

# Immunohistochemical analysis of photodamaged skin after treatment with delta 5-aminolevulinic acid

## Análise imunohistoquímica da pele fotoenvelhecida após tratamento com o ácido 5 delta aminolevulinico

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Received on: 14/06/2011  
Approved on: 15/12/2011

This study was carried out at the Hospital das Clínicas da Universidade de São Paulo (USP) - São Paulo (SP), Brazil.

Financial support: None  
Conflicts of interest: None

### ABSTRACT

**Introduction:** Photodynamic therapy destroys target cells by directly harming them, injuring the vascular stroma, and activating the immune system.

**Objective:** To study photoaged skin's immune response to photodynamic therapy.

**Methods:** Thirteen female patients underwent three treatment sessions with delta 5-aminolevulinic acid combined with 630 nm diode light. The immunohistochemical response of the skin's immune system was evaluated using the markers anti-CD1a, anti-CD4+, anti-CD8+, anti-TNF $\alpha$ , anti-IFN $\gamma$  and anti-IL4 before, 24 hours after the first session, and 21 days after the third session.

**Results:** At the final evaluation, patients demonstrated a decrease in CD4 and CD8 lymphocytes, an increase in interleukin-4 in the epidermis and dermis, and a reduction and an increase of TNF- $\alpha$  in the epidermis and dermis, respectively. The Langerhans cell and INF- $\gamma$  populations remained unchanged.

**Conclusion:** Regarding the immune system, photodynamic therapy did not cause changes in Langerhans cells; therefore, there was little local immunosuppression. CD4 and CD8 lymphocyte apoptosis was observed, suggesting that photodynamic therapy can be useful in treating inflammatory diseases. The changes in TNF- $\alpha$  and an increase of IL4 in the dermis and epidermis suggest a humoral type response, which was also evidenced by the absence of INF $\gamma$ .

**Keywords:** photochemotherapy; aminolevulinic acid; skin aging.

### RESUMO

**Introdução:** O mecanismo de destruição da célula alvo na terapia fotodinâmica compreende a destruição celular direta, a injúria ao estroma vascular e a ativação do sistema imune.

**Objetivo:** Estudar a resposta imune da pele humana fotoenvelhecida ao tratamento com terapia fotodinâmica.

**Métodos:** Treze pacientes femininas foram submetidas a três sessões de tratamento com o ácido 5 delta aminolevulinico associado à luz de diodo de 630nm. Foi avaliada a resposta imunohistoquímica do sistema imunológico da pele por meio dos marcadores: anti-CD1a, anti-CD4+, anti-CD8+, anti-TNF $\alpha$ , anti-IFN $\gamma$  e anti-IL4; antes, 24 horas após a primeira sessão e 21 dias após a terceira sessão. Os dados foram analisados por meio de estatística descritiva: média, desvio padrão, valor mínimo e máximo, e mediana.

**Resultados:** 21 dias após o último tratamento houve diminuição do número de linfócitos CD4 e CD8, aumento de interleucina 4 na epiderme e derme, diminuição do TNF- $\alpha$  na epiderme e aumento na derme. A população de células Langerhans e do INF- $\gamma$  não mudou.

**Conclusão:** Em relação ao sistema imune esta terapia não mostrou alteração das células de Langerhans e, por isso, pouca imunossupressão local. A apoptose dos linfócitos CD4 e CD8 foi evidenciada sugerindo que a terapia fotodinâmica possa ser útil em doenças inflamatórias. O estudo mostrou diminuição do TNF $\alpha$  na epiderme e aumento na derme além do aumento da IL4 em ambas, o que sugere resposta do tipo humoral neste protocolo, o que também pode ser evidenciado pela ausência do INF $\gamma$ .

