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Effects of Pringle maneuver and partial hepatectomy on the pharmacokinetics and blood-brain barrier permeability of sodium fluorescein in rats

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Abbreviations: AST, aspartate aminotransferase; AUC, area under the plasma concentration-time curve, BBB, blood-brain barrier; C_{br}^{30}, brain concentration at 30 min; $Cl_{bile,app}$, apparent biliary clearance; FL, Na fluorescein; FL-Glu, fluorescein glucuronide; f_u, unbound fraction in plasma; Hx, partial hepatectomy; HxIR, ischemia-reperfusion plus partial hepatectomy; IR, ischemia-reperfusion; K_{in}, apparent brain uptake clearance; Mrp2, multidrug resistance-associated protein 2; P-gp, P-glycoprotein; TBA, total bile acids.

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ABSTRACT

Liver diseases are known to affect the function of remote organs. The aim of the present study was to investigate the effects of Pringle maneuver, which results in hepatic ischemia-reperfusion (IR) injury, and partial hepatectomy (Hx) on the pharmacokinetics and brain distribution of sodium fluorescein (FL), which is a widely used marker of blood-brain barrier (BBB) permeability. Rats were subjected to Pringle maneuver (total hepatic ischemia) for 20 min with (HxIR) or without (IR) 70% hepatectomy. Sham-operated animals underwent laparotomy only. After 15 minutes or 8 h of reperfusion, a single 25-mg/kg dose of FL was injected intravenously and serial (0-30 min) blood and bile and terminal brain samples were collected. Total and free (ultrafiltration) plasma, total brain homogenate, and bile concentrations of FL and/or its glucuronidated metabolite (FL-Glu) were determined by HPLC. Both IR and HxIR caused significant reductions in the biliary excretions of FL and FL-Glu, resulting in significant increases in the plasma AUC of the marker. Additionally, the free fraction of FL in plasma was significantly increased by HxIR. Although the brain concentrations of FL were increased by almost twofold in both IR and HxIR animals, the brain concentrations corrected by the free FL AUC (and not the total AUC) were similar in both groups at either time points. It is concluded that Pringle maneuver and/or partial hepatectomy substantially alters the hepatobiliary disposition, plasma AUC, plasma free fraction, and brain accumulation of FL without altering the BBB permeability to the marker.

Keywords: Sodium fluorescein, Pharmacokinetics**,** Blood-brain barrier permeability, Hepatic ischemia-reperfusion injury, Pringle maneuver, Partial hepatectomy

1. Introduction

Partial hepatectomy (Hx) or liver resection is usually used for living donor liver transplantation and treatment of various liver diseases such as benign and malignant tumors. Due to the unique anatomical structure and function of the liver, hepatic surgery, including liver resection, is associated with a significant degree of risk (Huntington et al., 2014; Savage and Malt, 1991), including excessive bleeding. Pringle maneuver, developed by J. H. Pringle in 1908, is one of the major strategies to reduce excessive bleeding by completely occluding blood supply to the liver (Huntington et al., 2014; Yang et al., 2014). However, the resultant ischemia and subsequent reperfusion produces a series of events, leading to the so-called ischemia-reperfusion (IR) injury. One of the hallmarks of the liver IR injury is activation of Kupffer cells mediated by the release of gastrointestinal lipopolysaccharides (LPS), which leads to the synthesis and release of reactive oxygen species and pro-inflammatory mediators, such as tumor necrosis factor-α, interleukin-1β, and interleukin-6 (Jaeschke and Farhood, 1991; Liu et al., 1995; Wanner et al., 1996). Additionally, these pro-inflammatory mediators alter the expression and function of different drug transporters in the liver (Ikemura et al., 2009; Miah et al., 2014; Parasrampuria et al., 2012; Tanaka et al., 2006), as well as in the remote organs such as intestine (Ikemura et al., 2009) and kidneys (Tanaka et al., 2008). Therefore, Hx and/or Pringle maneuver could potentially alter the disposition of xenobiotics and endogenous compounds in patients who are subjected to these procedures.

Very recently (Miah et al., 2014), we demonstrated that the hepatic IR injury alters the expression and function of P-glycoprotein (P-gp) in the liver canalicular membranes as well as in the brain. Additionally, liver diseases are associated with increased plasma bile acids and ammonia (Tanaka et al., 2006; Wright et al., 2014), both of which have shown to increase the

BBB permeability (Quinn et al., 2014; Skowronska et al., 2012). A potential increase in the BBB permeability could lead to increased passage of neurotoxins into the brain, which may be responsible for different neurological complications that are associated with hepatic diseases (Leke et al., 2013). However, the effects of hepatic IR and/or partial hepatectomy on the integrity of the BBB and its paracellular permeability to xenobiotics and endogenous compounds have not yet been studied.

Sodium fluorescein (FL) is a hydrophilic, low molecular weight (376 Da) dye, which cannot cross BBB transcellularly. Therefore, it can enter the brain through paracellular route, which is very limited in normal brain due to the BBB tight junctions. Because of these characteristics, FL has been used extensively as an *in vivo* marker to determine the changes in the paracellular permeability of BBB in different diseases (Kaya and Ahishali, 2011). In most experiments, investigators use the absolute brain concentrations of FL as a measure of BBB permeability (Cao et al., 2015; Nishioku et al., 2010; Oppenheim et al., 2013; Tress et al., 2014). However, this approach may result in inaccuracies if the systemic exposure of the marker, such as area under the plasma concentration-time curve (AUC), is also altered by the disease or the intervention. Furthermore, previous studies have shown that a significant fraction of FL circulating in the plasma is bound to plasma proteins (Li and Rockey, 1982). Because only the free drug is expected to cross the membranes, including the BBB, a change in the free fraction of FL in plasma may also affect the degree of accumulation of the marker in the brain (Mandula et al., 2006). Therefore, the use of brain concentrations of FL alone, or even after its correction for the total (free plus bound) plasma concentrations of FL, may be a misleading indication of the BBB permeability when the plasma exposure and/or free fraction of the marker in plasma is altered.

Our main goal for the current study was to investigate the effects of hepatic IR (Pringle maneuver) alone or in combination with Hx (HxIR) on the paracellular blood-brain barrier (BBB) permeability using FL as a marker. Our hypothesis was that hepatic IR with or without Hx would increase the BBB permeability to FL. Additionally, because FL is both metabolized by the liver and excreted into the bile (Webb et al., 1962), we were interested in investigating the effects of IR and/or HxIR on the hepatobiliary disposition and systemic pharmacokinetics of the marker. Our studies show whereas Hx and HxIR significantly alter the hepatobiliary disposition, plasma free fraction, and absolute brain concentrations of FL, BBB permeability to FL corrected based on the systemic pharmacokinetics of the marker remains unchanged by these surgical interventions.

