Title Page

Title: Assessing steatotic liver function after ischemia-reperfusion injury by in vivo multiphoton imaging of fluorescein disposition

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DMD # 60848

Running Title Page

Running title: In vivo subcellular imaging of the liver

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Number of text pages: 13

Number of tables: 3

Number of figures: 6

Number of references: 32

Number of words in Abstract: 250

Number of words in Introduction: 730

Number of words in Discussion: 1034

List of abbreviations:

Ai = amount
ALT = alanine transaminase
AUC = area under the curve
Ci = concentration
CLuptake = uptake clearance into hepatocytes
FLIM = fluorescence lifetime imaging microscopy
GRIN = gradient-index
hep = hepatocyte
HPLC = high-performance liquid chromatography
I/R = ischemia-reperfusion
kbile, F = rate constant describing biliary excretion of fluorescein
kbile, FG = rate constant describing biliary excretion of fluorescein monoglucuronide
kmet = rate constant describing metabolism of fluorescein
MPM = multiphoton microscopy
MRP3 = multidrug resistance-associated protein 3
NASH = nonalcoholic steatohepatitis
SEM = standard deviation of the mean
sin = sinusoid
Vi = distribution volume
Abstract

Ischemia-reperfusion injury is a common complication during liver surgery, where steatotic livers are more prone to the injury and may become more prevalent in the growing obese population. This study aimed to characterize liver morphology and understand changes in subcellular function in steatotic livers exposed to ischemia-reperfusion injury through quantitative description of fluorescein distribution obtained by minimally-invasive in vivo multiphoton microscopy using a physiological pharmacokinetic model. Rats were fed a high fat diet for 7 days to induce liver steatosis. Partial ischemia was induced, following reperfusion for 4 hours, when fluorescein (10 mg/kg) was injected intravenously. Liver images, bile and blood were collected up to 180 min following injection. Ischemia-reperfusion injury was associated with an increase in alanine transaminase levels and apoptosis. In addition, steatosis had the presence of lipid droplets and an increase in the fluorescein associated fluorescence observed in the hepatocytes by multiphoton imaging. Analysis of the hepatic concentration-time profiles suggests that the steatosis induced increase in fluorescein associated fluorescence mainly arises by inducing the hepatic fluorescein metabolism. The combination of ischemia-reperfusion with steatosis exacerbates these effects further. This was confirmed by fluorescence lifetime imaging microscopy showing a decreased average fluorescence lifetime of the liver, indicative of increased production of the metabolite. Our results show the potential of non-invasive imaging of a dye to further improve our understanding of liver disease induced subcellular changes in vivo by also providing further quantitative measures of metabolic and biliary liver function and, hence, extending the qualitative liver function tests now available.
Introduction

Approximately 5-10% of the general population are diagnosed with cirrhosis, the end result of many types of liver disease. It accounts for 4.4% of all deaths among adults in developed countries (Lim and Kim, 2008) and liver transplantation is the only immediate treatment (Jonas et al., 2001). Fibrosis is treatable in its early stages, therefore accurate staging of the extent of fibrosis is important for optimal treatment (He et al., 2010). Liver function is traditionally assessed by static measures, which are not able to track changes in liver function quickly. Liver biopsy is the gold standard in evaluating fibrosis, however there is a risk of bleeding complications and false negative results. Liver biopsies provide only a snap shot image of the liver tissue and do not allow dynamic monitoring of morphological events (Goetz et al., 2008b). Variation in staining efficiency, expense and impracticality for mass screening, are some further disadvantages of liver biopsy (Lin et al., 2012). It is not ideal to perform serial liver biopsies in order to accurately determine disease progression or to monitor treatment effects (He et al., 2010). In addition, scoring systems to assess fibrosis use qualitative rather than quantitative measures. It is difficult to obtain highly reproducible results from these scores with a high degree of intra- and inter observer discrepancy (as high as 35%) (Tai et al., 2009).

