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BASIC STUDIES

Fluorescence spectroscopy to diagnose hepatic steatosis in a rat model of fatty liver

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Abstract

Background: Steatosis is diagnosed on the basis of the macroscopic aspect of the liver evaluated by the surgeon at the time of organ extraction or by means of a frozen biopsy. *Aim:* In the present study, the applicability of laser-induced fluorescence (LIF) spectroscopy was investigated as a method for the diagnosis of different degrees of steatosis experimentally induced in rats. *Material and methods:* Rats received a high-lipid diet for different periods of time. The animals were divided into groups according to the degree of induced steatosis diagnosis by histology. The concentration of fat in the liver was correlated with LIF by means of the steatosis fluorescence factor (SFF). *Results:* The histology classification, according to liver fat concentration was, Severe Steatosis, Moderate Steatosis, Mild Steatosis and Control (no liver steatosis). Fluorescence intensity could be directly correlated with fat content. It was possible to estimate an average of fluorescence intensity variable by means of different confidence intervals ($P = 95\%$) for each steatosis group. SFF was significantly higher in the Severe Steatosis group ($P < 0.001$) compared with the Moderate Steatosis, Mild Steatosis and Control groups. *Conclusion:* The various degrees of steatosis could be directly correlated with SFF. LIF spectroscopy proved to be a method capable of identifying the degree of hepatic steatosis in this animal model, and has the potential of clinical application for non-invasive evaluation of the degree of steatosis.

Liver transplantation is a therapeutic modality used for the treatment of end-stage hepatic insufficiency. The good results of this therapy have led to an increased demand for liver transplantation, although the supply of organ donors has not followed this demand. The need to increase the donor pool has led to the use of donors considered not to be ideal, as long as the procedure does not impair recipient or graft survival. Thus, there is an increased need for the establishment of the donor-related risk factors for primary graft dysfunction (PGD) (1–3). PGD manifests clinically as primary non-function (PNF) there or initial poor function (IPF) of the graft (4). Among the risk factors for PGD is hepatic steatosis (5, 6). Steatosis can be characterized quantitatively and qualitatively. Qualitatively, fat deposits in hepatocytes can be classified as macro- or microvesicular steatosis. In macrovesicular steatosis, hepatocytes contain one single vacuole of fat, which displaces the nucleus to the periphery of the cell. Macrovesicular steatosis has been rapidly identified as a risk factor for primary non-function. In contrast, microvesicular steatosis consists of many small fatty inclusions in the cytoplasm without displacement of the nucleus. Microvesicular steatosis is caused by dysfunction of the mitochondrial β-oxidation, which is usually related to toxins or metabolic disorder, and is owing to the low cellular energy levels, but its clinical relevance still remains unclear. The effect of microvesicular steatosis on liver injury remains controversial, a general consensus currently exists in the hepatic transplantation community that microsteatosis has less clinical importance than macrosteatosis, while moderate to severe ($\geq 30\%$) macrosteatosis increases the risk of post-operative complications and patient death after liver transplantation (7). The quantitative steatosis estimation is based on the percentage of hepatocytes containing cytoplasmic fat droplets (8). Moderate hepatic steatosis is a risk factor for IRF (9) and may also be a risk factor for PNF when it is associated with other adverse factors such as prolonged ischaemia time (2). Severe donor steatosis is associated with PNF (10–12) which, in turn, is correlated with increased mortality (13). An up-to-date steatosis diagnosis can be made on the basis of the macroscopic aspect of the liver assessed by the surgeon during organ removal from the donor (13, 14). Most transplant centres recommend the use of a frozen biopsy (4–6).

The laser-induced fluorescence (LIF) spectroscopy is an optical method that assesses the interaction between light at a given wavelength and tissue (15, 16), allowing tissue characterization based on fluorescence (17). Fluorescence has been applied in medicine as a diagnostic method for tumours, at several sites, premalignant lesions of the digestive tract, carotid atherosclerosis plaques and among other lesions (18–24).

The objective of the present study was to assess experimentally the applicability of LIF spectroscopy as a method to detect mild, moderate and severe steatosis quantitatively.