2. Results

2.1. Plasma concentrations of biochemical markers

The plasma concentrations of aspartate aminotransferase (AST), total bile acids (TBA), and albumin in both the 15-Min and 8-h groups are presented in Fig. 1. The plasma concentrations of the liver injury marker AST in the HxIR groups were significantly higher than those in their corresponding Sham groups in both 15-Min ($p < 0.05$) and 8-h ($p < 0.001$) reperfusion groups (Fig. 1a and 1b). However, the AST concentrations in the IR groups were significantly ($p < 0.001$) higher from those in the Sham group only in the 15-Min group (Fig. 1a and 1b). Although the TBA concentrations in the 15-Min IR and HxIR groups were higher than those in the corresponding Sham groups, only the difference between the IR and Sham groups were statistically significant ($p < 0.01$) at this time point (Fig. 1c). On the other hand, at 8 h post reperfusion, the TBA concentrations in the HxIR group, and not in the IR group, were significantly ($p < 0.0001$) higher than those in the corresponding Sham group (Fig. 1d). As for

the plasma albumin concentrations, there were no significant differences among the surgical groups at either reperfusion time points (Figs. 1e and 1f).

2.2. Plasma pharmacokinetics of FL

The plasma concentration-time courses of FL in different surgical groups after 15 min or 8 h of reperfusion are depicted in Fig. 2, and the corresponding AUC and free fraction in plasma (f_u) values are presented in Table 1. The plasma concentrations in the IR and HxIR groups were generally higher than those in the Sham groups (Fig. 2). However, only the AUC values for the 15-Min HxIR ($p < 0.05$) and 8-h IR ($p < 0.01$) groups were significantly higher than those of their corresponding Sham groups (Table 1). Additionally, the f_u values of FL in the HxIR groups were higher ($p < 0.01$) than those in their respective Sham groups for both the 15-Min and 8-h groups (Table 1).

2.3. Biliary excretion of FL and FL-Glu

The bile data for FL and its glucuronidated metabolite, FL-Glu, are presented in Figs. 3 and 4 for the 15-Min and 8-h reperfusion groups, respectively. For the 15-Min reperfusion groups (Fig. 3), IR and HxIR, respectively, caused 74% and 89% reductions ($p < 0.0001$) in the biliary recovery of FL (Fig. 3a), which was also reflected in its $Cl_{bile,app}$ (Fig. 3b). Similar patterns were also noted for the biliary recovery of FL-Glu (Fig. 3c) and the total biliary recoveries of FL plus FL-Glu (Fig. 3d). Additionally, the bile flow rates in the IR and HxIR groups were, respectively, 61% and 73% lower ($p < 0.0001$) than those in the Sham group (Fig. 3e). In fact, there were significant correlations ($p < 0.0001$) between the biliary recovery of FL or FL-Glu and the bile flow rate with r^2 values of 0.929 or 0.938, respectively (Fig. 3f).

After 8 h of reperfusion (Fig. 4), the bile flow rates and biliary recoveries of FL and/or FL-Glu for the IR group returned to values not significantly different from those in the Sham

animals. However, the corresponding values for the HxIR animals remained significantly lower than those in the Sham animals even after 8 h of reperfusion (Fig. 4). Similar to the 15-Min reperfusion groups, the correlation between the biliary recoveries of FL or FL-Glu and bile flow rate remained significant ($p < 0.0001$) for the 8-h groups (Fig. 4f).

2.4. Western blot analysis of liver transporters

Figure 5 depicts the representative Western blots (a and b) and individual densitometric data for Mrp2 (c and d) and Oatp1a4 (e and f) levels in the liver membrane fractions. The protein levels of Mrp2 in the IR and HxIR groups after 15 min of reperfusion were significantly higher than those in their corresponding Sham livers (Fig. 5c). However, there were no differences among the surgical groups for Mrp2 levels in the 8-h reperfusion groups (Fig. 5d). Additionally, the protein levels of Oatp1a4 were similar in all the surgical groups after 15 min (Fig. 5e) or 8 h (Fig. 5f) of reperfusion.

2.5. Liver concentrations of ATP

The concentrations (μmol/g tissue) of ATP in the right lobes of the livers in the IR group after 15 min of reperfusion (0.723 ± 0.414) were 65% lower ($p < 0.01$, unpaired t-test) than those in the 8 h IR group (2.08 ± 0.447).

2.6. Brain uptake of FL

The brain concentrations and apparent uptake clearance (K_{in}) values of FL, based on both the total (free plus bound) and free (unbound) concentrations of FL in plasma, are presented in Fig. 6. The brain concentrations of FL in the IR and HxIR were between 1.8 to 2.0 fold higher than those in their corresponding Sham animals after both 15 min (Fig. 6a) and 8 h (Fig. 6b) of reperfusion. The K_{in} values based on the total AUC values also showed similar patterns (Fig 7c

and d), although the surgically-induced increases in the total K_{in} values (between 1.35 and 1.50 fold, relative to the Sham groups) were less than the increases in the brain concentrations (1.8 to 2.0 fold). In contrast to the brain concentrations and total K_{in} values, the free K_{in} values, estimated based on the free FL AUC, were not significantly different from their corresponding Sham values for any of the surgical groups at either reperfusion times (Fig. 6e and f).

3. Discussion

Recent studies (Hashimoto and Sanjo, 1997; Ikemura et al., 2009; Parasrampuria et al., 2012; Shaik and Mehvar, 2011; Thorling et al., 2014; Tian et al., 2005a; Tian et al., 2005b) have shown that IR injury or Hx alters the disposition of drugs. In agreement with these studies, our current investigation showed that IR alone or in combination with Hx (HxIR) would change the systemic pharmacokinetics of FL (Table 1 and Figs. 2-4). Additionally, brain accumulation of FL, which is a marker of *in vivo* BBB permeability, was increased by almost twofold in the IR and HxIR rats (Fig. 6). Both hepatic IR and HxIR altered the systemic pharmacokinetics of FL by substantially reducing the biliary excretion of the marker and its glucuronidated metabolite (FL-Glu) in a reperfusion time-dependent manner (Figs. 3 and 4). Additionally, IR and/or HxIR increased the plasma concentrations, AUC, and f_u of FL (Fig. 2 and Table 1). Considering the brain concentrations of FL alone as the measure of BBB permeability, without regard for the changes in its systemic pharmacokinetics, one would conclude that IR and HxIR would increase the BBB permeability after both 15 min (Fig. 6a) and 8 h (Fig. 6b) of reperfusion. However, correcting the brain concentrations for the free FL AUC suggests that neither IR nor HxIR would alter the BBB permeability (Figs. 6e and 6f). It should be noted that correcting for the total (bound plus free) AUC of FL, although reducing the magnitude of the effect, still would suggest higher BBB permeability for the IR and HxIR animals (Figs. 6c and 6d). Therefore, in our

studies, different conclusions are reached whether the systemic pharmacokinetics of FL, including its degree of serum protein binding, are considered or not in the estimation of BBB permeability using FL.

It has been reported (Pardridge and Landaw, 1984; Tanaka and Mizojiri, 1999) that for some drugs the free fraction of the drug in the blood could not adequately explain their *in vivo* brain uptake. Therefore, it was suggested that an interaction between the plasma proteins and the brain capillary membranes would result in conformational changes in the proteins, resulting in enhanced dissociation of some drugs from the proteins and increased brain uptake. However, it was later suggested (Mandula et al., 2006) that the reported apparent enhanced dissociation effect might have been related to the mixing of the bolus dose solutions, containing different concentrations of proteins, with the circulating blood passing through the brain vasculature. Nevertheless, the BBB permeability of FL is very low, compared with the cerebral blood flow, suggesting that the K_{in} value of FL is directly proportional to its f_u and free permeability-surface area product. Collectively, our data suggest that when FL is used as an *in vivo* BBB permeability marker, one has to consider the potential changes in both the systemic exposure (AUC) and degree of protein binding of the marker in plasma, in addition to its brain accumulation.

