fibroblastos de quelóide irradiados nas doses estudadas ( $p=0,261$ ). Observou-se diferença nos fibroblastos de pele adjacente ( $p=0,025$ ), com relação às doses de 18 J e 6 J ( $p=0,03$ ). **Conclusões:** Houve redução dos fibroblastos de pele adjacente irradiados com LED azul de 470 nm na dose de energia de 18 J em relação à dose de 6 J. Não houve alteração na quantidade de fibroblastos de quelóide nas doses aplicadas após 24 horas da irradiação.

**Descritores:** Fibroblastos. Quelóide. Fototerapia. Lasers. Pele. Terapia a Laser de Baixa Intensidade.

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### Introduction

Keloid is a wound repair disorder resulting from excessive collagen deposition, which occurs in susceptible individuals, and its pathophysiology has not yet been fully elucidated<sup>1-3</sup>. The literature presents many options of treatment, alone or in combination, nevertheless they are not specific. Among the therapeutic modalities that have been most used, stand out the administration of topical and intralesional

corticosteroids, the use of silicon plates, pressotherapy and exeresis followed by betatherapy<sup>4,5</sup>.

Recently, high potency LASER (Light Amplification by Stimulated Emission of Radiation) and IPL (Intense Pulsed Light) were added to that arsenal. However, these resources of phototherapy have high cost and relative success, besides the fact that the applications are painful and involve risks of burns and hypochromia<sup>6,7</sup>.

Low-level LASER therapy (LLLT) is a modality of treatment that is being used for the improvement of impaired wound healing due to its biomodulatory character, with major action in mitochondrial metabolism<sup>8</sup>. In this sense, nowadays, LED (Light Emitting Diode) appears as an alternative to LLLT and differentiates itself by not showing collimated and coherent beam. However, both lasertherapy and LED may have similar biological effects due to absorption of photons by tissue chromophores. Such resources are already being used in many skin diseases like acne<sup>9</sup>, in which blue light exerts anti-bacterial effect stimulating bacterial membrane chromophores from *Propionum acnes* and releasing reactive oxygen species. Phototherapy is also used in tissue repair treatments, acting on fibroblasts proliferation<sup>10</sup>, collagen synthesis<sup>11</sup> and neovascularization<sup>12</sup>.

*In vitro* studies with red<sup>13</sup> and infrared LASER<sup>14,15</sup> showed that the highest doses investigated inhibited fibroblast proliferation. In hypertrophic scars and skin fibroblasts, *in vitro*, it was demonstrated that low intensity LASER with wavelength 680 nm (red) and energy (E) 0.5 J (31 s) and 0.8 J (52 s) had a stimulatory effect on both cell counts, with a more pronounced result obtained with the lowest dose used<sup>13</sup>. On the other hand, 880 nm infrared LASER at doses of 0.71 J (44.5 s) and 1.18 J (74 s) resulted in reduction of cell proliferation in both skin and hypertrophic scar fibroblasts, noting that the second dose led to a greater inhibition<sup>14</sup>.

Experimental studies with LED, in wavelengths corresponding to green and blue, reported effects in fibroblasts. In chicken embryos, it was observed that irradiation with green spectrum (532 nm) at a dose of 0.90 J, for 1 s caused an increase in the number of fibroblasts<sup>16</sup>, compared with the group of cells that was not irradiated. In blue spectrum of 470 nm, irradiation with LED for 180 s inhibited proliferation of gingival fibroblasts<sup>17</sup>. However, no studies were found using LED in blue spectrum, in keloid fibroblasts, which could be valuable as a prevention or treatment of this disorder. Therefore, the objective of this study was to quantify keloid fibroblasts after irradiation with 470 nm blue LED, *in vitro*.

## Methods

This study was approved by the Research Ethics Committee of the Federal University of Sao Paulo and the discarded parts were used after all patients signed a free and informed consent form.

### Collection of keloid samples

Keloid samples used were obtained from six female patients aged 18-50 years and with Fitzpatrick skin types II to VI. The scars had at least 1-year evolution, were in clinical activity, presenting hyperemia, pruritus and/or pain, and also central involution (*i.e.* a lower relief in the central compared with the peripheral area) and with minimum dimensions 3 cm at the longitudinal axis and 2 cm at the transversal axis. Keloid location was delimited in the thoracic region, between a transverse plane at the level of the acromion and a transverse plane at the level

of xiphoid process<sup>3</sup>. Were excluded keloids previously treated or patients with chronic dermatopathies, metabolic, collagen or degenerative / auto-immune diseases, malignant neoplasms, or patients submitted to systemic or topic treatment with corticosteroids.

