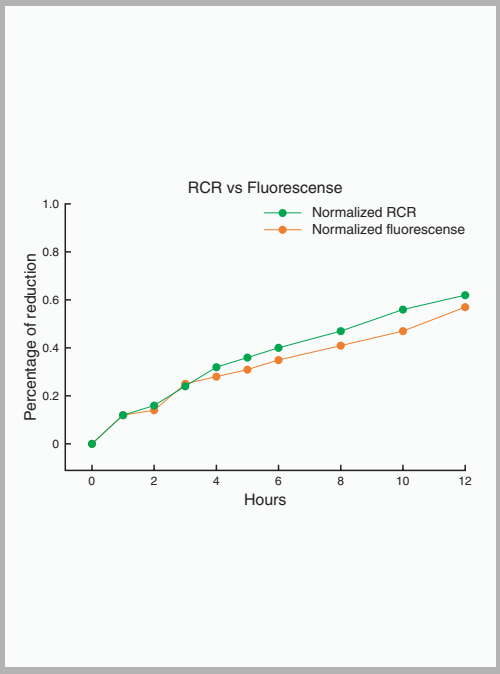
Abstract: There has been an increasing demand for liver transplantation, while the supply of liver grafts remains limited. Strategies to augment the donor pool include "liver-splitting", "domino" procedure, Living-related liver transplantation and the use of marginal donors. Simultaneously, there has been growing interest in Laser-induced fluorescence (LIF) as a diagnostic tool, mainly in oncology. The basis of all optical spectroscopic techniques is that physiological, morphological or biochemical changes associated with physical disorders, affect the interaction between light and tissue. The aim of this experimental study was to analyze the applicability of LIF in evaluating liver grafts, in rats. In this experiment, the animals were divided into two groups according to the temperature of the perfusion medium. The perfusion medium used was saline solution between 0–4°C in one group (CS group) and the same solution, between 22–26°C, in the other group (RT group). Mitochondrial respiratory capacity, swelling and membrane potential along with the cellular ATP content were used as the functional and metabolic indicators of viability. The Cluster-Russia fluorescence spectroscopy system was used to analyze the LIF. Deterioration of mitochondrial function and ATP content occurred progressively and relatively rapidly. LIF reflected the findings of the mitochondrial respiratory control ratio in the CS group, but not in the RT group. The ratio of the intensity of fluorescence detected at 636 nm and 600 nm showed rapid change 5 to 6 hours after perfusion in the CS group. Therefore LIF shows great potential as an auxiliary tool in the analysis of liver grafts, for transplantation.



Graph comparing the reduction of the mitochondrial respiratory control ratio (RCR) and hepatic fluorescence in the CS group, during the 12 hours after perfusion

Use of laser auto-fluorescence for evaluating liver grafts

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1. Introduction

The possibility of treating end-stage liver disease with transplantation is one the notable advances of the past century. Once accepted as a valid therapeutic option, by the National Institutes of Health of the United States of America in 1983 [1], there has been an exponential increase in the number of people being offered this treatment worldwide [2].

Unfortunately, the donor supply has not grown accordingly, leading to increasing waiting lists as well as increasing mortality rates while awaiting transplantation [3]. There can be no transplantation without a donor organ.

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The strategies used to increase the donor pool include "liver-splitting" [3], the "domino procedure" [4], living-related liver transplantation [5,6] and the use of marginal donors [7].

The advent of the University of Wisconsin (UW) cold storage solution improved preservation of the graft. Increased time of storage, provided by the UW solution, has several important benefits. Longer preservation time improves donor liver procurement and utilization, permits lengthy back-table procedures such as "liver-splitting", increases time for preoperative preparation of recipients and generally expands the availability of transplantation therapy [7]. Liver transplantation no longer needs to be performed on an emergency basis.

Livers stressed *in vivo*, prior to storage by hypoxia, ischemia or other metabolic perturbations are called marginal livers because of doubts concerning their suitability as donor organs. The criteria for accepting or rejecting marginal livers remain largely based on the clinical experience of the individual surgeon. In the United States, livers are recovered from about 80% of all organ donors (about 4800 livers from 5800 donors, yearly). The decision, not to use 1000 livers is made based on one exclusion criterion or another. Given the imprecision of the exclusion criteria, many rejected livers might, in fact, be suitable for transplantation [7]. Quantitative and rapid techniques to help with this decision are still to be developed.