**Palavras-chave:** fotoquimioterapia; ácido aminolevulinico; envelhecimento da pele.

Photodynamic therapy (PDT) is currently used to treat inflammatory diseases such as psoriasis, acne, sebaceous hyperplasia, and rosacea – and, more recently, photoaging. In addition, it has applications in the treatment of tumors.<sup>1-7</sup> PDT involves accumulating a specific photosensitizer agent in the target tissue, which is then activated by light. In the presence of oxygen, this process produces the singlet oxygen, which damages the target cells through direct cell destruction, injury to the vascular stroma, and activation of the immune system.<sup>1</sup> The role of PDT is to cause a cytotoxic reaction in the tissue with a delayed response (post-PDT), triggering an inflammatory immune response, which is innate and adaptive, to assist in the successful eradication of remaining residual cells.<sup>8</sup> Despite the lesser importance of the immune response compared to other effects in the cell ablation stage, this characteristic seems to be critical in maintaining the effects of the therapy over the long term.<sup>9</sup>

High-intensity PDT destroys important immune cells, while low-intensity PDT stimulates the immune system. On the one hand, this process takes place through the activation of monocytes and macrophages, and through the production of inflammatory mediators. On the other hand, it is caused by the massive attraction of neutrophils to the inflammation site, which stimulates the production of specific mediators.<sup>8,9</sup>

In the treatment of tumors, the main factors that seem to be involved in the induction of that response are the expression of several cytokines and other immunologically important genes. Among the cytokines, the expression of which is reportedly modulated by PDT, the most important are IL6, IL10 and TNF $\alpha$ , in addition to IL1 $\beta$ , IL2, the granulocyte colony stimulating factor, the epidermal growth factor, and the modulation of the expression of various genes involved in cellular adhesion and the presentation of antigens.<sup>8,9</sup>

## METHODS

Women ( $n = 13$ ) aged 50–78 (mean = 64) were prospectively studied. The inclusion criteria were females with phototypes I to IV<sup>10</sup> and Glogau photoaging classification Grade I–IV<sup>11</sup>. The exclusion criteria were: use of an abrasive method or keratolytic substance in the past month, systemic diseases and recent surgery, history of keloid formation and/or hypertrophic scarring, skin cancer, personal or family history of melanoma, and use of photosensitizers or immunosuppressive medications. The patients signed a term of free and informed consent, and the study was conducted according to the Declaration of Helsinki 2000 guidelines.

The patients underwent a biopsy in the right preauricular region, which was meticulously measured from the beginning to the end of the ear lobe and divided into 3 parts, where each biopsy was performed. The pre-procedure biopsy was carried out in the first region; 24 hours after the first session, a control biopsy was carried out in the second region; 21 days later, a control biopsy was carried out in the third region.

Three PDT sessions were administered at 15-day intervals. A control picture was taken 24 hours after the first application and a biopsy was carried out 24 hours after the second

application. Twenty-one days after the third session, a photographic control and biopsy were carried out in the third region. The face was first cleansed with alcohol, and 20% 5-delta-aminolevulinic acid (ALA) was applied evenly with the Levulan® Kerastick™ application stick (Laboratórios Stiefel, Guarulhos, São Paulo, Brazil). Two hours later, the ALA was irradiated with LED (light emitting diode) equipment-based light, with an output intensity of 3,100 mW/cm<sup>2</sup>, an optical intensity of 100 mW/cm<sup>2</sup>, and a 40x80 mm active surface emitting light at the 630 nm wavelength for 10 minutes on each side of the face. The patients were informed about possible side effects, instructed to use sunscreen, prohibited from using other chemical products without prior authorization, instructed not to remove peeling skin, and were required to return on preset dates.

The pre-procedure, 24 hours post-procedure and 21 days post-procedure histological samples of the patients' skin were embedded in paraffin wax and submitted to immunohistochemical reaction analysis using the avidin-biotin peroxidase complex method<sup>12</sup>. This method involves incubating primary antibodies at the following dilutions: 1/20 CD1, 1/400 CD4, and 1/50 CD8 (DAKO). The other antibodies used were: 1/30 IFN $\gamma$  (RD Systems – Code MAB285), 1/20 IL4 (RD System – Code AF-204-NA), and 1/20 TNF (RD System – Code AF-210-NA).

The immunohistochemistry analysis was conducted using a Zeiss light microscope with a 20x objective lens and 10x eyepiece. The quantitative evaluation (calculation of area, length, and particle count) was performed using the Image Analyzer System (Kontron Eletronic 300, Zeiss). All samples were evaluated by two independent researchers. The histomorphometric quantitative data regarding the number of CD1 positive cells and the semi-quantitative data from the inflammatory response – from the CD4 and CD8 positive cells, and from the IL4, IFN $\gamma$ , and TNF $\alpha$  positive reactions – were analyzed using descriptive statistics (mean, standard deviation, minimum and maximum, and median). The statistical tests were performed using the SigmaStat software (Jandel Cientific, CA, USA), with a significance level of  $p < 0.05$ .

