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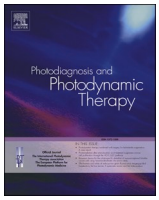

Neuroimmunomodulatory effect of Nitric Oxide on chronic wound healing after photodynamic therapy

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ABSTRACT

Neuroimmunomodulation is the capacity of the nervous system to regulate immune processes. The existence of neurotransmitter receptors in immune cells enables this phenomenon to take place. Neuronal mediators possess the capacity to direct and control several occurrences during the wound healing process. Nitric oxide (NO) functions as a neuromodulator, playing a crucial role in the regulation of vascular tone and blood pressure with antimicrobial properties. Photodynamic therapy has been shown to augment the function of immune cells involved in the healing process of venous leg ulcers. Nitric oxide can be secreted into the extracellular environment by these cells. In lesions treated with PDT, the synthesis of iNOs (the enzyme that releases NO) increased, as demonstrated by the experimental results. Therefore the significance of PDT in enhancing the clinical condition of the lesion is thus highlighted.

1. Background

According to international literature [1], chronic wounds are those that do not heal within six to eight weeks. Exaggerated matrix metalloproteinases (MMP) secretion, cellular infiltration, and infection affect this type of wound, making treatment difficult or impossible and expensive [1].

Photodynamic therapy (PDT) may be used to treat chronic wounds. It uses 5-aminolevulinic acid (ALA), a precursor of PpIX (protoporphyrin IX) [2–4]. However, there are also non-porphyrinoid ones, of which Methylene Blue is an excellent example [2–4].

Evidence links the action of PDT in chronic wounds to the activation of the immune system and consequently to the response of cellular infiltrates if this treatment is administered at sub-lethal doses [1,5-6]. Since the nervous system controls the immune system [7], a recent study demonstrated that PDT increased neuronal wound-healing mediators [8]. On this list, studies have added nitric oxide (NO) produced by NO synthase (NOs) in mammals. Inducible (i) NOs cause vasodilation, antimicrobial activity, and fibroblast function during wound inflammation [9,10]. Also, in one study, it was shown that in chronic wounds, the expression of iNOs increases after PDT treatment, as does in neurons, but it is reduced in mast cells (MC) even though their degranulation increases after this treatment [11].

To learn more about this issue, we looked at the expression of iNOs in different types of cells, of the cellular infiltrate, involved in the wound healing process [12], in both untreated and ALA-PDT treated chronic wounds. These cells included neutrophilic granulocytes, M1 and M2 macrophages, fibroblasts, and vessels.

2. Methods

2.1. Study population

A prospective cohort of 19 patients affected by chronic wounds was recruited from the Division of Dermatology of the Department of Surgery and Translational Medicine in a period from January 2009 to

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December 2014, University of Florence, at P. Palagi Hospital (Florence, Italy) after signing an informed consent. Approval for the study was obtained from the local ethical committee (progressive number 281/2008). As for clinical outcomes, refer to [5,8].

2.2. Photodynamic therapy

As the first step in the treatment, the photosensitizing agent (20% ALA in liposomal gel) was applied using pre-filled syringes for an incubation time of 3 h. At the end of the treatment, the dressing was removed, and the ulcer was cleaned of the excess product.

A red-light LED (630 nm) device (CE) 0434 PDT-CLD100 solid state light source, 230 V, Engineering Production Equipment Medical, EPEM srl, Florence, Italy), was used to light the area, and it reached a total of 80 J/cm^2 per single session of treatment. A 4-mm punch biopsy from the wound bed was taken before application of ALA 20% gel and repeated one hour after the first irradiation. Except for the weekly ALA-PDT application, wounds were managed with standard care. Illumination has been repeated on a weekly schedule up to three times. Patients were registered after a complete response, severe adverse events, or after the patient's retirement from the study [5,6,8].

2.3. Immunohistochemical analysis

The specimens were embedded in freezing tissue medium (Killik; BioOptica, Milan, Italy), quickly frozen, and sectioned by cryotome. Cryosections were post-fixed in cold acetone. Sections from each case (one section per staining) were stained with hematoxylin and eosin. In other sections, the following labels were combined with primary antibodies against iNOs (Abcam, Milan, Italy): SPM250 (marker for granulocytes, Genetex, Irwine, CA, USA), CD68 (marker for M1 macrophages, Abcam), CD163 (marker for M2 macrophages, Abcam), UEA (marker for vessels, Sigma, Milan, Italy), and HSP47 (marker for fibroblasts, Abcam). Omission of the primary antibody and substitution with an irrelevant one were used as controls for the immunohistochemical reaction. Photomicrographs were taken with an Axiophot microscope (Zeiss, Berlin, Germany) or a Leica TCS SP5 confocal microscope.