Material and methods

Animals and diet

Male Wistar rats weighing 200–250 g were housed in individual cages in a room with controlled temperature and a 12 h

light–dark cycle. The control group received a standard diet containing 69% carbohydrate, 18% protein, 4% fibre, 3% lipids, 4% mineral salts, 1% choline and 1% vitamins (25) for 7 days. The remaining animals received a high-lipid diet for 1, 2, 3, 4 or 7 days. The high-lipid diet, capable of inducing steatosis, contained 50% dextrose, 18% protein, 25.3% lipid, 4% mineral salts, 1% cholesterol, 0.5% sodium cholate, 0.2% choline and 1% vitamins (25). All animals received water *ad libitum*.

Steatosis grading

The animals were divided into groups according to the degree of induced steatosis determined by histology of haematoxylin–eosin (H&E)-stained material, regardless of the duration of the diet. Steatosis was graded as absent (Control Group), mild (up to 30% of the hepatocytes showing fat vacuoles), moderate (30–60% of the hepatocytes showing fat vacuoles) and severe (> 60% of the hepatocytes showing fat vacuoles) according to the classification proposed by Adam *et al.* (26).

Laser-induced fluorescence spectroscopy

After the diet period, the animals were subjected to laparotomy under general anaesthesia with xylazine/ketamine (80/16 mg/kg). Fluorescence spectroscopy was obtained by investigating three hepatic lobes at a wavelength of 532 nm (Nd^{3+}:YAG). The investigation probe is a Y-type one that delivers the excitation light through a central 110 μm fibre and collects the fluorescence through six 100 μm other ones. Fibres that collect the fluorescence exiting from the tissue surface are located around the excitation fibre (27) and this light is conducted to a spectrophotometer. A computer coupled to the spectrophotometer processes and stores the data. The interrogation probe was placed in gentle and perpendicular contact at the liver surface. The spectrum measurement was taken in < 3 s. The spectrum is generated in real time on the screen of the computer. For each hepatic lobe three measurements were taken, yielding a total of nine measurements per liver. The graphs generated are used to evaluate the pattern of the collected fluorescence, its behaviour and intensity. Evaluating the fluorescence data, we determined a steatosis fluorescence factor (SFF) obtained by the ratio of the backscattered amplitude at the backscattered excitation wavelength and the maximum fluorescence amplitude at the normalized spectrum. The results were calculated as the mean for the three hepatic lobes examined, analysed for each group according to the degree of steatosis and reported as mean ± standard deviation (SD).

Lipid extraction

Lipids were extracted by the Soxhlet method (28) and the results, reported as the amount of fat in mg per g of dry liver (mean ± SD), indicated the concentration of fat in the liver.

Statistical analysis

Data regarding hepatic fat concentration and SFF were analysed statistically by analysis of variance (ANOVA), with the level of significance set at $P < 0.05$. Data for the various groups were compared by the multiple-comparisons Tukey–Kramer test.

Fluorescence intensity data were analysed statistically by the non-parametric Kruskal–Wallis test and the data for the various groups were compared by the multiple-comparisons Dunn test. Although the fluorescence intensity was a variable showing a normal distribution, there was a statistically significant difference between the standard deviations of the groups studied, which prevented the application of a parametric test.

For the fluorescence intensity variable, the population mean was estimated by the confidence intervals using the Student *t*-distribution and establishing a probability of 95%.

Results

Histological evaluation and steatosis grading

The animals that received the standard diet did not show steatosis and were used as the Control group ($n = 7$). The Mild Steatosis group consisted of 13 animals, eight of which received the high-lipid diet for 1 day (8/8) and the other five received the same diet for 2 days (5/6). The moderate Steatosis group consisted of nine animals, with one animal receiving the high-lipid diet for 2 days (1/6), six receiving the same diet for 3 days (6/6) and two receiving the same diet for 4 days (2/7). Finally, the Severe Steatosis group consisted of 13 animals, five of which received the high-lipid diet for 4 days (5/7), while the remaining eight received the same diet for 7 days (8/8). The comparative macroscopic and microscopic characteristics of the Control group and of the Steatosis groups are presented in Figures 1 and 2.

Fat concentration in the liver

Fat concentration was significantly higher in the Severe Steatosis group (39.8 ± 3.75 mg/g liver) compared with the remaining groups ($P < 0.001$). The Moderate Steatosis group (29.22 ± 6.17 mg/g liver) presented a significantly higher concentration compared with the Mild Steatosis group and the Control ($P < 0.001$). There was no significant difference between the Mild Steatosis group (16.06 ± 3.75 mg/g liver) and the Control (11.5 ± 2.98 mg/g liver). The amount of fat and the degree of steatosis for the various groups are presented in Figure 3.

