

# Effects of hepatic ischaemia/reperfusion injury in a rabbit model of Indocyanine Green clearance

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## A B S T R A C T

Hepatic ischaemia/reperfusion (I/R) injury is a major cause of primary non-function of the graft after liver transplantation. The ability to assess the severity of ischaemic injury would be of prognostic value and allow the possibility of therapeutic interventions. Currently there is no reliable clinical method for assessing the severity of hepatic ischaemic injury. The hepatic handling of Indocyanine Green as a technique for monitoring the severity of I/R injury has been investigated in the present study. A rabbit model of lobar ischaemia was used. At laparotomy, left lobe hepatic ischaemia was produced for 30, 45 or 60 min, followed by 60 min of reperfusion. Liver function tests, bile excretion and flow in the hepatic microcirculation were measured in animals subjected to I/R injury and in controls. Indocyanine Green was given after reperfusion and its concentration was measured directly in the liver using near-infrared spectroscopy. Indocyanine Green hepatic uptake and excretion rates were calculated. I/R injury produced significant increases in hepatic serum enzymes and decreases in bile excretion and hepatic microcirculation in all I/R groups in comparison with controls. There was a significant reduction in Indocyanine Green uptake and excretion in the I/R groups in comparison with controls, which was correlated with the duration of ischaemia. Indocyanine Green uptake was correlated significantly with flow in the hepatic microcirculation, and its excretion was correlated significantly with the severity of liver damage, as reflected by the changes in serum enzymes and bile excretion. In conclusion, I/R injury affects the hepatic handling of Indocyanine Green, and direct quantification of the uptake and excretion of this dye by near-infrared spectroscopy may be used to objectively assess the degree of I/R injury.

## INTRODUCTION

One of the major problems accompanying liver transplantation is determining the viability of the grafted liver at an early stage. The ability to assess immediate graft function would be a major determinant of graft and patient survival. Many biochemical markers have been

used as indicators of ischaemia/reperfusion (I/R) injury after transplantation, including liver enzyme levels in the effluent perfusate [1], the liver's ability to restore its ATP content [2], the levels of purine compounds in the graft perfusate [3], the rate of bile flow [4] and hepatic protein synthesis [5]. Some of these methods are invasive, requiring a liver biopsy, or need complicated assays and

**Key words:** Indocyanine Green, ischaemia/reperfusion, NIRS methodology, rabbit model.

**Abbreviations:** ALT, alanine aminotransferase; AST, aspartate aminotransferase; HM, hepatic microcirculation; ICG, Indocyanine Green; I/R, ischaemia/reperfusion; LDF, laser Doppler flowmetry; LDH, lactate dehydrogenase; NIRS, near-infrared spectroscopy.

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considerable time to obtain the data. There is no reliable and clinically applicable technique for assessing the degree of hepatic I/R injury.

Near-infrared spectroscopy (NIRS) is a light-based technique that was used originally for the measurement of hepatic tissue oxygenation [6]. Indocyanine Green (ICG) has a characteristic maximum absorption peak at 805 nm in the near-infrared light region, which allows direct measurement of hepatic tissue ICG concentration using NIRS [7]. The rate of uptake of ICG is correlated with hepatic blood flow and flow in the microcirculation, and its rate of excretion correlates with hepatic function and biliary excretion [7,8]. The aim of the present study was to investigate the hepatic handling of ICG following I/R injury.

