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Enhancement of blood porphyrin emission intensity with aminolevulinic acid administration: A new concept for photodynamic diagnosis of early prostate cancer

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Aminolevulinic acid;
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Summary
Background: The objective of this paper was to verify if the oral administration of δ-aminolevulinic acid (ALA) in animals with prostate tumor can increase the sensitivity of cancer diagnosis by protoporphyrin IX blood autofluorescence. In this study, the autofluorescence of blood porphyrin was analyzed using fluorescence spectroscopy on healthy male NUDE mice and in those with prostate cancer induced by the inoculation of DU145 cells.
Methods: A total of 18 male NUDE mice, ~8 weeks old on arrival were divided into 3 groups: Control, Tumor and ALA Tumor. The autofluorescence of blood porphyrin of the groups was analyzed using fluorescence spectroscopy at different days after tumor induction, to monitor the tumor progression. Emission spectra were obtained by exciting the samples at 405 nm. The animals inoculated had their blood collected with and without oral ALA solution administration to compare PPIX endogenous (Tumor group) and exogenous (ALA Tumor group) signal intensity and to establish a method to diagnosis early prostate cancer.
Results: Significant differences were observed in autofluorescence intensities measured in the 575—725 nm spectral regions for the studied groups.
Conclusions: The results showed an enhancement of almost 100% in blood PPIX fluorescence, using the oral administration of δ-aminolevulinic acid on male NUDE mice with prostate cancer, making fluorescence measurements more accurate and sensitive since the first week after tumor induction.

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Introduction

The three most common forms of prostate disease are benign prostatic hyperplasia (BPH), prostatitis (inflammation of the prostate) and cancer. A man may experience one problem or a combination of two or more problems.

Prostate cancer is the most common non-skin related male cancer type in the world [1]. Prostate cancer is diagnosed using a variety of tests including biopsies of the prostate, digital rectal examination (DRE), transrectal ultrasonography (TRUS), and assaying prostate-specific antigen (PSA). DRE and TRUS are widely employed but are very limited in their ability to diagnose prostate cancer since they do not provide the ability to distinguish between benign enlargements of the prostate (BPH) and a cancerous prostate [2—4]. Although PSA measurement is regarded as the best conventional serum tumor marker currently available, it is not specific enough for a definitive diagnosis of prostate cancer because the PSA also increases in cases of BPH or prostate inflammation [5].

Biopsy of prostate tissue can definitely identify prostate cancer in most cases. This method results in a grading called the Gleason score which is based on the pattern of cancer tissue observed under a microscope [6,7]. Despite the reliability of this method, it is evident that it is extremely invasive.

An ideal method of cancer detection should be quick, cheap and reliable. Fluorescence detection of cancers, or photodynamic diagnosis (PDD), is the method of cancer detection based on light induced fluorescence (LIF) to characterize tissues. Fluorescence spectroscopy requires a fluorophore [8—13].

There are a number of studies about the exogenous administration of δ-aminolevulinic acid, ALA, to detect or treat early-stage cancers [14]. ALA is a precursor in the heme biosynthesis pathway and is metabolized to fluorescent Protoporphyrin IX or PPIX, before being converted to photoinactive heme products. The selective accumulation of PPIX in malignant tissue provides a strong color contrast between the intense red fluorescence of malignant lesions and the weak fluorescence of normal tissue [15—17]. Abnormal metabolism of PPIX has also been observed in total blood, plasma and erythroid cells of cancerous patients [18—20]. Nowadays, ALA is probably the photosensitizer (also called pro-drug) more selective to treatment of cancer currently known in oncology, because the accumulation of PPIX is higher in malignant cells than in normal tissues [21].

ALA can be delivered to the target tissue by topical application, oral administration, or by intravenous and intraperitoneal injection. Each technique delivers a different proportion of the total dose of ALA to the target tissues, and with different timing [22,23].

