8-2022

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American Journal of Physiology: Gastrointestinal and Liver Physiology, Vol. 323, No. 1 (August 2022): G126-G133. DOI.

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Abstract

Quantitative measurement of the degree of hepatic ischemia-reperfusion injury (IRI) is crucial for developing therapeutic strategies for its treatment. We hypothesized that clearance of fluorescent dye through bile metabolism may reflect the degree of hepatic IRI. In this study, we investigated sodium fluorescein clearance kinetics in blood and bile for quantifying the degree of hepatic IRI. Warm ischemia times (WITs) of 0, 30, or 60 min followed by 1 h or 4 h of reperfusion, were applied to the median and lateral lobes of the liver in Sprague-Dawley rats. Subsequently, 2 mg/kg of sodium fluorescein was injected intravenously, and blood and bile samples were collected over 60 min to measure fluorescence intensities. The bile-to-plasma fluorescence ratios demonstrated an inverse correlation with WIT and were distinctly lower in the 60-min WIT group than in the control or 30-min WIT groups. Bile-to-plasma fluorescence ratios displayed superior discriminability for short versus long WITs when measured 1 h after reperfusion versus 4 h. We conclude that the bile-to-blood ratio of fluorescence after sodium fluorescein injection has the potential to enable the quantification of hepatic IRI severity.

NEW & NOTEWORTHY

Previous attempts to use fluorophore clearance to test liver function have relied on a single source of data. However, the kinetics of substrate processing via bile metabolism include decreasing levels in blood and increasing levels in bile. Thus, we analyzed data from blood and bile to better reflect fluorescein clearance kinetics.

INTRODUCTION

A reliable diagnostic tool is lacking to evaluate liver viability after ischemia-reperfusion injury (IRI), which is necessary to reduce the risk of primary nonfunction after liver transplantation (LT) or posthepatectomy liver failure (1, 2). Hepatic IRI impacts multiple biological functions, and dysfunctional bile formation (i.e., cholestasis) has been considered an essential indicator of liver injury (3–8). However, investigations of the kinetics of markers through the bile formation machinery have not provided a reliable test in predicting hepatic viability after IRI (9–11). Notably, previous clinical studies utilizing substrate kinetics have relied on single data sources, either from blood sampling or liver imaging, and have attained limited success. As such, a fluorescent dye excreted from the blood into the bile may provide information on substrate kinetics during IRI (12–16).

Fluorescein is a Food and Drug Administration-approved fluorescent dye. Within 5 min of intravenous administration of fluorescein, clearance from the blood into the bile occurs in normal livers via hepatocyte membrane transporters (17, 18). As the kinetics of a substrate through bile metabolism consists of decreasing levels in the blood while increasing levels in the bile, we hypothesized that coupling the data measured by fluorometry from simultaneously collected blood and bile samples could enhance the accuracy of this approach and better reflect hepatic clearance (19, 20). As such, we sought to investigate whether the fluorescein clearance kinetics determined from blood and bile measurements could reflect the degree of hepatic IRI during the early phase of reperfusion.

MATERIALS AND METHODS

Animals

Male 7- to 8-wk-old Sprague-Dawley rats (median body weight of 243 g, interquartile range of 228–260 g, Charles River Laboratories) were housed in an animal facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The rats were fed Purina Lab Diet 5001 and reverse osmosis water ad libitum. All animals received humane care in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Only male rats were used in these experiments because multidrug resistance-associated protein 2 (MRP2), which is responsible for fluorescein excretion from the hepatocyte, is impaired by estradiol glucuronide (21). All experiments were approved by the Institutional Animal Care and Use Committee at the Medical College of Wisconsin (animal use Application No. 00004857).

Surgical Procedures

We evaluated the effect of hepatic IRI on sodium fluorescein clearance by applying different warm ischemia times (WITs; 0 min, 30 min, or 60 min) and reperfusion times (1 h or 4 h). Thus, there were five experimental groups (n = 5 each) based on ischemia and reperfusion times: no ischemia (control), brief ischemia (30 min) followed by 1 h of reperfusion, prolonged ischemia (60 min) followed by 1 h of reperfusion, brief ischemia (30 min) followed by 4 h of reperfusion, and prolonged ischemia (60 min) followed by 4 h of reperfusion (Fig. 1*A*).

