Chapter 5

Low Level Energy Photodynamic Therapy for Skin Processes and Regeneration

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Additional information is available at the end of the chapter

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Abstract

Skin is the largest human organ and displays multiple functions involving structure and protection against external agents that may affect the body. Solar radiation accelerates the normal aging process and may even cause great damage leading to many different cutaneous diseases and skin cancer. Moreover, a wound in the skin may be an open channel for the access of pathogens usually exposing blood vessels for infections and causing serious complications. For many reasons, regulatory skin processes are of great deal in different approaches: basic antiaging, wound healing, and skin cancer. In a good way, photoprocesses with specific wavelength at low energy levels associated with photoactive compounds are known to cause the opposite effect, promoting the healing of cutaneous diseases and leading to well-defined outcomes in rejuvenation and antiaging. This chapter will discuss the most relevant topics in photo skin regeneration using low energy levels associated with photodynamic therapy (PDT), which emerged as a combination to potentialize molecules with the already known effects of PDT and low level laser therapy (LLLT) in the treatment of skin pathologies, known as a photobiomodulation process.

Keywords: skin, wound healing, antiaging, photodynamic therapy, photobiomodulation

1. Introduction

The basic protocol of photodynamic therapy (PDT), the most classic of the photoprocesses applied to health treatment, involves the use of photosensitizer molecules (natural or synthetic) and visible light in a well-suited design for each application. In the presence of

molecular oxygen in the medium and under irradiation, reactive oxygen species (ROS) are generated in high amounts or low amounts, depending on the doses of light, promoting either the destruction of malignant cells, in the first case, or the induction of skin cells proliferation, leading to a stimulated wound healing process with promising results in antiaging effects [1, 2]. The effect known as "photobiostimulation," "photobiomodulation," or "biostimulation" is nondestructive at cell levels, in opposition to PDT main effect. Biostimulation has been applied in medical therapies in the treatment of severe wounds and the control of the pain, as well as in basic scientific research [3].

Studies have suggested that sufficiently decreasing the dose of laser light normally used in the basic PDT protocol could lead to a biostimulatory effect in cells, tissue, or organs, similar to what already happens with low level laser therapy (LLLT) [4]. In addition, cells enriched with low amounts of photosensitizer compounds can proliferate better after application of the LED or laser irradiation at the right wavelength and precise window of time, as the low concentration of generated ROS start a cascade of events leading to a proliferative cellular pathway [5]. Another recent approach in this research field is the laser stimulation at extremely low power densities (around 0.15 mW/cm^2) to enhance biological effects, known as ultralow level laser therapy (ULLLT) [6]. However, a laser power under 0.03 mW has not been proven any detectable response, and the effectiveness of ULLLT is only guaranteed when applied together with acupuncture and administered to acupoints. The tinny channels allow photons to be absorbed in the dermal layer, improving the technique [6].

The treatment with PDT requires, by definition, that parameters such as visible light and photosensitizer drug are simultaneously adjusted in a well-defined sequence of time and concentration to induce the desired effect. Important parameters to control *in vivo* studies include the time of drug uptake, time of treatment, number of applications, exposition time of the target tissue, administration route, and doses, which are critical to the success of this procedure. All parameters have been well evaluated during these long years of PDT studies around the world and have been setup under certain restrict intervals. Physical parameters of light include wavelength, irradiated area, power, energy, and frequency of irradiation [7]. The devices used to irradiate (LED, continuous wave laser or pulsed laser) have also been established, for many of the available setup in the market. The combination of all parameters is the major concern and uncertainty in studies of PDT for photoregeneration and may vary considerably when we move from *in vitro* to *in vivo* and clinical trial studies.

2. Mechanisms of PDT at low levels of energy

The mechanisms of light interaction in the organism and its response have been recently quite well understood, despite a long field to be elucidated [8]. An analogy to plants can be made, as via photosynthesis they absorb light energy by the chromophore groups in the chlorophyll and convert CO_2 and water in a sequence of reactions in the Calvin cycle. In eukaryotes, two mechanisms were suggested to be involved during light-cell interaction. One is acceleration of electrons transfer between redox pairs in some sections of the respiratory chain in the mitochondria [9]. The other is the production of small amounts of reactive oxygen species (ROS)

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induced by light absorption mainly by cytochrome c oxidase, a photoacceptor molecule in the mitochondria, accelerating ATP production and cellular proliferation [8].

When a photosensitizer molecule is present, as in PDT, it is first photoactivated to the right excited state of the molecule, by classic photochemical and photophysical pathways well defined by Jablonski diagram [10] and then the produced reactive species may accelerate the increments of ATP production. The most common ROS include superoxide anion (O_2^-) and peroxide hydrogen (H_2O_2) [6]. Briefly, the photosensitizer in the ground state (S_0) absorbs photons and is first excited to singlet state (S_1). The S_1 molecule can either return to its ground state as fluorescence emission or move to triplet excited state (T_1) through intersystem crossing, then forming free radical species by Type I reactions or transferring energy (Type II reactions) to molecular oxygen in the triplet state to the singlet state. The T_1 photosensitizer molecule can also return to S_0 through a process that lasts longer than fluorescence known as phosphorescence [7]. All processes are illustrated in **Figure 1**.