The increases in the f_u of FL, observed in the HxIR rats (Table 1), potentially could be due to a decrease in the concentrations of serum albumin and/or a decrease in the binding affinity of the marker to albumin. Because the plasma albumin concentrations were not altered in any of the surgical groups (Figs. 1e and 1f), HxIR most likely resulted in a decrease in the affinity of FL binding to albumin. Fluorescein is a weakly acidic drug, which is bound mainly to albumin in the plasma (Manzini and Crescenzi, 1979). Our recent *in vitro* studies (Shaik et al., 2013) showed that the f_u of FL increases with an increase in the concentrations of bile acids, such as lithocholic

acid, cholic acid, chenodeoxycholic acid, and taurocholic acid, which are also bound to albumin (Roda et al., 1982). Therefore, the increases in the concentrations of plasma TBA in the IR and HxIR rats (Figs. 1c and 1d) most likely have contributed to the higher f_u values of FL in these animals by competitive reduction of the binding of FL to albumin. However, our data would not preclude the contribution of other mediators, such as bilirubin, which also shows elevated plasma concentrations after IR injury and partial hepatectomy (de Graaf et al., 2012) and binds with a high affinity to plasma albumin (Sjoholm et al., 1979).

The other major effect of IR or HxIR on the systemic pharmacokinetics of FL was a significant reduction in the biliary excretion of the marker and its glucuronidated metabolite (Figs. 3 and 4). Whereas HxIR caused significant reductions in the biliary excretions of FL and FL-Glu at both reperfusion times of 15 min (Fig. 3) and 8 h (Fig. 4), the reductions in the IR groups were present only after 15 min of reperfusion (Fig. 3) as the corresponding values in the 8 h IR group were similar to those in the Sham animals (Fig. 4). The persistent reductions in the biliary excretion of FL and FL-Glu in the HxIR groups at both 15 min and 8 h of reperfusion (Figs. 3 and 4) are most likely due to reductions in the mass of the liver after Hx. However, the reductions in the biliary excretion of FL and FL-Glu, observed only in the 15 min IR groups (Figs. 3 and 4) should be related to temporal changes in the function of the liver as a result of IR. Indeed, the return of the biliary excretion of FL (Fig. 4a) and FL-Glu (Fig. 4c) to normal values at 8 h after the reperfusion in the IR group is in agreement with the return of the plasma TBA (Fig. 1d) and the liver injury marker AST to normal values in this group (Fig. 1).

To further explore the potential mechanisms of time-dependent changes in the biliary excretions of FL and FL-Glu in the IR animals, we investigated the effects of surgical procedures on the liver transporters responsible for the uptake and biliary excretion of FL and FL-Glu. In the

liver, FL reportedly enters the hepatocytes via human OATP1B (De Bruyn et al., 2011), which is similar to rat Oatp1a4. Once inside the hepatocyte, FL is metabolized to FL-Glu, and both the marker and its glucuronidated metabolite are excreted into the bile through the Mrp2 transporter (Mills et al., 1999; Thorling et al., 2013). The observed lack of change in the liver membrane Oatp1a4 protein as a result of IR or HxIR (Figs. 5e and 5f) suggests that the uptake of FL into the liver is not compromised in these surgical groups. However, the Mrp2 protein levels in the liver membranes were significantly increased in both surgical groups after 15 min of reperfusion (Figs. 5c and 5d). The increased Mrp2 levels at 15 min reperfusion time are in an opposite direction to the reductions in the biliary excretion of FL and FL-Glu in these animals (Figs. 3a-3d). However, the function of ATP-binding cassette transporters, such as Mrp2, is rate-limited by the availability of tissue ATP, which is substantially compromised by the IR injury (Bedirli et al., 2008). Therefore, the decreases in the biliary excretion of the marker and its metabolite in the 15 min IR animals (Figs. 3a and 3c), which return to normal values after 8 h of reperfusion (Figs. 4a and 4c), could be due to lower hepatic concentrations of ATP early after reperfusion, followed by its recovery at the 8 h reperfusion time. To test this postulate, we conducted an additional study using two IR groups at 15 min and 8 h of reperfusion (n = 4/group) to specifically collect and store the liver tissues for ATP measurements, which require strict handling and storage. Indeed, the ATP levels in the 15 min IR group were 65% lower than those with the 8 h of reperfusion, indicating that the low biliary excretions of FL and FL-Glu in the 15 min IR groups, which was not observed in the 8 h IR animals, are most likely due to lower ATP levels in these animals.

The IR-induced lower biliary excretion of FL and FL-Glu, despite an overexpression of the responsible transporter (Mrp2), is in agreement with our previous study (Miah et al., 2014)

using rhodamine 123, which is excreted into the bile by P-glycoprotein (P-gp). Whereas P-gp levels were overexpressed 24 h after 90 min of partial ischemia, the biliary excretion of rhodamine 123 was significantly reduced because of a significant reduction in the liver ATP concentrations. Although the ischemia model used in our current study (total ischemia for 20 min) is different from the reported model (90 min of partial ischemia), our data confirms the importance of the liver ATP concentrations in the biliary excretion of drugs by the ATP-binding cassette transporters.

Previous studies by us and others using partial (70%) hepatic IR model with 60-90 min of ischemia have shown a significant reduction in the mRNA (Tanaka et al., 2006) and protein (Miah et al., 2014; Tanaka et al., 2008) levels of Mrp2 at 24 h of reperfusion, without any significant changes at 3 or 6 h of reperfusion. The increased protein level of Mrp2 in our studies at much earlier reperfusion time (15 min) does not contradict these data. This is because the effects of IR on the protein levels of Mrp2 are most likely time dependent, and a later (24 h reperfusion) downregulation does not preclude a much earlier (15 min reperfusion) increased levels of Mrp2. Additionally, the effects of 60-90 min of partial (70%) ischemia on the expression of Mrp2, reported in the literature, might be different from those in a 20-min Pringle maneuver (complete ischemia), used in our current study. Nevertheless, the increased protein levels of Mrp2 in the liver membranes observed early after reperfusion (15 min) in our studies might be due to changes in the trafficking of the transporter between the canalicular membranes and cytoplasm, as reported before (Ban et al., 2009), rather than an increased expression of the transporter.

The substantial decreases in the biliary excretions of FL and FL-Glu in the IR and HxIR animals, observed in our study, were associated with significant decreases in the bile flow rates

(Figs. 3e, 3f, 4e, and 4f). The decreases in the bile flow rates of the HxIR animals at both 15 min (Fig. 3e) and 8 h (Fig.4e) are consistent with the substantial loss of liver mass in these animals. However, the lower bile flow rates in the IR animals, observed only after 15 min (Fig. 3e) and not after 8 h (Fig. 4e) of reperfusion, are most likely due to the lower ATP levels in the 15 Min IR group because lower ATP contents in the liver have been linked to the lower bile flow rates (Slater and Delaney, 1970). Indeed, the biliary excretions of FL and FL-Glu were significantly ($p < 0.0001$) correlated with the bile flow rates for both surgical groups and reperfusion times with very high coefficients of determination between 0.77 and 0.94 (Figs. 3f and 4f). The correlation between the biliary excretion of FL or FL-Glu and the bile flow rates are in agreement with the reported correlation between the biliary excretion of other organic anions, such as the antibiotic cefoperazone, and the LPS-induced reductions in the bile flow rate (Nadai et al., 1998).