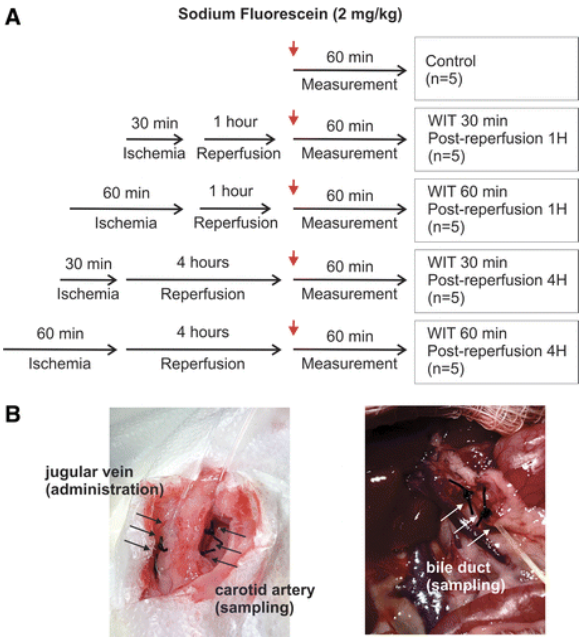

Figure 1. Study design. *A*: scheme of five experimental groups. All rats underwent intravenous injection of sodium fluorescein followed by a series of blood and bile samplings over 60 min to measure fluorescence. The rats in all groups, except the controls, underwent partial (70%) ischemia-reperfusion injury (IRI), starting with brief (30 min) or prolonged (60 min) ischemia, followed by 1-h or 4-h reperfusion, before sodium fluorescein injection. *B*: photos demonstrating vascular and biliary cannulation. Sodium fluorescein was administered through the right jugular vein. At each designated time point after the administration (0, 2, 5, 10, 20, 30, 45, and 60 min), blood and bile samples were collected from the left carotid artery and the bile duct, respectively. WIT, warm ischemia time.

Surgery was performed under isoflurane inhalation anesthesia (1%–3%; oxygen flow 1 L/min). Animals were placed on a warming pad set to obtain a temperature of around 39°C (Far Infrared Warming Pad, Kent Scientific, Torrington, CT). A transverse abdominal incision was made, and the pedicles to the median and lateral lobes of the liver (~70% of the volume of the liver) were temporarily occluded using a small vascular clamp (FE011K, Aesculap, Melsungen, Germany). This approach has been utilized for testing the effect of hepatic IRI on biliary excretion of fluorescein (16, 17). During the designated time period for hepatic IRI, the abdominal wall was temporarily closed to prevent dehydration and heat loss.

For injection and sampling, blood vessels and the bile duct were cannulated as follows: a midline incision was made on the neck, and 8- to 10-cm polyethylene tubes were cannulated to the right jugular vein [0.58 mm inner diameter (ID) × 0.97 mm outer diameter (OD); PE50, Braintree Scientific, Braintree, MA] and right carotid artery (0.28 mm ID × 0.61 mm OD; PE10, Braintree Scientific, Braintree, MA). The polyethylene tubes in the vessels were attached to a 30-gauge blunt needle (SAI Infusion Technologies, Lake Villa, IL) and filled with 20 U/mL heparin in 0.9% saline. Another polyethylene tube (0.28 mm ID × 0.61 mm OD; PE10, Braintree Scientific, Braintree, MA) was cannulated into the bile duct for bile sampling (Fig. 1*B*). The cannulation procedure was performed under a surgical microscope (Amscope, Irvine, CA).

Sample Collections and Fluorescence Measurements

Blood and bile were collected at eight time points over 60 min (0, 2, 5, 10, 20, 30, 45, and 60 min). At each time point, 180 µL of blood was collected by a heparin-coated syringe from the polyethylene tubing in the left carotid artery and mixed with 20 µL of heparin solution (10,000 U/mL). Of this 200-µL blood sample, 20 µL was used for whole blood analysis. The remaining 180 µL was centrifuged (3,000 rpm for 10 min), and 20 µL of supernatant was collected for plasma sample analysis. Likewise, 30 µL of bile was collected from the polyethylene tubing in the bile duct at each time point.

