J Fluoresc (2010) 20:665–669
DOI 10.1007/s10895-010-0600-x

ORIGINAL PAPER

Study of Blood Porphyrin Spectral Profile for Diagnosis of Chronic Renal Failure

Vivian Regina Tristão · Fernando Felippe de Carvalho · Cinthia Zanini Gomes · Adriana Regina Miranda · Cíntia C. Vequi-Suplicy · Maria Teresa Lamy · Nestor Schor · Maria Helena Bellini

Received: 17 September 2009 / Accepted: 29 January 2010 / Published online: 23 February 2010

Abstract The progression to end-stage renal failure is independent of the initial pathogenic mechanism. Metabolic acidosis is a common consequence of chronic renal failure that results from inadequate ammonium excretion and decreased tubular bicarbonate reabsorption. Protoporphyrin IX (PpIX) is the immediate metabolic precursor of the heme molecule. The purpose of this study was to evaluate the levels of erythrocytes protoporphyrin IX at an animal model during progressive renal disease. A total of 36 eight-week-old male Wistar rats were divided into six groups: Normal, 4 and 8 weeks after 5/6 nephrectomy (NX). Renal function was evaluated by creatinine clearance and plasma creatinine levels. The autofluorescence of erythrocytes porphyrin of healthy and NX rats was analyzed using fluorescence spectroscopy. Emission spectra were obtained by exciting the samples at 405 nm. Significant differences between normal and NX rats autofluorescence shape occurred in the 600–700 nm spectral region. A correlation was observed between emission band intensity at 635 nm and progression of renal disease.

Keywords Chronic renal failure · Protoporphyrin · Fluorescence

V. R. Tristão · F. F. de Carvalho · N. Schor · M. H. Bellini
Disciplina Nefrologia, Departamento de Medicina,
Universidade Federal de São Paulo—UNIFESP-EPM,
São Paulo, Brazil

C. Z. Gomes · A. R. Miranda · M. H. Bellini (✉)
Instituto de Pesquisas Energéticas e Nucleares, IPEN-CNEN-SP,
São Paulo, Brazil
e-mail: mhmarumo@terra.com.br

C. C. Vequi-Suplicy · M. T. Lamy
Instituto de Física da Universidade de São Paulo—USP,
São Paulo, Brazil

Introduction

Chronic renal failure is often a progressive rather than stable process that most frequently leads to end-stage renal disease (ESRD) [1]. The common destructive mechanism that leads any chronic renal disease to glomerulosclerosis and chronic tubulointerstitial damage has been extensively investigated [2]. Experimental studies incriminate glomerular hypertension in mediating progressive renal damage after any of a variety of initiating injuries [3]. As the renal function progressively declines, renal failure complications such as acidosis, uremia or volume overload become more and more significant and eventually may be the principal reason for the initiation of renal replacement therapy [1]. Experimental model of 5/6 nephrectomy or the remnant kidney model represents one of the most used animal models of progressive renal failure by reduced nephron number, best-characterized in rats. The reduction of renal mass is achieved by either infarction or surgical excision of both poles, with removal of the contralateral kidney. It enables to investigate the influence of pharmacological, nutritive and other factors on functional and morphological renal parameters [4]. Metabolic acidosis is a common consequence of chronic renal failure that results from an inadequate ammonium excretion and decreased tubular bicarbonate reabsorption [2].

Native cellular fluorescence represents the innate capacity of tissues to absorb and emit light of specified wavelengths [5]. Autofluorescence in living tissue is based on the presence of fluorophores such as elastin, collagen, tryptophan, flavins and porphyrins [6].

Many applications of native fluorescence spectroscopy of biomolecules are reported on the characterization of cellular metabolic pathways and the discrimination of malignant from normal conditions of tissues [7].

PpIX is a porphyrin derivative that combines with ferrous iron to form the heme of hemoglobin and with ferric or ferrous iron to form the prosthetic groups of substances, such as myoglobin, catalase, and the cytochromes. PpIX is present in all nucleated and also in mature human erythrocytes that are non-nucleated cells [6]. When PpIX is irradiated at wavelengths from 390 to 440 nm, it fluoresces at 635 nm (red) and can be used to differentiate normal from injured cells [8]. Variations of the peripheral constituents on the porphyrin ring often occur because of minor changes to the intensity and wavelength of these absorptions. Protonation of two of the inner nitrogen atoms or insertion of a metal into the porphyrin cavity also changes the visible absorption spectra [9, 10].