Another widely-used, low MW BBB permeability marker is ^{14}C-sucrose, which is not bound to plasma proteins. Therefore, in contrast to FL, the use of ^{14}C-sucrose is not complicated by the requirement for measurement of the free plasma concentration of the marker. However, similar to our studies with FL reported here, accurate measurement of the BBB permeability using ^{14}C-sucrose also requires estimation of K_{in} values using the terminal brain concentration and the plasma AUC (Bickel, 2005; Ohno et al., 1978). Although ^{14}C-sucrose is considered the gold standard for the measurement of the BBB permeability through tight junctions (Ohno et al., 1978), its use is limited to laboratories that can handle radioactive materials. Therefore, FL is still used by many investigators because of its ease of handling and detection. Our data, however, clearly show that the appropriate use of FL requires an additional step to measure the free drug concentration in plasma.

In conclusion, Pringle maneuver with or without partial hepatectomy caused decreases in the biliary excretion and increases in the plasma free fraction, AUC, and the brain concentrations of the BBB permeability marker FL. However, the increased brain concentrations of FL in IR and HxIR were due to the changes in the plasma AUC and free fraction of the marker. Therefore, when the brain concentrations of FL were corrected for the changes in its systemic pharmacokinetics, the surgical procedures did not have any impact on the BBB permeability to FL. These studies indicate that accurate estimation of BBB permeability using FL requires consideration of both brain and systemic pharmacokinetics, including degree of plasma protein binding, of the marker.

4. Experimental Procedures

4.1. Chemicals and reagents

Sodium fluorescein (FL) and β-glucuronidase (Type-LII) were obtained from Sigma–Aldrich (St. Louis, MO). For anesthesia, ketamine and xylazine solutions were purchased from Lloyd Laboratories (Shenandoah, IA, USA). Heparin solution was purchased from APP Pharmaceuticals (Schaumburg, IL, USA). Rabbit polyclonal anti-Oatp1a4 antibody was from LSBio (Seattle, WA, USA), and monoclonal anti-Mrp2 and anti-β-actin antibodies, both produced in mice, were purchased from Sigma-Aldrich and Enzo Life Sciences (Plymouth meeting, PA, USA), respectively. Microcon Ultracel YM-30 centrifugal devices, with a molecular weight cut-off of 30 kDa, were from Millipore (Billerica, MA, USA). PIC-A reagent (tetrabutylammonium dihydrogen phosphate) was purchased from Waters (Milford, MA). The kits for the measurement of plasma aspartate aminotransferase (AST) (Teco Diagnostics; Anaheim, CA, USA), total bile acids (TBA) (Crystal Chem; Downers Grove, IL), and albumin (Active Motif; Carlsbad, CA) were purchased from commercial sources. BCA kit for the

measurement of protein was purchased from Pierce Biotechnology (Rockford, IL, USA). All other chemicals were analytical grade and commercially available.

4.2. Animals

Our Institutional Animal Care and Use Committee approved the use of animals in the study. Additionally, all the procedures involving animals used in this study were consistent with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011). Adult, male Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA) and were acclimated with 12-h dark-light cycle in a temperature- and humidity-controlled room before surgery. Animals had free access to food and water before the experiments.

4.3. Ischemia-reperfusion and partial hepatectomy models

Animals (body weight: 240-300 g) were subjected to sham (Sham), hepatic ischemia-reperfusion (IR), or IR plus partial hepatectomy (HxIR) surgery. After an overnight fast, rats were anesthetized with an intramuscular injection of ketamine: xylazine (80:8 mg/kg), and the abdomen was opened using a midline incision. For the IR group, the total blood supply to the liver was occluded by placing a microvascular surgical clamp on the portal triad (portal vein, hepatic artery, and bile duct). After 20 min of ischemia, the clamp was removed to allow reperfusion of the ischemic liver with blood. In the HxIR group, immediately after the interruption of the blood supply with the clamp, partial (70%) hepatectomy of the liver was performed by ligating and excising the left and median lobes of the liver at their base, as described before (Emond et al., 1989). Similar to the IR group, after 20 min of ischemia, blood supply to the HxIR livers was reinstated by removing the clamp. Sham animals underwent only laparotomy and manipulation of the liver and blood vessels, without any interruption in the blood supply to the liver. During the surgery, rat body temperature was closely monitored and

maintained at 37^{O}C by using a combination of a heat lamp and a heating plate attached to a rectal temperature control probe.

4.4. Dosing and sampling

Three surgical groups of Sham, IR, and HxIR each with two reperfusion times of 15 min or 8 h were used in this study, resulting in a total of six experimental groups with 6-7 animals/group. To collect blood and bile samples, catheters were placed into the femoral artery and bile duct of the anesthetized animals, respectively. FL was then infused into the penile vein of the animals at a dose of 25 mg/kg (acid equivalent) over 5 min. Blood samples were collected at 0, 5, 10, 20, and 30 min. Each time after collection of blood samples, catheter was filled with heparin (10 U/ml) to prevent the formation of blood clot. Immediately after the collection of blood, plasma was separated by centrifugation for 5 min at 14000 rpm. At the end of experiments, the whole rat body was perfused, through a catheter inserted into the left ventricle, with an ice-cold saline solution at the rate of 25 ml/min for three min to remove the residual blood from the brain. Finally, brain samples were collected and snap frozen in isopentane (–80^{o}C). All the samples were kept at –80^{O}C until analysis.

4.5. Analysis of fluorescein in plasma, brain, and bile samples

Concentrations of FL in the biological samples were determined by minor modifications of a previously published paper (Selan et al., 1985), as described in detail recently (Shaik et al., 2013). Brain samples were homogenized in de-ionized water (1:4) using an electric homogenizer. All the other samples were diluted with a 4% bovine serum albumin (BSA) solution in water before being subjected to the sample preparation method (Shaik et al., 2013). The plasma samples were diluted 100 fold. Additionally, the terminal plasma samples (30 min) were also subjected to ultrafiltration using Microcon Ultracel YM-30 centrifugal devices, as

described in detail before,(Shaik et al., 2013) and were diluted 50 fold. Diluted (1000 fold) bile samples were analyzed twice in the absence or presence of β-glucuronidase, as described in detail before (Shaik et al., 2013).

4.6. Quantitation of plasma biochemical markers

The plasma concentrations of AST, TBA, and albumin were measured using commercially available kits, following the manufacturers' protocols.

4.7. Quantitation of liver ATP

ATP concentrations in the samples from the right lobe of the livers were measured using a reversed-phase, gradient HPLC method (Manfredi et al., 2002).

