

A novel method for the detection of liver damage using fluorescence of hepatic mitochondria in a rat model following ischaemia/reperfusion injury of the small intestine

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Abstract. We present a novel method of assessing damage to the liver using fluorescence analysis of hepatic mitochondria following ischaemia/reperfusion of the small intestine in a rat model. This work is of substantial importance in understanding the syndrome of multiorgan failure after ischemia/transplantation of the small intestine.

Mitochondria were isolated from six sample groups that had undergone three different experimental treatments: a control group; a treatment with ischaemia followed by reperfusion of the small intestine (IR_x); and, a one hour ischaemia followed by reperfusion after transplant of the small intestine (TR_x). The IR treatment was further subdivided into three groups: 1, 24 h and 30 days reperfusion – IR₁, IR₂₄, IR₇₂₀, respectively. Concomitantly, the TR treatment was further subdivided: one group underwent a 1 h reperfusion and another group a 6 h reperfusion following ischaemia and transplant – groups TR₁ and TR₆, respectively.

Once treatment had been undergone, mitochondria were isolated and all five experimental groups – IR₁, IR₂₄, IR₇₂₀, Tr₁, Tr₆ – and their emission matrices were analysed compared with that of the control group (C).

Comparing fluorescence values in zone A of all experimental groups with those of the control group indicated a reduction in aromatic amino acids in the mitochondria of all experimental groups. Comparison of fluorescent zone B of experimental groups with the control group identified a lack of oxygen in samples IR₁, IR₂₄, which was indicated though an increase in the fluorescence of the reduced pyridine nucleotide NADH + H⁺.

Keywords: Rat liver mitochondria, ischaemia/reperfusion of small intestine, small bowel transplantation, fluorescence excitation–emission matrix

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1. Introduction

Gastrointestinal ischaemia–reperfusion (I/R) can be a major complication in transplantation, and it is associated with many different clinical situations, ranging from surgery (abdominal aortic aneurysm repair) to hemorrhagic shock. Injury from gastrointestinal I/R can be divided into local, i.e., in the intestine, and remote organ injury, i.e., a second organ, e.g., the liver [6,9,10]. Reactive oxygen species (ROS) are formed in early phase of gastrointestinal I/R and lead to oxidant-dependent injury [5]. Mitochondria are known to be a major source as well as a target for ROS [1]. Mitochondria play a key role in hepatic I/R injury, since mitochondrial dysfunction is the main cause of hepatocellular death [2,5,8,11]. Metabonomics is a novel strategy for the study of intestinal I/R, which may be used as part of a systems approach for quantitatively analyzing the intestinal proteome in patients exposed to intestinal I/R [4].

The aim of our work was to study liver mitochondrial functions after ischaemia/reperfusion of the small intestine using fluorescence analysis. A 1 h ischemia followed by 1, 24 h or a 30-day reperfusion was performed on the small intestine (experimental groups IR₁, IR₂₄, IR₇₂₀). In parallel, a second treatment – small bowel transplantation with a 1 h ischaemia followed by a 1 or a 6 h reperfusion – was carried out (TR₁, TR₆). The mitochondria (IR₁, IR₂₄, IR₃₀, Tr₁, Tr₆) of all experimental groups were compared with those of control mitochondria (C).

2. Materials and methods

2.1. Materials

The respiration medium (pH 7.4), distilled water containing 0.78 mM ethylenediamine tetraacetic acid (EDTA), 6 mM magnesium chloride (MgCl₂), 15 mM Tris HCl, 0.08 M potassium chloride (KCl), 0.3 M dipotassium hydrogen phosphate (K₂HPO₄) and 0.3 M potassium dihydrogen phosphate (KH₂PO₄) was prepared by using bidistilled water, 1 mM sodium succinate (NaOOCCH₂CH₂COONa · 6 H₂O) and Tris HCl, which were obtained from Sigma, Germany.