Once the baseline (0 min) samples were collected, sodium fluorescein (Sigma-Aldrich, St. Louis, MO) solution in 0.9% saline (2 mg/mL) was injected as a bolus (~50 µL/s) into the polyethylene catheter in the right jugular vein at a dose of 2 mg/kg, selected based on previous studies (12, 18). At this dose, significant clearance of fluorescein from blood into bile could be observed immediately, whereas a dose of 10 mg/kg delayed the clearance by over 1 h, possibly due to oversaturation (17). After the injection, the catheter was flushed using 30 µL of heparin solution in 0.9% saline (20 U/mL).

The blood, plasma, and bile samples (20 µL each) were placed in a 384-well plate (Greiner Bio-one, Monroe, NC) and covered with plastic film to minimize evaporation. The fluorescence intensity was measured in the samples using a microplate reader (CLARIOstar, BMG Labtech, Ortenberg, Germany), with the excitation set at 478 nm and emission light collected within the 505–552-nm spectral range. Fluorescence gain was set at 1,000, 600, and 500 for whole blood, plasma, and bile, respectively. The emission peaks were analyzed for measuring the fluorescence intensity using software provided by the manufacturer (MARS, BMG Labtech, Ortenberg, Germany).

Statistical Analyses

The data were expressed as medians with interquartile ranges. $P < 0.05$ was considered significant. Correlations between two variables were determined by the Spearman rank correlation coefficient. Differences among the three groups were analyzed by the Kruskal–Wallis test and the post hoc Mann–Whitney test with Bonferroni's correction. The effects of a factor along the time-sequenced samples were assessed by repeated measures analysis of variance (ANOVA) with the Geisser–Greenhouse correction. Statistical analyses were conducted using Prism 7 for Windows (GraphPad Software, San Diego, CA).

RESULTS

Fluorescence Intensity Values in Plasma Strongly Correlated with Those from Matched Whole Blood Samples

The overall time-fluorescence curves of the samples from whole blood, plasma, and bile in the control group ($n = 5$) are depicted in Fig. 2*A*. Levels of fluorescence intensity were higher in bile compared with those in whole blood and plasma, especially at later time points of the measurement. Furthermore, plasma and whole blood showed different levels of fluorescence intensity, presumably due to different gain settings and the absence of background absorption and scatter from hemoglobin in plasma (22). However, both curves showed a similar pattern of kinetics, featuring an initial peak followed by a retention phase. The data pairs between values from whole blood and plasma showed a significant correlation ($R = 0.9442$, $P < 0.0001$; Fig. 2*B*). Thus, the fluorescence intensity values from plasma reliably represent those from whole blood.

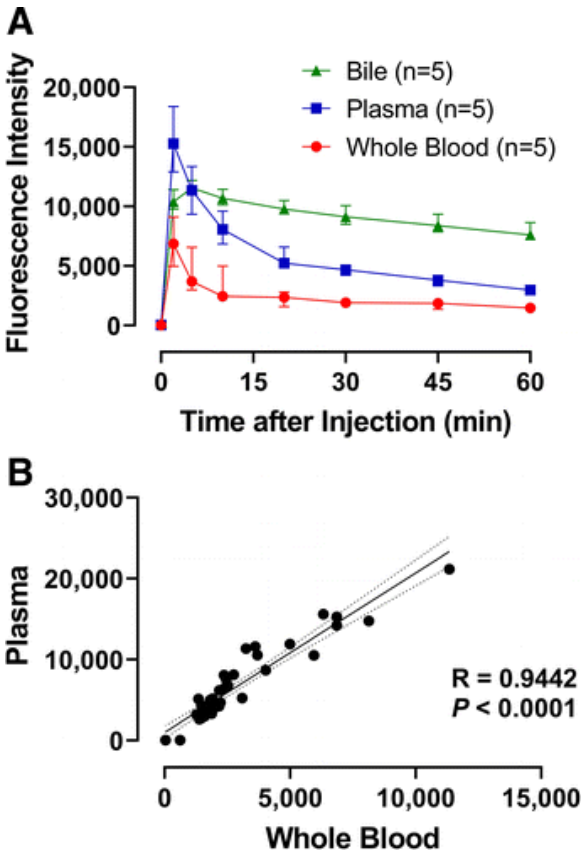

Figure 2. Data from whole blood, plasma, and bile in the control group. *A*: time course of fluorescence intensity changes, demonstrating overall differences between bile (green triangle), plasma (blue squares), and whole blood (red circles) from samples in the control group (n = 5). Values are presented as medians with interquartile ranges. *B*: the scatter plot demonstrating correlation between fluorescence intensity values from whole blood and plasma samples from 5 rats in the control group (n = 40; samples from 8 time points per animal). The correlation was determined by the Spearman's rank correlation coefficient (R).