Keloids were excised in the subcutaneous plane, under local anesthesia, with exceeding skin fragments represented by the "dog ears". After exeresis of the keloid lesions, samples were obtained from adjacent skin and peripheral keloid (between the excisional margin and the central umbilication) of the surgical specimens using a circular punch of 3 mm diameter, from the epithelialized surface of tissue containing epithelium and connective tissue<sup>18</sup>.

### Obtainment of the fibroblast cultures

Primary keloid fibroblast culture was done by explant<sup>19,20</sup> by which the fractions corresponding to samples of adjacent skin and keloid were obtained. Fractions were placed in 15 ml conic tubes and exhaustively rinsed (six times) with 10 ml PBS (Phosphate-Buffered Saline, Cultilab, Brazil) containing penicillin (100UI/ml, Gibco®) and streptomycin (100µ/ml, Gibco®) under vigorous agitation, changing tubes and PBS at each repetition. Then, fractions were transferred to 60 mm<sup>2</sup> diameter Petri dishes, in grid areas scratched with a scalpel, and plates were left semi-opened in the laminar flow for 30 min, for the fragments to adhere to its surface. Then, 6 ml of DMEM (Dulbecco's Modified Eagle's Medium, Cultilab, Brazil) supplemented with 15% FBS (Fetal Bovine Serum, Cultilab, Brazil), penicillin (100 UI/ml, Gibco®) and streptomycin (100 µg/ml, Gibco®) were carefully added to each plate. Plates were kept in humidified incubator at 37°C, 95% O<sub>2</sub> and 5% CO<sub>2</sub>.

Culture medium was changed every two days and a few days after establishing the primary culture, spindle-like cells were seen proliferating from the edges of the explanted tissue, regarded as culturing fibroblasts. Fibroblast satisfactory proliferation was observed in approximately 7-14 days and subculturing (passage) was performed when cellular confluence reached approximately 80% at the Petri dish.

For subculturing, the culture medium was aspirated and the keloid fragments, discarded with tweezers. The plate containing fibroblasts was washed with PBS, and then quickly rinsed with Versene™ (PBS with 0.05M EDTA, Ethylene Diamine Tetra Acetic, Sigma®). Versene™ was aspirated and 1 ml of trypsin 0.25% with EDTA 0.02% was added to the plate. The plate was kept for 2 min in the incubator and taken to the microscope to confirm fibroblast detachment from the dish surface. Trypsin was neutralized with 3.0 ml DMEM 10% FBS and the cellular suspension was centrifuged (100 g, 6 min). The pellet was resuspended in DMEM 10% FBS and antibiotics (penicillin 100 UI/ml and streptomycin 100 µg/ml) and 100,000 cells were seeded in each 75 cm<sup>2</sup> culture flask. For the experiments, cells were seeded in 30 mm<sup>2</sup> diameter (10 mm height) Petri dishes, in triplicates. In the first sample, 2x10<sup>5</sup> cells were seeded per plate and, for better confluence, in all other samples, 5x10<sup>4</sup> cells were seeded per plate, always in triplicates. Before irradiation, cells were kept for 48 h in the incubator, at 37°C.

Cell irradiation

The equipment Quasar Esthetique® was used for cell irradiation. The device has a handpiece with LEDs, covering the blue spectrum (470 nm) with 100 mW power, 125 mW/cm<sup>2</sup> power density, optical fiber diameter 1 cm and beam area of 0.8 cm<sup>2</sup>.

The samples of keloid and adjacent skin fibroblasts were divided into groups according to the total energy supplied: Control Group, received no irradiation; Group 1 min, received 6 J total energy (E) and energy density (ED) 59.87 J/cm<sup>2</sup>, Group 2 min received 12 J (E) and (ED) 122.3 J/cm<sup>2</sup>, Group 3 min, received 18 J (E), and (ED) 183.43 J/cm<sup>2</sup> (Table 1).

TABLE 1 – Irradiation parameters

Group	Control	1 min	2 min	3 min
<b>ED (J/cm<sup>2</sup>)</b>	0	59.87	122.30	183.43
<b>E (J)</b>	0	6 J	12 J	18 J
<b>t (s)</b>	0	60	120	180

ED: energy density, E: energy; t: time

The device tip was fixed at a distance of 2 cm in height from the plate's bottom. The culture medium was aspirated for 3 min in all groups, for homogenization of the samples. Irradiation was performed with the LED's collimator perpendicular to the Petri dish, which was placed on top of a black base (Figure 1).