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## MATERIALS AND METHODS

### Animal preparation and surgical procedure

The study was conducted under a license granted by the U.K. Home Office in accordance with the Animals (Scientific Procedures) Act 1986. New Zealand white rabbits ( $3.6 \pm 0.4$  kg;  $n = 24$ ) were used in this experiment. Anaesthesia was induced by 0.5 ml/kg Hypnorm® (fentanyl citrate and fluanisone; Janssen Animal Health Ltd, High Wycombe, Bucks., U.K.) and 2.5 mg/kg Diazemuls® (diazepam; Dumex Ltd, Tring, Herts., U.K.) intramuscularly, and maintained by halothane (May and Baker Ltd, Dagenham, Essex, U.K.) via a standard anaesthetic circuit. The animals were placed in the supine position on a heating pad (Harvard Apparatus Ltd, Edenbridge, Kent, U.K.) for maintenance of body temperature between 36 and 38 °C. Arterial oxygen saturation and heart rate were monitored continuously by a pulse oximeter (Ohmeda Biox 3740 pulse oximeter; Ohmeda Co., Louisville, KY, U.S.A.). Polyethylene catheters (PE-50; 0.58 mm inner diameter; Portex, Hythe, Kent, U.K.) were inserted into the femoral artery for monitoring of mean arterial blood pressure and for collecting blood samples, and into the femoral vein for saline infusion ( $10 \text{ ml} \cdot \text{h}^{-1} \cdot \text{kg}^{-1}$ ) to compensate for intraoperative fluid loss.

Laparotomy was done via a transverse incision. Ligamentous attachments from the liver to the diaphragm were divided for complete exposure and mobilization of the liver. Complete ischaemia of the median and left lateral lobes of the liver was produced by clamping the left portal vein, hepatic artery and biliary radicles using an atraumatic microvascular clip. This method induces ischaemia in the left and median lobes of the liver (approx. 70% of the liver), while leaving the blood supply to the

right and the caudate lobes uninterrupted [9,10]. At the end of the ischaemic period (30, 45 or 60 min), the vascular clip was removed and reperfusion was allowed for 60 min in all groups. During I/R periods, the animal's abdomen was covered with a plastic wrap to prevent fluid evaporation. At the end of the experiment the liver was taken and weighed, and the animal was killed by exsanguination.

### Measurement of liver function

Arterial blood samples (2 ml each) were used for measurement of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH). The blood volume taken was replaced with an equal volume of normal saline. The blood samples were taken at the beginning of the experiment and again after reperfusion. The measurements were done by a standard spectrophotometric method using an automated clinical chemistry analyser (Hitachi 747; Roche Diagnostics Ltd, Lewes, E. Sussex, U.K.).

### Measurement of bile excretion

The common bile duct was cannulated with a polyethylene catheter (PE-50; 0.58 mm inner diameter; Portex) for the continuous collection of bile. Bile was collected for 15 min before clamping (baseline), at the end of the lobar ischaemia period and after 60 min of reperfusion. Bile volume was expressed as  $\mu\text{l} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$  wet weight of liver.

### Assessment of the hepatic microcirculation (HM)

The HM was measured by surface laser Doppler flowmetry (LDF) (DRT4; Moor Instruments Limited, Axminster, Devon, U.K.), and is given in flux units; one flux unit is defined as the product of the total number of moving red blood cells in the measured volume (a few  $\text{mm}^3$ ) and the mean velocity of the red blood cells. LDF has been shown to be a suitable method for estimation of the HM [11]. The LDF probe was placed on a fixed site of the left lobe of the liver and was held in place by a probe holder. LDF measurements were calculated at baseline (before clamping), at the end of the ischaemia period and at the end of the reperfusion period for each animal, and the decrease in HM after reperfusion was calculated relative to the baseline value. LDF measurements at the relevant time points were collected as means of 1-min data.

### Measurement and analysis of hepatic ICG concentration curve

For measurement of hepatic ICG clearance in all groups, a bolus of 0.5 mg/kg ICG (Cardiogreen; 90% dye content; Sigma, Poole, Dorset, U.K.) was given to all animals. ICG was given 120 min after the start of the experiment in the control group or after 60 min of

reperfusion in the other groups. ICG was dissolved in sterile water (1 mg/ml) and given via the femoral vein over 20 s at the end of the reperfusion period. Hepatic tissue ICG was measured by NIRS (NIRO 500; Hamamatsu Photonics KK, Hamamatsu, Japan). NIRS probes were placed, with a separation of 10 mm, on the surface of the left lobe of the liver for continuous measurement of hepatic tissue ICG for 30 min after ICG injection.