2.2. Animal model of ischemia and experimental design

Adult male Wistar rats (mean body weight 320 g, total $n = 36$) used for the experiments were housed in a menagerie under standard conditions and maintained at a temperature of $22 \pm 2^\circ\text{C}$ with variation in light at 12 h intervals. Food and water were provided *ad libitum*. Ischaemia of the small intestine was induced via the occlusion of the *arteria mesenterica cranialis* for 60 min using a small atraumatic clip whilst the subject was under anesthesia. Normothermic conditions (37°C) were monitored using a microthermistor placed in the ear. Temperature was maintained using a homeothermic blanket. Control animals ($n = 4$ for each time interval) were prepared in the same way but without the occlusion of *a. mesenterica*. Subject animals then underwent a 60 min ischaemia followed by a 1, 24 or 720 h reperfusion (each group $n = 8$). Mitochondria were isolated and labelled as coming from control (C) or experimental groups (IR₁, IR₂₄, IR₇₂₀, TR₁, TR₆).

2.3. Mitochondria isolation and determination of proteins

Animals were decapitated, and their livers were quickly removed. Mitochondria were isolated according to [7]. Protein content of isolated mitochondria was determined by the method of [3]. The solution containing isolated mitochondria was diluted to a final concentration of 2 µg/ml using a respiration medium (pH 7.4) containing the substrate succinate.

2.4. Fluorescence spectroscopy

Fluorescence spectra were run on a Perkin-Elmer, Model LS 55, Luminescence Spectrometer using a 1 cm pathlength quartz cuvettes at ambient temperature. The wavelength scan speed of both monochromators was 1200 nm/min. The setting of the instrument's excitation slit was 10 nm and the emission slit was 15 nm. Data processing was managed by the FL Winlab (Perkin-Elmer) software package. The samples of mitochondria were analyzed in respiration medium containing the substrate succinate using excitation emission matrix (EEM). The EEM technique involves recording a fluorescence–emission matrix, which is composed of 10 emission spectra at 10 different excitation wavelengths, and concatenating them into a matrix with increments of 10. The fluorescence spectra of mitochondria were monitored in the range of wavelengths from 220 to 450 nm.

3. Results and discussion

3.1. Mitochondrial fluorescence

Mitochondria are highly organized multifluorescent systems of endogenous fluorescent molecules, which can be detected by fluorescence spectroscopy. In this study we investigated the endogenous fluorescence of mitochondria using fluorescence analysis of three-dimensional excitation–emission spectra (EEM). The topographic map of the EEM of control mitochondria displayed a characteristic shape with two fluorescence maximal peaks (Fig. 1). Rayleigh and Raman scatter depicted in EEM topographic maps also occur when endogenous molecules were excited to a higher energy state by a photon with insufficient energy to completely excite the molecules (Fig. 1). The higher state is unstable, and the molecules quickly relaxes, emitting light. Depending on the original and final vibrational energy level of the molecules, the emitted light may have higher, lower or the same energy as the excited light. In Rayleigh scatter, the emitted light is of the same energy as the excited light. This process, known as elastic scattering, is the most probable one of all possible processes, and it has the highest intensity. Rayleigh scatter in the EEM is an exactly diagonal structure occurring at $\lambda_{em} = \lambda_{exc}$.

The topographic graphical image of the mitochondrial experimental groups differed in fluorescence values in comparison with control group in both fluorescence zones (Figs 1–3). The horizontal cut of three-dimensional EEM at excitation wavelength $\lambda_{ex} = 240$ nm revealed a simple emission spectra with two fluorescence maxima A and B (Fig. 2). The maxima A and B were the result of endogenous fluorescence of the different fluorophores present in the mitochondrial outer membrane. The first fluorescent zone A represents a mixture of various fluorophores, the dominant proteins of which are mainly the aromatic amino acids (tryptophane, tyrosine and phenylalanine). Fluorescent zone B shows mainly reduced coenzyme NADH + H⁺. The fluorescence of fluorescent zones A and B in the control (C) and experimental mitochondria (IR₁, IR₂₄, IR₇₂₀, Tr₁, Tr₆) were compared mathematically, the results of which can be seen in (Fig. 3). Generally, the fluorescence intensity of zone A (fluorescence of proteins, aromatic aminoacids and their metabolites) was higher in control mitochondria (C) than in experimental samples (I/R₁, I/R₂₄, I/R₇₂₀, T/R₁, T/R₆). Our results showed that fluorescence zone A in experimental mitochondria I/R₁ was similar to that of samples T/R₆. The early stages of oxygen reperfusion of the small intestine lead to decrease of proteins [12,13]. A potential marker of this process is fluorescence of tryptophan, which is the main component, which shares of fluorescence of zone A (Fig. 3). The elimination of proteins in a remote organ, e.g., the liver can occur in a latter stage [6,9,10] where we observed the largest decrease of fluorescence of zone A in samples I/R₂₄, I/R₇₂₀ when compared with control