Fluorescence intensity

The fluorescence spectra for the various groups are shown in Figure 4. The first narrow peak corresponds to the backscattered excitation light. All spectra were normalized relative to

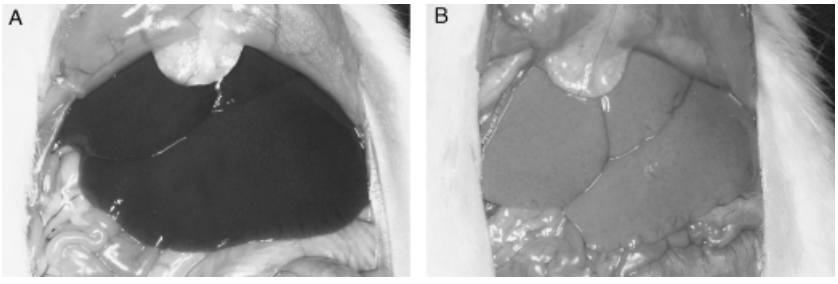

Fig. 1. Macroscopic aspect of the liver in the Control group (A) and the Severe Steatosis group after 7 days on the respective diets (B).

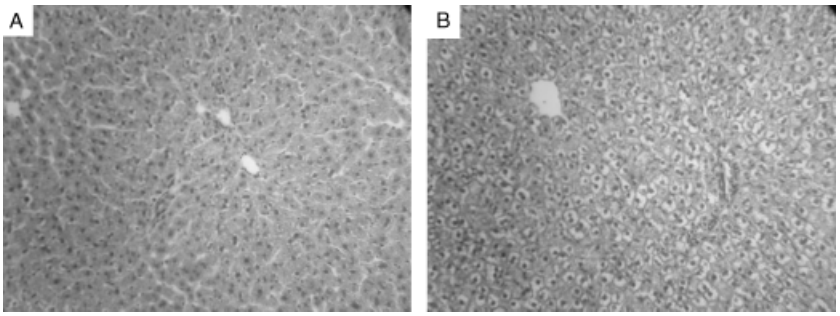

Fig. 2. Histological aspects of the liver of the Control group (A) and of the Moderate steatosis group (B). Haematoxylin–eosin staining; magnification × 250.

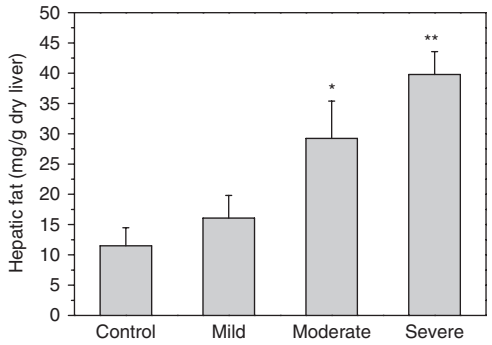

Fig. 3. Lipid concentrations in the different groups studied. *Moderate≠Mild and Moderate≠Control ($P < 0.001$). **Severe≠Moderate, Severe≠Mild and Severe≠Control ($P < 0.001$). ANOVA followed by the Tukey–Kramer test.

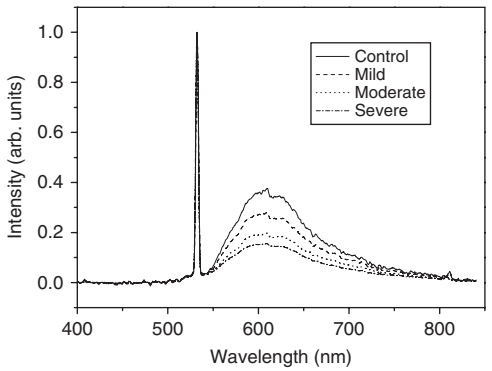

Fig. 4. Normalized fluorescence spectra for the investigated groups. Overall intensity changes can be discriminated.

this peak. The wider emission band corresponds to the collected fluorescence. The fluorescence behaviour was preserved for all investigated groups, as shown in Figure 4, no qualitative changes were evident.

Laser-induced fluorescence maximum relative intensity around 605 nm was significantly lower in the Severe Steatosis group ($P < 0.001$) and in the Moderate Steatosis group ($P < 0.05$) compared with the Control group. Relative fluorescence intensity was also lower in the Severe Steatosis group compared with the Mild Steatosis group ($P < 0.01$). The results are shown in Table 1. There was no significant difference between the Severe Steatosis group and the Moderate Steatosis group, between the Moderate Steatosis group and the Mild Steatosis group or between the Mild Steatosis group and the Control, although the typical spectra were clearly distinct. The confidence interval for the fluorescence intensity was calculated for each group, also shown in Table 1.