Continuous measurement of hepatic ICG by NIRS produces a concentration–time curve. This curve was analysed to produce two exponential rate constants, representing hepatic ICG uptake from the plasma to the hepatocytes ( $\alpha$ ) and hepatic ICG excretion from the liver due to cytoplasmic transport and biliary excretion ( $\beta$ ). These rate constants were calculated by fitting the ICG concentration–time curve to a two-compartment mathematical model, as defined by the sum of two exponential equations, as reported previously by Shinohara and colleagues [7]:

$$\text{ICG}(t) = -A\exp(-\alpha t) + B\exp(-\beta t)$$

where  $\text{ICG}(t)$  is the hepatic concentration of ICG at any time ( $t$ ), and  $\alpha$  and  $\beta$  ( $\text{min}^{-1}$ ) are the rate constants for hepatic ICG uptake and excretion respectively.  $A$  and  $B$  are the zero-time intercepts, both theoretically equal to the initial hepatic concentration. The assumption is that  $\alpha > \beta$  and  $A \approx B$ . The fitting to this model was done using a commercial computer package based on non-linear least-squares regression (Graph Pad Prism, Graph Pad Software Inc., San Diego, CA, U.S.A.). The iterative procedure of the program minimizes the reduced sum of squares. The goodness of the fit was evaluated by the  $R^2$  value.

### Experimental groups and protocol

Four groups of animals ( $n = 6$  each) were used. Group A contained sham-operated animals (controls). These animals underwent an identical experimental protocol, but without clamping of the hepatic blood vessels. For comparison with the other groups, bile excretion and HM measurements were taken in controls 60 min after the start of the experiment, and compared with the measurements after ischaemia in the other groups. Control measurements were also taken after 120 min, and compared with the post-reperfusion measurements in the other groups. Animals in groups B, C and D underwent ischaemia for 30, 45 and 60 min respectively. A 60 min reperfusion period was allowed in all of these groups.

### Data collection and statistical analysis

Values are expressed as means  $\pm$  S.D. One-way ANOVA and Student's  $t$  test with Bonferroni adjustment for multiple comparisons were used for statistical analysis between the groups.  $P < 0.05$  was considered statistically significant. The relationships between the hepatic ICG

uptake and excretion rates, the HM, serum enzyme levels and bile flow were evaluated using the Pearson correlation coefficient.

## RESULTS

### Haemodynamic parameters

There were no significant differences in heart rate or mean arterial blood pressure between the groups. These parameters did not change significantly throughout the experiment in either the controls or the groups subjected to lobar I/R.

### Hepatocellular injury

In all groups the baseline serum enzyme levels (ALT, AST and LDH) were within the normal range, with no significant differences between the groups (Table 1). After I/R these enzymes were significantly increased in comparison with controls (Table 1). There was no significant difference between groups undergoing 30 min (group B) and 45 min (group C) of ischaemia, but there were significant differences between both of these groups and that subjected to 60 min of ischaemia (group D).

### Bile excretion

In group A, bile volume was  $4.2 \pm 0.8 \mu\text{l} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$  liver weight, which did not change significantly throughout the experiment. In groups B, C and D the baseline bile values were within the normal range, with no significant differences between the groups (Table 2). During ischaemia there was almost no bile excretion. After reperfusion, incomplete recovery of bile excretion occurred, which remained significantly lower than in group A in all I/R groups (Table 2). There was no

**Table 1** Serum ALT, AST and LDH levels

Values are means  $\pm$  S.D. for six animals in each group. The experimental groups were: controls (group A), and 30 (group B), 45 (group C) and 60 (group D) min of ischaemia. Values are those before ischaemia (baseline) and after 60 min of reperfusion in the experimental groups. Significance of differences:  $^{**}P < 0.01$  compared with group A (Student's  $t$  test).