Spectroscopy has the potential to provide important diagnostic information about tissues. Light signals can be delivered and collected via optical fibers. Information can thus be gathered *in situ* instead of bringing the tissue to the instrument; part of the instrument can be brought to the tissue. Further, data can be collected and analyzed in a fraction of a second, allowing the possibility of real-time feedback. In addition, spectroscopic signals can indicate biochemical changes, and these generally precede the morphological changes observed in histology. But perhaps most important of all, the information provided by spectroscopy is quantitative, and thus it can bring an added degree of objectivity to the process of diagnosing disease [8].

Various spectroscopic techniques can be used to diagnose tissue. Reflectance, fluorescence, infrared absorption, Raman spectroscopy and others have been employed. Each of these has its own special features and potential applications [8]. Laser-induced Fluorescence (LIF) has been shown to be a very sensitive analytical technique for biochemical analysis at ultra trace levels. This technique has also been demonstrated to be a useful tool for monitoring DNA damage and for cancer diagnostics [9–11]. Recently LIF has been used experimentally to analyze the viability of porcine hearts after perfusion with storage solutions [12].

We proposed to analyze, experimentally, the applicability of LIF in evaluating the viability of rat liver once perfused with saline solution. Mitochondrial function and cellular ATP content of the liver were used as indicators of its metabolic status. The spectroscopic findings were then compared on a time scale with the mitochondrial function. Therefore, the aim of the study was to verify LIF's capacity to detect changes taking place in the organ. Structural changes usually occur at later stages and frequently denote irreversible damage.

2. Methodology

This study used Wistar rats weighing between 250 and 300 grams. They were treated according to the directives of the Canadian Council of Animal Care. The anesthetics used for the surgical procedure were ketamine/xylazine (80/16 mg/kg), administered by intramuscular injection. The animals were divided in two groups. In one group cold saline solution (CS group), with the temperature between 0 and 4° C, was used as the perfusion medium, while in the other group saline solution at room temperature (RT group), between 22 and 26°C was used. Perfusion of the liver was achieved by cardiac puncture with a 20 gauge catheter, according to the technique described by Lima in 1992. Adequate perfusion was achieved with 250 ml of the solution.

Laser Induced Fluorescence (LIF) was measured before and soon after perfusion with the storage solution. Thereafter, the measurements were taken hourly for a period of 12 hours. For each measurement, LIF was obtained from three different points of three lobes and the average of these nine points was used to plot the graph.

Like the LIF measurements, mitochondrial function and cellular ATP content of the liver were evaluated before and soon after perfusion, then hourly for 12 hours. Each measurement of mitochondrial function was obtained from 3 rats so that the average could be plotted.

2.1. Isolation of rat liver mitochondria

Mitochondria were isolated by conventional differential centrifugation [13]. The liver was immediately removed and cleaned in cold saline solution and homogenized three times at 1 min intervals in a Potter-Elvehjem homogenizer in 10 mL of a medium containing 250 mM sucrose, 1 mM EGTA, 10 mM Hepes-KOH at pH 7.2. Homogenates were centrifuged at 770 g for 5 min and the resulting supernatant further centrifuged at 9.800 g for 10 min. Pellets were suspended in 10 mL of a medium containing 250 mM sucrose, 0.3 mM EGTA and 10 mM Hepes-KOH at pH 7.2, and centrifuged at 4.500 g for 15 min. The final mitochondrial pellet was suspended in 0.5 mL of a medium containing 250 mM sucrose, and 10 mM Hepes-KOH at pH 7.2. All procedures were conducted at 4°C and all solutions were prepared using glass-distilled and deionized water.

2.2. Protein determination

Mitochondrial protein content was determined by biuret reaction [14].