Recently, the autofluorescence of blood protoporphyrin IX (PPIX) was analyzed using fluorescence and excitation spectroscopy on healthy male NUDE mice and in those with prostate cancer induced by inoculation of DU145 cells [18]. A significant contrast between the blood of normal and cancer subjects could be established. Blood PPIX fluorescence showed an enhancement on the fluorescence band around 632 nm following tumor growth.

The objective of this paper was to verify if the oral administration of δ-aminolevulinic acid (ALA) in animals with prostate tumor can increase the sensitivity of cancer diagnosis by protoporphyrin IX blood autofluorescence. In this study, the autofluorescence of blood porphyrin was analyzed using fluorescence spectroscopy on healthy male NUDE mice and in those with prostate cancer induced by the inoculation of DU145 cells

Materials and methods

Cell line and cell culture conditions

DU145 cells were cultured in DMEM containing high glucose (4.5 g/L at 25 mM) and supplemented with 100 units/mL Penicillin, 50 mg/mL Streptomycin, and 10% FBS. The cells were maintained in a humid chamber at 37 °C in an atmosphere of 5% CO_2.

Animals and tumor induction

A total of 18 male NUDE mice, ∼8 weeks old on arrival, were obtained from the IPEN-USP and housed in laminar airflow cabinets under pathogen-free conditions with a 12-h light/12-h dark schedule and fed autoclaved standard chow and water *ad libitum*.

The orthotopic tumor model of prostate cancer was used in 15 animals, where 1×10^5 cells were inoculated into the prostate gland in a volume of 10 μL of sterile phosphate buffered saline (PBS). These 15 animals were divided into 2 groups: 7 animals for the group without ALA (Tumor group) and 8 animals for the ALA group (ALA Tumor group). The control animals, three mice, have been inoculated with only PBS into prostate gland (without cells), and had their blood collected without ALA administration (Control group) and after, with oral ALA administration (called ALA Control Group).

Monitoring of tumor growth

The animals were monitored and blood samples were collected 7, 14, 21, 35 and 49 days after tumor induction. Approximately 250 μL of blood was collected with Heparin as anticoagulant by retro-orbital plexus with a glass capillary for each animal. All experiments were performed in accordance with the institutional guidelines animal care.

Protoporphyrin IX calibration curve

Protoporphyrin Standard (Sigma Porphyrin Products, Logan, Utah, USA) was dissolved in acetone (analytical purity) and solutions containing concentrations of 0.01, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1.5 μg/mL were prepared in triplicate. Emission spectra were obtained exciting samples at 405 nm. Average curves of each concentration were obtained and emissions between 575 and 725 nm were plotted as a function of protoporphyrin IX concentration to perform a calibration curve.

Oral administration of ALA solution

Solutions of ALA (Sigma) were freshly prepared, before each administration in every day of blood collection (at the 7th, 14th, 21st, 35th and 49th day after tumor induction, administrated to animals from ALA Tumor Group), by dissolving 0.05 g of ALA in 5 mL of PBS. The pH of the solutions was adjusted to ~7.2 by addition of NaOH solution. A total of 500 μL of ALA solution (5 mg per animal) were given to each animal from ALA Tumor group (on the days mentioned above) and ALA Control group through a bulbtip gavage needle. Blood was collected after 3 h 30 (±15 min) of ALA administration.

Porphyrin extraction

450 μL of analytical grade acetone were added to 150 μL of total blood collected and mixed well. The mixture was centrifuged at 4000 rpm for 15 min. The clear supernatant of mixture was stored in a clean tube and spectrofluorometer analyses were carried out at the same day.

Fluorescent spectral analyses

The emission spectra were obtained by exciting the samples at 405 nm, inside of a 1 mm optical path cuvette. The fluorescence of the samples was analyzed with a Horiba Jobin Yvon Fluorolog 3 Fluorimeter in the range of 575—725 nm. The emission peaks had been compared with the standard curve of PPIX in acetone solution.