Recently, it has been proposed that PDT stimulates proliferation of dermal fibroblasts caused by low amounts of ROS, which activates extracellular signal-regulated kinases (ERK) [11]. ERK contributes in the regulation of critical cell processes, not only in proliferation, but also differentiation, survival, and apoptosis. It is suggested that fibroblast growth factor (FGF) stimulation leads to the reactivation of ERK signaling [12]. This has been most observed for the treatment of acne, as the photobiomodulation (PBM) effect is associated with increased proliferation of extracellular matrix (ECM) components and gene expression of anti-inflammatory cytokines [13]. The ECM cells, such as fibroblasts and macrophages, produce growth factors, cytokines, and chemokines crucial for regeneration and repair [14].

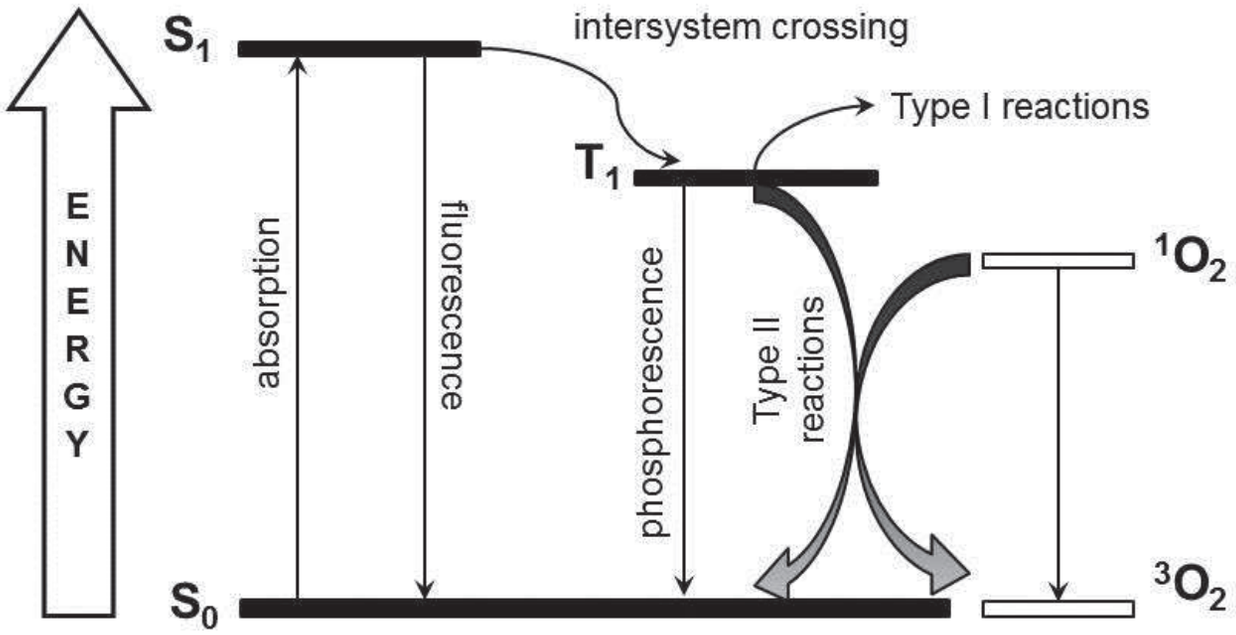

Figure 1. Simplified Jablonski diagram for energy transition between states and from photosensitizers in excited states to molecular oxygen, producing singlet oxygen and other reactive species.

It is important to note that most reactions in the organism are regulated by signaling, altering gene expression. In this concept, the nuclear factor kappa B (NF-kB), a transcription factor, plays an important role, as it regulates the expression of multiple genes and may govern inflammatory and stress-induced responses in the cell [15], activating the mitochondrial cytochrome c oxidase, which will generate ROS. The correlation between NF-kB and the production of ROS happens as NF-kB is activated when the redox state of the mitochondrial membrane is altered. NF-kB is a complex which contains its inhibitory protein, IkB, and for this reason it is inactive in the cytoplasmic medium. ROS are known to stimulate IkB-kinase, which inhibits the production of IkB by ubiquitination and proteasomal degradation with release of NF-kB. NF-kB is then transported to the nucleus and cause the expression of approximately 150 genes possibly involved in defense mechanisms against cell stress. Therefore, the concentration of ROS may contribute to benefit cells proliferation or be cytotoxic [13, 15]. The NF-kB activation can also be stimulated by ROS in other situations involving tumor necrosis factor alpha (TNFa), phorbol ester, and interleukin (IL)-1 [15].

Various signaling routes involved in metalloproteinases (MMPs) transcription control are regulated by redox reactions. This way, researchers propose a relation between MMP activity and production of ROS. It is suggested that ROS acts in the activation or deactivation of some MMPs in transcriptional level, acting in redox signaling routes [16]. Metalloproteinases from the extracellular matrix are calcium-dependent endopeptidases, which contain zinc, and are structural and functionally related to one another [17]. The main function of MMPs is on degradation and/or formation of extracellular matrix. More aspects of MMPs will be discussed in the wound healing section.