Non-invasive imaging has the potential to extract extra information over a long period of time over a larger sampling area and with minimal morbidity (He et al., 2010). Non-invasive biomedical imaging techniques, such as ultrasonography, computed tomography, and magnetic resonance imaging are widely used to detect liver disease, but they do not have sufficient sensitivity, spatial resolution and specificity to detect and stage liver disease at an early stage (Lin et al., 2012). Fluorescence imaging, on the other hand, can take several virtual biopsies to obtain more of an overall view of the liver (Goetz et al., 2008a), as well as providing dynamic information (Goetz et al., 2008b). It is useful regardless of sample
preparation, e.g. frozen or paraffin embedded tissue, easy and fast, no sampling bias (Gailhouste et al., 2010) and has the potential of quantifying liver disease such as fibrosis (He et al., 2010). However, in order to provide functional information regarding uptake, metabolism and excretion, a fluorescent dye must be administered.

Fluorescein is a non-toxic and FDA-cleared dye for diagnostic angiography of the retina and is mainly excreted by the kidneys and liver (Neumann et al., 2010). Fluorescein has been used to study liver function in humans by permitting visualisation of hepatocytes, bile ducts, sinusoids and collagen fibers \textit{in vivo} (Goetz et al., 2008a). Fluorescein rapidly diffuses through the body within seconds, is taken up by the hepatocytes, metabolised to fluorescein monoglucuronide and excreted into the bile by active transport (Sherman and Fisher, 1986). Tissue architecture and vessels can be well visualized, however nuclei are not well seen (Goetz et al., 2008b). Fluorescein and fluorescein monoglucuronide have overlapping excitation and emission spectra, but have different fluorescence lifetimes, making it possible for fluorescence lifetime imaging to detect these lifetime changes. Our group has showed that fluorescence lifetime imaging microscopy (FLIM) is able to study the metabolism of fluorescein to fluorescein monoglucuronide (Thorling et al., 2011a). In this study, we used the clinically approved fluorescein to understand liver disease induced changes in liver subcellular function. Due to an increasingly obese population, hepatic steatosis is growing and may impact upon the availability of suitable liver donors (Nativ et al., 2012). Steatotic liver grafts are more susceptible to ischemia-reperfusion (I/R) injury, with increased lipid peroxidation (Vairetti et al., 2009; Ben et al., 2010), increased release of pro-inflammatory mediators (Selzner and Clavien, 2001; Vairetti et al., 2009) and increased neutrophil infiltration (Nakano et al., 1997). In addition, steatosis increases the cellular volume resulting in narrowed microvessels and therefore reduced blood flow (Nakano et al., 1997; Ben et al., 2010). These factors put steatotic donor grafts at great risk of primary non-function (Busuttil
and Tanaka, 2003). A physiologically-based pharmacokinetic model was used to simultaneously fit biliary excretion and imaging data after injection of fluorescein. FLIM was used to validate the model, by studying the metabolism of fluorescein to fluorescein monoglucuronide, as previously described (Thorling et al., 2011a). This is to our knowledge the first study to quantitate subcellular functional changes in steatotic livers after I/R injury using minimally-invasive MPM.

**Materials and Methods**

**Chemicals and Reagents**

Sodium fluorescein and β-glucuronidase were obtained from Sigma Aldrich (St Louis, MO, US). Ilum xylazine and ketamine hydrochloride were purchased from Bayer Australia (Pymble NSW, Australia) and Parnell laboratories, Australia, respectively.

**Animals**

Male Wistar rats were purchased from the Animal Resource Centre (Perth, WA, Australia), and housed at Pharmacy Australia Centre of Excellence in the University of Queensland Biological Resources Facility, where they had unlimited access to food and water. The temperature was maintained at 20±1°C and humidity at 60-75%, with artificial light for 12 hours (h) (7 am to 7 pm) daily. All animals received human care and the study was approved by the Animal Ethics Committee at the University of Queensland.

**Surgical Procedures**

All rats were first anaesthetized by an intraperitoneal injection of ketamine hydrochloride (80 mg/kg) and ilum xylazil (10 mg/kg), which was maintained throughout the experiment by administering ketamine (2.2 mg/100g) and ilum xylazil (0.25 mg/100g). Their body
temperatures were controlled by placing them on a heating pad set to 37°C. Figure 1 shows a schematic flow chart of the experiments.