Tumor excision and histological analysis

At the 7th, 14th, 21st and 35th day, 3 animals with prostate tumor were sacrificed and at 49th day, the remaining animals used in the experiment, including animals from control group, were sacrificed following the American Veterinary Medical Association guidelines for euthanasia. The prostates were excised and washed in PBS, fixed in 10% PBS-buffered formalin for 24 h, and then routinely processed for paraffin-embedding. Histological analysis was performed in 4 μm sections stained with hematoxylin and eosin.

Results

In Fig. 1, the emission spectrum of 0.06 μg/mL PPIX acetone solution (Standard PPIX) is compared with the one obtained from PPIX of non-cancer animal total blood (Animal Blood PPIX) extracted with acetone. In these spectra we observed the two characteristic bands of PPIX, centered on 632 nm and 700 nm. A very similar emission profile can be observed, indicating that PPIX is the main factor responsible for the emission signal obtained in the blood. This fact is due to the extraction of total blood porphyrin using acetone, which eliminates the signal of other molecular species. Consequently, light scattering from blood samples is negligible.

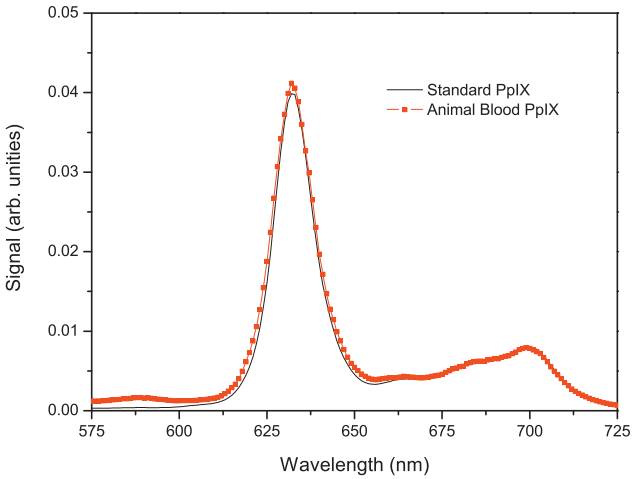

Figure 1 Comparison between standard PPIX (0.06 μg/mL) and endogenous PPIX fluorescence from animal blood extracted.

Obtaining PPIX calibration curve

To quantify PPIX concentration present in the blood, a PPIX calibration curve was obtained and is shown in Fig. 2. To obtain this figure, the area measured under the porphyrin emission curve in the spectral region of 575—725 nm was plotted as a function of PPIX standard acetone solutions concentrations ranging from 0.05 to 1.5 μg/mL. It can be seen that it increases linearly. A linear function was fitted to the experimental data to determine a calibration curve. Using this calibration curve it is possible to estimate blood porphyrin concentrations on biological samples.

Quantification of PPIX in blood

Nude mice bearing prostate tumors were investigated for their PPIX total blood accumulation response. For this purpose total blood porphyrin was extracted from male Nude mice, in which the tumor cells were inoculated. The results are shown in Fig. 3. In this figure, signal emission area, obtained integrating endogenous PPIX emission spectra in the range of 575—725 nm, was plotted as a function of days of tumor growth (7th, 14th, 21st, 35th and 49th day

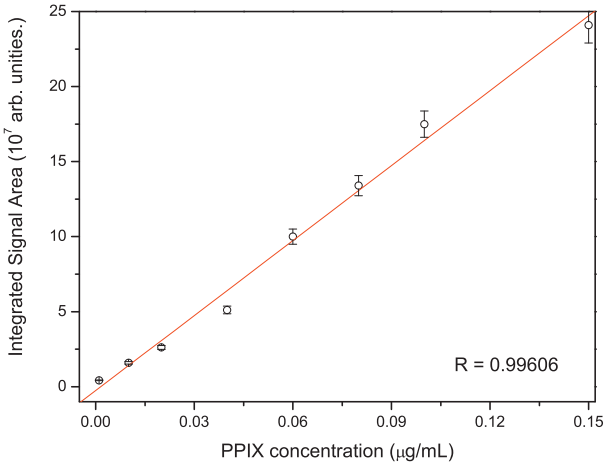

Figure 2 Calibration curve of PPIX acetone solutions.

