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Effect of photodynamic therapy on the skin using the ultrashort laser ablation

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Photodynamic Therapy (PDT) with 5-aminolevulinic acid (ALA) is known to be limited for applications in tumours of large volume mainly due to the limited penetration of topical photosensitization. The results show that micro-holes created using a femtosecond laser before PDT significantly increased the depth of PDT effect in the healthy tissue. The combination of ultrashort laser ablation technique with PDT showed an important scientific breakthrough related to transportation and delivery of drugs into the deeper regions of the tissue.

A representative image of a rat skin with the micro-drillings.

1. Introduction

Photodynamic Therapy (PDT) is a technique for the treatment of cancer and other pathological diseases based on the photoinduced cytotoxicity on the target cells [1]. In the presence of a photosensitizer, dysplastic cells or microorganisms will be destroyed when light of a certain wavelength mediates local cytotoxic effects caused by reactive oxygen species, especially singlet oxygen [2]. The choice of PDT is determined by many factors, like cost, instrumentation, the patient and the location of the lesions. The topical PDT of superficial tissues with 5-aminolevulinic acid, a precursor of an important photosensitizer...
used in PDT treatment (protoporphyrin IX or PpIX), has been achieving promising clinical results [3, 4]. A major advantage of topical PDT is the lack of systemic skin photosensitivity; a situation that obliges the avoidance of sun exposure by the patient. 5-ALA is a precursor of heme formed by 5-ALA synthase from glycine and succinyl-CoA, the rate-limiting step of the heme biosynthesis [5]. Once this step is bypassed by exogenous administration of 5-ALA, porphyrins and heme accumulate mainly in malignant or abnormal tissue, where metabolic activity is different.

In dermatology PDT is widely used for the treatment of premalignant lesion and basal cell carcinoma (BCC) [6–8]. The technique has some limitations in cases of injuries that are too thick, due to limited delivery of medication. A limiting factor of ALA-PDT is the drug penetration depth for the treatment of bulk tumoral lesions [9].

The drug delivery technology to deeper regions in biological systems is well-studied and of considerable interest for medical applications [10]. Many authors have investigated alternatives to improve drug delivery and increase the effects of PDT [11]. Experiments using nude mice, porcine skin and other biological targets have shown that silicon micro-needle puncture could reduce application time and the ALA dose required to induce high levels of the photosensitizer protoporphyrin IX in skin [12, 13]. The combination of iron-chelating agents increases and/or accelerates the accumulation of PpIX induced by the prodrugs ALA (aminolevulinic acid), MAL (methyl aminolevulinate), and HAL (hexyl aminolevulinate) in squamous carcinoma (A431), and glioma (U-87 MG) cells [14]. Clinical investigation of the novel iron-chelating agent, CP94, to enhance topical photodynamic therapy of nodular BCC also has been studied [15]. Other works have investigated the light fractionation effects. Vijlder et al. [16] observed that it significantly improves in the response of superficial BCC and Brujin et al. [17] demonstrated that light fractionation, with a long dark interval, significantly increases the response to ALA-PDT in pre-clinical models and in non-melanoma skin cancer.

One good way of improving the drug delivery is using a laser as a “photonic syringe”, a non-invasive system which can cause less pain to the patient. For this we used a technique known as laser ablation. This technique involves tissue removal by laser beam. Some author reported the importance and efficiency of ablative fractional laser for drug delivery enhancement [18, 19] and as pre-treatment in PDT [20, 21].

A precise tissue removal with minimal collateral thermo-mechanical damage is necessary in many laser-surgical applications, including neurosurgery, dentistry and cardiology [13, 22]. Laser ablation of biological tissue, in the ultra-short pulses regime, is a technique employed due to its characteristic light-tissue interaction, resulting in a precise ablation technique [22–29], and avoidance of thermal damage in the surroundings [26]. It has been applied to the processing of soft and hard biological tissues, such as tooth [25, 26], bone [26, 28, 29], skin and liver [9]. The laser ablation of soft tissues shows interesting mechanisms of laser-tissue interaction [9, 22–24, 27].