Ischemia-reperfusion injury model

Ischemia (70%) was induced by clamping the portal vein, hepatic artery and bile duct that supply the median and left lobes using a microvascular clamp. After 1 hour (h) the clamp was removed to allow reperfusion of the liver and rats woke up during this time under close observation. The liver was reperfused for 4 hours, where after fluorescein was injected. At 3 hours of reperfusion, rats were anaesthetized again, the jugular vein and carotid artery were cannulated for sodium fluorescein administration (bolus 10 mg/kg at 4 h of reperfusion) and blood collection, respectively. A midline laparotomy was performed to expose the liver. The bile duct was cleared of surrounding tissue and cannulated for bile collection. All cannulations were done using PE-10 tubing. The left lobe was slightly raised above the intraperitoneal cavity and 0.9% saline was administered continuously.

Steatosis model

Steatosis was induced in rat livers by feeding rats a high fat diet based on the diet used by Arnault et al. (Arnault et al., 2003) provided by Specialty Feeds Pty Ltd (SF12-014) Glen Forrest, Western Australia for 7 days before induction of ischemia.

Tissue collection

Blood was collected at 4 hours of reperfusion for analysis of alanine transaminase (ALT) using a Hitachi 747 analyzer (Hitachi Ltd., Tokyo, Japan). Blood and bile was collected for measurements of fluorescein and fluorescein monoglucuronide, respectively and the liver was collected for histological assessment.
**Histopathological analysis**

Fixed liver tissue was sectioned onto each slide, stained and scanned using an AperioScanScope XT slide scanning system (Aperio Technologies Inc., Vista, California, USA) as previously described (Thorling et al., 2011b). The number of monocytes and macrophages were recognized by counting ED1-positive cells in the sinusoids in ten random fields per slide. The number of apoptotic cells was identified by counting positive nuclei in ten random fields per region per liver slide per region (periportal, centrilobular and midzonal) of the liver.

**High-Performance Liquid Chromatography (HPLC)**

Concentrations of fluorescein and fluorescein monoglucuronide in each collected bile sample were determined by HPLC analysis (Shimadzu, Kyoto, Japan) as previously described (Thorling et al., 2011a). In brief, the mobile phase consisted of 50 mM NaH₂PO₄ and methanol (2:3, v/v). Flow rate was set at 1 mL/min flow through a C18 column (Agilent HC-C18 (2) 4.6×150 mm, 5 μm). Fluorescence excitation and emission wavelengths were 488 and 515 nm, respectively, with a retention time of 5 min.

**Multiphoton Microscopy**

MPM images were recorded by a DermaInspect system (JenLab GmbH, Jena, Germany) with an ultrashort pulsed mode-locked 80-MHz Titanium:SapphireMaiTai laser (Spectra Physics, 25 Mountain View, USA). The excitation wavelength was set to 740 nm for liver autofluorescence or 920 nm for fluorescein fluorescence, with an emission range of 350-650 nm. The laser power was set to 20 mW and the acquisition time was 7.4 s per frame. Images were recorded at intervals up to 180 minutes after fluorescein injection, using 10× and 40× objectives.
**Fluorescence lifetime imaging microscopy**

FLIM was performed using a TCSPC830 detection module (Becker and Hickl, Berlin, Germany) characterized by the time resolution of 100 ps. FLIM data of fluorescein and fluorescein monoglucuronide were obtained in the emission range of 515 to 620 nm. Fluorescence lifetime images were analyzed using SPCImage (Becker and Hickl, Berlin, Germany). Each pixel of a given fluorescence lifetime image contains photon decay data, to which an exponential function is fitted in order to retrieve the fluorescence lifetime value(s). These lifetimes characterize the decay and, by extension, the fluorophore(s) present in the sample. Fluorescence lifetimes reported here are the mean ± standard deviation of the mean (SEM) of the values measured at the highest-intensity pixel in all images.