2.3. Mitochondrial respiration

Mitochondrial respiration was monitored polarographically with an oxygraph equipped with a Clarck-type oxygen electrode (Gilson Medical Electronics, Middlenton, WI, USA). Assays were performed at 30 ° C using mitochondria energized by 5 mM potassium succinate. Respiration media contained 125 mM sucrose, 65 mM KCl, 0.1 mM EGTA, 1mM $MgCl_2$, 2 mM KH_2PO_4, and 10 mM Hepes–KOH at pH 7.4. The state 3 of mitochondrial respiration was determined by the addition of 400 nmoles of ADP while the state 4 of respiration was determined after the phosphorilation of the added ADP. The mitochondrial parameters were expressed in natoms of O/min/mg of protein. The respiratory control ratio, which is another mitochondrial parameter, is the relation between the velocity of respiration in state 3 and the velocity of respiration in state 4, indicates the degree of coupling of the mitochondria.

2.4. Electrical transmembrane potential

Electrical transmembrane potential difference ($\Delta\Psi$) was monitored spectrofluorimetrically using 5 μM safranine O as an indicator and a SLM-Aminco, Bowman, series 2 luminescence spectrophtometer at 495/586 nm excitation/emission wavelength pair. Assays was performed in a incubation media contained 200 mM sucrose, 1 mM $MgCl_2$, 2.5 μM NaH_2PO_4 and 10 mM Hepes-NaOH at pH 7.4. Mitochondria was energized by 5 mM sodium succinate. The $\Delta\Psi$ was expressed in mV (15).

2.5. Mitochondrial swelling

The mitochondrial swelling was estimated from the decrease in absorbance at 540 nm using a Model DU-640B Beckman spectrophotometer (USA). In a medium containing sucrose 125 mM, KCL 65 mM, hepes-KOH 10 mM, pH 7.2 energized by 5mM of potassium succinate, the mitochondrial protein was added (0.4 mg). Mitochondrial swelling was induced by adding Ca Cl_2 20 μM and KH_2PO_4 4 μM [16].

2.6. Fluorescence measurement

The fluorescence spectroscopy system is composed of: i) One spectrometer, which goes from 350 nm up to 850 nm; ii) One Y-shaped fiber, which delivers the laser light through one central fiber and collects the fluorescence from the tissue using six periferical fibers; iii) And an excitation sources, 532 nm (Nd:YAG). The laser power is in the order of a 5 mW, assuring no thermal effect on the incident spot. It should be pointed out that the signal from the back scattering (fluorescence at the same wavelength of incidence light) is about thousand times more intense than the one from the broad fluorescence peak. To simplify the analyses we used an optical filter to reduce it one thousand times; this way both parts of the spectrum present comparable intensity. The spectrum was collected by direct contact between the fibre and the liver. To protect the fiber and to avoid contamination from one measurement to the other, we used a flexible transparent plastic film between the liver tissue and the fiber. An extra experiment was carrieds out to confirm that the plastic film does not interfere with the measurements. After perfusion, the liver removed for the remaining measurements. Once the fluorescence spectrum is obtained, a mathematical procedure, presented in the discussion section, permitted the comparison of the fluorescence spectrum and the RCR index. Of particular interest is the percentage of reduction of the RCR level after perfusion and extraction of liver. Therefore, we concentrated on the evaluation of this parameter.

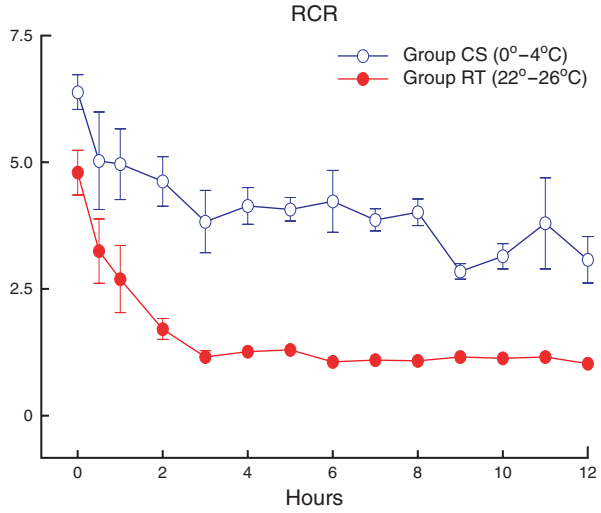

Figure 1 (online color at www.lphys.org) Graph showing the Respiratory Control Ratio (RCR) of the mitochondria in the liver of both groups, during the 12 hours after perfusion

This preliminary experimental study is a qualitative description of the Laser Induced Fluorescence obtained from the rat livers, which mimics the situation of liver grafts in clinical transplantation. It was designed to analyze the applicability of LIF in this context, as an auxiliary tool in evaluation of the liver grafts.