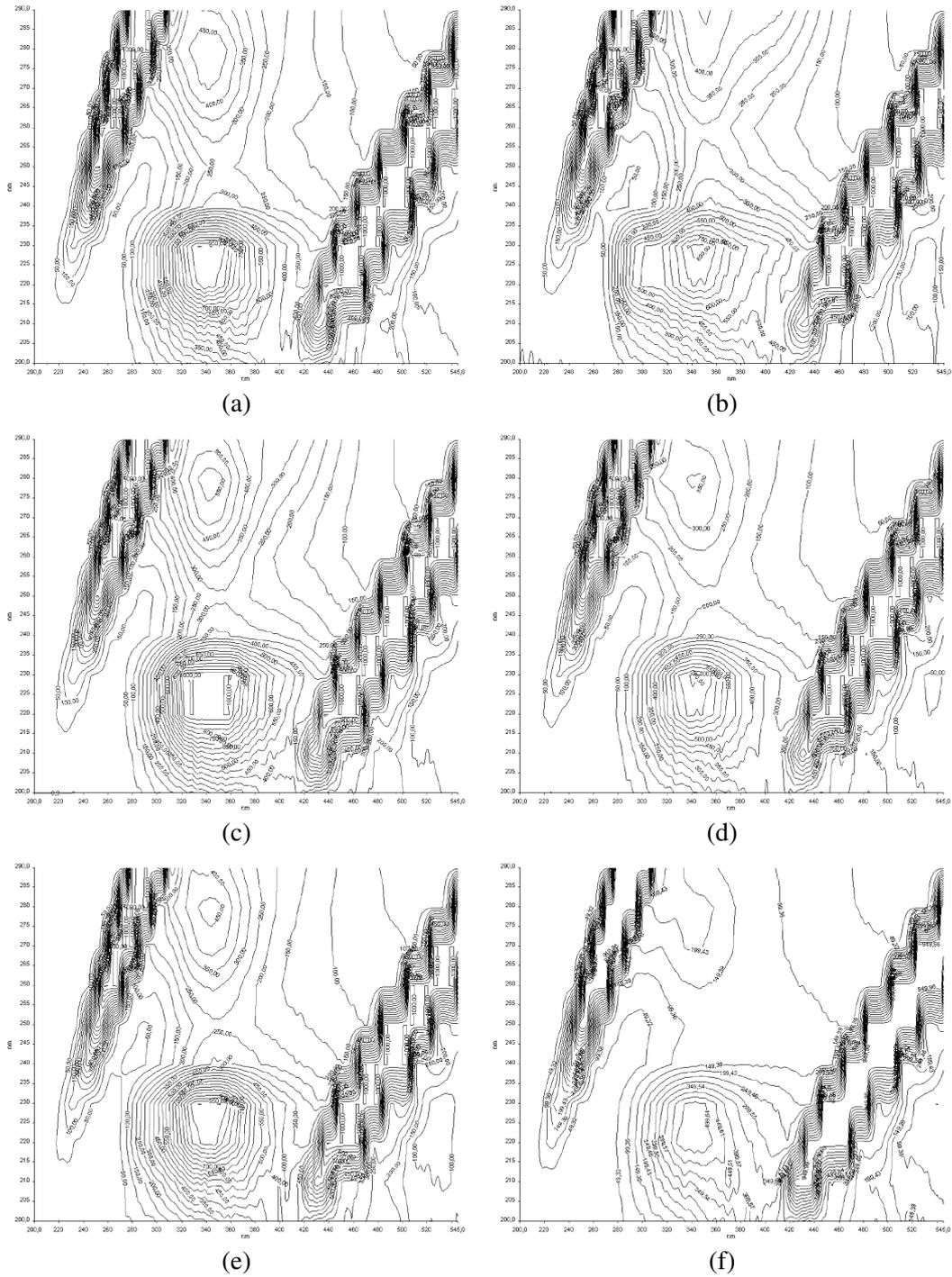


Fig. 1. The topographic maps of fluorescence excitation–emission matrix (EEM) (a) control rat liver mitochondria (C) and experimental rat liver mitochondria, (b) I/R₁ (c) I/R₂₄ (d) I/R₃₀, (e) T/R₁ (f) T/R₆.

