Biochemical changes in cutaneous squamous cell carcinoma submitted to PDT using ATR-FTIR spectroscopy

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ABSTRACT

Nonmelanoma skin cancers are the most common form of malignancy in humans. Between the traditional treatment ways, the photodynamic therapy (PDT) is a promising alternative which is minimally invasive and do not requires surgical intervention or exposure to ionizing radiation. The understanding of the cascade of effects playing role in PDT is not fully understood, so that define and understand the biochemical events caused by photodynamic effect will hopefully result in designing better PDT protocols. In this study we investigated the potential of the FTIR spectroscopy to assess the biochemical changes caused by photodynamic therapy after 10 and 20 days of treatment using 5-aminolevulinic acid (ALA) as precursor of the photosensitizer photoporphyrin IX (PpIX). The amplitude values of second derivative from vibrational modes obtained with FTIR spectroscopy showed similar behavior with the morphological features observed in histopathological analysis, which showed active lesions even 20 days after PDT. Thus, the technique has the potential to be used to complement the investigation of the main biochemical changes that photodynamic therapy promotes in tissue.

Keywords: ATR-FTIR spectroscopy, photodynamic therapy, squamous cell carcinoma, 5-aminolevulinic acid

1. INTRODUCTION

Nonmelanoma skin cancers are the most common form of malignancy in humans[1–3]. Among them, cutaneous squamous cell carcinoma (SCC) is the most worrying due to its aggressive pattern and potentially metastatic[4,5]. The traditional treatment forms of cutaneous cancers can be surgically administered (surgical excision, Mohs surgery, curettage and electrodessication, cryosurgery or laser surgery), as well as non-surgical procedures (radiotherapy and chemotherapy)[6]. Beyond these, the photodynamic therapy (PDT) is a promising alternative which is minimally invasive and does not require surgical intervention or exposure to ionizing radiation. PDT treatment involves administration of a photosensitizer substance (topically or systemically applied) which is activated by irradiation with light of specific wavelength and promote a series of biochemical events that lead to tumor cell death[7,8]. The understanding of the cascade of effects playing role in PDT is not fully understood, however, it is known that these anticancer effects result from three interdependent processes: direct cell damage (induced by cytotoxicity of reactive oxygen species (ROS)); vascular shutdown, and activation of a nonspecific immune response. The mechanisms of death induced by PDT (apoptosis, necrosis and autophagy) are not independent and can occur in the same time in a photosensitised lesion. The individual contribution of each mechanism to the overall result (cell death) is still subject of study, but it is known that the response to PDT may vary with the cell type (phenotype and metabolic potential), total fluence delivered, different types of photosensitizer and the time interval between the application of it and the light irradiation[9,10].

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Define and understand the biochemical events caused by photodynamic effect will hopefully result in designing better PDT protocols[9]. Nevertheless, most of the research about PDT effects are centered on clinical assessments of treated patients and relatively few studies have focused in the molecular effects[11]. Generally, cell damage induced by PDT are detected by analysis of morphological features in the histopathology or by the presence of specific cell event using immunohistochemistry. However, with respect to the biochemical characterization, the vibrational spectroscopy (represented by Raman scattering and Fourier transform Infrared (FTIR)) have obtained great success in the characterization of biological materials and provide important information about the biochemistry of the analyzed tissue[12–14].

2. OBJECTIVE

This is a preliminary study to investigate the potential of the ATR-FTIR technique in the study of biochemical changes caused by photodynamic therapy after 10 and 20 days of treatment using 5-aminolevulinic acid (ALA) as precursor of the photosensitizer photoporphyrin IX (PpIX).