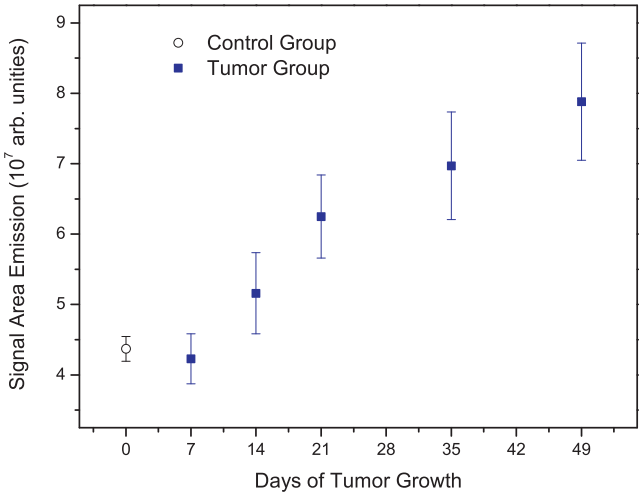

Figure 3 The area of emission spectra of PPIX extracted from animals' blood, in the range of 575—725 nm plotted as a function of the days of tumor growth). Day 0 corresponds to Control Group and Days 7, 14, 21, 35 and 49 correspond to Tumor Group (endogenous PPIX emission).

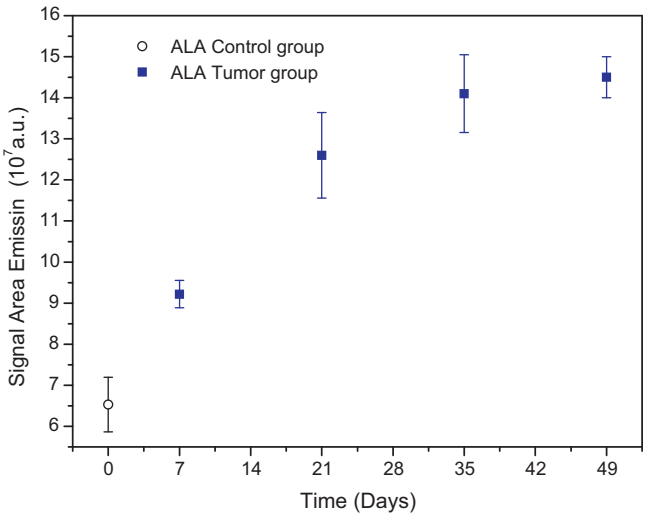

Figure 4 The area of emission spectra of PPIX extracted from blood of animals that received oral ALA plotted as a function of time (days of tumor growth). Day 0 corresponds to ALA Control Group (animals inoculated only with PBS and had received ALA) and Days 7, 21, 35 and 49 correspond to ALA Tumor Group (8 mice bearing prostate tumor that had received oral ALA).

after inoculation procedure). Each point corresponds to an average of signal from each studied group. For the Control Group, three animals were used and were inoculated only with PBS (without DU145 cells) to exclude enhancement of PPIX from possible inflammation, infection or surgery reaction and the result from this group was indicated at day 0. For the Tumor Group, seven animals were used and were inoculated with 1×10^5 DU145 cells and had their blood collected from 7 to 49 days after tumor induction. It can be clearly observed that the mean of the values increases in intensity as the tumor grows inside the animals, indicating that porphyrin is accumulating in their blood.

Although, the average of samples spectra shows different values in each week, some of the standard error (SE) bars coincide in the intervals analyzed. The mean ± SE can be clearly distinguished between control and tumor animals after the second week, but there are no statistical difference between the first 14 days after DU145 cells inoculation and the control animals.

Effects of ALA oral administration in the PPIX emission signal in the blood

We analyzed the possibility to enhance the PPIX emission intensity present in the blood upon ALA administration.