2.4. Morphometry and statistical analysis

Each skin sample was used as a sample unit to calculate the average value for each parameter. A one-way analysis of variance was conducted to compare the differences among all experimental groups, including Bonferroni-corrected *t*-tests or Tukey HSD tests. Significant results were compared to controls using a Student's *t*-test for unpaired values with two tails for each time frame. A significance level of $p < 0.05$ was considered. Besides the analysis considers the median values and the standard error (SE) of the percentage of cells labeled with the iNOs antigen from the total cells studied (i.e., granulocytes, M1 macrophages, M2 macrophages, vessels, or fibroblasts). The chi-square test was utilized to analyze values pertaining to percentages.

3. Results

Acanthosis, (i.e., a benign thickening of the epidermis' stratum spinosum layer [13]), diapedesis, (i.e., the movement of leukocytes from the circulation system to the site of infection or tissue injury [14]), and a rich inflammatory infiltrate distributed uniformly in both the papillary and reticular dermis were identified in sections of the treated chronic wounds. However, no apoptosis phenomenon was detected. The presence of a significant number of granulocyte populations in the cellular infiltrate of both chronic and treated lesions was identified using the SPM 250 marker. The use of the lectin UEA-1 allows the examination of the endothelium of blood vessels, which increases in both chronic wounds and those exposed to PDT. After further testing, it was found that the population of fibroblasts positive for HSP47 antigen increased in the treated lesion. There was also an increase in the numbers of M1 and M2 macrophages, that were marked with CD68 and CD163 antigens (data not shown). Finally, if the percentage of expression of iNOs in granulocytes significantly increases (Fig. 1A), this appears similar in M1-M2 type macrophages (Figs. 1B-1C) but decreased in blood vessels and fibroblasts (Figs. 1D-1E).

4. Discussion

This study was designed to evaluate the effects of a treatment with ALA-PDT in chronic wounds [1,2,5,6,8], in terms of cellular response in relation to the release of a neuronal mediator such as NO. The examined specimens show signs of acanthosis, diapedesis, and not apoptosis, a clear manifestation that the irradiation carried out occurred at sub-lethal doses [1,2,5,6,8,15,16].

In a previous study, neuronal mediators such as Substance P (SP), Neurokinin A (NKA), Calcitonin Gene Related Peptide (CGRP), Neuropeptide Y (NPY), Vasoactive Intestinal Peptide (VIP), Neuronal Growth Factor (NGF), and Protein Gene Product (PGP 9.5) were compared in chronic wounds before and after ALA-PDT treatment. These neuronal mediators were chosen for their distinct and established roles within the wound healing process [8]. There was an increase in the expression of several peripheral neuropeptides (except SP) by skin neuronal cells and MC that contain VIP and NGF [8].

Keeping this in mind, we have postulated that MC activity after therapy raises the release of NGF and VIP [8]. These mediators can then interact with neurons and nerve fibers in the dermis, leading to an improvement. In fact, nerve fiber activation may be associated to other phenomena such as enhanced extracellular matrix production by fibroblasts [6], an increase in TGFbeta [5], and cellular infiltration response [5].

Recently, NO has been identified as one of the mediators involved in the process of wound healing [9]. In response to stress, iNOs upregulate, producing this molecule. Besides, inflammatory cytokines, apoptotic bodies, or bacterial antigens all increase the production of this enzyme [10]. Therefore, it has been suggested that iNOs is also implicated in the inflammatory stage of wound healing, where it facilitates vasodilation and exhibits antibacterial properties [9,10]. A study that demonstrated how this mediator increases in chronic wounds following PDT treatment as well as its content in the neuronal population strengthened this idea [11]. On the contrary, the expression of iNOs in MC decreased following this therapy, despite the increase in their degranulation index [11].

This study demonstrates that PDT induces an elevated expression of iNOs in granulocytes. In addition, both M1 and M2 macrophages express iNOs at the same levels, while there are reductions in the presence of iNOs in blood vessels and fibroblasts.

The presence of this mediator in granulocytes and its greater concentration in this cell population are directly associated with the function that these cell types assume following treatment. This is because these cells increase in number after therapy, and the level of iNOs seems to be correlated with the severity of inflammation [17].

If there were almost no changes in the amount of this substance in M1 and M2 macrophages (which are both involved in inflammation) [18–20], then it shouldn't come as a surprise that the expression of iNOs dropped in fibroblasts and blood vessels.

This mediator actively promotes the generation of new cell progenitors [21–22]. If the mediator declines, it suggests that the stimulus responsible for promoting cell survival in these populations may have achieved a maximum capacity, as the number of the same cell types increases following the therapy [21–22].

These findings lead us to the conclusion that NO belongs on the list of neural mediators that aid in PDT-induced wound healing.