## RESULTS

Thirteen patients with facial photodamage received treatment with PDT and topical ALA. According to Glogau's classification,<sup>11</sup> six patients (46.15%) (patients 1, 4, 9, 10, 11, and 12) had grade III photoaging, five (38.46%) (patients 2, 3, 5, 6, and 13) had grade IV, and two (15.38%) (patients 7 and 8) had grade II. Two patients (15.38%) (patients 2 and 5) were classified as Fitzpatrick phototype I, five (38.46%) (patients 1, 3, 6, 9, and 12) presented phototype II, five (38.46%) (patients 4, 7, 10, 11, and 13) had phototype III, and one (7.69%) (patient 8) presented phototype IV.

The histological, immunohistochemical, and morphometric analyses' results of the immune system were evaluated in only 12 patients due to technical problems with the biopsy. The immunohistochemistry analysis of the histological sections for anti-CD8 revealed that CD8 positive mononuclear inflamma-

tory cells were present around blood vessels and annexes in the dermis. (Figures 1A, 1B). The morphometric analysis was semi-quantitative, and the individual results of each patient were studied.

Comparing the three time points evaluated in the same treatment (before, 24 hours after, and 21 days after the third session) using the Kruskal-Wallis nonparametric statistical test of variance analysis, a significant difference was observed ( $H = 10.185$ ,  $p = 0.006$ ). To better specify the differences, the Dunn's multiple comparison method was applied, revealing a highly significant reduction of CD8 cells after treatment ( $Q = 2.68$ ,  $p < 0.05$ ) (Graph 1).

Regarding the CD4 antibody (used to demonstrate lymphocytes), the analysis of histological samples submitted to immunohistochemistry reaction to anti-CD4 showed that CD4 positive mononuclear inflammatory cells were present around blood vessels and annexes in the dermis. The morphometric analysis was semi-quantitative.

Comparing the three time points studied for the same treatment regarding CD4+ cells in the dermis, the Kruskal-Wallis nonparametric statistical test of variance analysis found no significant differences ( $H = 4.821$ ,  $p = 0.090$ ).

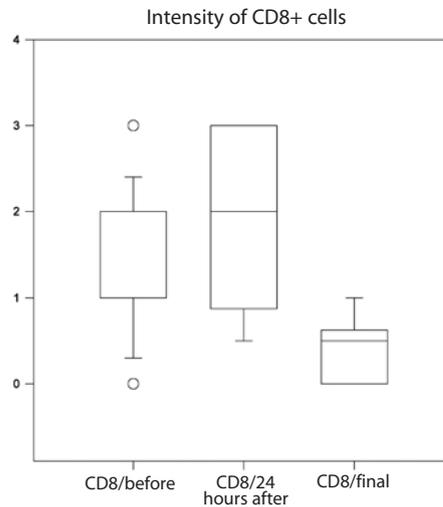
Regarding the antibody CD1 (which indicates the presence of Langerhans cells), Langerhans cells were present in the skin 24 hours after the PDT procedure and after the full treatment course had been completed (Figures 2A, B).

The quantitative morphometric analysis was used to evaluate the number of CD1+ cells per area of epidermis. The mean values and standard deviations were:  $CD1_{\text{before}} = 0.000458 \pm 0.000151$ ;  $CD1_{24h} = 0.000429 \pm 0.000255$ ;  $CD1_{\text{final}} = 0.000934 \pm 0.000810$ .

Comparing the three time points studied for the same treatment (before, 24 hours after, and 21 days after) for the presence of CD1+ cells in the epidermis using the Kruskal-Wallis nonparametric statistical test of variance analysis, no significant differences were observed ( $H = 1.977$ ,  $p = 0.372$ ).

Regarding the antibody IL4 (used to demonstrate the intensity of interleukin 4), it was verified that PDT treatment promotes the presence of IL4 in the epidermis (Figure 3) and in the dermis (Figure 4).

