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Identification of skin lesions through aminolaevulinic acid-mediated photodynamic detection

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KEYWORDS
Fluorescence; Photodynamic detection; Skin lesion; Aminolaevulinic acid; Diagnosis

Summary Non-melanoma skin cancer is the most common cancer lesion worldwide. In Brazil, it represents 95% of all skin cancer lesions, and 25% of all tumor types. Early diagnosis allows treatment at initial stages of the disease, improving patient’s prognosis. Thus, it is of great importance the development of techniques to aid diagnosis, such as marked fluorescence, which we propose here for early detection of skin cancer lesions. In this study, we use a photosensitive substance, aminolaevulinic acid (ALA), as biomarkers, and analyze its in situ fluorescence response to light excitation. The use of ALA as a biomarker precursor is interesting because it shows selectivity for protoporphyrin IX production/concentration in abnormal cells. Protoporphyrin IX shows high fluorescence yield when excited with UV-blue light. In this study, ALA solutions (at 5% and 10% concentrations) were applied to malignant (basal cell carcinoma) and potentially malignant skin lesions (actinic and seborrheic keratoses), aiming to investigate our ability in detecting and distinguishing them by using this technique. At regular time intervals (15, 30, 45 and 60 min), fluorescence images were collected with a prototype system for wide-field fluorescence imaging. ALA has provided a marked fluorescence that allowed significant discrimination of normal and tumor. Potentially malignant and benign lesions were all well-identified by their autofluorescence; photodynamic detection did not improve diagnostics. This technique also provided a better delineation of the lesion margins, which is very important for an effective treatment of malignant, potentially malignant and benign skin lesions.

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Introduction

Skin cancer can be classified in two major groups: non-melanoma and melanoma. Non-melanoma lesions are the most common ones, with estimative between 2 and 3 million cases per year around the world [1,2].

The most common non-melanoma skin cancer is the basal cell carcinoma (BCC), originated in the basal layer of epidermis. Its occurrence is higher in middle-aged and elderly people with skin phototype I or II, and its appearance is directly related to prolonged exposure to solar radiation throughout life. It is usually observed at body areas exposed to the sun, such as arms, face and neck [2–6].

Others non-malignant, but also important, skin lesions — due to the large occurrence and possibility of misdiagnosis — are actinic keratosis (AK) and seborrheic keratosis (SK). Actinic keratosis is a skin epidermal dysplasia that develops due to the cumulative effect of ultraviolet radiation from sun exposure. It is more prevalent at the same population group as for skin cancer and presents a higher incidence on face, scalp, arms and hands. The seborrheic keratosis, usually associated to genetic origin, affects mainly the chest and face, but it can occur also in the limbs. These lesions have a warty appearance, irregular surface and a soft consistency [3–5]. Therefore, being able to differentiate these lesions is of relevance for clinical diagnosis.

Prevention and detection are the major challenges in dealing with skin cancer: prevention depends mainly on the use of sunscreen and avoidance of exposure to high ultraviolet irradiation, and early diagnosis improves patient prognosis, and reduces patient morbidity, as well as treatment complexity and costs [4,5,7,8].

Skin diagnosis is initiated with whole body visualization under illumination with white light sources, during the clinical examination. If any lesion is identified, a dermatoscopic examination is performed to obtain additional details. This equipment magnifies the visualization field in 10 up to 40 times. When a malignant condition is suspected, a biopsy is performed. Histopathological analysis is “gold standard” for tissue diagnosis. Clinical characteristics of benign lesions and carcinomas at early stage are similar, and the large variability of professional skills to recognize early malignant features result in a non-efficient inter-lesion discrimination. Biopsy is an invasive technique which does not provide information of lesion margins, which is relevant for treatment planning, since surgical resection of the lesion has to be performed beyond the clinically apparent margin for the malignant conditions. For the lesions in areas of cosmetic relevance or in surgically complex anatomic regions, especially in face and scalp, the treatment must consider removing the least possible amount of healthy tissue [9,10].

The detection of lesions using illumination parameters distinct to the conventional white light, and the fluorescence visualization has the potential to increase in situ tissue discrimination. Among optical techniques for tissue diagnostics, one of the most investigated one is autofluorescence [11–14], because tissue biochemical and structural changes that take place during cancer development and progression alter the light—tissue interactions. Tissue fluorescence patterns also depend on these interactions. Thus, fluorescence is capable of detecting such changes. At skin, autofluorescence helps in the detection and delineation of tumor margins, limiting damage caused by the tumor recurrence due to an incomplete surgical removal [11]. The use of skin fluorescence also aids in distinguishing tumor from healthy tissue [12] and benign lesions [13].