Enzyme	Serum levels (units/litre)			
	Group A	Group B	Group C	Group D
<b>Baseline</b>				
ALT	38 $\pm$ 16	24 $\pm$ 18	25 $\pm$ 21	33 $\pm$ 25
AST	49 $\pm$ 11	39 $\pm$ 29	40 $\pm$ 19	42 $\pm$ 18
LDH	264 $\pm$ 78	260 $\pm$ 69	255 $\pm$ 71	259 $\pm$ 103
<b>Reperfusion</b>				
ALT	36 $\pm$ 13	116 $\pm$ 60 <sup>**</sup>	164 $\pm$ 60 <sup>**</sup>	427 $\pm$ 65 <sup>**</sup>
AST	51 $\pm$ 19	358 $\pm$ 89 <sup>**</sup>	587 $\pm$ 68 <sup>**</sup>	2370 $\pm$ 389 <sup>**</sup>
LDH	269 $\pm$ 48	563 $\pm$ 83 <sup>**</sup>	787 $\pm$ 92 <sup>**</sup>	1400 $\pm$ 177 <sup>**</sup>

**Table 2** Bile volumes

Values are means  $\pm$  S.D. for six animals in each group. The experimental groups were: controls (group A), and 30 (group B), 45 (group C) and 60 (group D) min of ischaemia. Significance of differences: \*\* $P < 0.01$  compared with group A (Student's  $t$  test).

Time	Bile volume ( $\mu\text{l} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ wet liver weight)			
	Group A	Group B	Group C	Group D
Baseline	$4.3 \pm 0.8$	$4.1 \pm 0.6$	$4.2 \pm 0.6$	$3.9 \pm 0.9$
After reperfusion	$4.4 \pm 0.7$	$2.9 \pm 0.4^{**}$	$2.3 \pm 0.4^{**}$	$1.2 \pm 0.5^{**}$

**Table 3** Effects of I/R on the HM

Values are means  $\pm$  S.D. for six animals in each group. The experimental groups were: controls (group A), and 30 (group B), 45 (group C) and 60 (group D) min of ischaemia. Significance of differences: \*\* $P < 0.01$  compared with group A (Student's  $t$  test).

	LDF (flux units)			
	Group A	Group B	Group C	Group D
Baseline	$234 \pm 48$	$225 \pm 35$	$231 \pm 40$	$221 \pm 34$
Ischaemia	$236 \pm 42$	$44 \pm 16^{**}$	$49 \pm 19^{**}$	$36 \pm 18^{**}$
Reduction after reperfusion	$17 \pm 8$	$43 \pm 16^{**}$	$61 \pm 18^{**}$	$96 \pm 19^{**}$