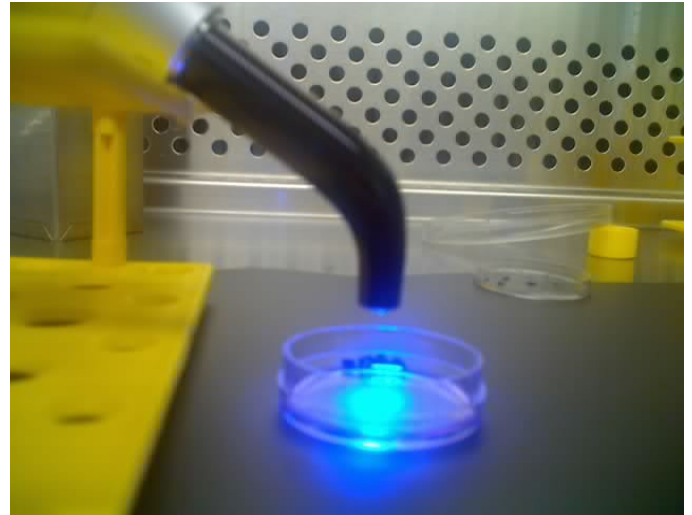


FIGURE 1 – Cell irradiation with 470 nm blue LED.

After irradiation, cells were put back in incubator for another 24 h. After this period, cell cultures were prepared for counting in Neubauer chamber. Each sample (adjacent skin and keloid fibroblasts) was counted three times, using a manual cell counter, and data were expressed as the average of the triplicates. Statistical significance was set at p<0.05.

Results

The values obtained after cell counting are shown in table 2. With the Kolmogorov-Smirnov test, it was verified that the samples did not show normal distribution (p=0.016).

TABLE 2 – Number of keloid and adjacent skin fibroblasts according to the dose, 24 h after irradiation (each value is the arithmetic mean of each triplicate culture)

Group	Control		1 min		2 min		3 min	
Energy	0		6 J		12 J		18 J	
	K	AS	K	AS	K	AS	K	AS
	Number of cells (triplicate mean)							
Sample 1	3.5 x 10 <sup>4</sup>	7.5 x 10 <sup>4</sup>	3 x 10 <sup>4</sup>	7 x 10 <sup>4</sup>	4.5 x 10 <sup>4</sup>	4.5 x 10 <sup>4</sup>	2 x 10 <sup>4</sup>	5 x 10 <sup>4</sup>
Sample 2	30 x 10 <sup>4</sup>	12 x 10 <sup>4</sup>	28.33 x 10 <sup>4</sup>	25 x 10 <sup>4</sup>	26.67 x 10 <sup>4</sup>	26 x 10 <sup>4</sup>	23.67 x 10 <sup>4</sup>	23 x 10 <sup>4</sup>
Sample 3	14 x 10 <sup>4</sup>	11.75 x 10 <sup>4</sup>	13.5 x 10 <sup>4</sup>	12.25 x 10 <sup>4</sup>	12.25 x 10 <sup>4</sup>	8.4 x 10 <sup>4</sup>	13.13 x 10 <sup>4</sup>	7.95 x 10 <sup>4</sup>
Sample 4	15 x 10 <sup>4</sup>	7.88 x 10 <sup>4</sup>	14.5 x 10 <sup>4</sup>	8.88 x 10 <sup>4</sup>	9.5 x 10 <sup>4</sup>	8.75 x 10 <sup>4</sup>	12 x 10 <sup>4</sup>	7.88 x 10 <sup>4</sup>
Sample 5	10.96 x 10 <sup>4</sup>	10.42 x 10 <sup>4</sup>	11.13 x 10 <sup>4</sup>	10.83 x 10 <sup>4</sup>	12.25 x 10 <sup>4</sup>	10.5 x 10 <sup>4</sup>	11.58 x 10 <sup>4</sup>	10.21 x 10 <sup>4</sup>
Sample 6	8.21 x 10 <sup>4</sup>	11 x 10 <sup>4</sup>	8.42 x 10 <sup>4</sup>	11.54 x 10 <sup>4</sup>	5.88 x 10 <sup>4</sup>	10.17 x 10 <sup>4</sup>	6.71 x 10 <sup>4</sup>	7.50 x 10 <sup>4</sup>

K: Keloid; AS: Adjacent Skin; 0: Control

Friedman's test showed no differences on the keloid fibroblasts amounts at the energy doses applied ( $p=0.261$ ) compared to the control group. However, the same test showed that there was a difference in the amounts of skin fibroblasts irradiated with 18 J (3 min) according to the energy doses supplied ( $p=0.025$ ), compared to the control group. Wilcoxon's test revealed that adjacent skin fibroblasts irradiated with 18 J

(3 min) presented a reduction of its total amount when compared to adjacent skin fibroblasts irradiated with 6 J (1 min) ( $p=0.03$ ) although there wasn't, in the control group, any significant difference on the number of irradiated cells between the groups irradiated with 18 J ( $p=0.5$ ) and those irradiated with 6 J ( $p=0.09$ ) (Table 3).