Fluorescein is Retained in Blood, and Its Biliary Excretion is Suppressed with Prolonged WIT

It is important to differentiate severe IRI from mild IRI. For example, 60 min of WIT was suggested as the extreme limit for hepatic pedicle occlusion to prevent bleeding during liver surgery (Pringle maneuver; 23). Likewise, in donation after circulatory death, a donor WIT of 30 min is often considered as the maximum that the liver graft can tolerate for LT, which will require additional WIT (often over 30 min) in the recipient for vascular anastomoses (24). Therefore, we designed this study to observe the hepatic metabolism of sodium fluorescein with 30 min versus 60 min of WIT.

We observed changes in fluorescence intensity in two compartments, namely, blood and bile, during the 60 min following sodium fluorescein injection. Fluorescence in the blood compartment was measured by two methods: one from whole blood and the other from plasma. Thus, time-fluorescence curves in whole blood, plasma, and bile could be obtained from the three WIT groups (0, 30, and 60 min) after 1 h (Fig. 3*A*) or 4 h (Fig. 3*B*) of reperfusion. There was no incidence of experimental failure in this study, and all data from 25 experimental rats were included in the analyses.

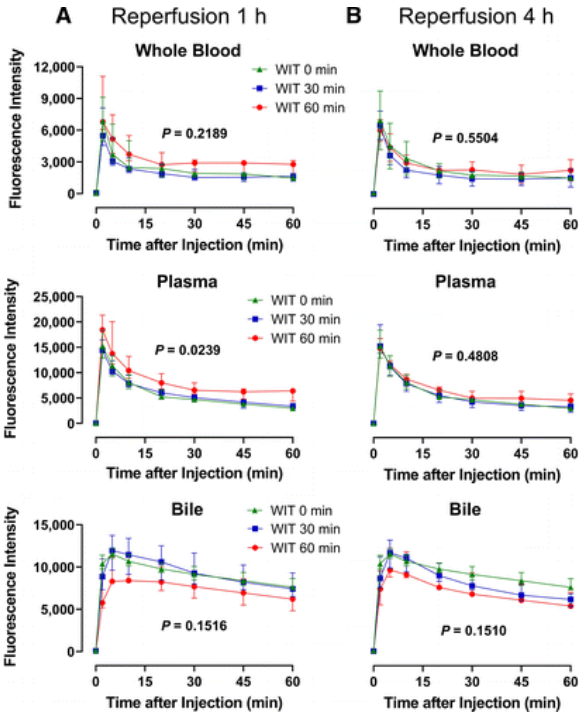

Figure 3. Fluorescence time course determined from whole blood, plasma, and bile samples in three warm ischemia time (WIT) groups: 0 min (control, green triangles), 30 min (blue squares), and 60 min (red circles). Values are presented as medians with interquartile ranges. Effects of WIT were determined by repeated measures ANOVA with the Geisser–Greenhouse correction. *A*: data measured after 1 h of reperfusion. *B*: data measured after 4 h of reperfusion. *n* = 5 each.

We used WITs of 60 min and 30 min as models for severe and mild IRI, respectively, and observed whether these degrees of injury could be distinguished based on their fluorescein kinetics curves. The curves from the control group (WIT = 0 min) were added as a baseline for comparison. Retention of sodium fluorescein in severe hepatic IRI (WIT = 60 min) was indicated by elevated fluorescence intensities in whole blood and plasma and by lower fluorescence intensities in bile compared with the control and mild IRI groups. The hierarchy of the curves was the same at 1 h and 4 h postreperfusion (Fig. 3, *A* and *B*). The curves for the 30-min WIT condition mostly overlapped with those from the control group (WIT = 0 min). However, the curves for 60-min WIT stood out from the other groups, especially with the shorter reperfusion time (i.e., 1 h). In particular, the differences of fluorescence intensities in the plasma curves among the WIT groups were significant at 1 h ($P = 0.0239$, Fig. 3*A*), but not at 4 h ($P = 0.4808$, Fig. 3*B*). Notably, the differences in bile curves did not reach statistical significance at either reperfusion time point.