3. Results and discussion

First we shall present the results of the mitochondrial function and cellular ATP content. As would be expected the ATP content diminished and mitochondrial function deteriorated progressively after perfusion. These changes occurred more rapidly in the RT group due to the lack of protection provided by low temperatures.

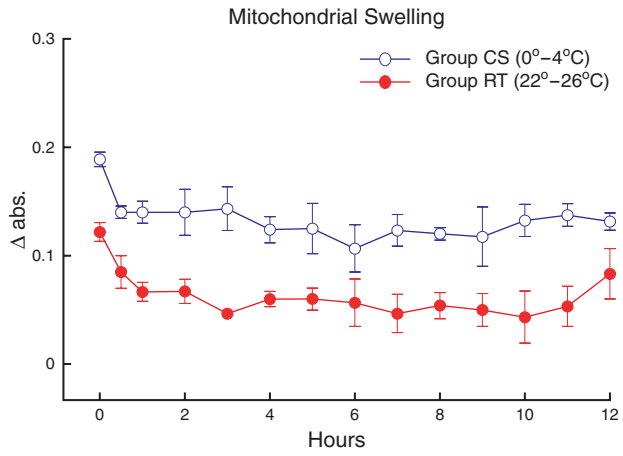

Figure 2 (online color at www.lphys.org) Graph showing the reduction in absorbance of the mitochondria in the liver due to osmotic swelling in both groups, during the 12 hours after perfusion

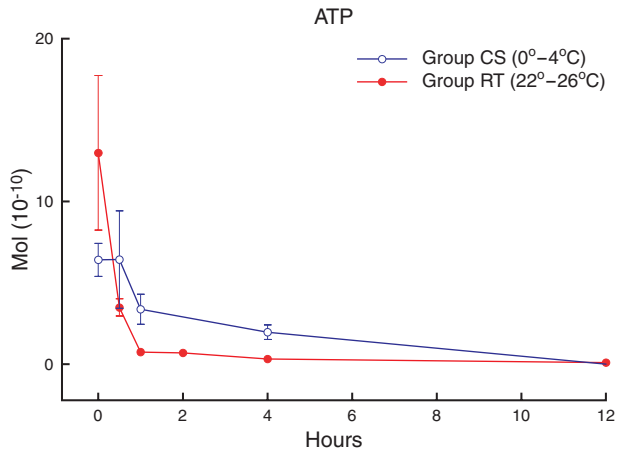

Figure 4 (online color at www.lphys.org) Graph showing the cellular ATP content of the mitochondria in the liver of both groups, during the 12 hours after perfusion

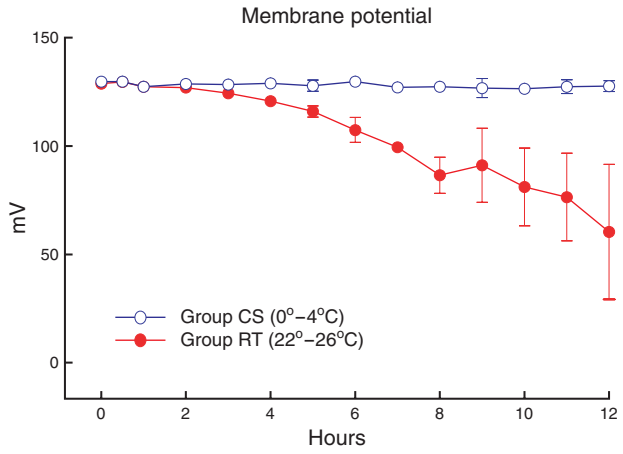

Figure 3 (online color at www.lphys.org) Graph showing the membrane potential of the mitochondria in the liver of both groups, during the 12 hours after perfusion