3. METHODS

2.1. Chemical Carcinogenesis

Cutaneous neoplastic lesions were induced in mice using a well-established in vivo model of chemical carcinogenesis[15]. For this, after approval by the ethics committee for research on animals (Comite de Etica no Uso de Animais, CEUA) of Instituto de Pesquisas Energéticas e Nucleares (IPEN) (project no. 71/10-CEUA-IPEN/SP, 21 December 2010), 20 Swiss female mice, aged from 8 to 10 weeks, with a weight of 20 g were submitted to chemical carcinogenesis consisted for two stages. In the first one, 50 mg of DMBA (7,12-dimethyl-benzanthracene) diluted in 100 mL of acetone was topically applied on the shaved back mice. The second phase of the protocol began a week later and consisted in a bi-weekly application of 5 g of TPA (12-O-tetradecanoyl-phorbol-13-acetate) diluted in 200 mL of acetone. After 28 weeks, the animals obtained visible single or multiple tumor nodules with verrucous aspect (Figure 5) and the mice were divided into 3 groups (Table 1).

Table 1. Experimental groups.

Group	Number of mice	Description
G1	13	Neoplastic tissue
G2	4	Neoplastic tissue + ALA + PDT (10 days after treatment)
G3	3	Neoplastic tissue + ALA + PDT (20 days after treatment)

After chemical carcinogenesis, the animals from group 2 and 3 were submitted to PDT, whereas the neoplastic lesions from animals of group one was extracted by biopsy and kept in formaldehyde for 24 h to the histological fixation. Once fixed, the biological tissues were diaphanized in two baths of pure xylol for 30 min and dehydrated with ethanol baths in increasing concentrations (50%, 70% and 100%). Samples were mounted in wax blocks and slices of 5 μm thickness were obtained from FFPP (Formalin-fixed paraffin-processed) sections using a microtome and placed in MirrIR low-E-coated slides (Kevley Technologies, Chesterland, OH, USA) for ATR-FTIR spectroscopy and histopathological analysis. Due to the spectral contributions of paraffin in the used wavenumber range, FFPP sections were submitted to dewaxing protocol, which was composed by two baths of xylene during 10 min and one bath of absolute ethanol during 5 min.

2.2. Photodynamic therapy

Animals from G2 and G3 were submitted to a single session of PDT. We used a homemade photosensitizer ointment, which base is prepared with lanolin and petrolatum and the active principle being either 5-aminolevulinic acid (ALA) (20%) and other ingredients kept confidential (patent pending PIN°0705591-9). The ointment was topically applied in the neoplastic lesions and irradiated with a prototype composed of a cluster of 30 LEDs emitting at 630 nm, power 180 mW and power density of 5 mW/cm². After irradiation, the animals of group G2 were confined for 10 days and the animals of groups G3 for 20 days, so that it was possible to analyze the changes caused by photodynamic effect at different times after treatment.

2.3. ATR-FTIR Spectroscopy

ATR-FTIR measurements were performed in the range corresponding to the medium infrared (4000–400 cm^{-1}), with a spectral resolution of 4 cm^{-1}. Spectra were obtained using an Attenuated Total Reflectance (Smart Orbit, Thermo Scientific, Waltham, MA, USA) sampling mode coupled to a Fourier transform infrared spectrometer (Thermo Nicolet 6700, Waltham, MA, USA) system. The samples were pressed into the diamond crystal of ATR with a standardized pressure using a manometer. FTIR spectrometer was fitted with a deuterated triglycine sulfate (DTGS) detector (Thermo Scientific). For each spectrum, 100 scans were co-added and the spectrum obtained for each sample represents the averaged from 10 replicates measured in each histological cut. Thermo Scientific system was controlled with Omnic software (Thermo Scientific). Due to the overlapping bands in raw spectrum, the amplitude of second derivative of spectra were obtained and used to compare the groups. The second derivative of each spectrum was obtained with Matlab R2013a and smoothed with a Savitzky–Golay filter with a polynomial of second order in an eleven points window.

4. RESULTS AND DISCUSSION

3.1. Histopathological Analysis

Once that histopathological analysis is the gold standard for tissue evaluation, we assessed the morphological features of H&E (hematoxylin–eosin) stained tissue, as shown in Figure 1.

