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Can measurement of the fluorescence lifetime of extracted blood PPIX predict atherosclerosis?

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ABSTRACT

In this work, fluorescence lifetime has been used to analyze protoporphyrin IX (PPIX) extracted from blood for diagnosing atherosclerosis. A total of 10 adult white male rabbits (New Zealand) were divided into the control group (CG), with a normal diet, and the experimental group (EG), subjected to a diet containing 1% cholesterol. Blood samples were collected from the animals, and protoporphyrin IX was extracted from the blood using acetone. The PPIX fluorescence lifetime (PPIXFL) was measured using time-correlated single photon counting, after excitation at 403 nm from a pulsed laser diode. It was found that the PPIX emission intensity was enhanced in the animals that had received a hypercholesterolemic diet. The CG and EG animal's fluorescence decays were fitted by three exponentials and the mean lifetimes were 4.0 ns and 9.5 ns, respectively. This lifetime dependence resulted in a calibration curve that allows the determination of the PPIX concentration with a temporal measurement. The obtained results show that fluorescence lifetime can potentially be used as a noninvasive, simple, rapid, and sensitive tool in atherosclerosis diagnosis.

1. Introduction

Atherosclerosis is characterized as a progressive disease in which the arterial wall thickens through a process of inflammation [1], oxidative stress [2] and flow restriction. The arterial plaques may also rupture leading to thrombosis and occlusion of the vessel, causing myocardial infarction, angina, ischemic stroke and some manifestations of peripheral arterial disease, such as acute limb ischemia [3].

The association between LDL (low density lipoprotein), HDL (high density lipoprotein) and total cholesterol levels with cardiovascular disease risk is well known [4]. However, the weak capacity to distinguish between patients who will and those who will not develop cardiovascular disease has prompted the search for further refinement of these traditional measures [5].

Existing screening and diagnostic methods are insufficient in identifying the disease before an event occurs. Diagnostic methods, including catheter-based techniques, can localize and characterize vulnerable plaques, but are invasive and require hospitalization [6].

Several optical spectroscopy techniques have previously been used in atherosclerosis characterization; these include fluorescence spectroscopy [7], optical coherence tomography, near-infrared absorption, Raman and diffuse reflectance spectroscopy [8–11].

Many authors have reported on the use of autofluorescence spectroscopy of tissues for distinguishing atherosclerotic plaques from normal tissue [12–14].

Time-Correlated Single Photon Counting (TCSPC) has usually been used to characterize biological tissue [15]. TCPSC generates decay curves by measuring single photons that are emitted from an excited fluorescent molecule, and creating a photon-intensity histogram [16,17]. The instrument registers the time between an excitation source pulse and the emitted photons, and after collecting a significant number of photons, a decay curve emerges from this data. The photon count is used because the emission intensity is directly proportional to the probability that a photon will be detected at a given time.

The lifetime of each fluorophore present in the tissue is highly sensitive to micro environmental changes. The decay dynamics are sensitive to intermolecular interactions and changes in the microenvironment, but are relatively independent of the artifacts existing in steady-state measurements. Several studies have shown that the techniques of time-resolved laser induced fluorescence [12] and fluorescence lifetime imaging microscopy [18] show potential for the characterization of the composition of atherosclerotic plaques, and to detect markers associated with plaque instability and rupture.

A potential marker for atherosclerosis in tissue and blood is protoporphyrin IX (PPIX). Spears et al. were the first to describe the accumulation of porphyrins in atheromatous lesions [19]. Peng et al.

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Table 1
Experimental Groups.

Groups	Number of Animals	Procedures
Control Group: CG	4	**Commercial diet washed with chloroform**
Experimental Group: EG	6	**Diet containing 1% cholesterol (Sigma Aldrich) diluted in chloroform**

previously detected PPIX in atheromatous plaques, after an intravenous administration of δ-aminolevulinic acid (ALA), in rabbits subjected to a hypercholesterolemic diet [20]. Silva et al. observed that PPIX that accumulates in atheromatous plaques transfers to the blood and can be analyzed by extracting porphyrin from total blood [21].

In the present study, we used the TCPSC technique to analyze blood PPIX for atherosclerosis diagnosis. Our work shows sufficient sensitivity and specificity to distinguish subjects with and without plaques, suggesting a new and noninvasive diagnostic method.

2. Materials and methods

2.1. Animal testing

A total of 10 adult white male rabbits (New Zealand species Orytolagus cuniculus, approximately 2.3 ± 0.1 kg, and ~3.5 months old) were divided into two experimental groups as shown in Table 1.

The animals were individually housed in a controlled environment that was maintained at 19 °C, and food and water were provided *ad libitum*. The Ethics Committee of UNIFESP approved the protocol of this study (Protocol no. 0374/12).