4.8. Western blot analysis

The protein contents of Mrp2, Oatp1a4, and the loading control β-actin were determined in crude membrane fractions of the liver, which were prepared according to standard methods. Briefly, liver tissues were homogenized in tissue homogenization buffer (1:4), containing 10 mM Tris-HCl (pH 7.4), 1 mM mercaptoethanol, 1 mM ethylene glycol tetraacetic acid, 1 mM $MgCl_2$, 1 mM DL-dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, and 1% glycerol. To 1 ml of the homogenate were added 40 µl of 0.5 M Tris-HCl and 1 µl of protease inhibitor cocktail for mammalian tissue extracts (Sigma). The homogenate was then centrifuged at 2000 g for 15 min ($4^{O}C$); supernatant was isolated and centrifuged again at 100,000 g for 30 min ($4^{O}C$); and the resultant pellet was re-suspended in 250 µl of homogenization buffer, containing 2.5 µl of the protease inhibitor phenylmethylsulfonyl fluoride (100 mM). Protein concentrations in the samples were measured by the BCA kit. Finally, 40 µg of liver proteins were electrophoretically resolved using 4-20% gradient SDS-polyacrylamide gels (Thermo Scientific, Rockford, IL,

USA), and the resolved proteins were transferred onto a polyvinylidene difluoride membrane. The membranes were blocked for 1 h at room temperature with 5% nonfat instant milk, followed by overnight incubation (4^{O}C) with primary antibodies anti-Mrp2 (1:1000), anti-Oatp1a4 (1:1000), and anti-β-actin. After washing, the membranes were incubated with secondary antibodies for 1 h, and band intensities were quantified using VersaDoc Image system and Quantity One software (Bio-Rad, Hercules, CA, USA).

4.9. Pharmacokinetic analysis

Linear trapezoidal method was used to calculate the AUC of FL from zero to 30 min (AUC_{0-30}). As a measure of the BBB permeability, apparent brain uptake clearance (K_{in}) of FL was determined by using the following equations (Bickel, 2005):

$$K_{in} = \frac{C_{br}^{30}}{AUC_{0-30}} \quad (1)$$

where C_{br}^{30} indicates the concentration of FL in the brain 30 min after its injection. The free fraction of FL in plasma (f_u) was estimated by dividing the filtrate (free) concentration by the total (free plus bound) plasma concentration for the terminal (30 min) sample. An estimate of the free K_{in} value of FL was then obtained by dividing K_{in} by f_u. The amount of FL and FL-Glu recovered in the bile during the sampling period (30 min) was estimated by multiplying their concentrations in the bile by the volume of the bile. The apparent biliary clearance of FL ($Cl_{bile,app}$) was calculated by dividing the amount of FL recovered into the bile by the corresponding plasma AUC during the sampling period.

4.10. Statistical analysis

All the results are presented as mean ± SD. The statistical differences among the surgical groups were analyzed using one-way ANOVA, followed by Tukey's multiple comparison test. The relationship between the biliary recoveries of FL or FL-Glu and bile flow rates was analyzed using linear regression analysis. A p values less than 0.05 was considered significant.

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FIGURE LEGENDS

Fig. 1 – Plasma concentrations of AST (a and b), total bile acids (c and d), and albumin (e and f) in the Sham, IR, and HxIR animals 15 min (left panels) or 8 h (right panels) after reperfusion (*n* = 6-7/group). The symbols and horizontal lines represent the individual and mean values, respectively. Statistical analysis is based on one-way ANOVA, followed by Tukey's post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Fig. 2 – Plasma concentration-time courses of sodium fluorescein in the Sham, IR, and HxIR animals 15 min (top) or 8 h (bottom) after reperfusion (*n* = 6-7/group). Symbols and bars represent mean and SD values, respectively.

Fig. 3 – Biliary recovery (a) and apparent biliary clearance (b) of sodium fluorescein (FL), biliary recovery of fluorescein glucuronide (FL-Glu) (c), total biliary recovery of FL plus FL-Glu (d), bile flow rates (e), and the correlation between bile flow rate and biliary recovery of FL or FL-Glu (f) in the Sham, IR, and HxIR animals 15 min after reperfusion (*n* = 6-7/group). The symbols and horizontal lines represent the individual and mean values, respectively. Statistical analysis is based on one-way ANOVA, followed by Tukey's post-hoc test. *** $p < 0.001$, **** $p < 0.0001$.

Fig. 4 – Biliary recovery (a) and apparent biliary clearance (b) of sodium fluorescein (FL), biliary recovery of fluorescein glucuronide (FL-Glu) (c), total biliary recovery of FL plus FL-Glu (d), bile flow rates (e), and the correlation between bile flow rate and biliary recovery of FL or FL-Glu (f) in the Sham, IR, and HxIR animals 8 h after reperfusion (*n* = 6/group). The symbols and horizontal lines represent the individual and mean values,

respectively. Statistical analysis is based on one-way ANOVA, followed by Tukey's post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Fig. 5 – Representative Western blots (a and b) and individual densitometric data for Mrp2 (c and d) and Oatp1a4 (e and f) in the liver membrane fractions from the Sham, IR, and HxIR animals 15 min (left panels) or 8 h (right panels) after reperfusion (n = 6-7/group). The symbols and horizontal lines represent the individual and mean values, respectively. Statistical analysis is based on one-way ANOVA, followed by Tukey's post-hoc test. ** $p < 0.01$.

Fig. 6 – Brain concentrations (a and b), apparent brain uptake clearance (K_{in}) based on the total AUC (c and d), and apparent brain uptake clearance (K_{in}) based on the free (unbound) AUC (e and f) of sodium fluorescein in the Sham, IR, and HxIR animals 15 min (left panels) or 8 h (right panels) after reperfusion (n = 6-7/group). The symbols and horizontal lines represent the individual and mean values, respectively. Statistical analysis is based on one-way ANOVA, followed by Tukey's post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 1