This is because during the therapy, NO stimulates the activation of several cells involved in the cellular response to an injury. This cellular reaction is likely triggered by MC, which rapidly release this mediator

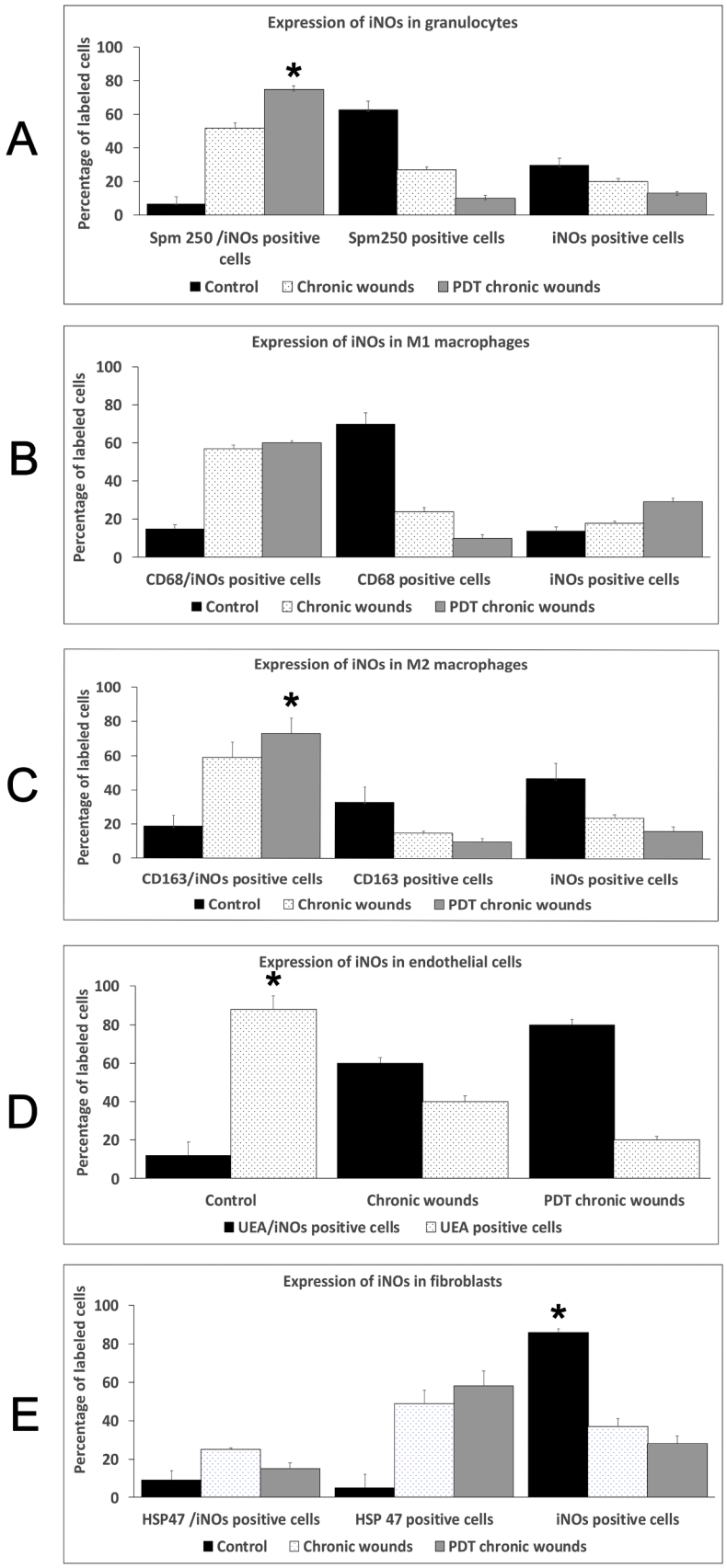

Fig. 1. Percentage of the expression of iNOs revealed in granulocytes (A), M1 (B) and M2 (C) macrophages, vessels (D) and fibroblasts (E) in normal skin, chronic wounds, and PDT treated chronic wounds. Data are expressed as mean ± SE: * $p < 0.05$ vs. control, $N = 19$.

[11] and play an important role in wound healing [23].

5. Conclusion

Therefore, this study demonstrates that neuroimmunomodulation is a possible mechanism involved in this therapy and that NO and other mediators influence the fine cellular mechanisms evoked by PDT treatment in chronic wounds.

Compliance with ethical standards

Role of funding source

This research was funded by the Italian Space Agency (Wound Healing and Sutures in Unloading Conditions ASI N. 2018- 14-U.0).

Ethical approval and informed consent

After giving informed consent, 19 CVU patients were recruited from the Division of Dermatology of the Department of Surgery and Translational Medicine at the University of Florence, P. Palagi Hospital (Florence, Italy), from January 2009 to December 2014. The Local Ethical Committee (progressive 281/2008) approved the study. This study's clinical outcomes are in Grandi et al., 2018.

CRediT authorship contribution statement

Patrizia Nardini: Methodology. **Lorenzo Notari:** Methodology. **Miriam Magazzini:** Methodology. **Bianca Mariani:** Methodology. **Federico Rossi:** Methodology. **Sofia Rossi:** Methodology. **Elisabeth Van Aardt:** Methodology. **Katarzyna Marszalek:** Methodology. **Vieri Grandi:** Methodology. **Alessandro Corsi:** Methodology. **Nicola Pimpinelli:** Supervision. **Stefano Bacci:** Writing – review & editing, Supervision, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare no conflicts of interest.

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