Biliary Excretion Relative to Plasma Retention of Sodium Fluorescein is Markedly Decreased in the 60-min WIT Group

Fluorescein excretion into bile relative to its elimination from blood was presented as a ratio of fluorescence intensity values in bile versus whole blood, or in bile versus plasma (Fig. 4). The curves for the 60-min WIT group were distinctly lower than the others, and this difference was more prominent in samples after 1 h of reperfusion (Fig. 4*A*) than after 4 h (Fig. 4*B*).

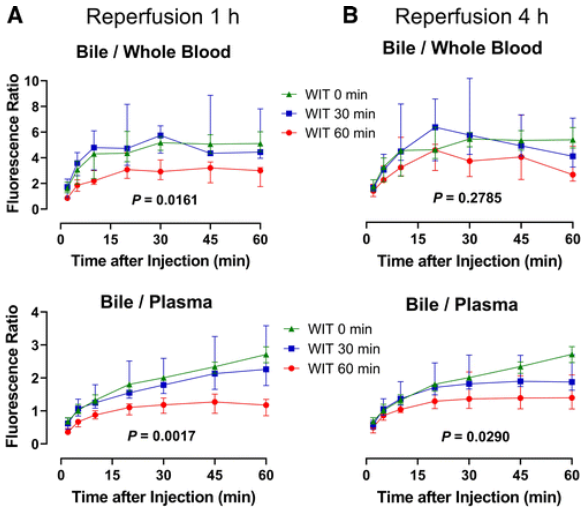

Figure 4. Relative fluorescence intensity values of bile to whole blood or plasma values in three warm ischemia time (WIT) groups; 0 min (control, green triangles), 30 min (blue squares), and 60 min (red circles). Values are presented as medians with interquartile ranges. The effects of WIT were determined by repeated measures ANOVA with the Geisser–Greenhouse correction. *A*: the bile-to-whole blood (*top*) and the bile-to-plasma (*bottom*) fluorescence ratios measured 1 h after reperfusion. *B*: bile-to-whole blood (*top*) and bile-to-plasma (*bottom*) fluorescence ratios measured 4 h after reperfusion. *n* = 5 each.

To test the clinical applicability of these procedures, we tested a very short postreperfusion time of 1 h, as opposed to the 4 h or 24 h that was suggested by previous studies using multiphoton microscopy (16, 17). We anticipated that 1 h may be sufficient to detect differences, since intracellular translocation of MRP2 has been observed within 1 h of reperfusion (25). Surprisingly, as shown in Figs. 3 and 4, the fluorescein kinetics data at this early reperfusion time point were not only significant but also allowed for more precise discrimination between severe IRI (WIT 60 min) and mild IRI (WIT 30 min). This observation is potentially impactful because fast decision making is critical in a clinical setting. However, it should be noted that this study was performed with partial (70%) IRI in normal livers and that whole liver IRI with underlying conditions may show different tissue responses.

Bile-to-Plasma Fluorescence Ratios Are Distinctly Lower in the 60-min WIT Group

To evaluate the ability of the bile-to-plasma fluorescence ratio to distinguish between 60-min WIT samples and others, bile-to-plasma fluorescence ratios obtained at 30, 45, and 60 min after sodium fluorescein injection were presented for each group (Fig. 5*A* for 1-h reperfusion and Fig. 5*B* for 4-h reperfusion). The median values showed a stepwise decrease among the three WIT groups at both 1 h and 4 h after reperfusion. Furthermore, in the 1-h reperfusion condition, when the highest value in the 60-min WIT group was defined as a cut-off for poor excretion of fluorescein, all but one value from the control and 30-min WIT groups were higher than the cut-off (Fig. 5*A*). However, most values from the control and 30-min WIT groups (23 out of 30) overlapped with the range of values from the 60-min WIT group (Fig. 5*B*). Notably, differences among groups were highly significant throughout the monitoring time (i.e., 30, 45, and 60 min) after 1 h of reperfusion. However, they were less significant or insignificant after 4 h of reperfusion. These data indicate that changes in fluorescein clearance in response to mild versus severe IRI are more clearly differentiated when samples are obtained earlier (1 h vs. 4 h postreperfusion).