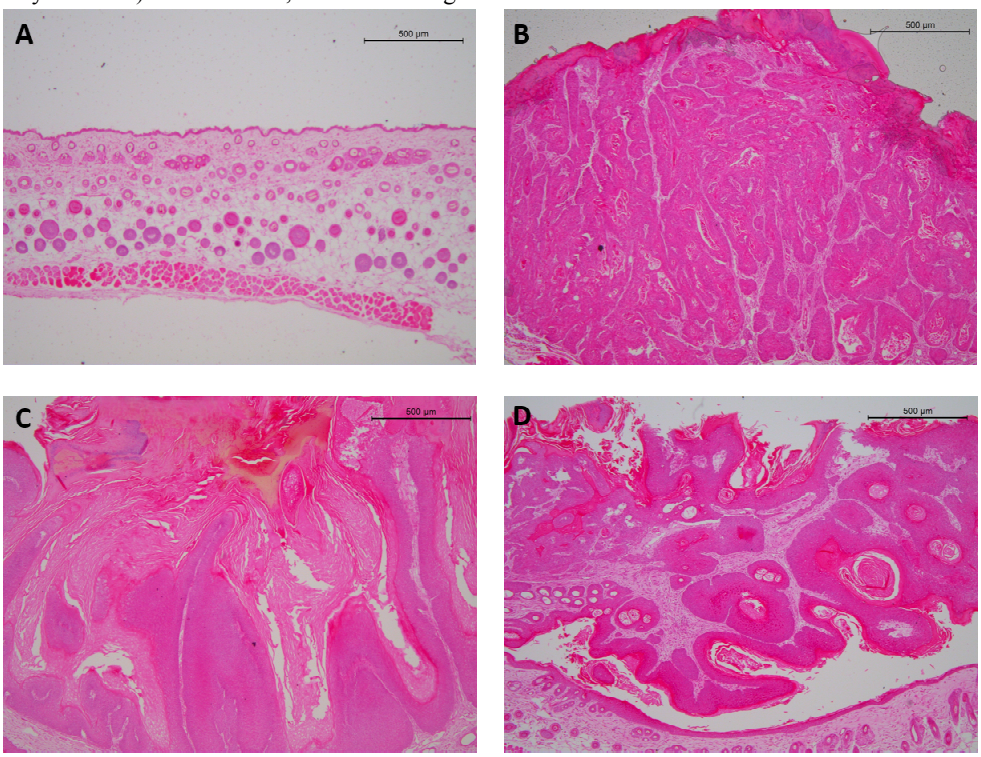

Figure 1. Light microscopy of representative histological sections hematoxylin–eosin (H&E) stained; (**A**) Healthy skin; (**B**) Neoplastic lesion; (**C**) Neoplastic lesion 10 days post-PDT; (**D**) Neoplastic lesion 20 days post-PDT

In general, the neoplastic lesions presented an intense proliferation of keratinocytes in an exophytic profile covered by a thick stratum corneum. Beyond this, moderate dysplasia was observed in epithelial basal layer, characterized by an intense nuclear hyperchromatism and cell pleomorphism. These findings classify the lesions obtained as papillomas and are in agreement with DMBA/TPA chemical carcinogenesis, which induces papillomatous lesions in a first stage and evolves in time for squamous cell carcinoma. After 10 days of PDT, some cases exhibited residual lesions with histopathological characteristics similar to the original tumors. At 20 days post-PDT the papillary pattern was not visible

and an intense reparative process of connective tissue was observed. However epithelial proliferation was also present in some cases indicating active lesions.

3.2. ATR-FTIR Spectroscopy

We used ATR-FTIR spectroscopy to compare non treated neoplastic lesions with treated lesions after 10 and 20 days post-PDT mediated by ALA as precursor of the photosensitizer PpIX. Figure 2 shows the spectra from 900–1800 cm^{-1} (fingerprint region), which provides information about vibrational modes associated with important cell content.