In this study we analyzed the progression of prostate tumor versus the enhancement of PPIX emission signal, after ALA administration, from ALA Control group animals (plotted as day 0) and ALA Tumor group animals (at days 7, 21, 35 and 49 after tumor induced). Since the first week of tumor growth, it is possible to observe a significant difference between the intensity of PPIX extracted from total blood from Control animals with ALA (ALA Control Group) and animals with prostate tumor that had received ALA (ALA Tumor group). These significant differences can be used to diagnosis early prostate cancer since the first's cancerous changes and help to determine the tumor stage.

In Figs. 3 and 4, data are presented as mean ± standard error (SE). Kinetics of fluorescence accumulation in the total blood from Control animals (with and without ALA), Tumor and ALA Tumor groups were assessed using Anova test to identify values deviating from the linear relationship, and p-value < 0.05 was considered statistically significantly different.

Table 1 represents the concentration of endogenous and exogenous (ALA-induced PPIX) porphyrin extracted from animals' blood, calculated from the calibration curve of Fig. 1.

Table 1 PPIX concentrations of animals' blood versus tumor growth (growth days after tumor induction) with and without ALA administration and the values of tumor mass area.

Groups	[PPIX] endogenous mean ± SE (μg/mL)	[PPIX] exogenous-ALA mean ± SE (μg/mL)	Tumor mass area (μm^2)
Control	0.0274 ± 0.004	0.0398 ± 0.009	–
Tumor 7 days	0.0270 ± 0.007	0.0568 ± 0.006	–
Tumor 14 days	0.0348 ± 0.006	–	1420.4
Tumor 21 days	0.0391 ± 0.010	0.0781 ± 0.014	4131.7
Tumor 35 days	0.0435 ± 0.013	0.0876 ± 0.017	18,520.2
Tumor 49 days	0.0486 ± 0.012	0.0893 ± 0.007	47,331.3

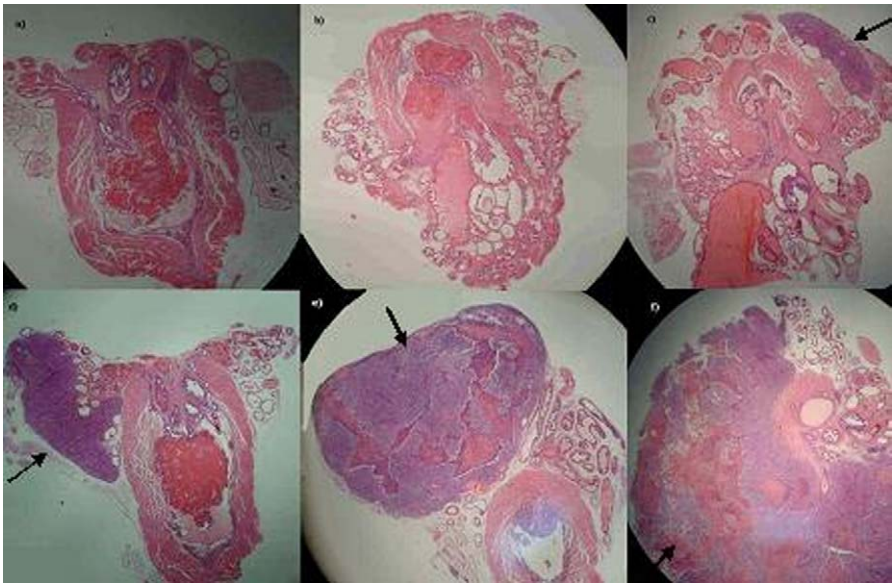

Figure 5 Histological prostate slides of all points of tumor progression. (a) The Control group prostate slide; (b) prostate tumor at 7th day; (c) prostate tumor at 14th day; (d) prostate tumor at 21st day; (e) prostate tumor at 35th day; (f) prostate tumor at 49th day.

Analyses of tumor-bearing prostate were performed at all points of the experiment, to evaluate the tumor progression and to investigate the correlation between tumor mass growth and PPIX fluorescence from blood. Analysis of tumor cells in situ, using HE (Hematoxylin and Eosin) staining, indicated the increases of the tumor area during the experiment, Fig. 5.