3. Topics in photoregeneration

The regeneration process is usually referred to the proliferation of cells and tissues to replace lost structures, as it happens in the growth of an amputated limb in amphibians, as an example. In mammals, whole organs and complex tissues are not fully regenerated; only parts of the liver can grow after partial resection or necrosis, which is known as compensatory growth. Tissues with high proliferative capacity can be continuously renewed after injury, as it happens in the hematopoietic system, the epithelia of the skin, and gastrointestinal tract. However, this is only possible if the stem cells of these tissues are not damaged [14]. These studies belong to the so-called regenerative medicine, an open multidisciplinary field of research involving many different aspects.

Photoregeneration consists in acting direct in the regeneration process using light itself or the combination of a light-based treatment with photoactive compounds in order to restore the lost structures, withhold water loss and decrease the exposition time of a wound or injury, reducing the risk of infection [18]. This is mostly applied in tissue repair in the wound healing process, but the principle can be expanded to regeneration of photodamaged skin, treatment of acne, and other skin pathologies, as well as in antiaging treatment [18]. In the wound healing process, over exposure of a wound can also cause the proliferation of bacteria, as it is an open piece of the organism to the environment. In this context, phototreatment is most considered

due to its antimicrobial properties. This section will discuss important aspects of photoregeneration in anti-aging and wound healing applications. All processes are regarded to skin and, therefore, it is significant to understand the constitution of the skin and its main components.

3.1. Skin

Skin is the largest organ in the human body, comprising about 16% of body weight. An average person can have about 1.8 m^2 of the body covered with skin, which may be submitted to harsh conditions [19]. The same way as other organs, it has the ability to grow, develop, and be repaired. The skin covers the surface of the body and is constituted of an epithelial portion from ectodermal origin, the epidermis, and a connective portion from mesodermal origin, the dermis. The skin plays multiple functions. Due to the corneum layers of epidermis, it protects the organism against water loss and friction, keeping the homeostasis of the system. Besides, the support function is crucial to keep structures such as blood vessels, innervation and muscle, as well as the sensitive function to psycho-emotion reactions, ultraviolet radiation, and endocrine functions [20].

The epidermis can be renewed every 2–3 weeks through the keratinocyte differentiation process [14]. Depending on the thickness of epidermis, varying from 50 to 150 μm, it can be distinguished the thin and thick skin. The thick skin is mostly found in the palms and soles, while the rest of the body is protected by thin skin. The junction between the dermis and epidermis is irregular, as dermis has projections, the dermal papillae, which fit in the indentations in the epidermis, increasing the cohesion between both layers. Hair, nails and sweat and sebaceous glands are attached skin structures [21].

The thickness of the dermis varies from 300 μm in the eyelids to 3 mm in the back. The dermis can be divided in papillary, situated right under the epidermis, and reticular dermis, located between the papillary and hypodermis. The hypodermis, also known as subcutaneous layer, is a loose connective tissue composed primarily by fat cells [22]. The main component of the dermis is the extracellular matrix in the connective tissue. The connective tissue is produced by fibroblasts and is composed of three major classes of biomolecules: glucosaminoglycans (GAGs), proteoglycans, structural proteins (collagen and elastin), and special macromolecules (fibrillin, fibronectin, laminin, and hyaluronan) [23].

3.1.1. Interaction of light in the skin

In PDT, it is important to be able to predict the spatial distribution of light in the target tissue. Light can be scattered or absorbed when it penetrates the tissue, and the extension of both processes depends on the type of tissue and the excitation wavelength. Absorption is mainly due to endogenous chromophores, such as hemoglobin, myoglobin, and cytochromes. Scattering is generally the most relevant factor for the determination of light penetration into the tissue. The combination of light absorption at short wavelengths by important chromophores in the tissue, such as oxy and desoxyhemoglobin and melanin, together with reduced light scattering in longer wavelengths and the occurrence of absorption by water in wavelengths longer than 1300 nm led to the concept of "optical window." In terms of PDT, the effective average of light penetration into the skin is approximately 1–3 mm in 630 nm [1].

The stratum corneum is a protective layer that consists of cells impregnated with keratin and considerably varies in thickness. Except by the scattered light, it is considered optically neutral. In the epidermal layer there is a slight scattering, with low amounts of light that pass through. The result is that all light not absorbed by melanin, main pigment, in the epidermis will pass to the dermis [24]. The dermis is composed by collagen fibers and, different from epidermal layer, it contains sensors, receptors, blood vessels, and nerve endings. Hemoglobin acts as a selective absorber of light. The dermis consists of two structurally different layers, papillary and reticular, which differ mainly in size of collagen fibers. The small size of collagen in the papillary dermis (with diameter smaller in magnitude than the incident light) promotes the back-scattering, in which light is totally directed back to the surface of the skin [24]. **Figure 2** shows an optical microscopy image (10× objective) of human skin biopsy stained by hematoxylin-eosin procedure showing the epidermal and dermal layers and the light pathways.

