

Assessment of hepatic ischaemia reperfusion injury by measuring intracellular tissue oxygenation using near infrared spectroscopy

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Abstract: *Aims/Background:* Hepatic ischaemia/reperfusion (I/R) injury is a major cause of liver damage during liver surgery and transplantation. The relationship between the severity of I/R injury and the degree of intracellular hypoxia has not been investigated. *Methods:* New Zealand white rabbits were used in 4 groups ($n=6$ each). At laparotomy, left lobe hepatic ischaemia was produced for 30, 45, or 60 min followed by 60 min reperfusion and compared with controls. Liver function, bile flow, and flow in the hepatic microcirculation (HM) were measured. Near infrared spectroscopy (NIRS) was used to monitor hepatic oxyhaemoglobin (HbO_2), deoxyhaemoglobin (Hb), and cytochrome oxidase (Cyt Ox). *Results:* I/R injury produced deranged liver function tests, reduced bile flow, and reduced flow in the microcirculation in comparison with controls. During ischaemia, HbO_2 and Cyt Ox were significantly reduced in comparison with controls. After reperfusion, a biphasic change in tissue oxygenation was observed, with an initial increase in HbO_2 and Cyt Ox followed by a progressive reduction. The reduction in tissue oxygenation with ischaemia and reperfusion paralleled the ischaemia time. After I/R, the changes in Cyt Ox (intracellular oxygenation) significantly correlated with the parameters of hepatocellular injury to a higher degree than HbO_2 (extracellular oxygenation). *Conclusion:* This study shows the potential of monitoring the degree of I/R injury by measuring hepatic tissue intracellular oxygenation.

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Despite the remarkable progress in liver transplantation, graft and patient loss still occurs as a result of post-transplant complications such as hepatic artery occlusion, portal vein thrombosis, primary non-function, and acute graft rejection (1, 2). Impairment of the liver graft microcirculation and tissue hypoxia are common pathology in all these complications with eventual loss of the graft without early intervention (3). Early detection of this impairment could reduce the overall morbidity and mortality of liver transplantation by allowing earlier treatment. Measurement of hepatic tissue oxygenation has been shown to correlate significantly with the microcirculatory impairment and liver dysfunction induced by I/R injury (4, 5).

Near infrared spectroscopy (NIRS) measures oxyhaemoglobin (HbO_2) and deoxyhaemoglobin

(Hb) as indicators of extracellular tissue oxygenation, and cytochrome oxidase (Cyt Ox) oxidation as an indicator of intracellular tissue oxygenation (6–8). The significance of the changes in Cyt Ox oxidation in relation to ischaemia reperfusion (I/R) injury has not been established. This study investigated the potential of using the changes in Cyt Ox as an indicator of the severity of hepatic I/R injury.

Materials and methods

Animal preparation and surgical procedure

The study was conducted under a licence granted by the Home Office in accordance with the Animals (Scientific Procedures) Act of 1986. New Zealand white rabbits (3.6 ± 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., Buckinghamshire, UK) and 2.5 mg/kg Diazemuls[®] (Diazepam, Dumex Ltd., Hertfordshire, UK) intramuscularly and maintained by halothane (May and Baker Ltd, Dagenham, UK) via a standard anaesthetic circuit. The animal's body temperature was maintained at 36–38 °C using a heating pad (Harvard Apparatus Ltd., Kent, UK). The arterial oxygen saturation and heart rate (HR) was continuously monitored by a pulse oximeter (Ohmeda Biox 3740-pulse oximeter, Ohmeda Co., Louisville, USA). Polyethylene catheters (PE-50, 0.58 mm inner diameter, Portex, Kent, UK) were inserted into the femoral artery to monitor the mean arterial blood pressure (MABP) and for the collection of blood samples, and into the femoral vein for saline infusion (10 ml · kg⁻¹ · h⁻¹) to compensate for intraoperative fluid loss.