Quantitative analysis of these sections showed that within 7 days from inoculation the tumor mass had not reached a detectable area yet, using HE staining (Fig. 5b), but the emission intensity of the blood from animals that received ALA (within 7 days) was almost 1.5-fold that of ALA Control group intensity. This result shows how sensitive ALA induced PPIX photodynamic diagnosis is.

The calculated areas of tumors at 14, 21, 35 and 49 days are show in Table 1.

A panel of histological prostate slides (a—f) of control (inoculated PBS) and tumor (inoculated DU145 cells after 7, 14, 21, 35 and 49 days) groups are shown in Fig. 5. The Control group samples (Fig. 5a) show normal prostate gland, and any inflammation characteristics were observed. Fig. 5b represents animal tumor group at 7 days from tumor induced. At this point of tumor progression no evidence of tumor cells could be observed by HE staining.

Histological analysis of the samples at 14th (Fig. 5c), 21st (Fig. 5d), 35th (Fig. 5e) and 49th (Fig. 5f) day evidenced proliferating tumor cell areas.

Discussions

It is our understanding that this is the first study that analysis PPIX accumulated in the blood of animals with cancer after ALA administration. PPIX accumulated in tumor cells is transferred to blood and can be analyzed by porphyrin extraction from total blood. Although, the results, in Fig. 4, indicate an increase of PPIX production in both, control and tumor animals, since almost all cells can synthesize PPIX, mice bearing prostate tumors present the largest concentration of PPIX.

As ALA is a precursor in the heme biosynthesis pathway, it is metabolized to fluorescent PPIX. The ALA solution will reach tumors cells and the tumor tissues accumulate temporarily more PPIX than normal tissues. The selective accumulation of PPIX in malignant tissue provides a contrast between control animals and those induced with tumors. This contrast was reported by several studies to detect surgical margins during radical and partial prostatectomy in patients and animals with carcinoma of the prostate and photodynamic therapy for prostate cancer and many other cancers [7,14].

The results indicated that the increase in PPIX blood emission accompanied the growth of the tumor mass was at all points significantly different from the values obtained for the normal prostate (control group animals), when animals have received ALA. These findings demonstrate a true exogenous accumulation of PPIX by prostate cancer cells caused by tumor-specific metabolic alterations and these PPIX molecules are transferred to blood.

Our results indicated that although at the first week histological slice did not show tumor mass yet, we observed it was already possible to identify the tumor presence on the samples of blood collected from animals that have received ALA. This fact indicated that this method can aid the early diagnosis of prostate tumors with high sensibility.

At 35th and 49th we observed the same fluorescent intensity. It may occurs because the necrosis areas are bigger and death cells cannot synthesize PPIX and as ALA has a limitation to penetrate deeply into tissue, it cannot reach all tumor cells to synthesize more PPIX.

The use of ALA esters with high lipophilicity would improve the effectiveness of ALA. Methyl and Hexyl Aminole-

vulinic (h-ALA) acid have shown the best results. A concentration of 100-fold lower than h-ALA compared to ALA increases the emission intensity by 2—3 fold than that induced by ALA [24,25].

ALA solution has no emission signal. The oral administration doses of ALA did not result in any adverse reaction and returning to background levels in up to 24 h [26—29].

Larger and better-designed studies are being developed to elaborate more on this matter, to verify the most appropriate concentration of ALA solution and the best time for ALA administration in animals.

Conclusions

This study shows that the administration of ALA in animals with prostate tumor leads to an increase in emission signal of PPIX extracted from their blood, making fluorescence measurements accurate and sensitive since the first week after tumor induction. This type of ''mass screening'' can aid the early diagnosis of prostate tumors to corroborate with digital rectal examination.

Although the study has been performed exclusively for identifying a prostate tumor in an animal model, we believe this enhancement of PPIX fluorescence after ALA administration occurs in all kinds of cancer that accumulate endogenous PPIX and this method can be used to diagnosis other cancers in an early stage, in animal and human models.

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