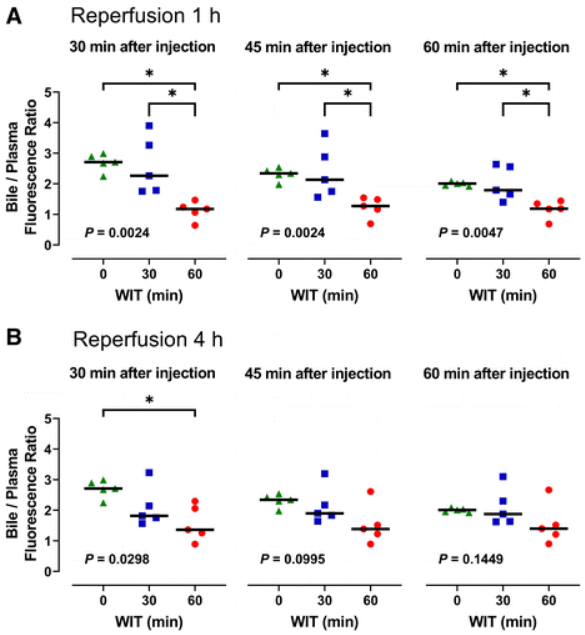

Figure 5. Bile-to-plasma fluorescence ratios in three warm ischemia time groups (green triangles, 0 min; blue squares, 30 min; red circles, 60 min). Data collected at selected time points (30, 45, and 60 min after sodium fluorescein injection) are presented. *A*: data in the control and 1 h postreperfusion groups. *B*: data in the control and 4 h postreperfusion groups. *n* = 5 each. Black bars represent median values. The differences among the groups were evaluated by the Kruskal–Wallis test (*P* values in each panel) and the post hoc Mann–Whitney test with Bonferroni's correction (* between the groups). *n* = 5 each. WIT, warm ischemia time.

DISCUSSION

Bile synthesis has long been considered a liver viability biomarker (4). However, conventional assessment methods, such as measuring bile volume or the levels of endogenous components (i.e., bilirubin and bile acids), are affected by IRI-independent conditions (26). Therefore, the kinetics of an exogenous marker through bile formation machinery may more accurately reflect liver function and viability (10). For example, in patients with bile duct obstruction, biliary indocyanine green excretion correlated with the hepatic ATP levels (27). In this regard, fluorometry is an attractive approach, as portable fluorescence-monitoring devices can be developed as a relatively small and accurate measurement tool to allow for convenient and real-time measurement (28). Intriguingly, sodium fluorescein, an FDA-approved fluorescent dye, is excreted from the blood into the bile through the liver, and like bile synthesis, this process is mediated by hepatocyte membrane transporter proteins (17), namely organic anion-transporting polypeptides 1B1/3 (OATP1B1/3 [OATP1B2 in rats]) on the basolateral membrane and multidrug resistance-associated protein 2 (MRP2) on the canalicular membrane (17). Thus, clearance of sodium fluorescein may objectively reflect the function of hepatocyte membrane transporters, and the kinetics data are presumed to reflect liver viability more precisely than measuring bile amount.

Our data clearly demonstrate that hepatic clearance of fluorescein is suppressed in hepatic IRI. We found that this phenomenon was more prominent when ischemia time was prolonged (60 min vs. 30 min) and when reperfusion time was brief (1 h vs. 4 h). These results imply that a diagnostic system

based on fluorescein clearance could discriminate between livers with extreme injury (equivalent to 60-min WIT) from those with more tolerable injury (equivalent to 30-min WIT). Furthermore, in clinical settings, timely test results are of the utmost importance in making critical decisions. Our data demonstrate that results may be even more accurate when samples are obtained sooner after reperfusion (1 h vs. 4 h), which may facilitate data acquisition in future clinical studies. The immediate effect of IRI on fluorescein clearance at this early time point can be attributed to a unique regulatory mechanism of membrane transporters involving endocytosis-mediated translocation (29, 30). These transporter proteins exist in a recycling pool for rapid mobilization and insertion between the submembrane vesicle and the cell membrane (26, 31, 32). Among the various mechanisms that can regulate the function of transporters, endocytosis-mediated translocation has been suggested as the main contributor to cholestasis in acute stress such as IRI, due to its rapid response to insult (26, 31, 33–40). Although further studies are required, the acute fluorescein clearance observed 1 h after reperfusion can be explained by the internalization of transporters during the early phase of hepatic IRI (25, 32).