The blue light penetrates less efficiently into the tissue, while red and infrared radiations penetrate deeper. No source of light is ideal for all indications of PDT, even with the same photosensitizer molecule. The choice of the source of light must be based on the absorption by the photosensitizer drug, the purpose of the action (treatment of a small cancerous lesion or wound healing stimulation), characteristics of the lesion or wound (local, size, accessibility, and characteristics of the tissue) costs and size [7]. A wide number of photosensitizers in different drug delivery systems (DDSs) have been tested for PDT [25–29]. Among them, phthalocyanines

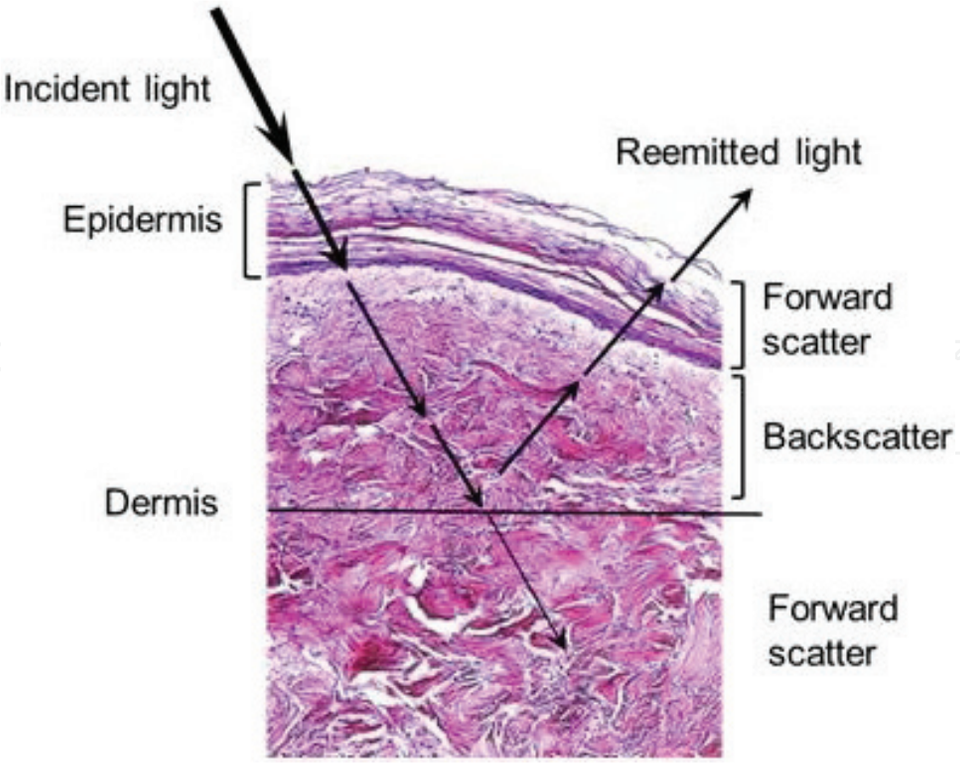

Figure 2. Histological image of human biopsy stained by a hematoxylin-eosin procedure, evidencing the light pathway through epidermal and dermal layers (adapted from Ref. [24]).

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(Pc) have been under investigation as promising second-generation photosensitizers, not only for PDT, but also for other applications. Because of their strong red-shifted absorption band, associated with their photophysical response, they are well suited to take advantage of maximal tissue penetration and low-light scattering properties of organelles and tissue at the ideal phototherapeutic window (600–800 nm).For instance, aluminum chloride phthalocyanine presents an intense absorption peak at 670 nm, and, therefore, diode laser operating at this wavelength is required [30, 31].

Mostly, the photobiomodulation effect has been observed when near-infrared light (NIR) is used at low levels; however, there have been reports of red, far-infrared (FIR) [13], green light [32], and specific types of lasers or LED. Normally, PDT requires a source of light in the visible range of light a source of light in the visible range of light spectrum between 600 and 800 nm called "therapeutic window." Today, there are some sources producing visible light in the range of 400–580 nm, due to some natural compounds that absorb light in this range and claim to induce some biological responses, such as curcuminoids and xanthene [33, 34]. Both compounds are phenolic structured in highly conjugated systems and can be used as dyes for drugs, cosmetics, and food. The dyes are primarily photoactivated, affecting enzymes, membrane lipids, and nucleic acids. The ability of these compounds to suppress the superoxide production by macrophages seems to modulate the secretion of protein kinases in tumor cell proliferation and induce the expression of anticarcinogenic enzymes [34, 35]. Recent studies have demonstrated that PDT is not restricted to visible light; deep tissue penetration in PDT could also be achieved with near-infrared (NIR) laser radiation, followed by upconversion to higher-energy visible or ultraviolet light in the biological medium [36, 37].

3.1.2. Collagen and elastin

Collagen constitutes a family of selected proteins during evolution to play different functions, mainly structural. During the organism evolution process, a family from a group of structural proteins influenced by the environment and by organism functional necessities have been modified and acquired variable degrees of stiffness, elasticity, and tensile strength. These proteins are collectively known as collagen, and the main examples of the many types of collagen are the ones found in the skin, bones, cartilage, smooth muscle, and basal lamina [21].

Collagen synthesis was initially associated with a restricted group of cells of the connective tissue, such as fibroblasts, chondroblasts, and osteoblasts. Currently, however, there is sufficient evidence that many types of cells produce this protein. Collagen fibrils are formed by the polymerization of molecular elongated units known as tropocollagen, and various types of collagen result in differences in the chemical structure of these polypeptide chains. In types I, II, and III collagen, the tropocollagen molecules aggregate in subunits to form fibrils. In types I and III collagen, these fibrils associate to form fibers. Type II collagen, present in cartilage, form fibrils but do not form fibers. Type IV collagen, present in basal lamina, form neither fibrils nor fibers [21, 38].