Laparotomy was done via a transverse incision. 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 produces ischaemia to the left and median lobes of the liver (about 70% of the liver) while leaving the blood supply to the right and caudate lobes uninterrupted (9, 10). At the end of the ischaemia period (30, 45, or 60 min), the vascular clip was removed and reperfusion was allowed for 60 min in all the groups. During ischaemia and reperfusion periods, the animal's abdomen was covered with a plastic wrap to prevent fluid evaporation. At the end of the experiment, the animal was killed by exsanguination.

Measurement of liver function

Arterial blood samples (2 ml each) were taken for measurement of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactic dehydrogenase (LDH) before clamping (baseline) and after reperfusion. An equal volume of normal saline was used to replace the blood taken. The measurements were done using an automated clinical chemistry analyser (Hitachi 747, Roche Diagnostics Ltd., Sussex, UK).

Measurement of the bile flow

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

Assessment of the hepatic microcirculation

Hepatic microcirculation (HM) was measured continuously by a surface laser Doppler flowmeter (LDF) (DRT4, Moor Instruments Limited, Axminster, UK) in flux unit (11). The LDF probe was placed on a fixed site on the left lobe of the liver and was held in place by a retort holder. LDF measurements were calculated as a mean of 1-min data before clamping (baseline), at the end of ischaemia, and at the end of reperfusion for each animal. The reduction in microcirculation after reperfusion was calculated relative to the baseline.

Measurement of hepatic tissue oxygenation

NIRS (NIRO-500, Hamamatsu Photonics K.K., Hamamatsu, Japan) was used to monitor hepatic tissue oxygenation. An NIRS algorithm was developed to measure continuously hepatic HbO₂, Hb, and Cyt Ox concentration changes in $\mu\text{mol/l}$ (7, 8). NIRS probes were placed at 10-mm intervals on the surface of the left lobe of the liver for continuous measurement of the hepatic tissue oxygenation. A flexible probe holder was used to ensure a satisfactory contact with the liver surface and a fixed inter-probe spacing.

NIRS measurements during ischaemia and reperfusion were expressed relative to baseline before vascular occlusion. For comparison between the different groups, NIRS measurements (at baseline, ischaemia and reperfusion) were calculated as the mean of 1-min measurements at the end of each period.

Experimental groups and protocol

Four groups of animals ($n=6$ each) were used. Group A was 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, measurements taken 60 min from the start of the experiment in controls were compared with the ischaemia measurements in the other groups. Control measurements taken after 120 min were compared with the 60-min post-reperfusion measurements in the other groups. Animals of group B, C, and D underwent ischaemia for 30, 45, and 60 min, respectively. A 60-min reperfusion period was allowed in all the groups.

Data collection and statistical analysis

The values are expressed as mean \pm SD. One-way analysis of variance (ANOVA) and Student's *t*-test with Bonferroni adjustment for multiple compari-

sons were used for statistical analysis between the groups. $p < 0.05$ was considered statistically significant. The relationship between the hepatic tissue oxygenation changes with I/R and hepatic microcirculation, serum enzyme levels, and bile flow was evaluated by the Pearson correlation coefficient.

Results

Haemodynamic parameters

In all animals in the sham-operated control group as well as animals subjected to lobar ischaemia, the heart rate and blood pressure did not change significantly throughout the experiment. Also, there was no significant difference between the I/R groups and controls.

Table 1. Serum ALT, AST and LDH levels (U/l)

Serum enzymes	Group A	Group B	Group C	Group D
ALT (baseline)	38±16	24±18*	25±21*	33±25*
AST (baseline)	49±11	39±29*	40±19*	42±18*
LDH (baseline)	264±78	260±69*	255±71*	259±103*
ALT (reperfusion)	36±13	116±60**	164±60**	427±65**
AST (reperfusion)	51±19	358±89**	587±68**	2370±389**
LDH (reperfusion)	269±48	563±83**	787±92**	1400±177**

Values are mean±SD of 6 animals in each group. The experimental groups are: controls (group A), 30 min (group B), 45 min (group C) and 60 min (group D) ischaemia. * p =non significant and ** $p < 0.01$ vs. group A (Student's t -test).