The semi-quantitative morphometric analysis was used



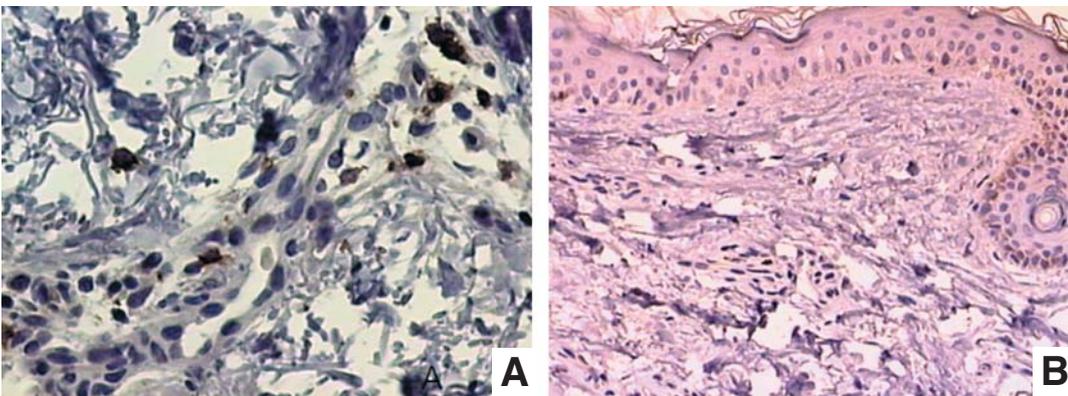
**Graph 1:** Intensity of CD8+ cells in the dermis before, 24 hours after, and at the end of the treatment.

to evaluate the intensity of the immunohistochemical reaction in the epidermis and dermis separately. Comparing the intensity of IL4 in the epidermis for the same patient for the three time points studied (before, 24 hours after the procedure, and 21 days after the treatment) using the Kruskal-Wallis nonparametric statistical test of variance analysis, a significant difference was observed ( $H = 16.090$ ,  $p < 0.001$ ). To better specify the differences, the Dunn's multiple comparison method was applied, which revealed a highly significant increase in IL4 in the epidermis after treatment ( $Q = 4.00$ ,  $p < 0.05$ ) (Graph 2).

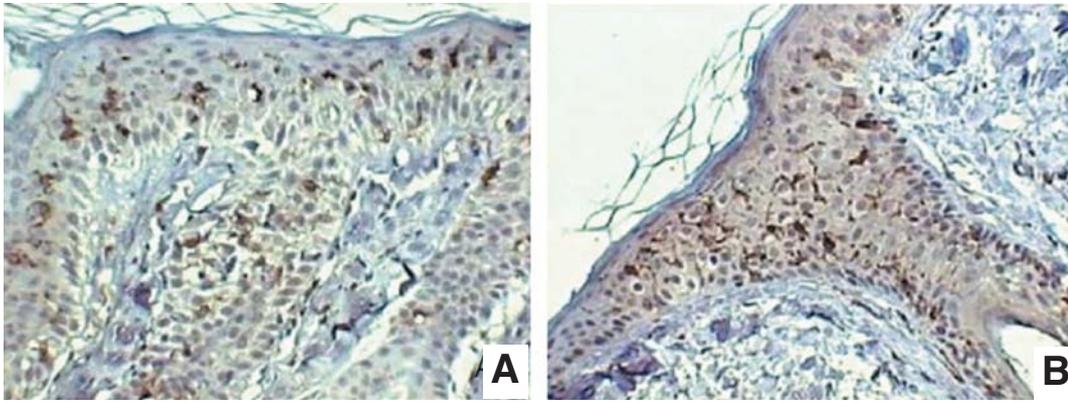
Comparing the intensity of IL4 in the dermis for the same patient for the three time points studied (before, 24 hours after the procedure, and 21 days after the treatment) using the Kruskal-Wallis nonparametric statistical test of variance analysis, a significant difference was observed ( $H = 13.77$ ,  $p = 0.001$ ). To better specify the differences, the Dunn's multiple comparison method was applied, which revealed a highly significant increase in IL4 in the dermis after treatment ( $Q = 3.71$ ,  $p < 0.05$ ) (Graph 3).

Regarding the  $TNF\alpha$  antibody, it was observed that PDT treatment reduces the amount of  $TNF\alpha$  in the epidermis and increases its intensity in the dermis (Figure 9B).

The semi-quantitative morphometric evaluation assessed the intensity of the immunohistochemical reaction in the epi-



**Figure 1:** Histological cut of the skin exposed to anti-CD8 immunohistochemical reaction before treatment. Mononuclear inflammatory cells with positive reaction (brown pigment) around the vessel (A: before treatment). Note the absence of CD8+ cells in the dermis (B: after treatment) X340.