The photodetection using an exogenous marker can increase the contrast resolution between abnormal and healthy tissue. In photodynamic diagnosis, a solution of aminolaevulinic acid (ALA) is topically placed on lesion surface. ALA is a protoporphyrin IX (PpIX) precursor which is an endogenous photosensitizer (PS) that naturally occurs in the cellular heme biosynthetic pathway. After ALA is localized into the cell, it enters in the heme biosynthesis and stimulates the PpIX production. This P5 has fluorescence properties, and accumulates preferentially in abnormal tissues, allowing its use as a complementary diagnostic tool [14,15]. A higher ALA-PpIX production at malignant cells has been observed at several studies [14,16,17], supporting ALA use as a potential marker for photodynamic diagnosis.

For diagnostic applications, after ALA solution is administered to the target tissue, a time interval is required for enough PpIX production and accumulation allowing its fluorescence visualization at the interrogated tissue. Then, skin is illuminated using light at the UV-blue spectral range, in order to excite the PpIX molecules inside the tumor cells and to induce their fluorescence emission. Under this excitation range, the PpIX shows an intense orange-red fluorescence, which strongly contrasts with the green autofluorescence of the biological tissues.

In this clinical study, the efficacy of ALA photodynamic detection (ALA-PDD) of malignant, potentially malignant and benign skin lesions was investigated. To the best of our knowledge, there is no analysis evaluating the best ALA concentration for the photodynamic detection of skin lesions. The aim of the present study was to evaluate the efficacy of a clinical protocol for the ALA-PDD for the detection of skin lesions.

Material and methods

Patient accrual

In this study, 43 patients of both genders, Caucasian, between 45 and 91 years old, with BCC, AK, and SK were investigated between May 2010 and June 2011. A total of 71 lesions were imaged: 29 BCC, 31 AK, and 11 SK.

All lesions were clinically diagnosed, and BCC was confirmed by histopathology. All patients were evaluated at the Skin Department of Amaral Carvalho Hospital, and all underwent surgery after fluorescence evaluation. This research was approved by the Review Board on Ethics in Research from the Amaral Carvalho Hospital Foundation (CEPFFAC 123/2010). Patients and their respective caregivers signed an informed written consent for participation in the clinical study.

Photosensitization

The pro-drug used was 5-aminolevulinic acid (5-ALA — FSUESCC “NIOPIIK” B SADAVAYAd. 1k4, Moscow, Russia) manipulated at concentrations of 5% and 10% in Milli-Q water solution with 3% of dimethylsulfoxide (DMSO) and
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White light  Autofluorescence  60 min after ALA

BCC

AK

SK

Figure 1  Skin lesions images using white light illumination (left column), autofluorescence (central column) and 1 h after application ALA (right column). The lines show typical images for: BCC (top), AK (center), and SK (bottom).

1 mM ethylenediaminetetraacetic acid (EDTA). DMSO is an enhancer of ALA penetration in tissue, and EDTA inhibits the enzyme ferrochelatase, resulting in a higher concentration of PpIX in cells. The PpIX production occurs inside the mitochondria, and it is induced by the ALA present in the biosynthetic heme pathway [18–20].

Fluorescence device

The fluorescence evaluation was performed using a homemade handheld device for widefield fluorescence imaging. The illumination system is composed of a set of LED arrays with emission between 380–420 nm, with peak in 400 nm and irradiance at 50 mW/cm². Three optical components are used: a filter at the excitation path (band-pass filter between 390 nm and 460 nm), a dichroic mirror (reflection of the blue spectrum and transmission of the green–red spectrum), and a filter at the collection path (long-pass filter, over 475 nm). This optical arrangement was designed to improve the contrast visualization (difference between colors and intensities) of light in green and red regions of the visible spectrum, main fluorescence emissions of the biological tissue and PpIX, respectively. A digital color camera (Sony DSC-H50) was coupled at the device through an adapter for image acquisition [21].

Clinical protocol

The skin lesions were initially evaluated under conventional white light and dermatoscopy by a certified dermatologist (AGS), and the clinical characteristics described at the patient chart.

Solutions of ALA 5% or 10% were prepared, and immersed in a warm bath for total dissolution of ALA. After that, the solution was kept at room temperature, protected from light until the moment of use.

Lesions were cleaned with chlorhexidine soap, and cleansed with NaCl 0.9% solution. No curettage procedure was performed in the interrogated lesions, because our aim was to analyze the efficacy of the photodynamic diagnostics without any invasive procedure, so variations due to the inherent lesion characteristics, like surface keratin and dead cell layers, could not be avoided.

Five percent ALA solution was applied on 54 lesions (21 BCCs, 22 AK, and 11 SK). In order to determine if a higher pro-drug concentration could improve the photodynamic detection, 10% ALA solution was applied on 17 lesions (8 BCCs and 9 AK), and the same analysis parameters were performed.