TABLE 3 – Comparison of energy doses applied on groups of adjacent skin fibroblasts (AS)

Energy Doses on AS	6 J x 0 J	12 J x 0 J	18 J x 0 J	12 J x 6 J	18 J x 6 J	18 J x 12 J
Significance Level (p)	0.09	0.92	0.5	0.12	0.03	0.12

AS: Adjacent Skin; 0 J: control; 6 J: 1 min; 12 J: 2 min; 18 J: 3 min

## Discussion

Nowadays, some alternative therapies, such as Pumped Dye Laser<sup>6</sup> and IPL (Intense Pulsed Light)<sup>7</sup>, are being used in keloid. Success has been relative concerning reduction of scar height and scar lightening, yet it involves risks such as dyschromia, burns and discomfort during application, as those are high potency equipments. However, in the arsenal of phototherapy there are LLLT, widely used in tissue repair process<sup>11</sup> and LED, recently investigated as a low cost, alternative source of light<sup>12</sup>. However, there are still no researches with LED and fibroproliferative scars, a fact that motivated the present study.

It is known that the mechanism of action of low intensity phototherapy depends on the wavelength used and on the total energy supplied, which directly affects mitochondrial metabolism, acting on the chromophores of the respiratory chain<sup>8</sup>. Wavelengths above 500 nm are widely used to stimulate wound healing, specially red and infrared bands<sup>11</sup>. In the blue spectral region, flavoproteins such as NADH dehydrogenase and succinate dehydrogenase, and also porphyrins can work as photoacceptors<sup>8,21</sup>. This enzyme acts in the oxidation of ketone bodies and its suppression leads to a pH decrease, which can lead to cell death (apoptosis)<sup>21</sup>. In the researched literature, the studies found, concerning blue spectrum, reported the inhibition of cell proliferation or no change in fibroblasts<sup>17</sup>. In addition, this wavelength also exerts an inhibitory effect on acne<sup>9</sup>, characterized by neurogenic inflammation, which is exacerbated in keloid<sup>5</sup>.

*In vitro* studies with LASER or LED often use well plates, in which one well is next to the other, eventually dissipating energy to each other. In this study, cells were irradiated in Petri dishes, to ensure that light scattering from one dish wouldn't interfere with the cells in the other ones. Besides that, a black base was used under the Petri dishes to make sure that light wouldn't be reflected. The culture medium was also aspirated prior to irradiation, to avoid the light to be refracted before reaching the cell layer on the bottom of the Petri dish. These measures were adopted to ensure the best possible use of the energy supplied and

to keep each sample of cells completely isolated from the others. There is no consensus concerning the distance from the light source to the cell layer. Therefore, to avoid contact of the LED's device tip with the cell layer, we standardized in this study the distance of 2 cm height from the plate's bottom, considering that the plate's border is 1 cm height and leaving the device tip 1 cm higher, as a safety margin.

Wataha *et al.*<sup>22</sup>, irradiated gingival fibroblasts, with LED between 400 and 500 nm (blue), with ED 60 J/cm<sup>2</sup> for 60 s and verified, after 24 h, suppression of the enzyme succinate dehydrogenase and as a consequence, inhibition of cell proliferation. One of the exposure times used in the present study was 60 s, with energy similar (59.87 J/cm<sup>2</sup>) to that used by Wataha *et al.*<sup>22</sup>; nevertheless there was no significant changes in the number of keloid or skin cells. On the other hand, Taoufik *et al.*<sup>17</sup> irradiated gingival fibroblasts with a wavelength of 470 nm for 180 s, which was also similar to our study, and they performed counting after 24 h and 1 week. After 24 h, they didn't detect any changes, corroborating with the present study, when compared to keloid fibroblasts which also didn't show any changes with the same irradiation times and counting. However, those authors' data confront the results obtained here in this study with adjacent skin fibroblasts, since the cell counting performed 24 h after irradiation for 180 s (18 J) showed a reduction in the number of cells compared to the group irradiated for 60 s (6 J). Taoufik *et al.*<sup>17</sup> observed inhibition of gingival fibroblasts irradiated for 180 s, after a week. Since Wataha *et al.*<sup>22</sup> found cell alterations as early as 24 h after light exposure, and Taoufik *et al.*<sup>17</sup>, after seven days, it leaves opened the perspective to perform cell counting with different intervals of time so that it could be checked the inhibitory effect on cell growth, depending not only on the dose applied, but also on the interval of time. Both cited studies did not provide sufficient data to calculate the total energy supplied, which would be ideal to compare with our study. However, exposure times were similar: 60 s<sup>22</sup> and 180 s<sup>17</sup>. It is necessary a