Laser-induced fluorescence (LIF) is a promising tool for differentiating fluorescing molecules, and it appears to be very important in the diagnosis of many diseases. Fluorescence detection has advantages over other light-based investigation methods: high sensitivity, high speed, safety, and the possibility of use for real-time diagnosis [9, 10].

The objective of this study was to examine the correlation of red fluorescence and the progression of renal disease.

Material and methods

Animals

Two-month old, 250–280 g body weight Wistar rats from CEDEME UNIFESP-EPM (Federal University of São Paulo, São Paulo, Brazil) were maintained under specific pathogen-free conditions with a 12-h light/12-h dark schedule, and fed with autoclaved standard chow and water ad libitum.

NX rat model

A total of 36 male Wistar rats were divided into six groups: one normal and five groups, which were sacrificed after, 4 and 8 weeks from the 5/6 nephrectomy. The rats were anesthetized with ketamine (40 mg/kg body weight, Dopalen; Vetbrands; Ceva Santé Animale; Libourne, France) and xylazine (5 mg/kg body weight; Anasedan; Vetbrands; Ceva Santé Animale; Libourne, France), and approximately 1 mL of blood was collected via orbital sinus into heparin (used as an anticoagulant). This procedure was immediately followed by cervical dislocation and kidney harvest. The experimental procedure was approved by the Federal University of São Paulo Committee for the Use of Live Animals in Teaching and Research.

Chemicals and renal function

Plasma creatinine (pCr) and creatinine clearance levels were determined using commercial kits (Creatinina K—Cat. 96; Labtest Diagnostics, Brazil).

Urine protein levels were determined using commercial assay kits (DC-Protein; Bio-Rad; Hercules; CA, USA).

Porphyrin extraction

Collected blood was centrifuged at 2,500 rpm for 5 min. The supernatant plasma was removed completely and three volumes of analytical grade acetone were added in the formed elements and mixed well. The mixture was centrifuged at 4,000 rpm for 15 min. The clear supernatant of mixture was collected in a clean tube and maintained at 4 °C before spectrofluorometer analysis.

Standard curve of PpIX

Predetermined amounts of commercial metal-free PpIX (Sigma Chemical Company, St. Louis, Mo., USA) had been used dissolved in 20% of NaOH 0.2 M and 80% of acetone PA. A PPIX standard curve over a concentration range of 0.01 μg/mL to 2 μg/mL was constructed for quantification of PpIX in samples.

Fluorescent spectral analyses

The analysis of the commercial PpIX and samples was carried through by measures of luminescence in the equipment Cary Eclipse ® (Varian, NY, USA). The emission spectra were obtained by exciting the samples in 405 nm. The emissions had been carried through between 610 nm and 730 nm. The emission peaks had been detected by the system detector PMT of the proper equipment and had been compared with the standard curve of PpIX.

Statistical analysis

Statistical analysis of the differences between the experimental groups was performed by applying Student's t test. Significance was set at $P<0.05$. Data are expressed as mean±standard deviation (SE).

Results

Renal function

The renal mass reduction model (5/6 nephrectomy) was used as a model of progressive renal disease. Creatinine