**Image analysis and physiological pharmacokinetic modeling**

Liver images were obtained over time. The fluorescence intensity of fluorescein in sinusoid and hepatocyte was determined separately using ImageJ 1.44p (National Institutes of Health, USA) and was converted into concentrations using a standard curve prepared in homogenized liver. The fluorescein concentrations in the sinusoid ($C_{\text{sin}}(t)$) and hepatocyte ($C_{\text{hep}}(t)$) together with the fluorescein amounts excreted into the bile as parent compound ($A_{\text{bile}}(t)$) or as metabolite ($A_{\text{bile,met}}(t)$) were measured over time and were fitted simultaneously into a compartment model (Fig. 4b) modified from previous one (Weiss et al., 2013). The uptake clearance ($CL_{\text{uptake}}$) controls transport of fluorescein from sinusoid into hepatocyte, while fluorescein elimination from hepatocyte is governed by its metabolism to fluorescein monoglucuronide ($k_{\text{met}}$) and biliary excretion of fluorescein ($k_{\text{bile,F}}$) and fluorescein monoglucuronide ($k_{\text{bile,FG}}$). The time course of fluorescein amount in sinusoids was described by a biexponential function with parameters of $A$, $B$, $\alpha$, and $\beta$ and used as a fixed forcing function. The differential equations for the model are as follows:
\[
\frac{dA_{\text{sin}}(t)}{dt} = Ae^{-\alpha t} + Be^{-\beta t} - \frac{CL_{\text{uptake}}}{V_{\text{sin}}} A_{\text{sin}}
\] (1)

\[
\frac{dA_{\text{hep}}(t)}{dt} = \frac{CL_{\text{uptake}}}{V_{\text{sin}}} A_{\text{sin}} - (k_{\text{met}} + k_{\text{bile,F}})A_{\text{hep}}
\] (2)

\[
\frac{dA_{\text{hep,met}}(t)}{dt} = k_{\text{met}}A_{\text{hep}} - k_{\text{bile,FG}}A_{\text{hep,met}}
\] (3)

\[
\frac{dA_{\text{bile}}(t)}{dt} = k_{\text{bile,F}}A_{\text{hep}}
\] (4)

\[
\frac{dA_{\text{bile,met}}(t)}{dt} = k_{\text{bile,FG}}A_{\text{hep,met}}
\] (5)

With \(C_{\text{sin}}(t) = A_{\text{sin}}(t)/V_{\text{sin}}\) and \(C_{\text{hep}}(t) = A_{\text{hep}}(t)/V_{\text{hep}}\)

**Data analysis**

A population approach was used to estimate the various rate constants, the uptake clearance and other parameters used to describe the model \((A, \alpha, B, \beta, CL_{\text{uptake}}, k_{\text{bile,F}}, k_{\text{met}}, k_{\text{bile,FG}}\) and \(V_{\text{hep}}\)) (Fig. 4b) using the package ADAPT 5 (D'Argenio et al., 2009). The maximum likelihood expectation maximization program available in the package provides estimates of the population mean and inter-subject variability as well as of the individual subject parameters (conditional means). Model parameters were assumed to be log-normally distributed. The standard deviation of measurement error was assumed to be a linear function of the measured quantity. All data sets including \(C_{\text{sin}}(t), C_{\text{hep}}(t), A_{\text{bile}}(t)\) and \(A_{\text{bile,met}}(t)\) were fitted simultaneously to estimate all parameters.

**Statistical analysis**
An unpaired, nonparametric t-test (Mann-Whitney) was used to compare the population mean of each parameter to evaluate the steatosis and I/R injury effect. Results were considered significant with a p-value $\leq 0.05$.

Results

ALT and morphological changes

Serum ALT levels were significantly elevated in normal (1330.8±280.2 U/L) and steatotic (556±81.3 U/L) rats subjected to I/R injury compared to sham (54.3±7.1 U/L) as shown in Table 1.