Collagen is the most abundant protein in the organism, representing 30% of its whole dry weight. The types of collagen in vertebrates constitute a family of proteins produced by different cells and are distinguished by their chemical composition, morphological characteristics,

distribution, functions, and pathologies. As collagen is the main structural protein and composes 70–80% of the skin dry weight, the modulation of the collagen metabolism in the skin by therapeutic irradiation has clinical importance. The skin collagen synthesized by fibroblasts comprises 80–85% of type I collagen and 10–15% of type III collagen [39]. Type I collagen is easily visualized by optical microscopy after *Picrosirius Red* stain method as long red fibers. However, type III collagen can only be detected by cross-polarized microscopy as short green fibers. **Figure 3** represents both histological procedures [18].

Elastin is the main ECM protein that provides strength and elasticity to many flexible and mechanically active tissues, including skin, lungs, and vocal chords [40]. The most elastin producer cells are fibroblasts and the cells of smooth muscle of blood vessels. Previous to mature elastin, proelastin is formed, a globular molecule of 70 kDa of molecular weight that polymerizes in the extracellular space to form elastin, a rubber-like glycoprotein predominated by elastic fibers. Elastin is resistant to boil, alkaline, and acid extraction and digestion with usual proteinase, but is easily hydrolyzed by pancreatic elastase. The same way as collagen, elastin is rich in glycine and proline. In addition, elastin contains two more uncommon aminoacids, desmosine, and isodesmosine, formed by covalent bonds between four lysine residues. The cross linking seems to be responsible for the elastic consistency of elastin, which is five times more extensible than rubber [21]. Elastin is easily visualized by optical microscopy after *Orcein* stain method as red fibers forming a network, as shown in **Figure 4** [18].

3.2. Antiaging

The aging process of the skin can occur intrinsically and extrinsically. The first refers to chronological changes because of genetic and hormonal modifications with age, and the last is related to external factors, such as UV radiation, smoking, diet, and chemicals [20]. However, among these factors, the most concerning is the prolonged exposure to sunlight during the course of life, promoting serious damage to the skin, and accelerating the regular aging process. This is normally referred to as photoaging, due to the importance of such health care [20]. UV radiation, particularly UVB, is most responsible for direct damage, reaching not only the stratum corneum, but also viable epidermal cells [23]. This way, measures that retard the

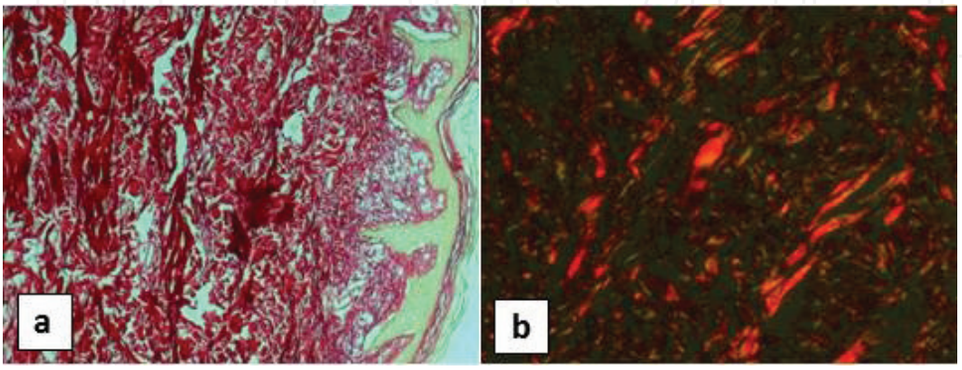

Figure 3. Histological images of human skin biopsies stained by *Picrosirius Red* and visualized by bright field microscopy (a) and cross-polarized microscopy (b).

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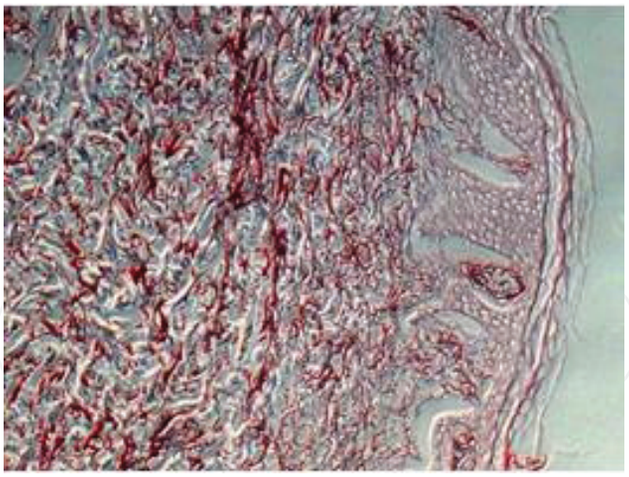

Figure 4. Histological images of human skin biopsies stained by *Orcein* and visualized by bright field microscopy, evidencing elastin fibers.

normal aging process are considered antiaging, and in this context it is relevant to understand the intrinsic changes that happen in the skin during this process.