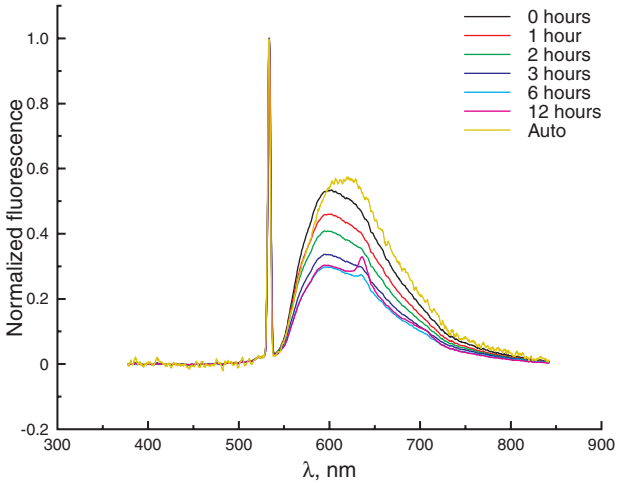

Figure 5 (online color at www.lphys.org) Graph showing the spectrum of fluorescence obtained after perfusion of the liver, in the CS group

Mitochondrial respiratory capacity represented by its Respiratory Control Ratio (RCR) diminished progressively in both groups as shown in Fig. 1. Mitochondrial swelling worsened in a similar manner and both groups are represented in Fig. 2. Progressive reduction in mitochondrial membrane potential can be seen in Fig. 3. Cellular ATP levels diminished soon after perfusion in both groups are presented in Fig. 4

Compared on the some time scale, the biochemical analysis show that initially there seems to be a reduction in the cellular ATP content, followed by progressive mitochondrial swelling, diminished RCR and finally by the loss of mitochondrial membrane potential.

In each graph of mitochondrial function as well as in the one of ATP content, time zero was considered as the moment soon after perfusion. This coincides with the knowledge that the differences in temperature of the perfusion solution can cause a difference in the degree of damage to the organ.

The fluorescence spectra obtained from the two groups were different. The spectra of the two groups are shown in Figs. 5 and 6.

Each spectrum has a common peak centered at the excitation wavelength followed by a broad peak at longer wavelengths, which extends from just above 532 nm to around 800 nm. This broad peak of fluorescence has vari-

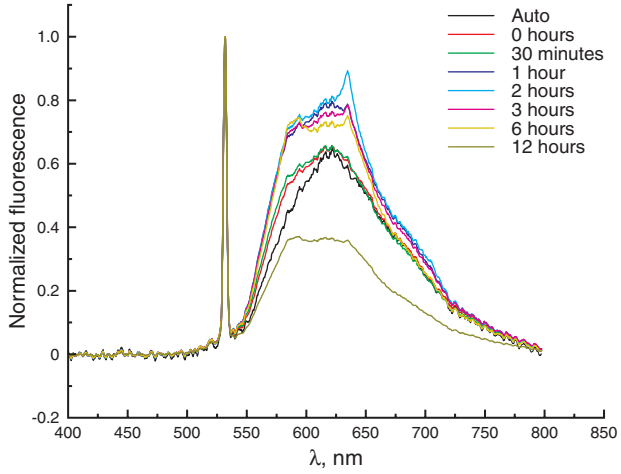

Figure 6 (online color at www.lphys.org) Graph showing the spectrum of fluorescence obtained after perfusion of the liver, in the RT group