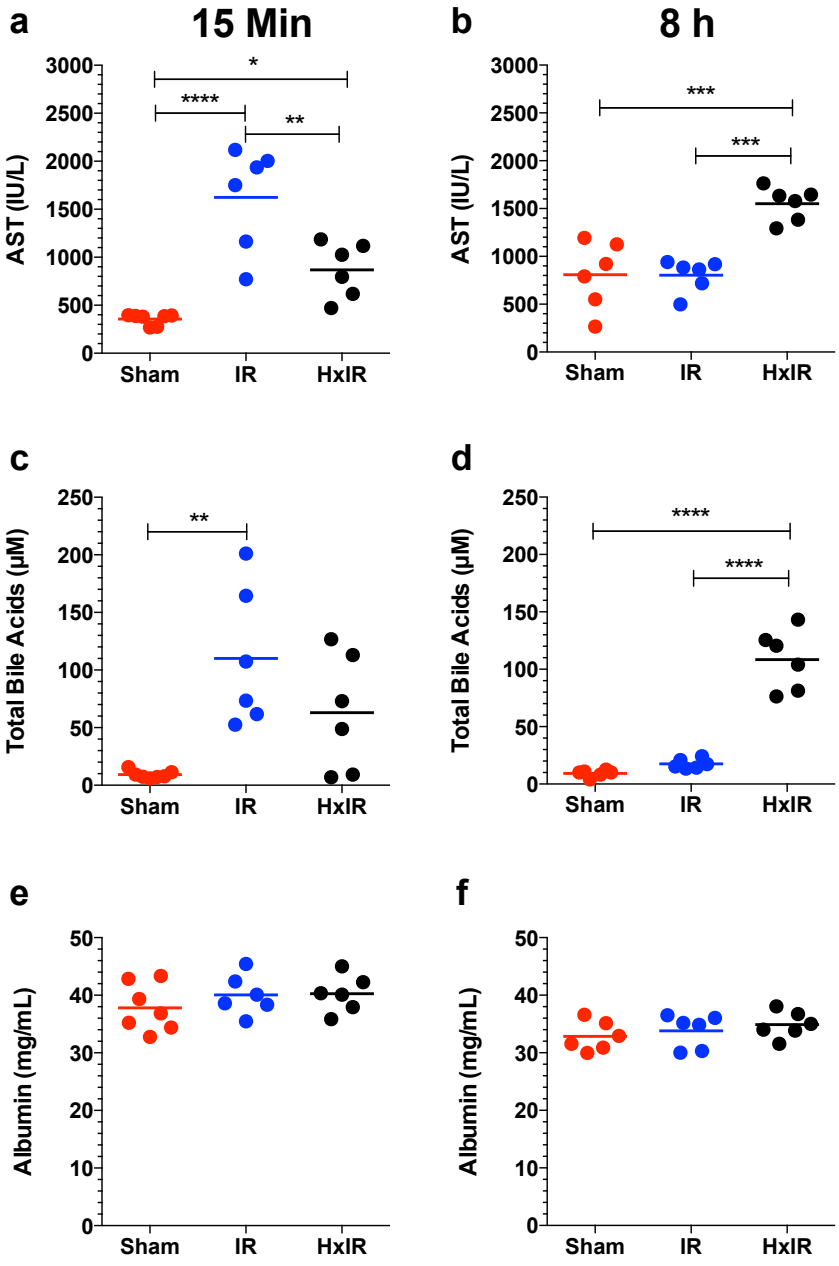
a
15 Min
AST (IU/L)
3000
2500
2000
1500
1000
500
0
*
****
**
Sham
IR
HxIR
b
8 h
AST (IU/L)
***
***
c
Total Bile Acids (µM)
250
200
150
100
50
0
**
d
Total Bile Acids (µM)
****
****
e
Albumin (mg/mL)
50
40
30
20
10
0
f
Albumin (mg/mL)

Figure 2

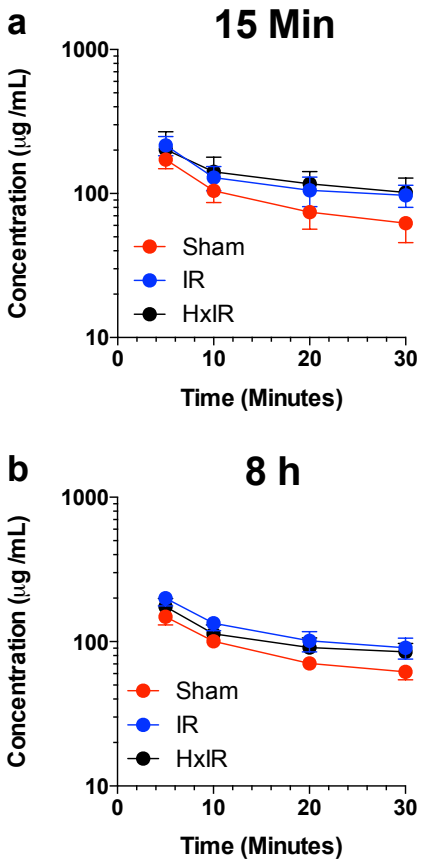
a
15 Min
1000
100
10
Concentration (µg /mL)
Sham
IR
HxIR
0
10
20
30
Time (Minutes)
b
8 h
1000
100
10
Concentration (µg /mL)
Sham
IR
HxIR
0
10
20
30
Time (Minutes)

Figure 3

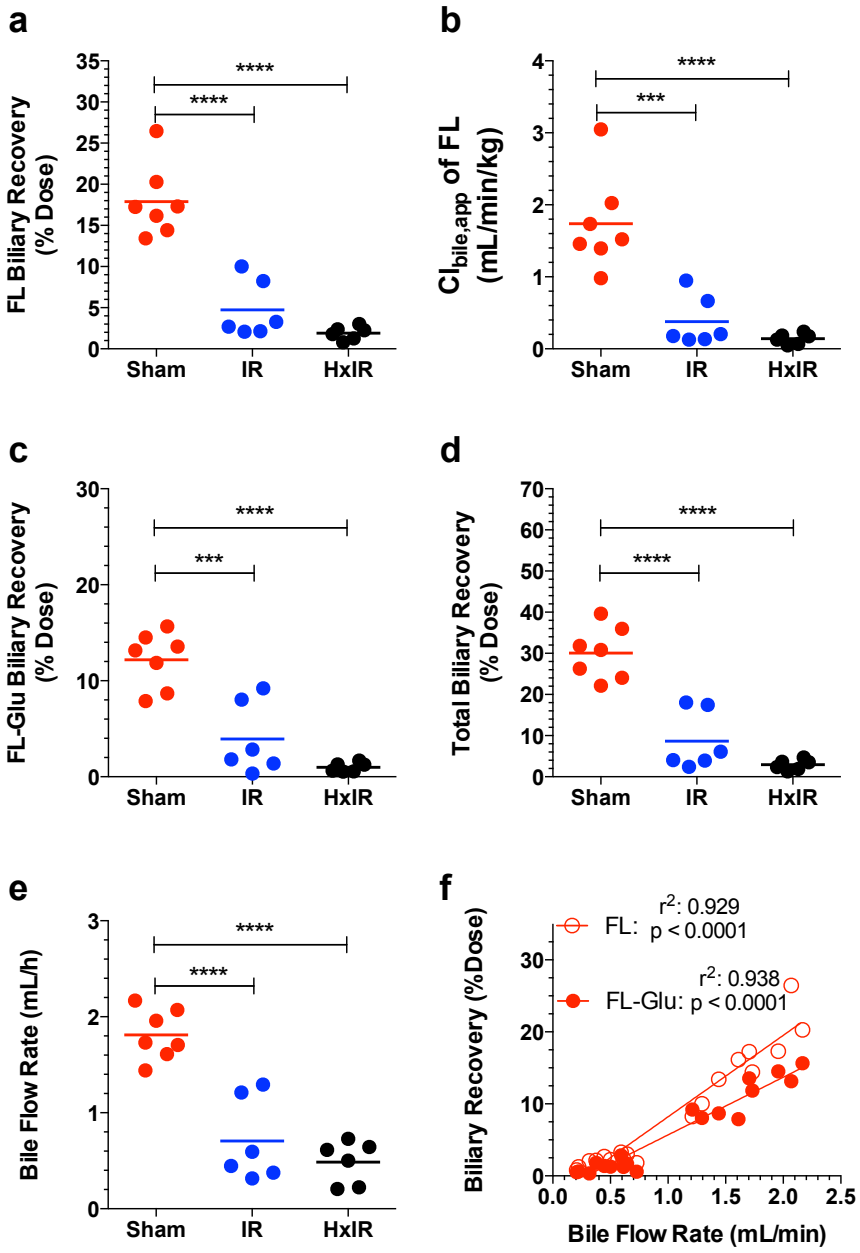
a
FL Biliary Recovery (% Dose)
35
30
25
20
15
10
5
0
****
****
Sham
IR
HxIR
b
$Cl_{bile,app}$ of FL (mL/min/kg)
4
3
2
1
0
****
***
Sham
IR
HxIR
c
FL-Glu Biliary Recovery (% Dose)
30
20
10
0
****
***
Sham
IR
HxIR
d
Total Biliary Recovery (% Dose)
70
60
50
40
30
20
10
0
****
****
Sham
IR
HxIR
e
Bile Flow Rate (mL/h)
3
2
1
0
****
****
Sham
IR
HxIR
f
Biliary Recovery (%Dose)
r^2: 0.929
FL: p < 0.0001
r^2: 0.938
FL-Glu: p < 0.0001
30
20
10
0
0.0
0.5
1.0
1.5
2.0
2.5
Bile Flow Rate (mL/min)