The first visible and easily recognized aspects that appear in aged skin are the presence of wrinkles, decrease in thickness, transparency, dryness, and irregular hair growth. In deeper level, aged skin has difficulties to sweat and loses subcutaneous fat tissue, which leads to hollowed cheeks and eye sockets [23]. Dermis and epidermis appear to decrease in thickness with age, and their junction may flatten [19]. Despite visible changes, no significant change seems to occur at molecular level, as histology of photoaged skin only presents signs of chronic inflammation [20]. However, at this level it is important to understand the role of stem cells in mitochondria, since stem cell factors regulate mitochondria during aging [41].

The changes in the composition of extracellular matrix constitute an important factor in the aging process. Collagen fibers associated with proteoglycans are important components of dermis, and the healthy skin is dependent on the balance of synthesis and degradation of collagen [42]. In young skin, collagen fibers are more organized, as they are arranged to be extended or stay in the normal configuration. With age, collagen fibers in the skin become denser, decreasing their extensible capacity and being fragmented, disorganized, and less soluble [20].

Not only the fragmentation of ECM occurs, but also in the epidermis the turnover rate of keratinocytes decreases due to decrease of mitotic activity, a process called cellular senescence [23]. This can be explained as the constantly shedding of dead corneocytes in the outer surface and formation of new keratinocytes in the basal layer. Besides, protein composition of cornified envelope, responsible for barrier function, changes. The metabolism of calcium is strictly related to the aging process, especially in the epidermis. Reduction of calcium production with age inhibits full differentiation of keratinocytes in the stratum granulosum [19].

The same way, elastin fibers suffer considerable changes in variation of density, as they are intertwined with collagen forming the ECM. The UV radiation in contact with skin and in the presence of oxygen produces high concentrations of ROS or free radicals, which induce

gene expression resulting in collagen degradation and elastin accumulation. The remodeling process of such network is primarily controlled by the activity of matrix metalloproteinase (MMPs). In photodamaged skin, ROS induces the production of MMPs that degrade collagen while the expression of the MMP inhibitors (TIMP) is reduced [20, 23]. These events triggered by ROS can be reduced by the use of antioxidants. In the opposite way, low concentrations of ROS are thought to inactivate MMPs and reduce the formation of wrinkles [43, 44].

Studies examined PDT for the treatment of photoaging in different levels, from *in vitro* assays using fibroblast cells [11] to animal studies using hairless mouse model [45] and clinical assays [43]. There are a few published reviews about photodynamic rejuvenation, most exposing the use of MAL (methyl aminolevulinic acid) or 5-ALA (5-aminolevulinic acid) as photosensitizers [46]. They reported excellent cosmetic effects, sustaining the hypothesis of induction of collagen formation in the dermal layer [44], even in the treatment of patients with severe photodamaged skin [43]. The changes in collagen and skin texture are good parameters for histological examination. The photodynamic rejuvenation technique seems to show excellent short-term efficacy and tolerability.

3.3. Wound healing

In the ancient Egypt, from a paraphysiological perspective, a wound was considered an opening in the body where fiends could enter or leave [47]. With development of medicine, the treatment of wounds consisted in the application of bandages containing medicine, herbs with healing properties, over the lesion. Until not long ago, about 50 years, this was the most used method, considering the advances in medicines and medical techniques.

Wound healing is a natural process to keep the integrity of the skin of those who have been submitted to surgery or that simply have been injured [48, 49]. The concept comprises not only the skin; it can be expanded to treatment of periodontal diseases [50] and cartilage regeneration [51]. Various methods have been adopted to improve the healing of wounds of many types, including the potential use of irradiation at low doses [52]. Some of the biostimulatory effects were confirmed *in vitro* for the proliferation of fibroblasts, collagen synthesis, stimulation of macrophages, and higher rates of extracellular matrix production [53].

Epidermal regeneration in mammals was conventionally described by histologists consisting of three phases: mitosis, migration, and differentiation [54]. Only epidermis is able to regenerate, while complex healing process of dermis and its development can be summarized in three steps: an initial inflammatory stage, followed by proliferation and finally repair, tissue remodeling stage (maturation), although they are not strictly separated from each other [55, 56]. However, regeneration and scar formation depend on the extent of the injury and the ability of the organism to initiate such processes. For instance, a superficial wound only requires reepithelialization, while a deep and extensive wound initiates a series of events essential for the organism survival, which occur right after any indication of tissue destruction [14].

3.3.1. Wound healing phases

The inflammatory phase depends on the inflammatory cells, such as polymorphonuclear leukocytes (PMN), macrophages, and lymphocytes, besides numerous chemical mediators.

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PMN acts from the moment of tissue injury for a period that varies from 3 to 5 days, and is responsible for bacteria phagocytosis. Macrophage is the most important cell in this phase and remains in the wound from the 3rd to the 10th day [57, 58]. Besides bacteria phagocytosis, it debrides foreign bodies and directs the development of granulation tissue. Also, they secrete cytokines, compounds that coordinate immune responses, besides recruiting further immune cells to the infection local [50]. Lymphocytes appear in the wound in approximately 7 days and their roles are not fully defined, although lymphokines produced by these cells influence macrophages. In addition to these cells and chemical mediators, the inflammatory phase also counts on the important role of fibronectin, which is synthesized by a variety of cells, such as fibroblasts and endothelial cells, working as an adhesive to strengthen the fibrin clot, the cells, and extracellular matrix components [59].