The hypothesis tested in this paper was that the delivery of the drug to deeper regions of the tissue could be enhanced by a mechanical mechanism, where transversal micro-channels are produced in the region where the drug will be applied. Thus, the micro-channels assist in the delivery of drugs which have difficulty in penetrating the skin, as is the case for compounds that have large proteins. The drug cream reaches the deep regions of the tissue through these micro-channels and, consequently, results in deeper photosensitization. Few studies have discussed new laser ablation parameters to improve the drug (ALA) penetration and damage depth in biological tissues. The combination of ultrashort laser ablation technique with PDT is an important scientific breakthrough related to transportation and delivery of drugs into the deeper regions of the tissue.

2. Experimental

2.1 Animal procedure

Fifteen male Wistar rats of approximately 300 g each were used in this study. The study was approved by the Local Institutional Ethics Committee. Animals were anaesthetized with an 0.08 ml/100 g ketamine solution (Ketamine Agener 10%, Agener União, Brazil), associated with xylazine 0.04 ml/100 g, an analgesic, sedative and muscle relaxant (Xylazine 2%, Coopazine, Schering-Plough, United States) intramuscular injection. The trichotomy was performed and the skin surface exposed for irradiation. The rats were positioned and moved in a linear translation stage (x − y − z). A study was made of the optimal parameters for laser ablation in the skin.

2.2 Laser ablation and PDT parameters

A Ti: Sapphire femtosecond (Libra-S, Coherent, Palo Alto, CA, USA) laser emitting pulses of approximately 70 fs, emission centered at a wavelength of 801 nm and operating at a pulse repetition rate of 100–1000 Hz was used in this study to produce the micro-channels.
After laser ablation, the surfaces of the rat skin were photosensitized with cream containing 20% Aminolevulinic acid (PDTPharma, Cravinhos-SP, Brazil). After 2 hours of occlusion, the skin was irradiated in a fluence of between 50 and 100 J/cm² at an intensity of 50 mW/cm². A 630 nm diode laser, Eagle Heron (Quantum Tech, São Carlos-SP, Brazil), was used as a PDT light source and an optical fiber of 300 μm diameters with coupled micro-lenses was used to obtain a uniform irradiation profile.

The pieces obtained were fixed in 10% formalin for 24 h. After this, they went through routine laboratory procedures until they were set in paraffin, and 5-mm-thick sections were obtained and stained with hematoxylin and eosin (HE) for histological analysis. The image analysis was done using a Zeiss microscope, model Axioskop 2 plus, and the image captured with an AxioCam Mrc digital camera and using a 100× objective. A semi-quantitative analysis was performed for the following histological aspects: edema, necrosis, inflammatory infiltrate and hemorrhagic foci.

In the first part of this paper we performed a comparative study using the laser operating in different regimes of average power and irradiation time. An experimental setup was developed in order to find the appropriate parameters for a tissue ablation free of charring and other secondary damage. Wister rats were placed in an x–y–z translator and micro-channels were made in different areas of the skin, approximately 7.0 mm × 5.0 mm spaced 0.25 mm, using an average power of 200 mW (Figure 1). The average power values were determined using a power meter (Coherent LM10, USA). After this, the skins were used to quantify the change in depth of tissue damage in PDT as a function of the micro-channels obtained by laser ablation. The vertical micro-channels were produced perpendicularly to the skin surface. The beam waist on the surface of the skin was approximately 50 μm.

The animals used in this study were separated into four different groups containing three animals each: a control group that represents only micro-drilling (CG); micro-drilling + PDT 50 J/cm² (MP50J); micro-drilling + PDT at 100 J/cm² (MP100J); PDT 50 J/cm² (P50J); PDT 100 J/cm² (P100J).

The conditions for the PpIX production were standardized to achieve the drug-light interval (DLI), and determined based on a previous study where the authors evaluated the production of PpIX by fluorescence spectroscopy as a function of time. With this technique we can non-invasively investigate the superficial presence of the PpIX in real time. The DLI was determined based on the time necessary for the generated PpIX to reach the plateau and before the clearance becomes more important than the production, i.e., at the decrease of fluorescence emission. After 2 hours of occlusion with 100 mg ALA cream 20% (w/w), a maximum of PpIX was observed using fluorescence spectroscopy.