Figure 4

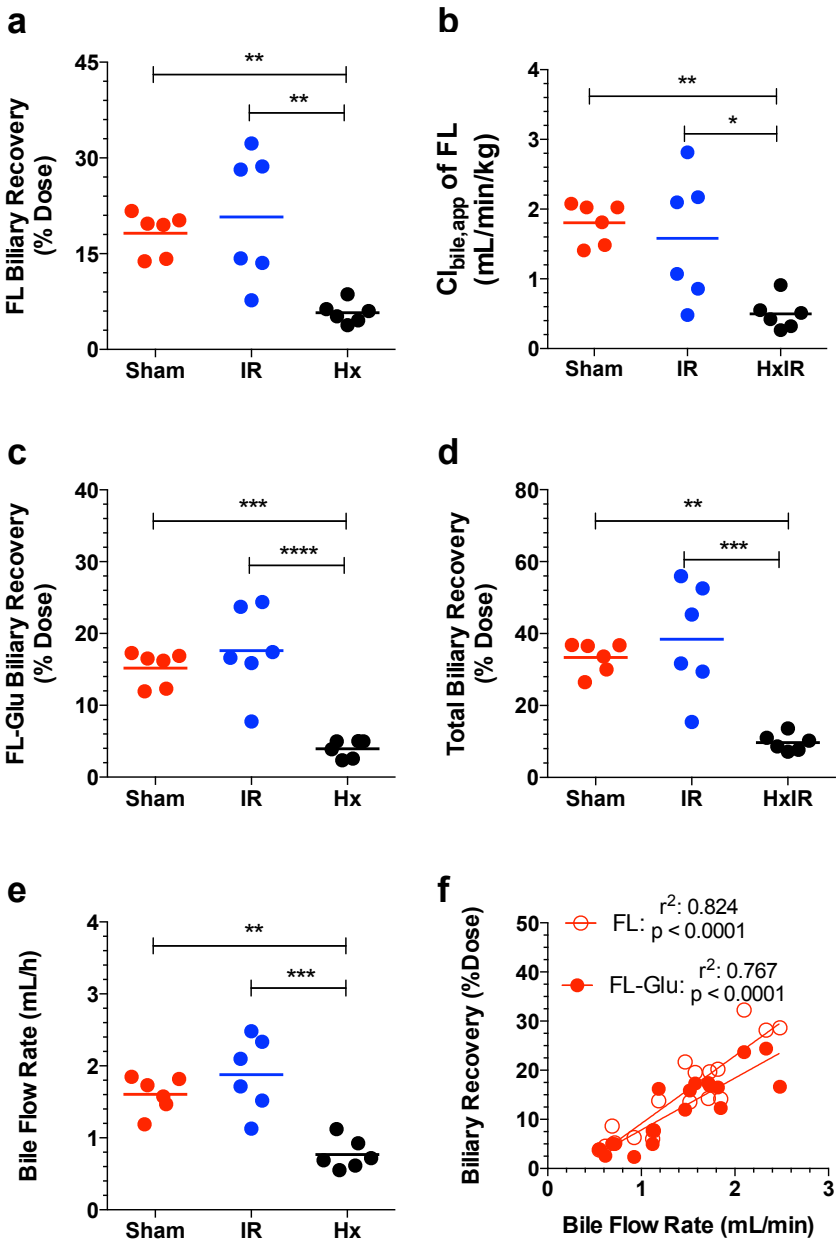
a
FL Biliary Recovery (% Dose)
45
30
15
0
**
**
Sham
IR
Hx
b
$Cl_{bile,app}$ of FL (mL/min/kg)
4
3
2
1
0
**
*
Sham
IR
HxIR
c
FL-Glu Biliary Recovery (% Dose)
40
30
20
10
0
***
****
Sham
IR
Hx
d
Total Biliary Recovery (% Dose)
80
60
40
20
0
**
***
Sham
IR
HxIR
e
Bile Flow Rate (mL/h)
4
3
2
1
0
**
***
Sham
IR
Hx
f
Biliary Recovery (%Dose)
50
40
30
20
10
0
FL: r^2: 0.824 p < 0.0001
FL-Glu: r^2: 0.767 p < 0.0001
0
1
2
3
Bile Flow Rate (mL/min)