Table 1. Fluorescence intensity at 605 nm and the confidence interval determined for Control and different degrees of Steatosis groups

Group	Intensity at 605 nm	Confidence interval
Control	0.38 ± 0.07	0.31–0.45
Mild steatosis	0.28 ± 0.06	0.25–0.32
Moderate steatosis	0.20 ± 0.03	0.18–0.23
Severe steatosis	0.16 ± 0.01	0.15–0.17

Steatosis fluorescence factor

The calculated SFF was significantly higher in the Severe Steatosis group (6.05 ± 0.58; $P < 0.001$) compared with the Moderate Steatosis (4.40 ± 0.46), Mild Steatosis (3.21 ± 0.49) and Control (2.69 ± 0.66) groups. The SFF was significantly higher in the Moderate Steatosis group compared with the Mild Steatosis group and the Control ($P < 0.001$) group and was significantly higher in the Mild Steatosis group compared with the Control ($P < 0.01$). The SFF values for the various groups are presented in Figure 5.

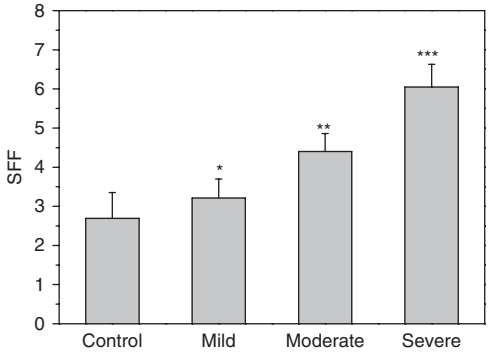

Fig. 5. Hepatic steatosis fluorescence factor for the different groups studied. *Mild≠Control ($P < 0.01$). ** Moderate≠Mild and Moderate≠Control ($P < 0.001$). ***Severe≠Moderate, Severe≠Mild and Severe≠Control ($P < 0.001$).

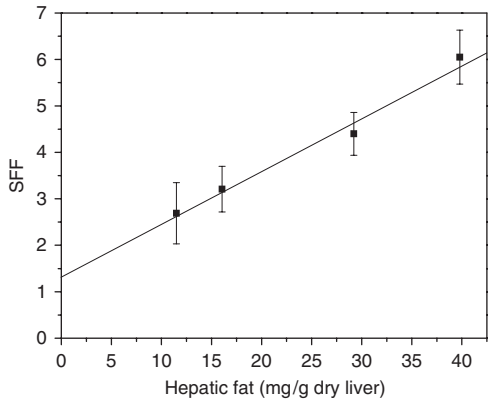

Fig. 6. Steatosis fluorescence factor (SFF) as a function of hepatic fat concentration.

In order to evaluate whether the LIF spectroscopy changes could be correlated to the hepatic fat concentration, the SFF was plotted as a function of the amount of fat (Fig. 6). A linear correlation was observed and the following equation set:

$$\mathrm{SFF} = 0.114[\mathrm{HF}] + 1.352.$$

The correlation factor between these two variables obtained by the above equation was 0.983. Thus, the SFF varied according to the fat concentration in the liver, allowing an estimation of the fat liver amount: the higher the hepatic fat concentration, the higher the SFF, in a linear correspondence.

Discussion

The high-lipid diet used in the present study was effective in inducing hepatic steatosis. The presence of a high lipid content and of dextrose, which are lipogenic, and of sodium cholate, which increases bile salt excretion and then the absorption of the diet offered, allowed effective induction of steatosis within a short period of a few days. Our results are in agreement with Arnault *et al.* (25), who obtained severe steatosis in 100% of the animals that received this diet for 7 days. However, in the present study, the intention was to induce steatosis of a moderate and mild degree with the same diet. This was achieved by administering the diet during different periods of time. Because the groups were set up after histological evaluation with the degree of steatosis being unpredictable for the different periods of high-lipid diet administration, except for the induction of severe steatosis over a period of 7 days as described previously (25), the groups resulted in a variable number of animals, i.e., $n = 13$, 9 and 13 for Mild, Moderate and Severe Steatosis respectively.