The animals were kept alive for exactly 30 hours to allow the establishment of necrosis; they were killed with anesthesia overdose and their skin was removed. The tissue damage was macroscopically evaluated, and samples were prepared for histological analysis (HE staining method). The aspect of the necrotic tissue and the depth of tissue damage (the measurement of the damage length from the irradiated surface to the limit between necrotic and healthy tissues) were the main analyzed characteristics. The histopathological analyses were based on the qualitative observation of the slides on an optical microscope.

3. Results and discussion

Figure 2 shows profile pictures of damage in rat skin after femtosecond laser irradiation, in accordance with the details of Figure 1.

The samples were irradiated with a femtosecond laser, using pulse energy of 52 μJ to 110 μJ, repetition rate of 1 kHz, and irradiation time of 5 seconds. After this, a study of histological slides of the tissue was made, and it was observed that the energy density applied on the tissue was not sufficient to induce an ablation, because a significant change in morphology of the tissue was observed (Figure 2A).

This change is associated with tissue damage caused by thermal laser-tissue interaction, resulting in the destruction of tissue cells, becoming more evident by the purple color of the image. In Figure 2B the energy density applied on the fabric was twice that in Figure 2A and, thus, it was possible to obtain an “optical breakdown”, leading to a tissue ablation.
The parameters used have caused a major change in the peripheral tissue (Figure 2A and B). Around the cavity, regions with strong staining in purple and pink, associated with altered thermal tissue, were observed, indicating cell destruction (Figure 2B).

The animals of the group treated with PDT and previous micro-drilling showed a pattern of lesions with the presence of hemorrhagic foci (hypodermis and dermis) and inflammatory infiltrate, edema and areas of necrosis (Figure 3A and B).

In the Figure 3, panels A and B, a greater intensity of MP100J was used (panel B) in comparison with MP50J (panel A). In the MP100J observed lesions distributed throughout the epidermis (necrosis), the papillary dermis, the hypodermis (among adipocytes) and muscle tissue, whereas group MP50J showed evidence in the dermis, hypodermis and muscle fibers. Two animals of the Group MP50J presented little dermal inflammatory infiltrate and hypodermic without bleeding.

Finally, the next figure shows the histological results of the skins of healthy animals treated with PDT, without micro-drilling (Figure 4A and B).

Weaker effects in the dermis and hypodermis were observed (edema and necrosis), with few hemorrhagic foci and inflammatory infiltration. Both animals of Group PDT50 (Figure 4A) as the Group PDT100 (Figure 4B) demonstrated a few spots of hemorrhage and inflammatory infiltration in the papillary layer of the dermis, compared to the Group MP50J and MP100J (Figure 3, panels A and B).

After photodynamic therapy occurs, the intensive damage in the epithelial cell and inflammatory infiltrate is finally observed, thus leading to necrosis of the irradiated tissue [30].

The histological analysis of this experiment shows that the micro-holes created using a femtosecond laser before PDT increase significantly the depth of your effect to healthy tissue. Skin treated with PDT after micro-drilling presented hemorrhagic foci in muscle tissue and hypodermis, abundantly in the group MP100J and moderately in MP50J (this was especially evident in the hypodermis), suggesting a greater depth in delivering the drug (cream containing ALA) in tissue and consequently a higher photochemical reaction, demonstrating a greater

**Figure 2** Histological images of damage profile in healthy rat tissue after irradiation with a femtosecond laser with 52 μJ and 5 s (Panel A), and 110 μJ and 5 s (Panel B).

**Figure 3** Photomicrographs of healthy rat tissue in the micro-drilling + PDT group (MP). Panel A: MP50J; panel B: MP100J. Smaller lesions intensity were observed in panel A. Dermis (D); edema among adipocytes (E); hemorrhagic foci in the hypodermis and between fibers muscle (*); perivascular inflammatory infiltrate in the hypodermis (II); muscle fibers (M); hypoderm (H). 100×. H.E.
inflammatory process in the different layers of the skin.

In the hypodermis region there was also found abundant inflammatory infiltrate in all groups, with a higher intensity in the groups with micro-drilling. The papillary dermis inflammatory infiltrates were also seen in all groups, mainly in the group MP100J, in which edema in the papillary dermis was observed.