As we have shown in this study, coupling data from bile and blood can significantly enhance the accuracy of the test. Nonetheless, the kinetics of fluorescent dyes in bile has not yet gained attention in clinical studies, presumably due to difficulties in obtaining bile samples from patients. However, liver surgery and transplantation practices are evolving, and new paradigms have emerged in recent years. In particular, novel approaches in liver surgery provide an opportunity to assist in making critical decisions regarding surgical commitment. For example, when the risk for posthepatectomy liver failure is high, two-stage hepatectomy [e.g., associating liver partition and portal vein occlusion for staged hepatectomy (ALPPS)] can be considered to promote liver regeneration before the second stage of the surgery. However, it has been reported that the risk of liver failure after the second stage of ALPPS remains high (41). In this context, the patient could be reassessed for tolerability during the first stage of the surgery, and biliary cannulation for sampling could allow for rapid testing of liver function. Another important application for these methods would be in the normothermic machine perfusion system for LT. The traditional static cold storage method for organ preservation does not allow for the monitoring of graft viability, however, normothermic machine perfusion systems simulate physiological conditions that allow the liver to produce bile. It was expected that biochemical assays of perfusate or bile during machine perfusion might predict outcomes after LT (4, 42, 43). However, time required for the biochemical analysis of samples could be an obstacle to rapid evaluation. Moreover, currently available biochemical tests have shown insufficient discriminatory power to predict organ viability for LT (44–47). We believe that our approach can be complementary to current practices and that the normothermic machine perfusion system is ideal for this purpose because it provides a closed circuit, samples from perfusate and bile, and access for administering fluorescein. To this end, future studies using ex vivo machine perfusion will provide critical data on the correlation between the currently available viability indicators such as lactate clearance (44) and the sodium fluorescein clearance kinetics. Importantly, a more accurate characterization of the kinetics of sodium fluorescein on passage through the liver would require additional data including kinetics data with different doses of sodium fluorescein. Furthermore, it is unknown whether the hepatocyte transporter function represented by sodium fluorescein clearance can predict the occurrence of IRI-associated liver damage, such as ischemic cholangiopathy. As such, a comprehensive tool to monitor hepatobiliary function will be required to accurately assess liver viability. Although further studies are required to test the feasibility of this approach, this study is the important first step in developing a real-time spectroscopy system as a liver viability monitoring device.

In summary, we found that sodium fluorescein clearance by the liver from blood into bile could be presented as bile-to-plasma fluorescence ratios, and these values displayed significant differences between short versus long WITs. The possibility of using sodium fluorescein kinetics to differentiate livers with mild hepatic IRI from those with severe IRI is worth investigating in future studies. The bile-to-blood ratio of fluorescence intensity after sodium fluorescein injection has the potential to determine the degree of hepatic IRI. Our data strongly suggest that the measurement of fluorescein kinetics in blood and bile has potential applicability to clinical liver surgery and transplantation.

GRANTS

This study was supported in part by The Kevin T. Cottrell Memorial Fund for Organ Transplantation and The J. Scott Harkness Organ Transplantation Research and Education Fund (to J. C. Hong).

DISCLAIMERS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

J.K. conceived and designed research; Y.Y. performed experiments; J.K. and Y.Y. analyzed data; J.K., Y.Y., S-K.H., J.Z., R.K.D., S.H.A., S.N.K., A.J., and J.C.H. interpreted results of experiments; J.K. and Y.Y. prepared figures; J.K., Y.Y., and S-K.H. drafted manuscript; J.K., Y.Y., R.K.D., S.H.A., M.A.Z., and J.C.H. edited and revised manuscript; J.K., M.A.Z., and J.C.H. approved final version of manuscript.

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