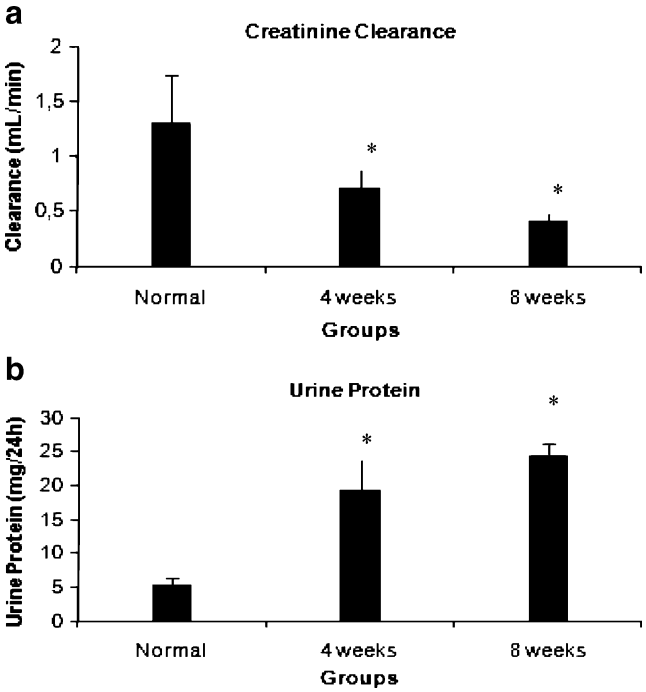

Fig. 1 Renal function measured in Wistar rats: **a** Creatinine clearance levels and **b** Urine protein levels. Data are reported as mean±SD (n= 6) (*P<0.05)

clearance and urinary protein excretion (proteinuria) were evaluated throughout the entire experimental period. Following 5/6 nephrectomy, rats developed severe proteinuria with a significant fall in creatinine clearance compared to the normal rats (P<0.05). (Fig. 1a and b).

Fluorescent spectral analysis

Standard curve of PpIX

According to the fluorescence intensity of a known gradient concentration of PpIX, the standard curve was plotted and the relative analysis equation was: $y = 433.31x + 29.856 (R^2 = 0.9995)$. (Fig. 2).

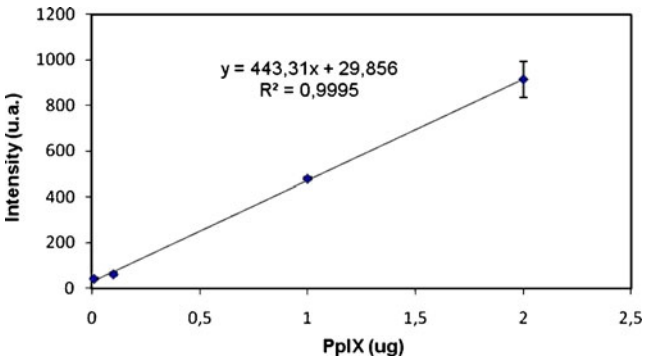

Fig. 2 Standard Curve of PpIX: Correlation between fluorescent intensity (~630 nm) and PpIX concentration (0.01 μg, 0.1 μg, 1 μg and 2 μg). PpIX concentration is expressed as μg/mL. (* arbitrary unit)

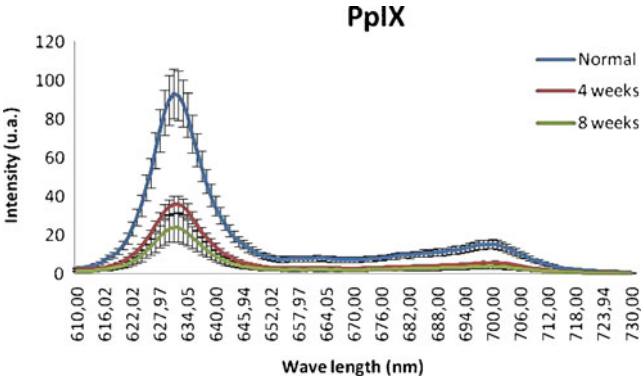

Fig. 3 Determination of the erythrocytes PpIX levels in the normal group, 4-week and 8-week groups, after excitation at 405 nm. Express values on Mean±SD. (* arbitrary unit)

Samples analyses

The PpIX levels were determined in samples of all nephrectomized groups and in the normal group. The samples were excited at 405 nm and the emission spectra obtained presented fluorescence from 610 nm to 700 nm. In fact, a most intense fluorescence peak was observed around 630 nm. Figure 3 shows the emission spectra of the normal group, the 4-week group and the 8-week group.

Table 1 shows the fluorescent intensity of PpIX (~630 nm) in the normal group, 4-week group and 8-week group. Data were expressed as mean±standard deviation (SE). The table sets out the minimum and maximum values of fluorescence presented for each group.