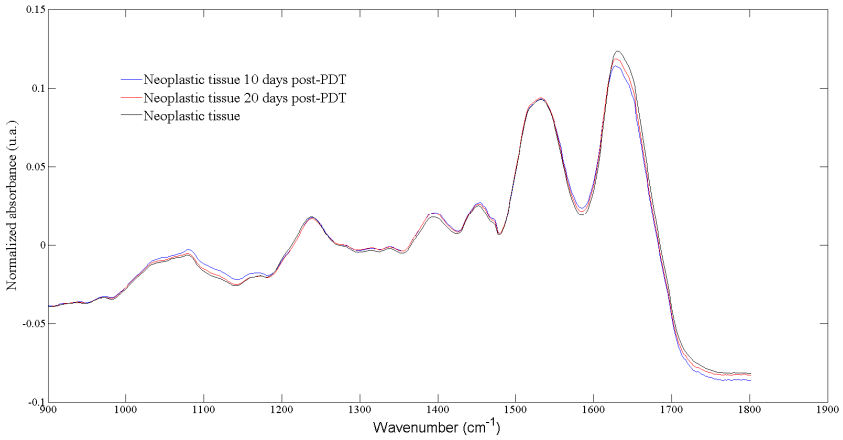

Figure 2. Fingerprint region (900–1800 cm^{-1}) of neoplastic tissue (blck line) and neoplastic lesions 10 days post-PDT (blue line) and 20 days post-PDT (red line).

As previous described, due to the overlapping of sub-bands in the row spectra, we calculated the second derivative of absorbance to compare the averaged spectra, as shown in Figure 3.

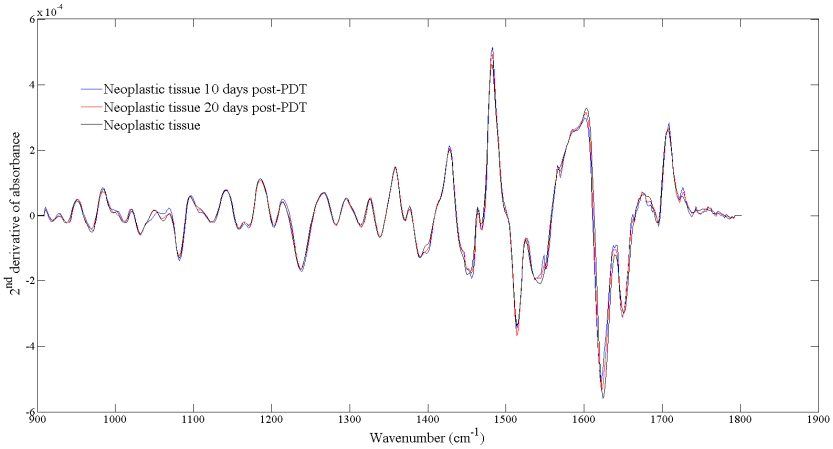

Figure 3. Second derivative of averaged spectra

Figure 3 depicts that all groups presented similar vibrational modes, which assignments are shown in Table 2.

Table 2. Band position and assignments

Band (cm^{-1})	Assignments [16]
1032	Glycogen absorption due to C-O and C-C stretching and C-O-H deformation motions
1082	PO_2^- symmetric; Glycogen absorption due to C-O and C-C stretching and C-O-H deformation motions
1151	Glycogen absorption due to C-O and C-C stretching and C-O-H deformation motions
1203	Collagen
1236	Collagen; Stretching PO2 asymmetric
1282	Collagen
1338	Collagen
1517	Amide II
1634	Antiparallel β-sheet structure of amide I
1657	α-helical structure of amide I
1696	Parallel β-sheet structure of amide I

The amplitude values of second derivative from vibrational modes identified in Table 2 presented normality using Shapiro–Wilk test and the comparison between the groups were performed by ANOVA one-way statistical technique. The histograms in Figure 4 shows the mean and standard error obtained for each group and data presented non statistically significant difference.