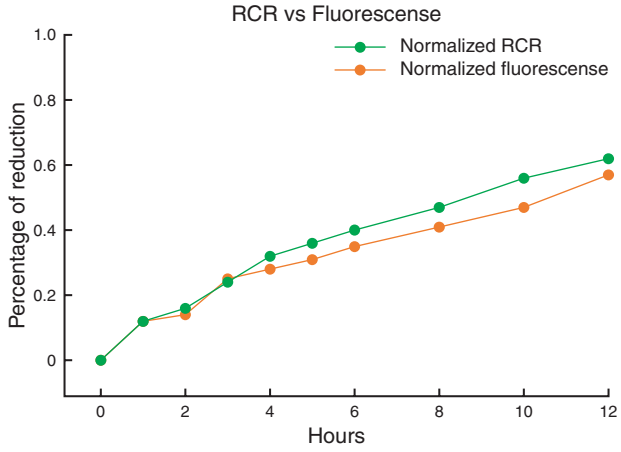

Figure 7 (online color at www.lphys.org) Graph comparing the reduction of the mitochondrial respiratory control ratio (RCR) and hepatic fluorescence in the CS group, during the 12 hours after perfusion

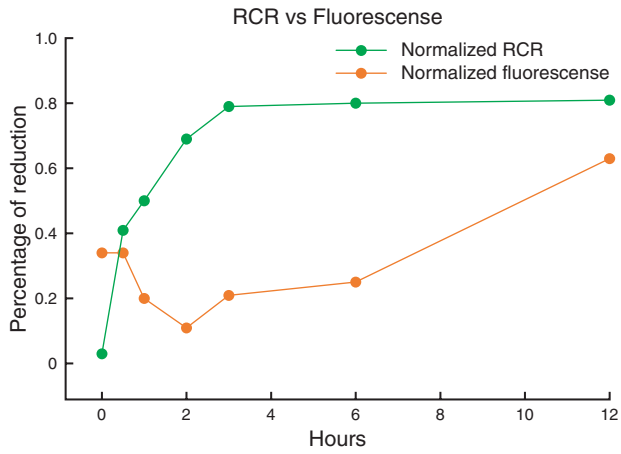

Figure 8 (online color at www.lphys.org) Graph comparing the reduction of the mitochondrial respiratory control ratio (RCR) and hepatic fluorescence in the RT group, during the 12 hours after perfusion

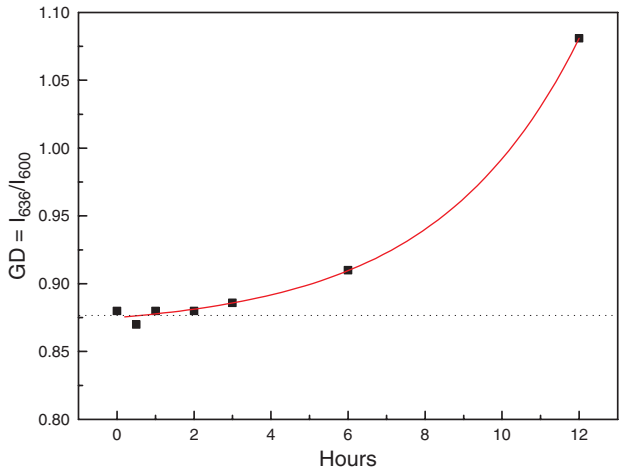

Figure 9 (online color at www.lphys.org) Graph showing the ratio between the intensity of fluorescence detected at 636 nm and 600 nm, during the period after perfusion of the liver, in the CS group. (GD – degree of degradation)

ous contributions. We believe that many endogenous chromophors contribute to this peak. The fluorescence of reduced nicotinamide adenine dinucleotide (NADH) may have special contribution as it is an essential part of the cellular energy metabolism, and it could therefore reveal the energy status of the cell. Although isolated NADH does not show prominent fluorescence under green (532 nm) excitation, in the intact organ (hepatocellular medium) NADH fluorescence may behave in a significantly altered manner, to the point of responding to green excitation. This, however, remains to be investigated. In order to obtain better organ preservation and consequently increase the donor pool, there is a lot still to be learned about the metabolism after organ perfusion and removal as well as about how individual chromophors in a cellular environment contribute to the fluorescence spectrum.