**Figure 2: A and B** - Histological cut of the skin 24 hours after treatment subjected to anti-CD1 immunohistochemistry. Langerhans cells are marked in brown in the epidermis and in hair follicles (A: before treatment, B: after treatment) X340.

dermis and dermis separately. Comparing the intensity of TNF $\alpha$  in the dermis for the same patient for the three time points studied (before, 24 hours after the procedure, and 21 days after the treatment) using the Kruskal-Wallis nonparametric statistical test of variance analysis, significant differences were observed ( $H = 12.895$ ,  $p = 0.002$ ). To better specify the differences, the Dunn's multiple comparison method was applied, which revealed a highly significant reduction in the intensity of TNF $\alpha$  in the epidermis after treatment ( $Q = 3.57$ ,  $p < 0.05$ ) Graph 4 shows the results, represented by the mean values of each case, depicted in a box plot graph.

Comparing the intensity of TNF $\alpha$  in the dermis for the same patient for the three time points studied (before, 24 hours after the procedure, and 21 days after the treatment) using the Kruskal-Wallis nonparametric statistical test of variance analysis, significant differences were observed ( $H = 11.78$ ,  $p = 0.003$ ). To better specify the differences, the Dunn's multiple comparison method was applied, revealing a highly significant increase in the intensity of TNF $\alpha$  in the dermis after treatment ( $Q = 3.61$ ,  $p < 0.05$ ). Graph 5 shows the results, represented by the mean values of each case, depicted in a box plot graph.

Regarding IFN $\gamma$  antibody, there was no positive reaction in either the dermis or the epidermis at the three time points studied (Figure 5A and B).

The analysis of the Hematoxylin-Eosin. stained histological sections revealed that mononuclear inflammatory cells were present around vessels and annexes in the dermis. Comparing the intensity of the inflammatory infiltrate in the dermis for the same patient at the three time points studied (before, 24 hours after the procedure, and 21 days after the treatment) using the Kruskal-Wallis nonparametric statistical test of variance analysis, no significant differences were observed ( $H = 1.11$ ,  $p = 0.572$ ).

## DISCUSSION

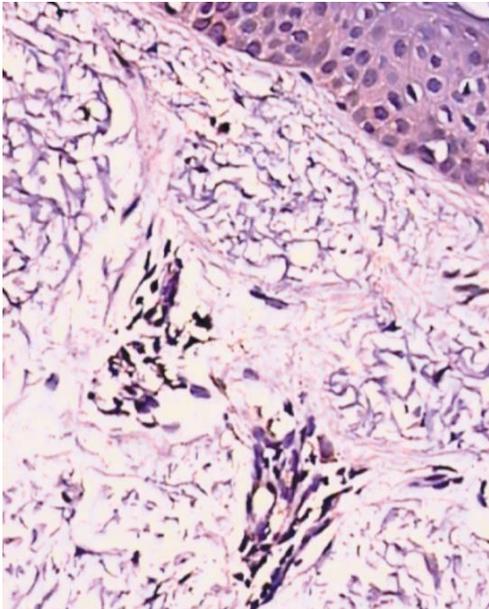
From the histological point of view, after 5-15 minutes of PDT, vasodilation and perivascular acute inflammation with neutrophils, eosinophils, and mastocytes takes place – nevertheless, the tumor does not change<sup>1,13</sup>. One to three days after, vacuolar degeneration of the basal layer occurs with foci of epidermal necrosis, acute perivascular inflammation, and dispersed

neutrophils and invasion of neutrophils in the epidermis. There is prominent vasodilation and edema in the dermis. Acute neutrophilic infiltrate can be seen in the sebaceous glands, which can present degeneration of their nuclei. During this period, the tumor still does not present alterations. On the third day, there is hydropic degeneration of the basal layer and intracellular edema of keratinocytes, and the nuclei becomes hyperchromatic. The inflammatory infiltrate then begins to exhibit acute and subacute characteristics. On the fourth day, keratinocyte necrosis begins to result in reticular degeneration, and tumoral necrosis and the regeneration of sebaceous glands starts. On the seventh day, the regeneration of the skin takes place, and is completed 4-6 weeks after PDT-ALA. Specific staining indicate reparation of the subepidermal zone with collagen fibers parallel to the epidermis, reducing the pre-existent elastotic mass<sup>13</sup>.