The cells proliferation phase happens over the 5th to 14th days and initiates the repair process of both dermis and epidermis [59]. It is crucial for the formation of granulation tissue, which is a combination of cell elements, including fibroblasts, inflammatory cells, and endothelial components of ECM, such as fibronectin, glycosaminoglycans, and collagen. The formation of granulation tissue depends on the action of fibroblasts to produce collagen, elastin, fibronectin, glycosaminoglycans, and proteinases, responsible for debridement and physiological remodeling. During proliferation, it also occurs the angiogenesis, essential to oxygen, and nutrients supply for scar formation [60].

The last two phases, contraction and remodeling, are later processes, responsible for maturation of wounds. The first occurs with intense participation of myofibroblasts with consequences on lesion contraction. The latter, provided by bridge formation between collagen fibers, results on the development of mature scar [61]. Synthesis of structural proteins, such as collagen, remains at high levels for 6–12 months, although the scar reaches up to 70% of the tensile strength of intact skin [62].

Fibroblasts play an important role in a series of physiological events, such as the wound healing process. In this case, fibroblasts of the side connective tissue become activated, proliferate, migrate to the clot in reabsorption, and start to synthesize the ECM components, such as collagen and elastin [60]. During wound healing process, the portion of collagen increases with time, and by two weeks their fibers dominate the ECM. Phagocytic cells slightly disappear and the granulation tissue is progressively constituted by a denser and less vascularized tissue, located right under the regenerated epidermis [59]. Collagen degradation initiates early and is more active during inflammatory process. Therefore, the development of ECM is a result of balance between collagen deposition and degradation.

3.3.2. The role of matrix metalloproteinases (MMPs)

Matrix metalloproteinases are a family of secreted or transmembrane endopeptidases with similar structural domains which degrade ECM components. They can be classified in groups based on substrate specificity. One group comprises the interstitial collagenases MMP-1, 8 and 13, that recognize collagen types I, II, and III. Another one is composed of the stromelysins MMP-3, 10, and 11, with specificity for laminin, fibronectin, and proteoglycans. The gelatinases MMP-2 and 9 comprise another group, and cleave collagen types IV and V. Regulation of gene expression of most MMPs occurs by transcription factors [62, 63].

As previously discussed, MMPs can influence various cellular properties, such as growth, death, and migration [64]. In the tissue repair process, MMPs directly interfere in molecular and cell events. In the inflammatory phase, MMP-2 and MMP-9 act as chemical mediators on the stimulation steps in phagocytic phase. In granulation phase (proliferation), MMPs act along with other biomolecules with regulating activity in the formation of collagen and elastin matrix, reepithelialization, and vascularization [65].

Gelatinase B (MMP-9) is a typical MMP, important on the migration of different cell types, such as leucocytes and tumor cells, due to its ability in degrading basal membranes and ECM components, such as collagen and elastin. Gelatinase B promotes migration of new bone marrow leucocytes to blood vessels, and then to the tissue infection sites [66]. MMP-9 expression is normally constant during whole tissue remodeling phase. In addition, it is directly related to the cell migration during morphogenesis and organogenesis processes [67]. Equally, MMP-9 has been considered during creation and development of fibrillar network, associated with the production of elastin network particularly due to the presence of type I fibrillin [68].

Analogous to MMP-9, the expression of MMP-2 (gelatinase A) in the ECM is observed after collagen remodeling phase. Previous studies have demonstrated that dermal fibroblasts promote increase of MMP-2 expression from the first to the 21st day of development [61]. Once such enzymes degrade ECM and are synthesized by cells, there is an important control of their production, which is the latency. These enzymes are synthesized as proenzymes, which are inactive until being activated by (auto)proteolysis. This mechanism initiates a cascade of events in which each enzyme is activated by its precedent and, at the same time, activates the one that succeeds it. This way, the conversion of progelatinase B to its active form is catalyzed by MMP-3 or MMP-2 [66].

Nelson and Melendez [16] demonstrated that the expression and activation of MMPs are regulated via oxidative processes from redox reactions involving ROS in aerobic organisms, by mitochondrial mechanisms and cell modulation. Monochromatic irradiation and photodynamic mechanisms alter ROS intra/intercellular concentration, affecting the electric-physiological metabolism of mitochondria, which leads to acceleration of transcription expression of MMPs and other ECM biomolecules [67].

Studies [44] have demonstrated that ROS generated after UV irradiation initiate the increase of MMPs by a complex signaling mechanism, decreasing the expression of procollagen I and procollagen-III, culminating on reduction of dermal matrix generation. Some physiological processes, such as aging and wound healing, can be evaluated relating collagen synthesis to levels of MMPs expression. The deficiency of collagen due to natural aging derives from its reduced synthesis with increasing of degradation and concomitant elevation of MMP expression. UV radiation induces MMP synthesis in human skin, and the destruction of collagen mediated by MMP counts mostly by the damage in connective tissue with photoaging [69]. It suggests that not only the synthesis of proteins in the ECM, but also their degradation is increased in irradiated skin [39].