To assure comparability in equivalent conditions of light and tissue coupling, we normalized all spectra at the excitation wavelength (reducing the background scattering). In other words, in each spectrum, the excitation peak received the value 1. After this normalization procedure, we notice that the overall intensity of fluorescence decreased with time. That indicates a possible variation in

biochemical activity and/or a possible modification of the cellular structure. To quantify the fluorescence variation, we calculated the area of the broad fluorescence spectra and calculated its reduction as time passed. Defining:

$$A_t = \int_{\lambda_1}^{\lambda_2} I(\lambda) d\lambda \,, \tag{1}$$

where $A_{\rm t}$ is the integration of the spectrum ($I(\lambda)$) from $\lambda_1 = 550$ nm to $\lambda_2 = 800$ nm, for the observation taken place at a time t after perfusion. The percentage of fluorescence reduction (PFR) observed in the tissue as the time pass by can be obtained calculating

$$\mathrm{PFR} = 100(A_{\rm t} - A_0)/A_0 \,, \tag{2}$$

where A_0, stands for the time equal zero value. The RCR has reduced to about 60% of its original value in 12 hours. This does not necessarily mean that the organ has undergone permanent irreversible damage. At present, we do not know at what RCR level the damage is still reversible. That will be the subject of future studies.

The focus of comparison with the fluorescence spectrum was the RCR as it showed the greatest similarity with the changes in spectral analysis on a time scale.

Considering that fluorescence is related to tissue activity, the PFR can be compared to the RCR. The behavior is very similar to the RCR, indicating that there is a real correlation between the time variation of RCR and that of fluorescence. RCR represents only the mitochondrial activity, whereas the fluorescence has innumerous contributions, i.e. as long as there are molecules fluorescence will always exist. Therefore a numerical constant must correlate both quantities and this can be obtained in the following manner:

$$(\mathrm{RCR})_{\rm F} = g\mathrm{PFR} \,, \tag{3}$$

where the subscript F represents the value of RCR obtained by fluorescence. In our case the numerical constant $g = 1.3$.

Fig. 7 compares the $(\mathrm{RCR})_{\rm F}$ with the RCR obtained from the respiratory assay in the CS group. The similarity between the values is very remarkable proving the viability of using fluorescence spectra as a tool for following changes in liver grafts.

On the other hand, there is no correlation between the RCR and fluorescence in the RT group, as shown in Fig. 8. This is probably due to rapid deterioration of the organ with the accumulation of metabolites allied to cellular structural changes resulting in the differences in the fluorescence spectrum

In this experiment, focus of analysis was for a period of 12h. Nevertheless, we have observed that the fluorescence spectrum changes dramatically after longer periods of observation, when new peaks start to appear. After longer periods of observation, there is not only a variation in the overall fluorescence but also the appearance of new peaks that suggest that tissue modification at cellular level is taking place. The appearance of such features may indicate that the integrity of the organ is lost.

The initial analysis of the spectrum used in this study is a relatively simple one in which only the intensity and the wavelength of the fluorescence was considered. Other means of analysis could be employed. As mentioned earlier and can be seen in graph 5, after longer periods of ischemia, in the CS group, a second peak of fluorescence begins to appear at 636 nm. This seems to occur sooner in the RT group. This environment may represent what is happening in earlier in the RT group and could symbolize an important cellular event. In a speculative manner, to investigate what this could represent, we plotted a graph of the ratio between the fluorescence values obtained at 636 nm and 600 nm (Fig. 9). Interestingly, this ratio remains stable for around 5 to 6 hours, in the CS group, and then undergoes a rapid change. This ratio was named the degree of degradation. We are not sure what this means but it surely raises some questions. Does this mean that around 5 or 6 hours, in the CS group, is the point of no return, when the organ would no longer function if implanted? This and many other question remain to be answered and will be the object of future studies.

4. Conclusions

We have presented results that show that fluorescence spectroscopy is potentially a powerful tool to be employed in evaluating the level of tissue integrity. This could be of tremendous value in liver transplantation by providing means for evaluating the organ to be implanted. Not only could this technique offer information on the organ, it would do so non-invasively, in real-time and as well as give the results quantitatively. This is of great value as it would remove the subjective ness of the surgeons evaluation without increasing the time necessary for analysis. The results also indicated that fluorescence may detect the appearance of new substances resulting from the decomposition and/or modification of the cellular structures, which could be important in analyzing the integrity of the tissue structure and function. Much remains to be done in order to turn these promises into reality in the surgical theater.

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