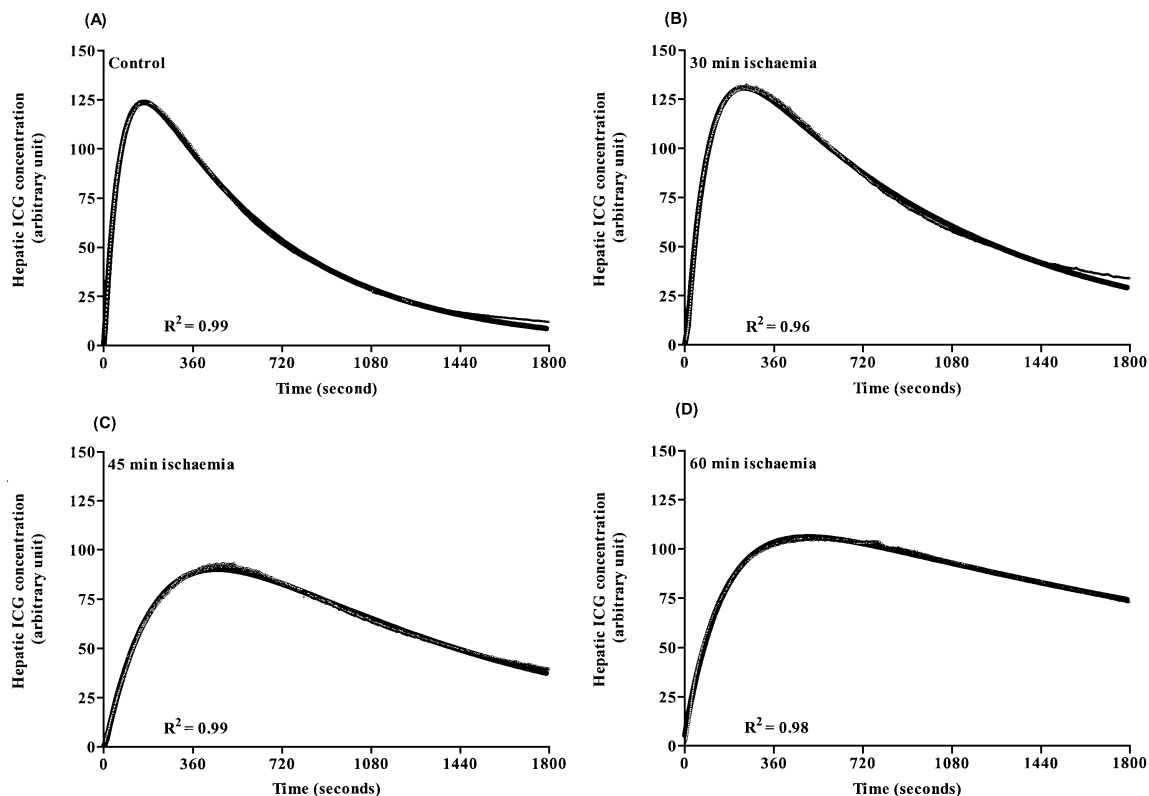
significant difference in bile excretion between groups undergoing 30 min (group B) and 45 min (group C) of ischaemia, whereas there were significant differences between both of these groups and that subjected to 60 min of ischaemia (group D) ( $P < 0.05$  for groups B and C compared with group D).

### HM measured by LDF

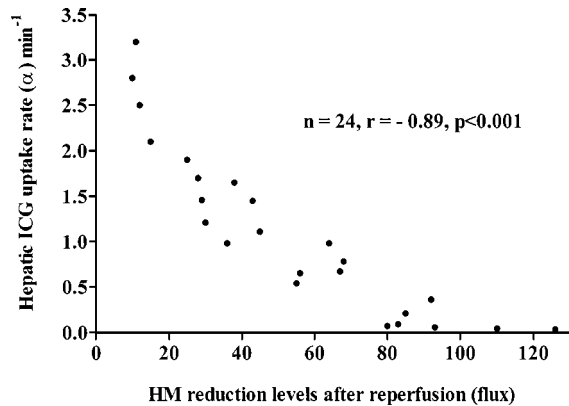
There were no significant differences in baseline HM between the experimental groups (Table 3). During ischaemia, flow in the HM was reduced significantly in all I/R groups, with no significant differences between the groups (Table 3). After reperfusion a significant decrease in the HM occurred in all I/R groups in comparison with controls (Table 3). There was no significant difference between groups undergoing 30 min (group B) and 45 min (group C) of ischaemia, whereas there were significant differences between both of these groups and that subjected to 60 min of ischaemia (group D) ( $P < 0.01$  for groups B and C compared with group D).

### Hepatic ICG clearance measured by NIRS

After reperfusion there was a significant reduction in ICG uptake rate ( $\alpha$ ) from the group A value of  $2.37 \pm 0.57 \text{ min}^{-1}$  to  $1.12 \pm 0.39$ ,  $0.85 \pm 0.40$  and  $0.084 \pm 0.065 \text{ min}^{-1}$  in groups B, C and D respectively ( $P < 0.01$

**Figure 1** ICG concentration–time curves

Shown are typical examples of ICG concentration–time relationships and their fitted curves in the experimental groups: (A) controls; (B–D) after I/R.