Topical drug delivery is essential in dermatology and the efficacy of topically applied drugs is crucially dependent on their ability to penetrate the outermost skin barrier of stratum corneum (SC) and epidermis. Some studies have been developed in this context. Høderdal et al. [31] observed that, from the creating of deep channels into skin through which direct penetration of drugs can occur, AFR appears to not only bypass the SC barrier, but to provide substantial delivery of MAL into deep dermis, in vivo. Lee et al. [32] also showed a greater enhancement effect on skin permeation of 5-FU, when the SC layer in the skin was partly ablated by an erbium:YAG (Er:YAG) laser. Penetration enhancement of two topical 5-aminolaevulinic acid formulations for photodynamic therapy by Er:YAG laser ablation of the SC was observed by [19]. Different laser systems have been used to pre-treat tissue prior to topically applied ALA, as the fractionated CO2 laser [18]. Another work compares the 5-year lesion response rates of superficial BCC treated with topical aminolaevulinic acid (ALA)-PDT using a single illumination (75 J/cm² delivered 4 hours after 20% ALA cream application) vs. ALA-PDT using a 2-fold illumination scheme (20 and 80 J/cm² delivered 4 and 6 hours after 20% ALA cream application, respectively). The complete response rate was significantly greater following the 2-fold illumination than the single illumination [16].

Fractional ablative lasers are commercially available as CO2 lasers (10,600 nm) and Er:YAG lasers (2,940 nm) [16–21, 31, 33]. Tissue absorption at these wavelengths is dominated by water. Deep tissue holes are created by a dynamic laser tissue interaction in which water absorption coefficients and pulse widths are responsible for the formation of (microscopic treatments zones) MTZ with different dimensions of ablation and thermal coagulation zones (TCZ) [31]. Potentially, bleeding through the laser holes may decrease drug uptake after AFR. On the other hand, a thick residual TCZ may inhibit the diffusion of some drugs from the laser hole into surrounding dermis [31]. Appropriate laser parameters are essential in order to obtain a desired penetration depth of the epidermis without destruction of viable tissue that may impair the formation of PpIX [21]. Femtosecond lasers are presented as great candidates for this purpose, because of the mechanisms involved in the ablative processes in biological tissue with minimal thermal damage [9, 22–29]. Furthermore, they do not form an epidermal necrotic zone, a potential obstacle for drug penetration, which is usually seen in erbium glass–based fractional lasers [20].

Other techniques that enhance drug penetration of the skin include micro-needling and microdermabrasion [12, 13, 33]. Micro-needling offers a simple method to increase the superficial penetration of ALA. Donnelly et al. [12] evaluated micro-needle mediated intradermal delivery of 5-ALA, which enhanced ALA delivery to superficial regions of ex-
cised porcine skin, but not to deeper skin layers. There are important differences between micro-needle puncture and AFR. AFR removes tissue, leaving an open channel into which topically applied drugs can migrate. AFR is hemostatic, such that there is little or no bleeding or fluid loss through the laser ablated holes, which could impede drug uptake [31].

Togsverd-Bo et al. [21] demonstrated that the PDT with MAL serves as an intensified treatment modality with significantly better efficacy compared with conventional PDT. They concluded that the AFXL-PDT represents a promising and intensified treatment modality for AK with a strong clinical potential to improve future PDT of especially thick lesions in field-cancerized skin. YOO et al. [20] in their pilot study reported the potential of the combination of ablative (CO\textsubscript{2}) fractional laser and MAL-PDT for synergistic effects in recalcitrant periungual warts. As for the results, a mean clearance of 100% was achieved in 36 (90%) warts, with a shorter incubation time, lower treatment number, no recurrences, and no severe side effects. Therefore, this pilot study supports the examination of the potential of the combination of ablative CO\textsubscript{2} fractional laser and MAL-PDT in recalcitrant periungual warts for synergistic effects.

4. Conclusion

In this paper we concluded that the combination of an ultra-short laser ablation technique with PDT, aiming to use the laser as a tool to assist in the delivery of drugs into the deeper regions of biological systems, is a good alternative to other forms of delivery. We observed that the tissues previously irradiated with a femtosecond laser indirectly showed that the drug reached the deepest regions and made a significant process of acute inflammation in the tissues after PDT. However, we believe that further studies should be conducted to analyse the PpIX in micro-perforated tissues, since these results represent an important scientific breakthrough related to transportation and delivery of drugs into the deeper regions of the tissue.

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Author biographies Please see Supporting Information online.

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