Erythrocytes protoporphyrin IX concentration was calculated from a standard curve of PpIX using the standard curve-derived equation. As noted in Table 2, the PpIX concentration, expressed in μg/mL in the samples, was gradually reduced with the progression of the renal disease.

Discussion

The progression of the renal disease is a matter of high interest in Nephrology. Every year many articles are published focusing on this subject, but just a few ones

Table 1 Fluorescence intensity (arbitrary unit) for the animal groups: normal, 4 and 8 weeks

Groups	Mean±SD	Minimum	Maximum	N
Normal	93.03±22.33	70.7	115.36	3
4 weeks	35.96±7.73*	28.23	43.69	3
8 weeks	23.20±12.99*	10.21	36.16	3

*p<0.02, Student's t test

Table 2 Concentration of PpIX (μg/mL) in the normal group, 4 and 8-week groups

Groups	Intensity (u.a.[a])	Concentration (μg/mL)
Normal	93.03	0.15
4 weeks	35.96	0.014
8 weeks	23.20	<0.01

[a] arbitrary unit

relate the chronic renal failure (CRF) with levels of erythrocytes protoporphyrin IX. In this study, Wistar rats were submitted to 5/6 nephrectomy, the most used method in the majority of studies in the literature, for its effectiveness in CRF induction. Progression of the disease was confirmed in this study by the biochemical analyses carried through in the experimental groups. In recent years the urine protein levels and creatinine clearance levels had gained place of prominence in some papers as an indication of renal failure. The increase in urine proteins is likely to have happened because of the loss in the renal function [11]. The results showed an increase of the urine protein levels at the nephrectomized groups vis-à-vis the normal group with significant difference between them, similar to Nakagawa's results [12]. The creatinine clearance levels—used as an estimate of the glomerular filtration rate, according to other studies [13, 14], is indicated as the most efficient way to evaluate the renal function. In this study, the data analysis showed significant difference in values of the 4-week group and the 8-week group when compared, separately, to the normal group. In the comparison between the nephrectomized groups, a significant difference was between the 4-week group when compared, individually, to the 8-week group. These data showed the evolution of the renal insufficiency, evidenced in 4 weeks and 8 weeks after the 5/6 nephrectomy was carried out.

The free erythrocytes PpIX levels were determined in all nephrectomized groups and in the normal group. The spectra of PpIX emission shown in Fig. 3 demonstrated characteristic fluorescence of the PpIX with the peak around 630 nm when excited at 405 nm. The results obtained were similar to those identified in previous studies [9, 10, 15, 16], showing that regardless of the origin of the sample (normal tissue, tumor tissue or blood), with the correct excitation, the PpIX emits fluorescence in the same region of the spectra.

In this study—carried through with Wistar rats—results demonstrated a reduction in PpIX levels with the progression of the CRF. A significant difference decrease was observed, between the 4-week group and the 8-week group when compared, separately, to the normal group. Our results are in agreement Vlassopoulos and collaborators who showed a significantly reduction of erythrocyte protoporphyrin IX in patients suffering from chronic renal failure when compared to healthy subjects [17]. The decreased levels of erythrocytes IX could be explained by gradual loss of renal mass in the CRF reduces the erythropoietin, a hormone produced by the kidney that promotes the formation of red blood cells in the bone marrow, production. Insufficiency of erythropoietin could affect erythroblasts and young red cells impairing haem synthesis [17, 18]. In contrast, El-Sharabasy [19] attained in studies with end-stage renal failure patients, reported an increase of PpIX levels. This study did not provide the relationship between erythrocyte protoporphyrin levels and the progression of renal insufficiency.

Conclusion

Based upon the results of this study, it is possible to confirm the effectiveness of 5/6 nephrectomy in inducing chronic renal insufficiency, and to differentiate the stages of the CRF presented for the animals. Moreover, one can affirm that PpIX levels were reduced with the progression of the renal disease.

In conclusion, these results show that the longer the time after 5/6 nephrectomy, the lower the erythrocyte protoporphyrin IX concentration.

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