**Figure 2** Relationship between the HM and ICG uptake

Shown is the correlation between the rate of uptake of ICG and the decrease in the HM as measured by LDF in controls and after 60 min of reperfusion following 30, 45 or 60 min of lobar ischaemia. Each point represents the change at the end of the reperfusion period in one animal.

for each compared with group A). There was no significant difference between group B and group C, whereas there were significant differences when comparing groups B and C with group D ( $P < 0.05$ ).

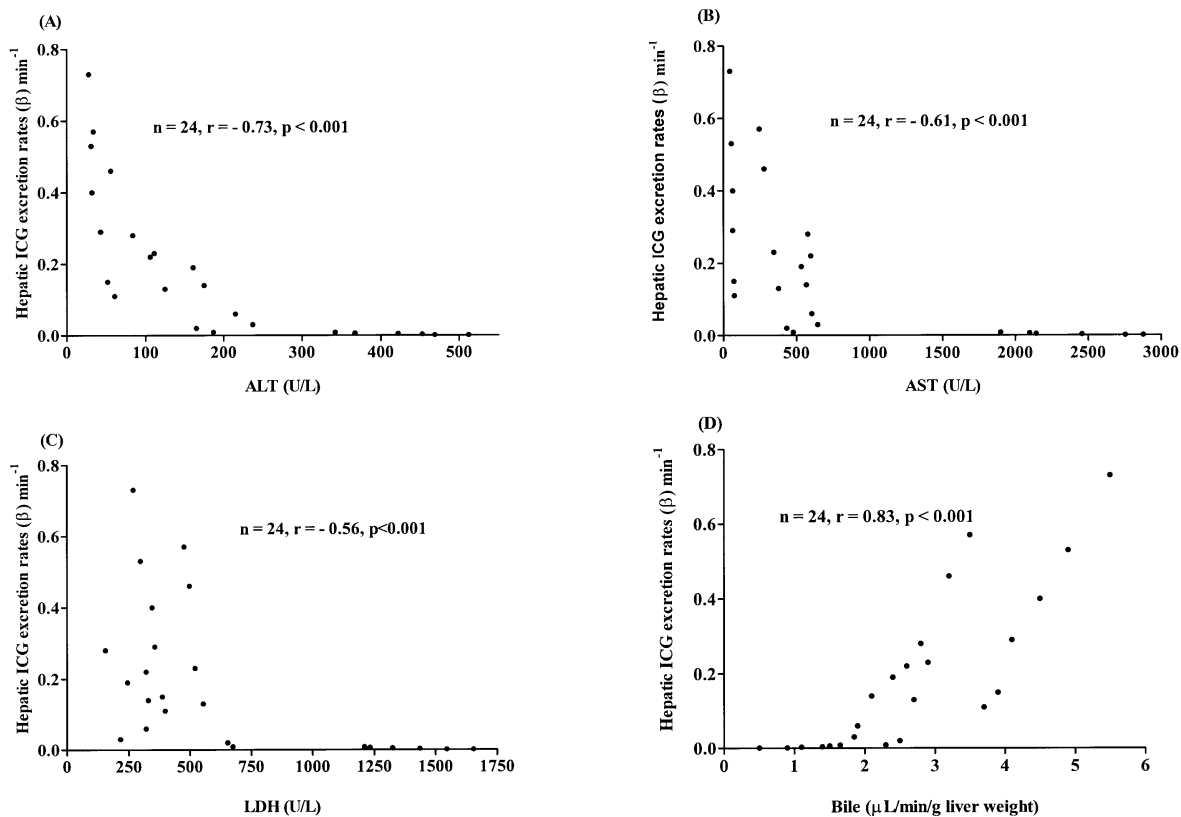
The ICG excretion rate ( $\beta$ ) was reduced from the group A value of  $0.37 \pm 0.24 \text{ min}^{-1}$  to  $0.24 \pm 0.23$ ,  $0.15 \pm 0.09$  and  $0.004 \pm 0.003 \text{ min}^{-1}$  in groups B, C and D respectively ( $P < 0.05$  for each compared with group A). There was no significant difference between group B and group C, but there were significant differences when comparing groups B and C with group D ( $P < 0.05$ ). Examples of ICG clearance curves and their fitting curves are shown in Figure 1.