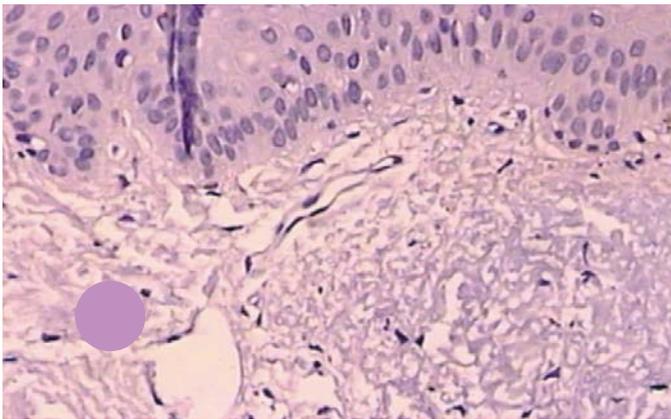
The progression of histological alterations observed in this study was similar to those reported in the literature: edema and vacuolization of the basal layer were observed after 24 hours, with the complete restructuring of the epidermis and formation of new collagen observed 21 days after the procedure – nonetheless, there was no significant alteration in the inflammatory infiltrate. The acute inflammation that is formed after PDT was not observed in this study, suggesting that this may occur more frequently in the treatment of tumors, where both the time of exposure to ALA and the systemic administration are longer; the destruction affect of the topical therapy occurs with the formation of singlet oxygen. The immunologic markers were chosen in order to define a tumoral and cellular immunity profile for the treatment.

The study of Langerhans cells' morphology and behavior after thermal injury, exposure to UV and trauma caused by adhesive tapes shows that the first disappear within two days and return after 11-15 days<sup>14-16</sup>. Although the majority of patients presented a decrease in the number of CD1, the changes were not significant in the present study. This result is in line with those cited in the literature, in which the change in number of Langerhans cells is a function of the procedure's degree of ablation.

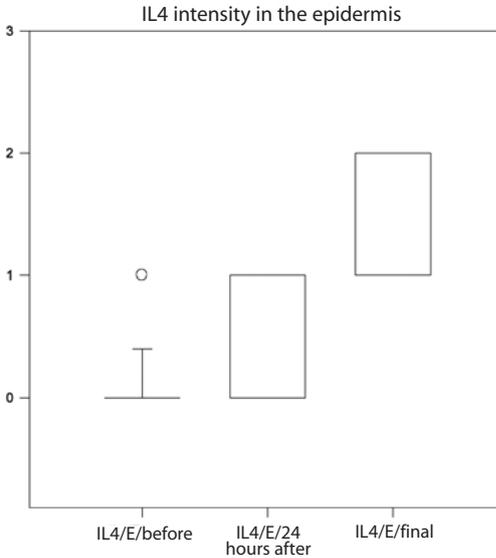
The decrease in the CD8 count 24 hours after the first session was not significant, however it became significant at 21 days after the third session. This reduction was not obtained by



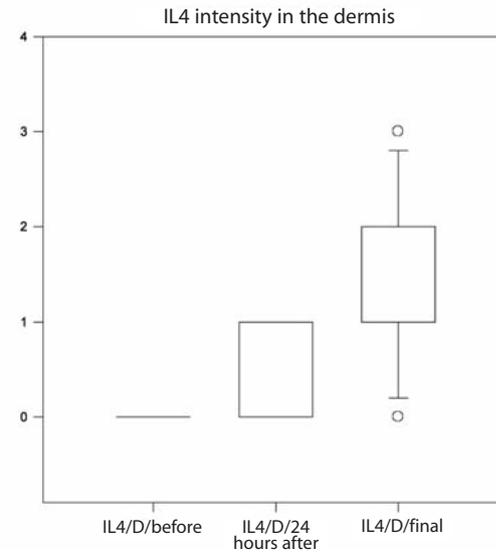
**Figure 3:** Histological cut of the skin before treatment, after anti-IL4 immunohistochemical reaction. Note the absence of positive reactions in the epidermis and dermis X340.



**Figure 4:** Histological cut of the skin subjected to anti-IL4 immunohistochemical reaction after treatment. Note the positive reaction (brown) in the epidermis and inflammatory cells around the dermis' vessels X340.