Apoptotic cells were counted in each of three different regions of the liver acinus (periportal, centrilobular and midzonal) (Fig. 2B, Table 2). Black arrows indicate positively stained cells. The number of positively stained cells was significantly increased in the centrilobular and midzonal regions in normal (89.5±19.5) and steatotic (84.5±21.5) livers exposed to I/R injury (p≤0.05) compared to sham (5.2±1.7) and steatosis (5.3±2.1). In addition, hollow black arrows shows lipid droplets, characteristic of steatotic livers. These lipid droplets were also seen in the MPM images (white arrows, Fig. 2B). Figure 2b shows a representative imaged liver for each group, at 10× and 40× magnification, respectively. While sham livers have well defined liver acinus and hepatocytes, the structure becomes less defined when exposed to I/R injury. In steatotic livers, we begin to see lipid droplets (white arrows), which are better seen in the higher magnification images.

Fluorescein disposition in normal and steatotic livers – In vivo multiphoton imaging

Images of fluorescein disposition were continuously recorded in vivo after bolus injection of fluorescein to monitor uptake and excretion of the dye (Fig. 3). The distribution of fluorescein at 2 min is irregular in the steatosis group compared to sham. At 40 min the
fluorescence intensity is higher in steatotic livers with or without I/R injury and at 180 min the distribution of fluorescein is higher in the diseased groups compared to sham.

A physiological pharmacokinetic model of fluorescein disposition in normal and steatotic livers after ischemia-reperfusion injury

The various substructures in the liver were represented as sub-compartments as seen in Figure 4A. The plasma concentrations of fluorescein did not differ significantly between the 4 groups (Fig. 4B). The concentrations of fluorescein in the sinusoidal and hepatocyte subcellular compartments are shown in Figure 4C. The fitting curve shows the goodness of the fit. The corresponding area under the curve (AUC) in sinusoids and hepatocytes showed no significant change (Fig. 4D). However, the rate constant for metabolism in the liver cytosol, $k_{met}$, was significantly increased in steatotic livers with and without I/R injury compared to sham (*) and compared to I/R injury (%) in the steatosis+I/R group (Table 3). Additionally, the average fluorescence lifetime ($\tau_m$) was significantly decreased in steatotic livers at 1 min and 40 min after injection of fluorescein compared to sham (*) and in steatotic livers exposed to I/R injury at 20 min after injection compared to sham (*) and I/R injury (%) (Fig. 5A). This decrease is also seen in the color coded images in Figure 5B, where the decrease in lifetime is indicative of an increase in blue color (red arrow).

Fluorescein biliary excretion in normal and steatotic livers after ischemic reperfusion injury

The biliary excretion rate of fluorescein was significantly decreased in steatotic livers at 40 min after injection and in steatotic livers exposed to I/R injury at 30, 40, 50 and 60 min after injection compared to sham (*) (Figure 6A). The total recovery of fluorescein in bile was also significantly lowered in steatotic livers at 60 and 120 min after injection and in steatotic livers exposed to I/R injury at 40, 50, 60 and 120 min after injection compared to sham (*) (Figure 6B). However, the biliary excretion rate and cumulative amount recovered in bile for
the fluorescein monoglucuronide metabolite were not significantly different between sham and the other groups (Figure 6A and B). The average bile flow rate up to 180 min post-injection rate was significantly decreased (p<0.05; n=3-6) in steatotic livers with I/R injury (0.008±0.001 ml/min) compared to sham (0.02±0.004 ml/min), steatosis (0.02±0.001) and I/R of normal livers (0.02±0.004 ml/min) (Figure 6C).