Figure 5

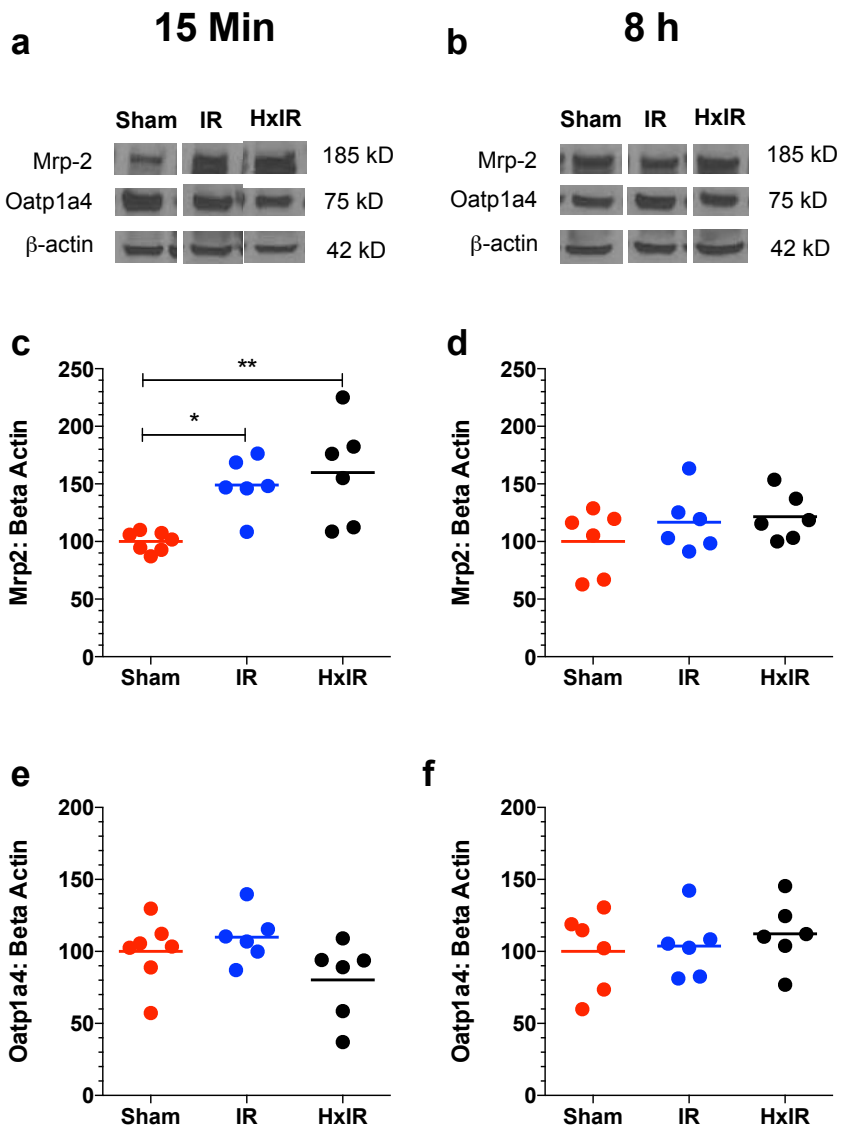
a
15 Min
Sham
IR
HxIR
Mrp-2
Oatp1a4
β-actin
185 kD
75 kD
42 kD
b
8 h
Sham
IR
HxIR
Mrp-2
Oatp1a4
β-actin
185 kD
75 kD
42 kD
c
Mrp2: Beta Actin
250
200
150
100
50
0
**
*
Sham
IR
HxIR
d
Mrp2: Beta Actin
250
200
150
100
50
0
Sham
IR
HxIR
e
Oatp1a4: Beta Actin
200
150
100
50
0
Sham
IR
HxIR
f
Oatp1a4: Beta Actin
200
150
100
50
0
Sham
IR
HxIR

Figure 6

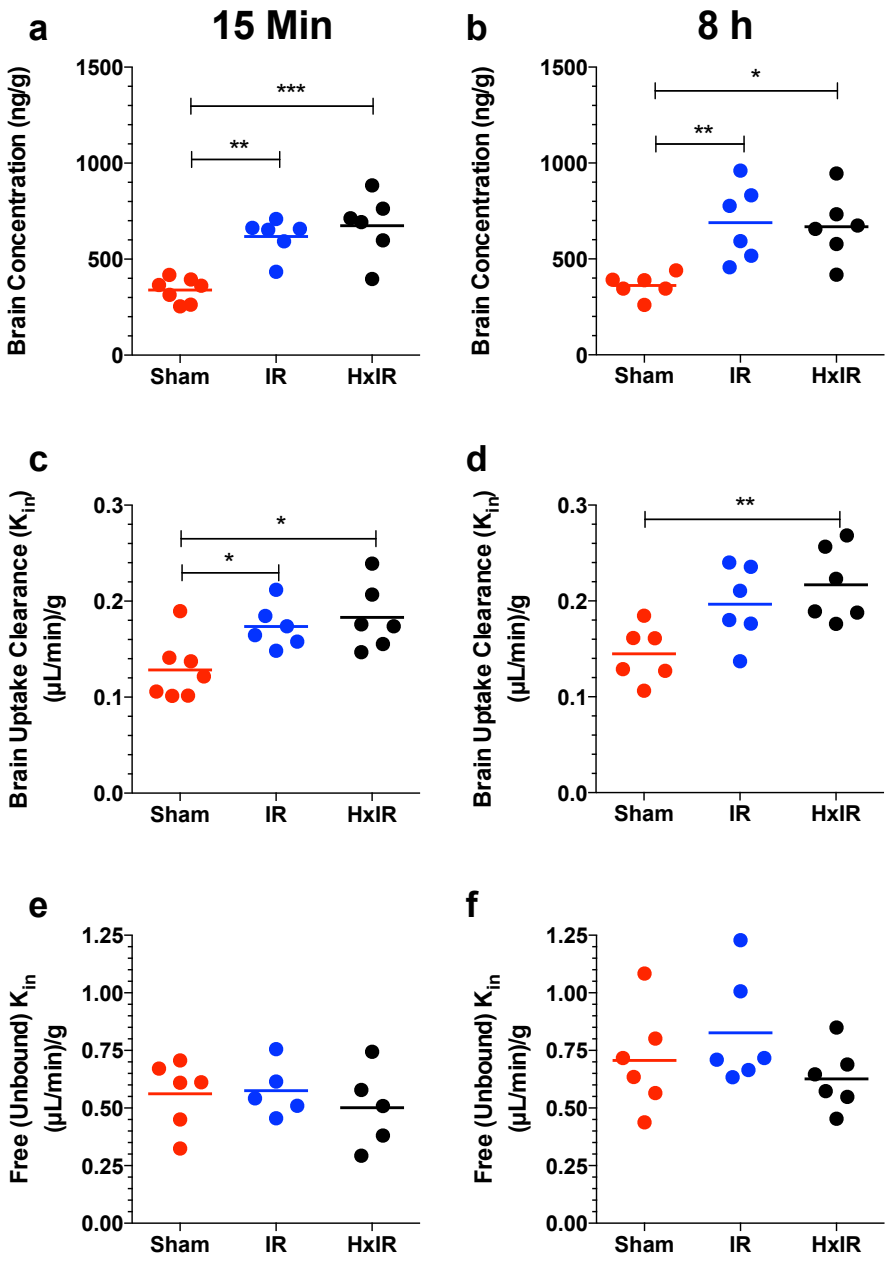
a
15 Min
b
8 h
Brain Concentration (ng/g)
1500
1000
500
0
***
**
*
**
Sham
IR
HxIR
c
d
Brain Uptake Clearance (K$_{in}$) (µL/min)/g
0.3
0.2
0.1
0.0
*
*
**
e
f
Free (Unbound) K$_{in}$ (µL/min)/g
1.25
1.00
0.75
0.50
0.25
0.00

Table 1 – Plasma AUC and free fraction values (Mean ± SD) of fluorescein after a short (5 min) intravenous infusion of the marker (25 mg/kg) in rats subjected to Pringle maneuver without (IR) or with (HxIR) partial hepatectomy or sham surgery (Sham), followed by 15 min or 8 h of *in vivo* reperfusion.

Treatment	*AUC, µg.min/ml*	f_u
15-Min groups		
Sham (*n* = 7)	2700 ± 490	0.233 ± 0.056
IR (*n* = 6)	3590 ± 594	0.301 ± 0.034
HxIR (*n* = 6)	3750 ± 971*	0.408 ± 0.112**
8-h groups		
Sham (*n* = 6)	2510 ± 145	0.215 ± 0.049
IR (*n* = 6)	3470 ± 432**	0.245 ± 0.052
HxIR (*n* = 6)	3060 ± 446	0.356 ± 0.082**,¶

* $p < 0.05$, ** $p < 0.01$: Significantly different from the corresponding Sham group (ANOVA, followed by Tukey's post-hoc analysis)

¶ $p < 0.05$: Significantly different from the corresponding IR group (ANOVA, followed by Tukey's post-hoc analysis).