**Graph 2:** IL4 intensity in the epidermis before, 24 hours after, and at the end of the treatment.



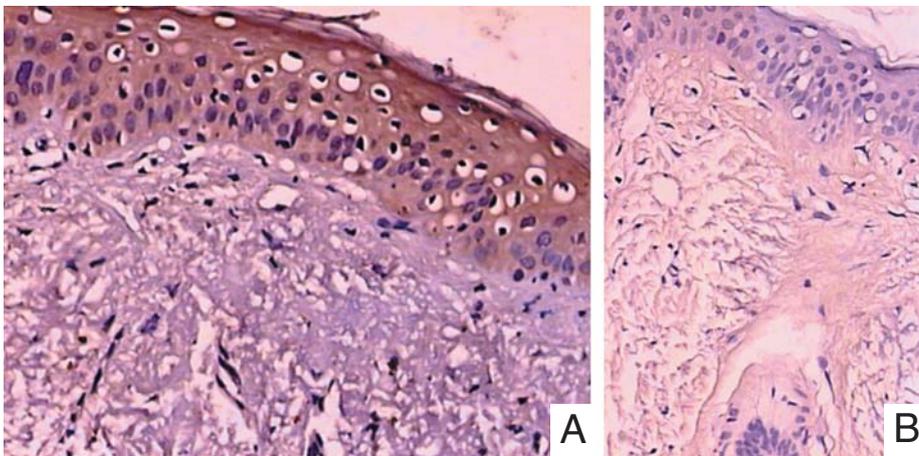
**Graph 3:** IL4 intensity in the dermis before, 24 hours after, and at the end of the treatment.

Abdel-Hady and others<sup>17</sup>, who found an increase in CD8 in treated areas that presented a positive response compared to treated areas that yielded negative results. On the other hand, Hryhorenko and colleagues<sup>18,19</sup> reported that PpIX accumulates in the activated lymphocytes and Gad (2001) cited Bissonette<sup>20</sup> reported apoptosis in those cells after PDT. Grebenova and colleagues<sup>21</sup> reported a 75% reduction in lymphocytes after PDT. The decrease in the CD4 cell count was not significant.

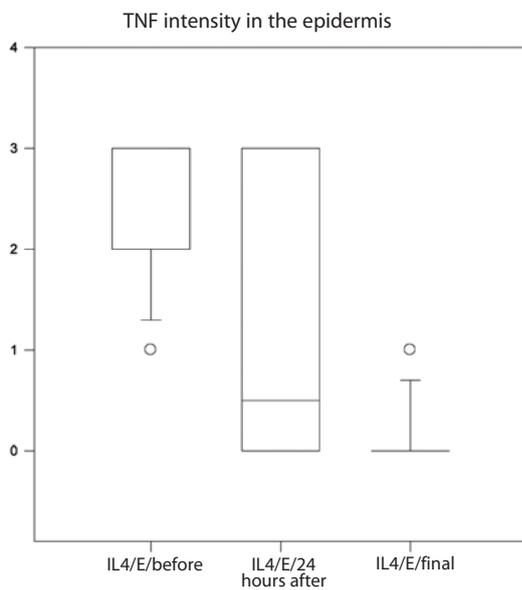
This reduction in the CD4 and CD8 cell counts suggests the apoptosis of lymphocytes following PDT, which is also reported by Grebenova<sup>21</sup> and Bissonette<sup>20</sup>. Notwithstanding, these studies involve treating tumors rather than photoaging; there are no reports in the literature describing alterations in CD4 and CD8 cells in the treatment of tumors. The increase in CD8 reported by Abdel-Hady<sup>17</sup> may be related to the control of the cellular response in cervical intraepithelial neoplasia; the tumor loses control when the treatment is successful.

TNF $\alpha$ , an important cutaneous inflammation mediator, is expressed in all inflammatory processes of the skin, but particularly in two types of reactions: pro-inflammatory effects and induction of death by apoptosis. Keratinocytes and dermal fibroblasts synthesize large amounts of TNF $\alpha$ . In the present study, the expression of TNF $\alpha$  suggested a significant decrease in that cytokine in the epidermis and a significant increase in the dermis. The increase in the dermis could explain the fibroblasts' stimulus, as evidenced by the increase in the collagen and elastic fiber fraction. Nevertheless, its reduction in the epidermis can only be justified by the increase in IL4 in the epidermis. The increase in IL4 in the dermis may have not been sufficient to inhibit fibroblasts, since this IL does not act only in the dermis, but rather in the epidermis. The CD4 and CD8 cells' apoptosis could also be related to the increase of TNF $\alpha$  in the dermis.