### Correlation between ICG uptake rate and HM

After reperfusion, the ICG uptake rate was correlated significantly with the decrease in the HM ( $r = -0.89$ ,  $P < 0.001$ ) (Figure 2).

### Correlations of ICG excretion rate with liver enzymes and bile excretion

After reperfusion, the ICG excretion rate was correlated inversely with the levels of ALT, AST and LDH ( $r = -0.73$ ,  $-0.61$  and  $-0.56$  respectively;  $P < 0.001$ ) (Figures 3A–3C). Also, a significant positive correlation ( $r = 0.83$ ,  $P < 0.001$ ) was observed between the ICG excretion rate ( $\beta$ ) and bile excretion (Figure 3D).



**Figure 3** Correlations between ICG excretion and liver function tests

Shown are the correlations between the rate of excretion of ICG and (A) ALT, (B) AST, (C) LDH (all in units/litre) and (D) bile volume in controls and after 60 min of reperfusion following 30, 45 or 60 min of lobar ischaemia. Each point represents the change at the end the reperfusion period in one animal.

## DISCUSSION

This study has investigated a possible role for the quantification of ICG handling by the liver as a method of determining the severity of I/R injury. We have confirmed preliminary findings that ICG uptake and excretion are affected by I/R [7,8], and have demonstrated that both ICG uptake and excretion rates are correlated directly with the severity of warm ischaemia.

Two distinct phases of reperfusion injury have been identified [12,13]. The early phase (< 2 h after reperfusion) is characterized by Kupffer cell-induced oxidative stress [14]. The late phase (> 6 h) is due mainly to the accumulation of neutrophils [15]. Recently, in a rat I/R injury model, a significant increase in hepatocyte apoptosis was demonstrated after 60 min of reperfusion [16]. Furthermore, a blood-oxygenation index measured after 60 min of reperfusion has been significantly correlated with serum ALT levels at 120 min of reperfusion [17]. These data indicate that a decline in hepatic tissue oxygenation during the early phase of reperfusion may be indicative of subsequent liver injury and prognosis. The present study was designed specifically to investigate the correlation between hepatic ICG clearance and hepatocellular function during the *early* stage of I/R injury.

The study involved a rabbit model of lobar I/R. Blood flow to the median and left lateral lobes was interrupted, inducing complete hepatic ischaemia in part of the liver while maintaining normal blood flow to the right and caudate lobes. This model maintains splanchnic blood flow and prevents portal vein stasis and intestinal venous congestion, which can result in portal bacteraemia with subsequent haemodynamic instability [9,10]. The systemic haemodynamic parameters remained stable with this lobar I/R model, which excludes any systemic influence on the extent of the liver injury.

The markers of liver I/R injury used in the present study were the liver function tests and bile excretion. I/R increased serum levels of hepatic enzymes (AST, ALT and LDH) due to rupture of the hepatocyte plasma membrane [18,19]. Bile excretion was also used as an index of I/R injury which reflects changes in the cellular ATP level [20,21]. Bile excretion decreased with I/R injury, and the extent of the decrease was correlated with the duration of ischaemia. This decrease could be secondary to decreased hepatic tissue blood flow and oxygenation [22], a reduction in the cellular ATP level [20,21] or occlusion of the bile canaliculi by cell swelling [19].