Histological evaluation was performed under H&E staining, which was the criterion used for the classification of the animals according to the degree of steatosis. The determination of hepatic fat, which was a variable analysed later in an objective manner, confirmed the specificity of the histological technique used for the identification of the degree of steatosis. Marsman *et al.* (29) detected a good correlation between the amount of liver fat and histological evaluation. Garcia Ureña *et al.* (30), in a study comparing various histological techniques for the diagnosis of hepatic steatosis, had already demonstrated 90% specificity of the H&E technique. In the present study, a mixed pattern of hepatic steatosis with the predominance of microvacuoles was detected, similar to that reported by Arnault *et al.* (25), who used the same experimental model. The classification used to grade steatosis according to the percentage of hepatocytes with lipid vacuoles is the one used most frequently (26).

Liver fat concentration was significantly different between the Severe and Moderate Steatosis groups and between them and the remaining groups (Mild Steatosis and Control). This confirmed what was expected, i.e., a direct correlation between the degree of steatosis and liver lipid concentration. There was no difference between the Mild Steatosis group and the Control, probably owing to the overlapping of the degree of steatosis in these two groups predicted by the classification used (26), in which steatosis involvement ranges from 0 to 30% of the hepatocytes.

Severe steatosis and moderate steatosis are recognized as risk factors for PGD in liver transplantation (4–6, 9). Briceno *et al.* (1) showed that moderate and severe steatosis increases by 3.63 times the risk of severe preservation lesion in the graft, which can be a cause of PGD. Adam *et al.* (26) reported that the incidence of the absence of primary graft function was significantly higher in the group of patients receiving grafts from donors presenting moderate and severe steatosis (13%) compared with grafts from non-steatotic donors (2.5%). Hepatic steatosis is a recognized risk factor for the primary lack of graft function and initially delayed graft function (31).

On this basis, the diagnosis of hepatic steatosis in the donor liver is an important procedure for the transplantation success. Computed tomography and magnetic resonance are methods with 100% specificity and 75% sensitivity for the diagnosis of hepatic steatosis (32). However, owing to cost and availability, they are not used as methods for the diagnosis of hepatic steatosis in cadaver donors. Two methods for the evaluation of steatosis of the donor liver have been used at transplant centres. One is the evaluation of the macroscopic aspect of the liver by the surgeon during the removal of the organ from the donor and before and after perfusion (13, 14), which shows 75% specificity and 73% sensitivity (33). Another routine method considered to be the gold standard for the diagnosis of donor steatosis is hepatic biopsy (34), where the sensitivity rate varies according to the histological technique used (30). Depending on the hospital where the donor surgery is performed, a biopsy may not be possible owing to available facility. This situation implies performing a biopsy at the transplant centre, causing an increase in the time of cold ischaemia. Despite the greater tolerance of the graft for cold ischaemia after the preservation solution of the University of Wisconsin (35) became available, this ischaemia may have negative results in a country with a large territorial area, such as Brazil, in which the donor is often very far from the transplant centre. Alternative methods have been applied for the diagnosis of steatosis in the liver of a cadaver donor, such as the measurement of blood flow in the hepatic microcirculation by Doppler flowmetry (36), which is decreased in severe steatosis (37). This method is non-invasive and provides results in real time, but does not discriminate between the various degrees of steatosis. In a study by Seifalian *et al.* (36), statistically significant differences were observed when the group with Steatosis was compared with the Control group, but not when the groups with different degrees of steatosis were compared. This method has limitations for clinical application aiming at the identification of moderate and severe steatosis that will affect the prognosis of transplantation. The development of a method for *in situ* diagnosis of different degrees of hepatic steatosis would be very useful for the operational system of liver transplants and would contribute to a reduced time of cold ischaemia.

In the present study, we investigated the applicability of LIF spectroscopy to the diagnosis of different degrees of steatosis aiming at a future clinical application in liver transplantation. LIF spectroscopy is a method used in medicine, especially in the area of cancer diagnostics. Its application requires contact between the investigation probe and the tissue surface of interest. This limits its use to superficial alterations, such as malignant and premalignant lesions of the digestive tube, the skin, the bladder and the lung (18–24). Concerning the interrogated volume of tissue provided by 532 nm excitation, in a previous work (38), the specific penetration of light at different wavelengths shows that around 532 nm, an effective penetration of 0.9 mm at rat liver was observed. This length can provide an idea for the volume of tissue sampled during the light excitation; with such a short penetration the information is quite superficial. In the transplantation area, Sankarankutty *et al*. (39) assessed the viability of hepatic grafts during the period of cold ischaemia using mitochondrial function and ATP production as biological parameters and correlating them with the study of fluorescence. The author observed that the fluorescence pattern, in an animal model, was altered before the deterioration of mitochondrial function. An advantage of the use of fluorescence as a diagnostic method is that it allows the identification of tissue changes without tissue removal and it provides the result in real time. Fluorescence is specific for each biochemical tissue composition, thus being quite sensitive to abnormalities. The result is obtained by means of an 'optical biopsy', i.e., without removing tissue fragments but by determining physical properties by means of fluorescence.