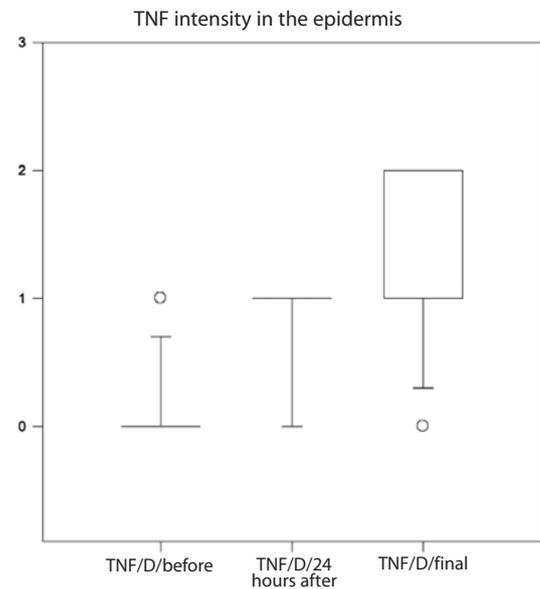
The IL4 acts on the growth and differentiation of Th2 cells. It inhibits IL1, IL6, and TNF $\alpha$ , and the expression of



**Figura 5:** Histological cut of the skin subjected to anti-TNF immunohistochemical reaction, before (A) and after (B) treatment. Note the positive reaction (brownish hue) in the epidermis and its absence in the dermis X340.



**Gráfico 4:** Intensity of TNF in the epidermis before, 24 hours after, and at the end of the treatment



**Gráfico 5:** Intensity of TNF in the dermis before, 24 hours after, and at the end of the treatment

ICAM1. IL4 expression promotes the development of the Th2 response, which is related to the B cells' responses. This study demonstrated an increase in IL4 in the epidermis and dermis, suggesting a Th2-type response.

In the present study, there was no expression of IFN $\gamma$ . The production of IFN $\gamma$  is restricted to the "natural killer" cell, the CD8 lymphocytes, a subset of CD4s that produces type I cytokines (Th1 cells). It modulates the immune response, activates the macrophages' properties, increases the capacity of antigen presenting cells, and induces adhesion molecules such as ICAM1 and VCAM1<sup>14-16</sup>. An absence of IFN expression can be related to a decrease in CD4 and CD8 lymphocytes and an increase in IL4, which determines Th2-type responses (while IFN $\gamma$  determines Th1-type responses).

Further investigation of ALA-PDT-based photoaging treatment is required to better understand the modulation of the immune system.

## CONCLUSIONS

Treating photoaged skin with the topical application of 20% ALA-PDT Levulan® Kerastick™ (LED equipment, 630 nm wavelength, output intensity 3100 mw/cm<sup>2</sup>, optical intensity 100 mW/cm<sup>2</sup>, 40x80 mm active surface, 2 x 10 minutes on each side of the face) using three 20-minute sessions with 15-day intervals presented the following outcomes:

1. The Langerhans cells did not change, suggesting that the procedure caused little local immunosuppression.
2. There was a significant decrease in the CD8 population count and an insignificant decrease in the CD4 population count, suggesting the apoptosis of lymphocytes.
3. There were significant differences in the expression of TNF $\alpha$ , which was reduced in the epidermis and increased in the dermis; the increase in the dermis has the potential to stimulate fibroblasts.
4. There was a significant increase in the expression of

interleukin 4 in the epidermis and dermis.

5. The IFN $\gamma$  did not present alterations in this protocol, which could be related to the lymphocytes' apoptosis.

6. The positive expression of IL4 and the negative expression of IFN suggests a Th2-type response.

7. The study's parameters – three sessions, 15-day interval, prior exposure time of 2 hours, 20% concentration ALA and the use of 630 nm red light – effectively treated photoaging. In addition, it was effective in stimulating the synthesis of collagen and elastic fibers, and in acting on the immune system. ●

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