better standardization of data provided by phototherapy studies, such as time, energy density, beam area and power for greater reliability when comparing, for example, the energy doses used in different studies.

Webb and Dyson<sup>13</sup> irradiated hypertrophic scars and skin fibroblasts, using LLLT at a wavelength of 660 nm with energies of 0.52 J (31 s) and 0.8 J (52 s), and performed differential counting during 5 consecutive days. They observed an increase of the cell numbers in all counting periods, comparing to the groups of non-irradiated cells. This is an expected result when using red spectrum, which increases mitochondrial activity and ATP synthesis in respiratory chain<sup>8</sup>. Even though, despite the wavelength, Webb and Dyson<sup>13</sup> irradiated hypertrophic scar fibroblasts using the same conditions of cultivation and preservation of cells and obtained results after one day of irradiation, the present study was performed under similar conditions of cultivation, irradiation and counting, but there was no change in the number of keloid or skin fibroblasts regardless of the dose used.

After that, Webb *et al.*<sup>14</sup> investigated the effect of 880 nm infrared LASER in hypertrophic scar and skin fibroblasts at doses of 0.71 J (44.5 s) and 1.18 J (74 s). 24 hours after irradiation, they didn't find any difference in cell counts compared with the controls. However, from the second day on, they reported a reduction in the number of cells of both skin and hypertrophic scar. It can be confirmed at the literature that high doses used in *in vitro* experiments ranging from 1.19 J<sup>14</sup> to 145.2 J<sup>15</sup>, especially with infrared, cause inhibition of cell proliferation<sup>15</sup>. As a result, some authors suggest this wavelength as a possible alternative for the treatment of hypertrophic scars<sup>14</sup>. In the present study, in addition to the different wavelength, the doses used (6 J, 12 J and 18 J) were higher than those used by Webb *et al.*<sup>14</sup> and applied in keloid cells. Nevertheless, it is worth noting that there is a consensus nowadays in literature<sup>3,23</sup> that keloid fibroblasts and hypertrophic scars fibroblasts have the same pathophysiology, *i.e.*, they would be both fibroproliferative scars with different expressions. Webb *et al.*<sup>14</sup>, using infrared laser observed, after 48 h, a reduction on the cell number with a smaller energy and time of exposure and thus, differently from this study, using blue led, which observed no change in the keloid fibroblast number, regardless of the dose of energy supplied. On the other hand, the skin fibroblasts response goes towards those authors' findings, since the greater dose used caused cell inhibition.

It becomes essential to have more studies comparing the effects on keloid and hypertrophic scar fibroblasts, in the infrared and blue spectra, since both are the most frequently reported, according to the literature, as potential inhibitors of mitochondrial metabolism, especially in high doses. Another perspective with blue LED would be its application in preoperative and postoperative groups in experimental studies, to verify its action in the wound healing process, and more specifically in the neurogenic inflammation phase, and thus be able to raise hypothesis of its use in clinical trials in the prevention of fibroproliferative scars.

Even more, the fact that this study verified a reduction in the skin fibroblasts counting, might open the possibility of applying pre-operative blue LED on the adjacent skin of patients undergoing excision of fibroproliferative scars.

Shi *et al.*<sup>24</sup> recently demonstrated that the metabolic activity of keloid fibroblasts is increased when compared to skin fibroblasts, and that their rate of apoptosis is lower. This could explain why keloid fibroblasts didn't show any changes after being irradiated with blue LED at the doses used in this study. Perhaps, with doses above 18 J, keloid fibroblasts could have showed some inhibitory response, similar to those observed in skin fibroblasts.

## Conclusions

This study demonstrated that a single *in vitro* application of 470 nm blue LED caused, after 24 h, a decrease in the total amount of adjacent skin fibroblasts at a dose of 18 J when compared to a dose of 6 J. However, at the doses tested, there were no significant differences in the amount of keloid fibroblasts.

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