**Discussion**

A number of studies have reported the non-invasive sub-surface imaging of the liver after laparotomy both in animals and in human patients. One of the earliest animal studies used fluorescence microscopy with fluorescein as an indicator (Hanzon, 1952). In this work, we have gone a step further in evaluating the changes in the subcellular function of the liver in steatosis with I/R injury using multiphoton subsurface *in vivo* imaging in combination with physiological pharmacokinetic modelling. The animal model of steatosis with I/R injury was developed on the advice of our liver surgeons, who were seeking to better understand the impact of steatosis and I/R injury on their human liver transplantation procedures. Prior to any human studies, we needed to define the likely outcomes in an animal model. The ischemic damage associated with this model was similar to that previously reported (Thorling et al., 2011b; Thorling et al., 2013) and was characterized with significantly elevated serum ALT, increased number of DNA damaged hepatocytes and lower bile flow. In this study we found that the liver acinus and hepatocytes are less defined when exposed to I/R injury in both normal and steatotic livers. This could be an effect of mitochondrial depolarisation, leading to depleted ATP, which could result in apoptosis and necrosis. This is confirmed by a significantly increased number of apoptotic cells in normal and steatotic livers exposed to I/R injury compared to sham, however not in the group of rats having steatosis without I/R injury as shown in Table 2.
The salient new observation in this work is the irregular distribution of fluorescein in the in vivo images of steatosis 2 minutes after injection, relative to normal livers (Fig. 3). I/R injury in steatotic livers appears to dampen this irregularity but is associated with a loss of the distinctive sinusoidal structures and the appearance of lipid droplets. The lipid droplets are still visible at 40 min and 180 min post-injection in steatotic livers exposed to I/R injury. These results are consistent with traditional morphology, as seen in Fig. 2A, and are in concordance with another study showing lipid droplets in steatotic livers (Hui et al., 1994). Images showed a higher fluorescence intensity in the liver at 180 min after fluorescein injection in all diseased groups compared to sham (Fig. 3). These results indicate an impaired excretion of fluorescein or change in metabolism. Our previous in vivo MPM studies also showed that the excretion of fluorescein was delayed in liver with I/R injury (Thorling et al., 2013). In order to better define the changes in liver function, the sinusoidal and hepatocyte concentrations of fluorescein acquired from the images and the amount of drug excreted in the bile were fitted by a physiologically based pharmacokinetic model (Fig 4B) to obtain kinetic parameters characterizing hepatic transporter and metabolism function. Intravital MPM imaging allows the direct observation of fluorescent compound concentrations in both space and time simultaneously under in vivo conditions (Liu et al., 2012). The model allows the fitting of naive data from all rats simultaneously (Weiss et al., 2013). Our results show a significantly increased rate constant of fluorescein metabolism ($k_{met}$) in steatotic livers with and without I/R injury compared to sham and also in the steatosis+I/R injury group compared to I/R injury of normal livers, which indicates an increased metabolism of fluorescein within the hepatocyte (Weiss et al., 2013). Since fluorescein is mainly metabolized in the hepatocyte to fluorescein monoglucuronide through glucuronidation, conjugating to a single glucuronic acid molecule (Chahal et al., 1985), it is likely that the increased metabolism (increased $k_{met}$) reflect an increased glucuronidation of fluorescein in the diseased liver. This
result was consistent with the previous report of the increased glucuronidation in obese humans (Hanley et al., 2010) and in hepatic I/R injury (Parasrampuria et al., 2012). Moreover, the increased metabolism of fluorescein in diseased rat liver found in this study coincides with a significant increase in intrinsic metabolic clearance ($CL_{int}$) of other compounds found in a recent study using another model of steatosis, namely nonalcoholic steatohepatitis (NASH) (Li et al., 2011). $CL_{int}$ represents the metabolic activity of the hepatocytes (Barbare et al., 1985), similar to $k_{met}$. To further confirm these results we tested our previously published model of measuring average fluorescence lifetime to determine whether metabolism of fluorescein to fluorescein monoglucuronide was increased (Thorling et al., 2011). Fluorescein and fluorescein monoglucuronide have two distinct lifetimes, however our analysis yielded one single average lifetime ($\tau_m$), which is a combination of the lifetime of fluorescein and fluorescein monoglucuronide. Since the lifetime of fluorescein monoglucuronide is significantly lower than that of fluorescein in vitro, 2.3 ns and 3.8-4.1 ns, respectively, the average lifetime ($\tau_m$) decreases when more fluorescein monoglucuronide is formed in the liver. Our results show a significantly decreased average lifetime in the liver in steatosis with and without I/R injury, which confirms the pharmacokinetic model of increased $k_{met}$ presented here. In addition, we report a decreased rate of excretion of fluorescein in this work. Rate of excretion ($R$) is dependent on clearance into the bile ($CL_{bile}$) and concentration of drug ($C$) in the hepatocyte ($R=CL_{bile}\times C_{hep}$) (Yamazaki et al., 1997). Since the metabolism is increased (increased $k_{met}$), the concentration of fluorescein in the hepatocytes is decreased, hence a decrease of the excretion rate is expected.