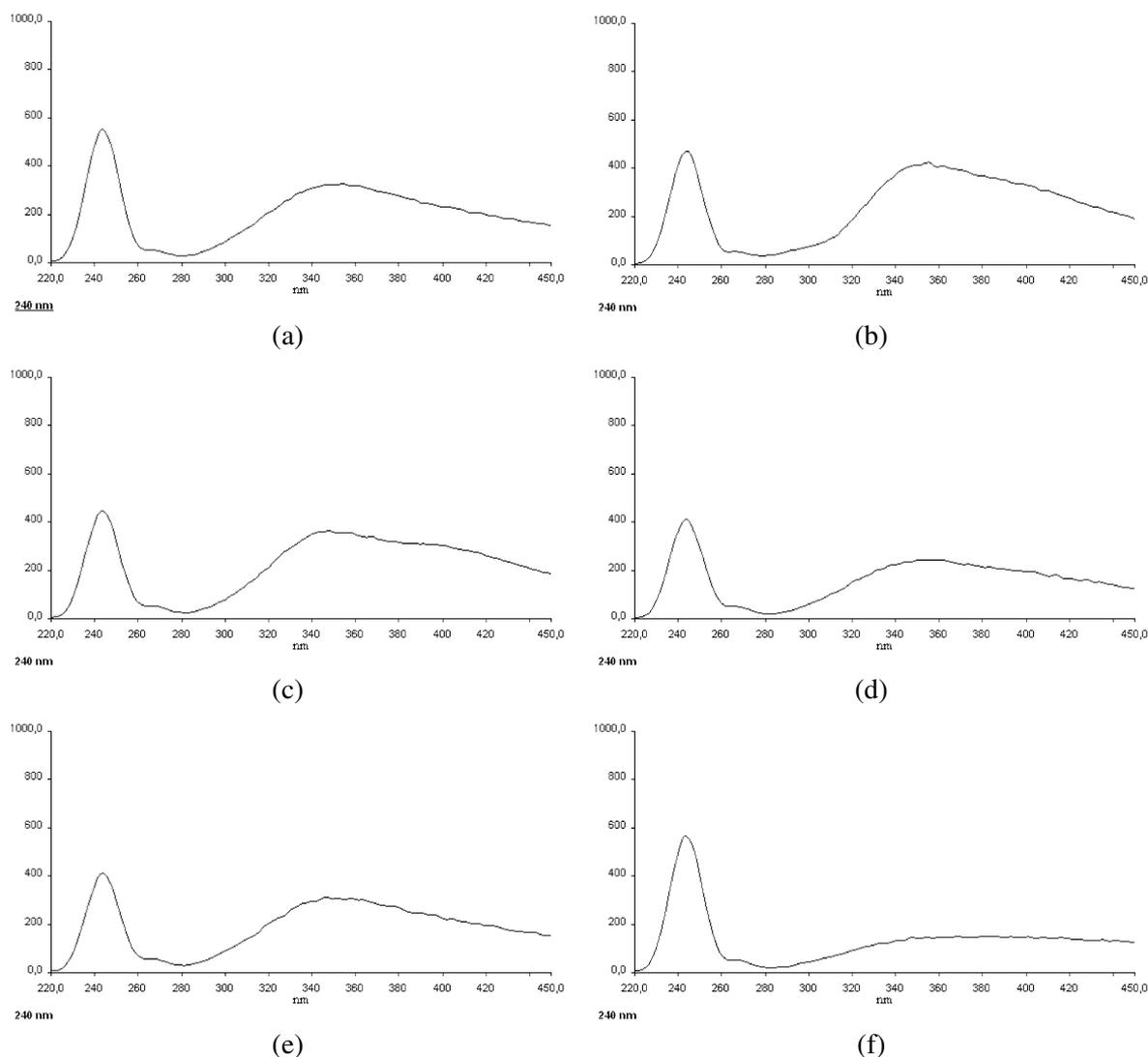


Fig. 2. Simple fluorescence emission spectra with two fluorescence maxima A and B of (a) control rat liver mitochondria (C) (A: $\lambda = 244$ nm, $F = 553$; B: $\lambda = 354$ nm, $F = 327$) and experimental rat liver mitochondria (b) I/R_1 (A: $\lambda = 244$ nm, $F = 555$; B: $\lambda = 361$ nm, $F = 412$), (c) I/R_{24} (A: $\lambda = 244$ nm, $F = 442$; B: $\lambda = 348$ nm, $F = 364$), (d) I/R_{720} (A: $\lambda = 244$ nm, $F = 413$; B: $\lambda = 357$ nm, $F = 244$), (e) T/R_1 (A: $\lambda = 243$ nm, $F = 484$; B: $\lambda = 352$ nm, $F = 325$), (f) T/R_6 (A: $\lambda = 243$ nm, $F = 471$; B: $\lambda = 357$ nm, $F = 148$).

groups (C). Groups I/R_1 , I/R_{24} displayed higher fluorescence of zone B (reduced pyridine nucleotide showed lower mitochondrial consumption of oxygen) in comparison with the control group (C). Fluorescence of zone B – a marker of higher mitochondrial respiration – of the experimental group T/R_1 was the same as the control group (C), while the fluorescence of experimental mitochondria from T/R_6 was decreased, what is the marker of higher mitochondrial respiration compared to control samples (C). Oxygenation during reperfusion restores metabolic processes by supplying oxygen to ischaemic tissue. The highest consumption of oxygen was shown by the mitochondrial experimental groups of T/R_6 and I/R_{720} in comparison with control mitochondria (C). The result of increasing oxygen consumption by

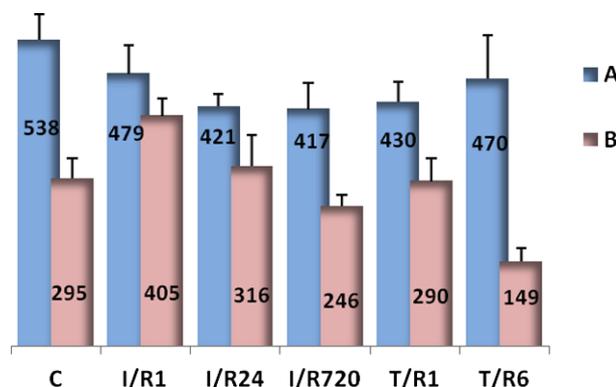