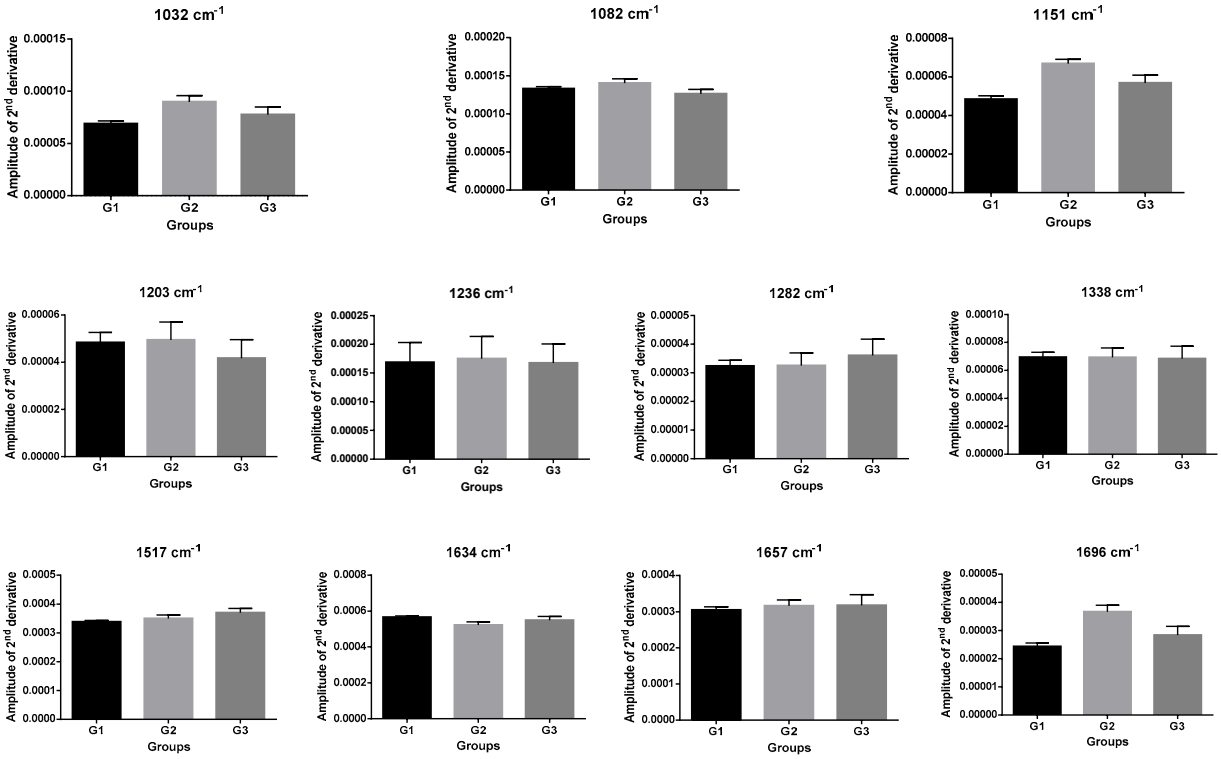

Figure 4. Mean and standard error of the amplitude values of secondary derivative from vibrational modes

The findings in Figure 4 show that amplitude values of second derivative from vibrational modes have similar behavior for all groups studied. The spectroscopic findings are in agreement with morphological features observed in histopathological analysis. In a previous study we reported significant clinical signs in neoplastic lesions as response for the photodynamic therapy mediated by ALA. However, as demonstrated with the histopathological analysis and FTIR spectroscopy showed active lesions even 20 days after PDT, which leads us to conclude that a single PDT session is not

enough to completely destroy the cancer cells. In this sense, for a treatment with higher effectiveness it would be necessary multiple sessions of PDT

5. CONCLUSIONS

The analysis of the results suggests that the findings observed for treated and non-treated neoplastic lesions by FTIR spectroscopy can be applied to the study of biochemical events caused by photodynamic therapy. Despite the fact that a statistical difference was not observed, the results were compatible to the morphological features observed in histopathological analysis. Thus, the technique has the potential to be used to complement the investigation of the main biochemical changes that photodynamic therapy promotes in tissue.

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