All 10 rabbits completed the experimental process. Ninety days after the start of the experiment, the animals were euthanized according to the American Veterinary Medical Association guidelines for euthanasia.

2.2. Porphyrin extraction

The animals' blood samples were collected 90 days from the start of the experiment. Three parts by volume of analytical grade acetone (P.A.-A.C.S. 790 g, 100% - SYNTH) were mixed with one part of the total blood collected. The mixture was centrifuged at 4000 rpm for 15 min. The clear supernatant of the mixture was stored in a clean tube and kept refrigerated, in the dark, until spectroscopic characterization was performed.

2.3. Fluorescence analyses

The emission spectra were obtained under excitation at 405 nm using a 1 mm optical path cuvette (Hellma). The sample fluorescence was measured from 550 to 750 nm using a Horiba Jobin Yvon Fluorolog 3 Fluorimeter.

The PPIX fluorescence lifetimes were obtained using a homemade system equipped with a pulsed diode laser (PDL 800-B, PicoQuant) that provides 45 ps pulses, centered at λ_{exc} = 403 nm, in an 8 MHz repetition rate pulse train. This yields a temporal instrument response function of, typically, (248 ± 1) ps FWHM, obtained by measuring the excitation light scattered by the cuvette. The average power was fixed at 0.1 mW. Detection was performed using a photomultiplier (Hamamatsu PMA 182-PM) and a RG610 longpass colored glass filter. A reflective ND filter ND30A (ThorLabs) was used to reduce the background noise. The obtained data was processed using PicoQuant PicoHarp 300 (TCSPC system connected to a PC through a USB 2.0 interface), and then analyzed using Mathematica 10.

The data are expressed as mean ± standard error of the mean (S.E.M.).

2.4. Artery excision and histological analysis

At the end of the experiment, the arteries were excised and washed with phosphate-buffered saline (PBS). Cryosections of the aortic arch specimens were cut in the vertical plane to a 10 μm thickness on a cryostat, and then mounted on glass slides and stained with Oil Red O (O0625 SIGMA-ALDRICH). Since lipids are soluble in the solvents used in standard histological tissue processing, fresh or fixed tissues were used for the cryostat sections. Oil-soluble dyes were used to stain and visualize the lipids in the sample sections, since the dyes have a greater solubility in the lipids than in their original solvents. Images were obtained using a Leica DMI6000 CS fluorescence microscope, a Leica DFC450FX digital video camera and Leica AF6000 software.

2.5. PPIX lifetime calibration curve

Protoporphyrin IX (Sigma CAS Number 553-12) was dissolved in acetone P.A. (Synth) and solutions containing concentrations ranging from 0 to 100 μg/L were prepared. Fluorescence lifetimes were obtained by the system already described.

3. Results and discussion

It was observed that within the 90-days duration of the experiment, atherosclerotic plaques were deposited in the arteries of rabbits fed the experimental diet containing 1% cholesterol. The images obtained from aortas stained with Oil Red are shown in Fig. 1, indicating a type IV, in

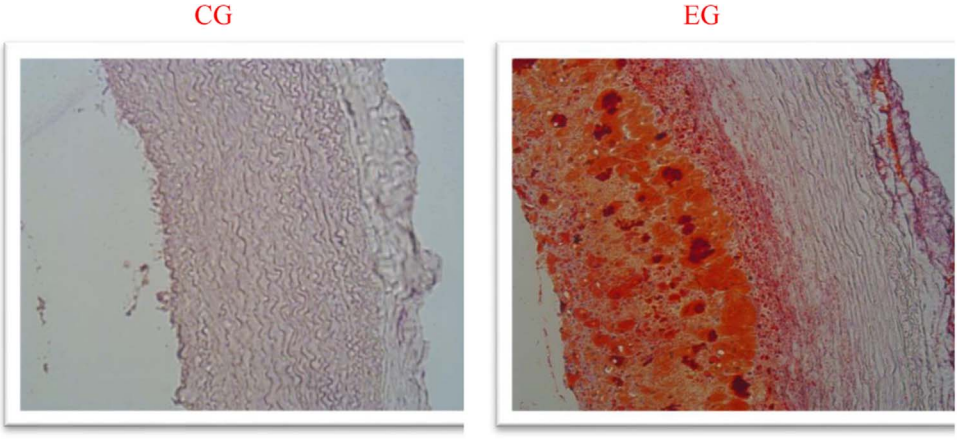

Fig. 1. Cross section of thoracic aortas from the control group (CG) and experimental group (EG) stained with Oil Red, indicating a type IV atherosclerotic plaque.