*In vivo* imaging has the potential to replace or guide for biopsies, however it is restricted to the surface or internal organs after invasive surgery. Therefore microendoscopy is a better choice, combining intravital microscopy and miniature endoscopy (Kim et al., 2008), sometimes in combination with a rodlike gradient-index (GRIN) lens.
In conclusion, our work shows a novel method of quantitatively assessing liver subcellular function in liver steatosis by non-invasive \textit{in vivo} multiphoton imaging, supplemented by a physiological pharmacokinetic model to interpret the liver functional changes. The model enabled the precise estimation of changes in uptake, metabolism and excretion resulting from liver steatosis alone and with I/R injury and can offer more quantitative measures of liver function. By administering a fluorescent probe in combination with microendoscopy or GRIN lenses opens up the door for more in depth information regarding liver functionality and has the potential to replace the traditional static liver biopsy methodology now currently used.
Acknowledgements

We thank Mr Clay Winterford and colleagues at Queensland Institute of Medical Research (QIMR) for their histology and immunohistochemistry preparation and Mr Goce Dimeski and colleagues at Queensland Pathology Services, Princess Alexandra Hospital, for conducting ALT measurements.

Authorship Contributions:

Participated in research design: Thorling, Roberts, Crawford, Burczynski, Liu X, Weiss


Contributed new reagents or analytic tools: Thorling, Liu X, Jin

Performed data analysis: Thorling, Jin, Liu D, Weiss

Wrote and contributed to the writing of the manuscript: Thorling, Jin, Liu X, Roberts, Crawford, Burczynski, Weiss, Wang, Liu D
References


DMD # 60848

Footnotes:

Camilla A Thorling and Lu Jin contribute equally to the manuscript

This work was supported by National Health and Medical Research Council [Grants 569710, APP1002611].

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Legends for Figures

**Fig. 1.** Flow chart of the different steps and time points of the experiment.

**Fig. 2.** Morphological changes associated with I/R injury in steatosis. (A) Positively stained apoptotic cells, in the periportal, centrilobular and midzonal regions of the liver acinus. Black arrows indicate positively stained cells as measured by ApopTag. Black hollow arrows indicate lipid droplets. (B) MPM images of the liver at low (10×) and high (40×) magnification before injection of fluorescein. White arrow points to a lipid droplet inside the hepatocyte.

**Fig. 3.** MPM images showing fluorescein distribution in sham and steatotic livers with and without I/R injury, recorded at 920 nm at various time points using low (10×) magnifications. Scale bar length is 200 µm.

**Fig. 4.** Physiologically-based pharmacokinetic model. (A) Schematic overview of compartmental model describing hepatic uptake and elimination kinetics of fluorescein. (B) Plasma concentration-time profiles of fluorescein in normal (sham) rats and rats with steatotic livers with and without I/R injury. The symbols represent mean values and the error bar represents SEM (n=3-4). (C) Fluorescein concentration-time profiles in the sinusoids and hepatocytes compartments. (D) Corresponding accumulation of fluorescein in the sinusoids and hepatocytes compartments.

**Fig. 5.** Average fluorescence lifetime in the liver. (A) Average fluorescence lifetime ($\tau_m$) obtained from *in vivo* fluorescence lifetime imaging microscopy of the liver over time after injection of fluorescein intravenously (mean ± SEM, p<0.05, n=3). (B) Color coded images showing change in average fluorescence lifetime represented in color. Red arrows show an increased blue color indicative of decreased average fluorescence lifetime.
**Fig. 6.** Bile data of fluorescein and its metabolite, fluorescein monoglucuronide. (A) Biliary excretion rate of fluorescein and fluorescein monoglucuronide. (B) Cumulative amount of fluorescein and monoglucuronide recovered in bile. (C) Average bile flow rate in normal and steatotic livers with and without I/R injury (mean ± SEM, p<0.05, n=3-6).
Tables

Table 1. Alanine transaminase (ALT) levels in plasma and inflammatory cell infiltration (monocytes/macrophages) in the liver in normal and steatotic livers with and without I/R injury (mean ± SEM, n=5-6).