The ALA solution was applied over the lesion surface, covering all clinical margins. After the pro-drug application, the lesions were protected from light exposure using an occlusive curative with PVC plastic film, aluminum foil and bandage. Images were collected at regular time intervals (15, 30, 45 and 60 min) in a dark room. At each time interval, the curative was removed for image collection, and replaced just after imaging.

The images were processed using an algorithm written in Matlab® 7.5 (MathWorks, USA) for evaluation of PpIX fluorescence emission. This algorithm determines the number of pixels for the component R ("red") in the RGB matrix for each image, attributing them to red light intensity, which was associated to the photosensitizer fluorescence.
Statistics

Data are expressed as average values and standard deviations. The Shapiro–Wilks test was used to determine the normality of the data. To compare differences between lesions and normal tissue, before and after ALA application paired Student’s t-test was used, and for discrimination between lesion and normal tissue, unpaired Student’s t-test was applied.

The software Statistica for Windows, release 7 (Statsoft, Tulsa, OK) was used for the statistical analysis and the significance level was set at 5% (p < 0.05).

Results

ALA 5%

The image sequence in Fig. 1 shows the PpIX production over time in a BCC at right temporal (Fig. 1a–c), an AK at the right forearm (Fig. 1d–f) and a SK at the chest (Fig. 1g–i). The left column shows the image of skin lesions under white light illumination (Fig. 1a, d, g); the central column their autofluorescence (Fig. 1b, e, i); and the right column, typical lesion photodynamic fluorescence, one hour after ALA application.

Healthy tissue shows fluorescence at the green region of the spectrum, and abnormal tissues, as BCC, AK, and SK, show loss of fluorescence emission (LOF), visualized as a darker region. The red fluorescence at Fig. 1c, is observed at the same regions previously associated with LOF. The visualized bright spots correspond to areas of high fluorescence emission (Fig. 1e and f) from hyper-keratinized areas of the lesion surface. It can be noted that SK does not fluoresce (Fig. 1h), visualized as a dark region with several fluorescent points. This autofluorescence pattern is related to a high melanin content that extensively absorbs both excitation and fluorescence lights, and to the corneal structures inside the lesion, resulting in LOF and highly fluorescent spots, respectively [9,22]. No qualitative visualization of the PpIX production could be observed at the AK and SK.

To quantify the PpIX showed in the images, regions at both normal and lesion tissue were selected, and the intensity of red fluorescence was calculated for each image. Then, intensity was normalized by its background red intensity (the red emission present at tissue autofluorescence), to obtain only the contribution of the PpIX emission in the tissue fluorescence after the ALA treatment. Fig. 2 shows graphs of PpIX intensity emission as a function of time of the three lesions shown in Fig. 1. It is possible to observe that the PpIX fluorescence intensity increases with time for BCC, while remains almost constant for the other lesions, enhancing the qualitative analysis.

Statistical tests were performed to determine whether there was significant difference between the fluorescence of the lesion and normal tissue (before and after ALA application) and between these lesions before and after ALA application. In order to show that the PpIX production occurs preferentially in abnormal cells, tests were also performed by evaluating normal tissue before and after ALA application.

In the comparison between lesion and normal tissue prior to ALA application (autofluorescence), there was significant difference in all cases (p < 0.01). The same was true when comparing the lesion and normal tissue 60 min after ALA application (p < 0.01).

When the lesion autofluorescence was compared with the values of fluorescence 60 min after ALA application, there was significant difference (p < 0.01) only for the BCC. Comparison between normal tissue before and 60 min after

![Figure 2](image-url)
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**Figure 3** Skin lesions images using white light illumination (left column), autofluorescence (central column) and 1 hour after application ALA (right column). The lines show typical images for: BCC (top) and AK (bottom).

ALA application showed no statistical significance for any group.

**ALA 10%**

The images in Fig. 3 show PpIX fluorescence emission over time in a BCC lesion (Fig. 1a–c) and an AK lesion (Fig. 1d–f). The left column shows the skin lesions under white light illumination (Fig. 1a and d); the central column, their autofluorescence (Fig. 1b and e); and the right column, lesion fluorescence one hour after ALA application.

For AK lesion, the result was similar to 5% ALA. For BCC lesion, however, a reduced production of PpIX was observed, although the quantitative analysis showed an increase in PpIX fluorescence intensity (Fig. 4), it was preferentially located at the lesion borders as showed in Fig. 3c. Here it is important to point out that this BCC lesion, in comparison to the one in Fig. 1a, showed a higher necrotic tissue at lesion surface, preventing an efficient penetration of the ALA solution, due to a non-curettage of these lesions, the PpIX emission was observed at the lesion regions of less keratinized surface.