During excitation of the liver tissue at 532 nm, there is a broad fluorescence spectrum emitted from 550 to 750 nm. There are many possible contributions to this fluorescence band. According to Beuthan (40), coenzymes such as flavins, proteins and acid arachidonic, present in the liver, may show a response to this excitation wavelength. Besides liver tissue, blood constituents are known by having relevant absorption at the green spectrum and consequently fluorescence. The observed fluorescence is a net result of several contributions, not only fluorescence itself but also scattering and absorption tissue characteristics. During excitation at 532 nm there are many components responsible for the observed fluorescence variation. Riboflavin, when excited around 530 nm presents an extended fluorescence band from 540 to 620 nm (41). Coincidently, riboflavin levels appear to be connected to some degree with mitochondrial alterations (42). This may have contributed to the observed decrease in fluorescence level at a high degree of steatosis. There are many other lipopigments, lipids associated with liver tissue that show variations during injuries. Lipofuscin present in the liver (43, 44) has strong emission bands in the region of 500 to 700 nm when excited at 370 nm, but it mostly probably may also be present on excitation at 532 nm. This lipopigment certainly contributes to the fluorescence band of healthy liver and it may present a strong variation during steatosis, where the presence of fatty acids may deplete their concentration, altering the fluorescence level. The exact determination of the contribution of all biomolecules involved in the final fluorescence spectrum and their variation presented at distinct steatosis degrees could not be obtained in this present study. However, the results may provide an indication of the viability of using fluorescence spectroscopy for the measurement of steatosis degree. From an absolute viewpoint, fluorescence intensity was inversely proportional to the degree of steatosis, thus showing the lowest amplitude in the group with severe steatosis. However, it was not possible to distinguish statistically the Severe Steatosis group from the Moderate Steatosis group or the Moderate Steatosis group from the Mild Steatosis group by the multiple-comparisons Dunn test. The estimation of the population mean for the fluorescence intensity variable by means of the confidence intervals allowed us to distinguish the groups with Severe and Moderate Steatosis, which were the target of interest in the present study owing to their association with complications in liver transplantation. The relative imbalance observed at the spectrum between backscattered light and fluorescence is the main effect that allows quantification of steatosis. The reason for such an effect relies on the fact that lipids are good light scatterers but poor absorbers of visible light. Therefore, the presence of fat mainly induces an increase of backscattering and a decrease of fluorescence, contributing towards increasing the SFF as the steatosis degree increases. There were overlapping values for the Mild Steatosis group and the Control group, a fact that may not have clinical implications because the incidence of PGD is similar in these two groups (10). Analysis of fluorescence by means of the SFF, which correlated the backscattered amplitude and the maximum amplitude of fluorescence, revealed an increase in this factor with increasing hepatic steatosis. There was a statistically significant difference of SFF in all comparisons made between the various groups studied, allowing the discrimination and identification of each degree of steatosis. The SFF was directly correlated with hepatic fat concentration; a linear correlation could be defined. The resulting equation was $SFF = 0.114[HF] + 1.352$, with HF being the concentration of hepatic fat. This equation shows a correlation factor of 0.983, which indicates a strong correlation between SFF and the concentration of hepatic fat. Thus, LIF spectroscopy was able to define specific confidence intervals of fluorescence intensity for moderate and severe steatosis and to distinguish all degrees of steatosis by means of the SFF. The positive correlation between SFF and hepatic fat concentration demonstrated by the line in the graph and its resulting equation supports the hypothesis that LIF is a method applicable to the identification and grading of liver steatosis.

Conclusion

Laser-induced fluorescence spectroscopy proved to be a diagnostic method capable of identifying and quantifying hepatic steatosis experimentally, in real time, on the basis of the confidence intervals for the amplitude of fluorescence and of the SFF. The applicability of fluorescence as a method for the diagnosis of hepatic steatosis should be evaluated in clinical studies as an auxiliary tool for liver surgery or during the graft evaluation part of the liver transplantation procedure.

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