<table>
<thead>
<tr>
<th></th>
<th>ALT (U/L)</th>
<th>ED1 (monocytes/macrophages)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>54.3±7.1</td>
<td>310±68</td>
</tr>
<tr>
<td>I/R</td>
<td>1330.8±280.2*</td>
<td>240±54</td>
</tr>
<tr>
<td>Steatosis</td>
<td>40.3±4.8</td>
<td>320±30</td>
</tr>
<tr>
<td>Steatosis+I/R</td>
<td>556±81.3*</td>
<td>260±10</td>
</tr>
</tbody>
</table>

* p<0.05 vs sham
Table 2. DNA damaged cells measured by ApopTag in the periportal, centrilobular and midzonal regions of the liver acinus (mean ± SEM, n=5-6).

<table>
<thead>
<tr>
<th></th>
<th>Periportal</th>
<th>Centrilobular</th>
<th>Midzonal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>7.4±2.5</td>
<td>2.0±0.6</td>
<td>5.2±1.7</td>
</tr>
<tr>
<td>I/R</td>
<td>17.8±4.9</td>
<td>43.2±12.8*%</td>
<td>89.5±19.5*%</td>
</tr>
<tr>
<td>Steatosis</td>
<td>8.6±4.2</td>
<td>2.0±0.5</td>
<td>5.3±2.1</td>
</tr>
<tr>
<td>Steatosis+I/R</td>
<td>7.9±3.2</td>
<td>25.5±6.7*%</td>
<td>84.5±21.5*%</td>
</tr>
</tbody>
</table>

* p<0.05 vs sham; % p<0.05 vs steatosis
Table 3. Pharmacokinetic parameters of fluorescein derived from the compartmental in normal and steatotic livers with and without ischemia reperfusion (I/R) injury (mean ± SEM, n=3-6).

<table>
<thead>
<tr>
<th></th>
<th>$CL_{uptake}$ (ml/min)</th>
<th>$k_{met}$ (min$^{-1}$)</th>
<th>$k_{bile, FG}$ (min$^{-1}$)</th>
<th>$k_{bile, F}$ (min$^{-1}$)</th>
<th>$CL_{bile}$ (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>4.9±2.4</td>
<td>0.07±0.06</td>
<td>0.001±0.002</td>
<td>0.08±0.08</td>
<td>1.1±1.0</td>
</tr>
<tr>
<td>I/R</td>
<td>8.4±3.8</td>
<td>0.1±0.1</td>
<td>0.004±0.003</td>
<td>0.08±0.06</td>
<td>2.3±1.1</td>
</tr>
<tr>
<td>Steatosis</td>
<td>8.5±6.2</td>
<td>0.6±0.2*</td>
<td>0.005±0.004</td>
<td>0.3±0.08</td>
<td>0.9±0.6</td>
</tr>
<tr>
<td>Steatosis+I/R</td>
<td>6.1±4.6</td>
<td>1.6±0.8**$^S$</td>
<td>0.0003±0.0004</td>
<td>0.9±0.6</td>
<td>1.0±0.8</td>
</tr>
</tbody>
</table>

$CL_{uptake}$ is the uptake clearance, $k_{met}$ is the rate constants of metabolism, $k_{bile}$ is the rate constant of biliary excretion, and $CL_{bile}$ is biliary clearance.

* $p<0.05$ vs sham, ** $p<0.01$ vs sham $^S$ $p<0.01$ vs I/R
Induce steatosis (high fat diet) or sham (normal diet) operation.

Induce ischemia or sham operation.

Surgery (jugular vein and bile duct cannulation, blood collection for ALT measurements).

Fluorescein injection (10 mg/kg).

Imaging and bile collection.

Liver collected for histology.

ALT measurement, liver histology, HPLC of bile samples, data analysis, PK modelling.

Figure 1
Figure 2