Fig. 3. Mathematical comparison of the fluorescence intensities between fluorescent maxima A and B of the control (C) and experimental mitochondria (I/R₁, I/R₂₄, I/R₇₂₀, T/R₁, T/R₆). The first fluorescent zone A represents a mixture of various fluorophores that are dominated mainly by aromatic amino acids (tryptophane, tyrosine and phenylalanine). Fluorescence intensity in zone B mainly represents reduced coenzyme NADH + H⁺. (Colors are visible in the online version of the article; <http://dx.doi.org/10.3233/SPE-2011-0545>.)

mitochondria and cells is accompanied by damage to cells. Cell damage occurs in close correlation with the recovery process of energy production after reoxygenation and is referred to as the “oxygen paradox” [12,13].

Fluorescence spectroscopy using emitted fluorescence signal of different fluorophores as one complex is a quantitative and rapid technique that has the potential to provide important diagnostic information about tissue hypoxia or ischaemia. Spectroscopic signals can indicate biochemical changes, and these can generally predict the morphological changes observed in histology [1].

Our results showed that the metabolism of liver mitochondria improved significantly after transplantation of the bowel in recipient rats. The damaging effects of intestinal ischemia–reperfusion (I/R) on the small intestine and the mitochondria of a remote organ, e.g., the liver was observed using fluorescence spectroscopy as a decrease in proteins in experimental samples of mitochondria. Based on our observations of endogenous mitochondrial fluorescence, we suggest a novel model for the diagnosis of intestinal ischaemia.

Acknowledgement

This work was supported by grant APVV-0252-07 “Syndrome of multiorgan failure after ischemia/transplantation of small intestine”.

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