The statistical tests for comparison between lesion and normal tissue, both prior to ALA application (autofluorescence) and 60 min after, showed significant differences in all cases ($p < 0.01$). When lesion and normal tissues’ autofluorescence was compared to fluorescence intensity values 60 min after ALA application, no significant difference was observed.

**Discussion**

The observed LOF from the abnormal skin has been also described in other studies [12,23,24], and this is mainly justified by the decrease in collagen fluorescence caused by fiber linkage breakdown [25]. Others skin components such as flavins do not have a great contribution due to the excitation wavelength, which is not within the absorption wavelengths of these components [26].

Fluorescence images after the ALA application show preferential accumulation of PpIX in skin areas associated or within the BCC lesions, representing a potential aid to the cancer diagnosis, since it increases the visualization contrast between normal and abnormal tissue. With this increase in contrast, an improved discrimination of the lesion superficial margins is provided, information highly important for the treatment planning and execution.

The difference between the autofluorescence from normal and abnormal tissue is noticeable. This observation is in agreement with other studies in literature which demonstrate a reduction of the green fluorescence at skin, and an increase in the red fluorescence for lesions [16,17,27]. After application of the ALA solution, the difference between tissues is more evident with time, when considering the red fluorescence emission. The use of the photodynamic diagnosis only showed a response for BCC lesion, but there was no improvement at the visual discrimination to the autofluorescence detection.

A possible reason for the lesions in which there was no increase in the red fluorescence emission is the inefficient penetration of ALA solution; mainly caused by areas of hyper-keratinization and necrotic tissue.

Several lesions of BCC and AK present extensive keratinization on the surface, which behaves as physical barrier to the penetration of ALA solution [28]. The result is a large number of lesions which showed no increase in PpIX fluorescence even 60 min after ALA application. However, this keratin layer can be removed by curettage, which is a simple medical procedure. Indeed, the curettage is performed in ALA-photodynamic therapy (PDT) for the removal of the keratin and dead cells layers to improve the pro-drug penetration [29,30]. In the investigated protocol, the curettage was not performed, since the aim was to verify the efficacy of the photodynamic detection using a procedure with the least possible number of steps. Based on the present results, an improved performance and diagnostic resolution may be achieved, if the curettage is performed previously to ALA solution placement.
For SK, the high light absorption by melanin hinders fluorescence visualization, in both autofluorescence and photodynamic detection.

However, for SK and AK lesions, the autofluorescence detection presents already an improved visualization of the lesion. The photodynamic detection did not show a significant increase in the red fluorescence intensity. Therefore, sensitization has shown to be important only for BCC lesions.

The autofluorescence for SK lesions may help on the differential diagnostics to melanoma. The corneum pseudocysts, a tissue structure that differentiates the SK from melanoma, become evident in autofluorescence images [22,31]. In melanoma lesions, the autofluorescence pattern is an evident LOF, visualized as a dark area without any fluorescent spots.

The anatomical site also plays an important role on the penetration of the ALA solution. Lesions that are located in the arm and forearm usually present a thicker layer of keratin than those located in the head and neck, reducing the pro-drug penetration. In arm/forearm cases, the PpIX fluorescence emission was less intense in the lesions borders, as well as less intense when compared to cases in head and neck.

Even when the ALA concentration is increased from 5% to 10%, most of the lesions did not show enough accumulation of PpIX, reinforcing the hypothesis of the physical barrier impairing an efficient tissue photosensitization [15,32,33].

Although curettage has been already used for other protocols as a safe and effective practice [29,30], this study aimed to verify how feasible this technique could be with no application of any invasive procedure.

**Conclusion**

Fluorescence investigation of BCC lesions, using both autofluorescence and photodynamic detection techniques, showed ability to significantly differentiate normal from tumor tissue. PpIX accumulation in skin lesions increases visual contrast between healthy and abnormal tissues. This contrast allows for improved identification of lesion margins.

For detection of non-malignant skin lesions, AK and SK, the use of autofluorescence images only proved to be insufficient; no additional information was provided by using photodynamic diagnosis. In the case of AK, due to excessive keratin at lesion surface, ALA solution did not adequately penetrate into the tissue to induce PpIX production. For SK lesions, with higher melanin content, autofluorescence images result in tissue LOF, with highly fluorescent spots inside the lesion. These spots are corneum pseudocysts, which are characteristic structures at those lesions. Therefore, observing them contributes to diagnosis, especially to differentiate them from melanoma.

Increasing concentration of ALA form 5% to ALA 10% for BCC and AK showed that the low PpIX production was not due to low concentration, but to the limited penetration of the pro-drug solution, which might be managed by performing curettage on lesions prior to ALA administration.

Widefield fluorescence imaging is, therefore, a potential auxiliary tool for skin diagnosis, helping to define the lesions margins; and ALA-mediated photodynamic detection showed to be able to increase visual contrast of BCC lesions.

**References**


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