Hepatic sinusoidal perfusion failure secondary to I/R has been shown to be a key factor in the pathogenesis of I/R injury [9,23]. Hepatic microvascular flow during I/R was measured by LDF. LDF signals were observed during ischaemia despite total lobar ischaemia. This has been reported in other studies [24,25], and this para-

doxical LDF signal when blood inflow is zero is called 'physiological zero' [26]. This signal is due to the random wandering motion of residual red blood cells and the influence of breathing movements. After reperfusion there was an increase in blood flow in the HM, but it did not return to baseline values and the initial increase was followed by a progressive reduction in the HM. The reduction of flow in the HM after I/R in this experiment was the result of sinusoidal perfusion failure [9,23].

Several mechanisms may contribute to this impairment of sinusoidal perfusion, including sinusoidal endothelial cell swelling with luminal narrowing [25] and sinusoidal vasoconstriction mediated by an altered endothelin/nitric oxide balance [27,28]. The increased expression of adhesion molecules, with the subsequent leucocyte and endothelial cell interactions, may also play a crucial role in this sinusoidal perfusion failure [29].

ICG is a synthetic dye that has been used for many years to measure hepatic blood flow and in tests of liver function [30,31]. In liver transplantation, ICG clearance has been used for evaluation of the donor graft [32]. Measurement of its blood clearance post-transplantation showed a significant correlation between clearance and graft function, as represented by prothrombin time and acidosis [33]. In addition, it has been shown that ICG clearance is correlated with clinical and histological features of acute rejection, as well as the successful treatment of an episode of rejection in human liver transplantation [34].

Recently, Shinohara and co-workers [7] demonstrated that the elimination of ICG by the rabbit liver depends on two exponential rate constants; the first reflects uptake of the dye from plasma to hepatocytes, and the second represents removal of the dye from the liver via cytoplasmic transport and biliary excretion. By direct measurement of ICG in the liver it was possible to distinguish between these two factors [8]. The rate of uptake of ICG was found to correlate with hepatic blood flow and tissue microcirculation, and the rate of its excretion reflected hepatic function and biliary excretion [8].

The application of the direct measurement of hepatic ICG using NIRS to assess the severity of I/R injury has not been studied previously. In the present experiment the rate of ICG uptake decreased with I/R, and the extent of the reduction paralleled the duration of ischaemia. A significant correlation was found between the changes in ICG uptake rate and the reduction in the HM measured by LDF. The decrease in ICG uptake with I/R injury occurs secondary to impairment of sinusoidal perfusion and tissue blood volume, as shown by LDF measurements.

The rate of excretion of ICG decreased with I/R, and the reduction again paralleled the duration of ischaemia. Significant correlations were observed between the rate of ICG excretion and liver enzyme levels and bile excretion, which were used in this experiment as indica-

tors of the severity of tissue injury. I/R injury could reduce the rate of excretion of ICG via several different mechanisms. For example, cellular microtubule damage can lead to the subsequent impairment of ICG removal from hepatocytes into bile canaliculi [35] and hepatocyte necrosis [36], as ICG clearance is correlated significantly with hepatocyte volume, which indicates the hepatic functional reserve [37]. Also, ICG removal can be reduced secondary to a decrease in cellular ATP levels after reperfusion [38,39], resulting in the impairment of bile excretion [39].

The equipment used in this study is relatively expensive, as it is currently used as a research tool. Were the techniques to be adopted in clinical practise, costs would fall rapidly. The technique itself is relatively simple, with optodes being placed on the liver surface and the ICG being administered by intravenous bolus via a peripheral vein.

The present study showed a significant correlation between the ICG uptake rate ( $\alpha$ ) and flow in the HM, and between the ICG excretion rate ( $\beta$ ) and hepatocellular function and bile excretion. Direct measurement of liver ICG handling by NIRS could be used at the time of liver transplantation to assess I/R injury and to predict graft function postoperatively.

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