The activities of MMP-2 and MMP-9 can be experimentally detected and measured by gelatin zymography followed by data analysis for quantification. As gelatinases are secreted enzymes, the culture media in experiments involving cells proliferation can be collected for

the assays, being possible to evaluate the presence of such enzymes during different periods of treatment. The basic procedure consists in electrophoresis in polyacrylamide gels containing gelatin, inoculated with the media samples and revealed by an enzyme substrate buffer (50 mM Tris-HCl pH 8.0, 5 mM $CaCl_2$, 0.02% NaN_3), followed by staining protocols. The non-colored areas are quantitative related to the degradation activity of MMP-2 and MMP-9, in both pro and active forms [18, 70].

3.3.3. PDT on wound healing

Studies have evidenced that the treatment with He-Ne laser in cutaneous lesions would accelerate wound healing process [71]. As no increase of temperature was observed during light irradiation, the effects at cell level were considered more biochemical than thermal. Besides, no stress was expected during irradiation [22]. Combinations of light and drug, such as ALA/He-Ne laser and HpD/He-Ne and Nd:YAG lasers were tested in rats and demonstrated good performance in biostimulation [52].

Tissue stimulation was tested in human skin biopsies using the photosensitizer silicon-naphthalocyanine in lipossomal formulation and irradiation with laser at 670 nm and doses of 0.5, 1, 3, and 5 J/cm^2 [67]. This study has initiated a new approach in the application of photodynamic processes in cutaneous remodeling. It was found that the stimulatory effects were enhanced when PDT at low doses was applied instead of light itself. One study that evaluated the influence of continuous irradiation of diode laser (670 nm), with daily doses of 30 J/cm^2 in rat skin reported that the most significant morphological changes occurred during the first 7 days of wound healing [56]. A recently published study tested the effects of PDT using aluminum chloride phthalcyanine in nanoemulsion as photosensitizer and laser light at doses of 70, 140, and 700 mJ/cm^2 in human skin biopsies [18]. PDT at the intermediary dose promoted the increase of approximately 20% in collagen and elastin, as well as in MMPs expression, which was not reached using only light. The photobiomodulation effect was observed even in the first 7 days after irradiation, but the most prominent was reached after 14 days [72].

Here, we present the results of many photobiological studies evaluating the effects of low visible light at 685 nm, alone or combined with a phthalocyanine-derived photosensitizer dye (NzPC), on the healing process of cutaneous wounds using 3D dermal fibroblast collagen lattice as dermal equivalent (DE). Irradiations were carried out at 685 nm and, first, doses of 5, 10, and 20 J/cm^2 were applied 1 hour after the dye uptake, developed 24 hours after DE preparation. Contraction was reduced in all days of analysis (1, 3, and 7) and the number of cells decreased very fast. Then, lower doses (1, 3, and 5 J/cm^2) were tested and a new parameter was introduced, the effect of a delay time between dye andligth application (1–72 hours). The results showed that for doses higher than 1 J/cm^2 some toxicity were still present (>50%), but the delay in the ligth application seems to be crucial to a positive effect.

In the same studies, gelatin zymography was performed as previously described. Concerning metalloproteinase expressions, interestingly, MMP-9 expression was constant within fibroblasts during the collagen lattice remodeling and its progressive diffusion to the extracellular matrix occured later. The constant value for MMP-9 expression early observed as major lattice remodeling could thus be responsible for the later appearance of type I fibrillin.

Similarly, MMP-2 expression both within the extracellular matrix and the secretion in the culture medium was also observed after the early remodeling phase of the collagen lattice. The observations on the decreasing levels of active MMP-2 are in accordance with the findings of indirect immunodetection analysis comparing the expression of α-actin, fibrillin, and type I and III collagen (data not shown). In addition, it was found that for both types of treatment, the activated form of MMP-2 becomes higher than the proform of the enzyme after 12 days of culture.

Recent publications also reported the use of PDT with different photosensitizers and applications. Regarding oral diseases, PDT with methylene blue (MB) was tested on wound healing of rat buccal mucosa as a promising antimicrobial modality. The study qualitatively showed that MB-mediated PDT would have an inhibitory effect on healing process after 14 days of the wound creation [73]. In the same field, a review study evaluated current evidence and focused on gaps in knowledge to identify potential paths forward for clinical translation with photobiomodulation (PBM) therapy with an emphasis on craniofacial wound healing. They concluded that PBM offers a novel opportunity to examine fundamental nonvisual photobiological processes as well as develop innovative clinical therapies in clinical dentistry [74].

Regarding severe wounds, PDT with MB delayed reepithelialization in rats' wounds on the 7th day and interfered in standard healing. However, when used separately, MB and LLLT had a significant effect in the analyzed periods (1, 3, 14, and 21 days), when compared to control group [75]. Other studies evaluated third degree burns with lesions in mice treated with LED. The results showed that the LED-irradiaton was able to accelerate the wound healing process. In addition, a statistically significant microbial reduction was obtained with photodynamic inactivation compared to chemical decontamination [76]. Other types of model of study have been tested, such as the adult human skin wound healing organ culture (WHOC) treated with PDT. It demonstrated increased reepithelialization and extracellular matrix reconstruction and remodeling [77].

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