ALT: alanine aminotransferase. AST: aspartate aminotransferase. LDH: lactic dehydrogenase.

Table 2. Bile volume ($\mu\text{l} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ wet liver weight)

Bile	Group A	Group B	Group C	Group D
Baseline	4.3±0.8	4.1±0.6*	4.2±0.6*	3.9±0.9*
Reperfusion	4.4±0.7	2.9±0.4**	2.3±0.4**	1.2±0.5**

Values are mean±SD of 6 animals in each group. The experimental groups are: controls (group A), 30 min (group B), 45 min (group C) and 60 min (group D) ischaemia. * p =non significant and ** $p < 0.01$ vs. group A (Student's t -test).

Table 3. Hepatic microcirculation in flux unit.

	Group A	Group B	Group C	Group D
Baseline	234±48	225±35*	231±40*	221±34*
Ischaemia	236±42	44±16**	49±19**	36±18**
Reduction after reperfusion	17±8	43±16**	61±18**	96±19**

Values are mean±SD of 6 animals in each group. The experimental groups are: controls (group A), 30 min (group B), 45 min (group C) and 60 min (group D) ischaemia. * p =non significant and ** $p < 0.01$ vs. group A (Student's t -test).

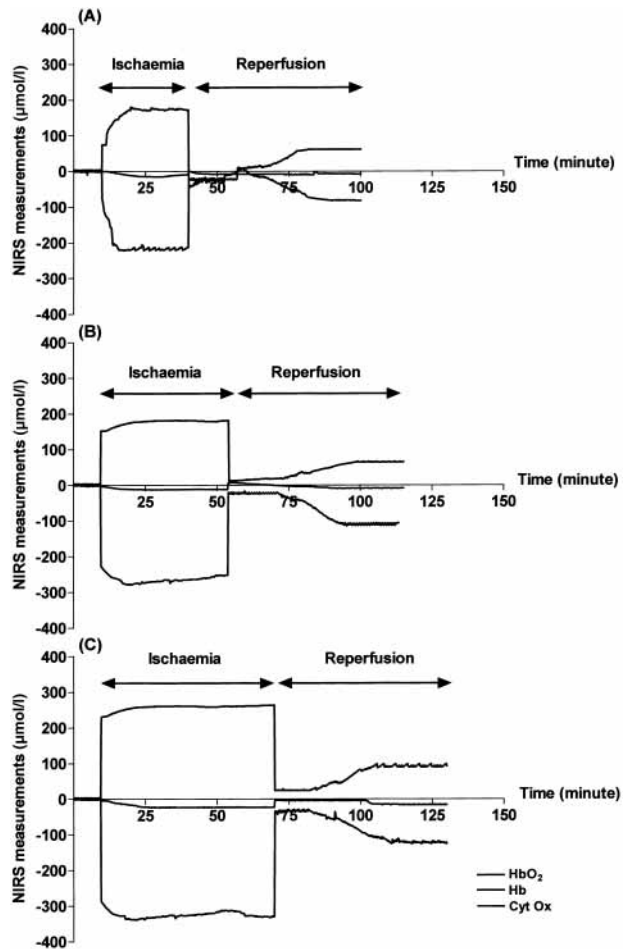


Fig. 1. Typical examples of NIRS measurements with ischaemia reperfusion in (A) 30 min, (b) 45 min, and (C) 60 min of lobar ischaemia followed by 60 min reperfusion in all groups.

Hepatocellular injury

In all groups the baseline serum enzymes (ALT, AST and LDH) were within the normal range, with no significant differences between the groups (Table 1). After I/R they were significantly increased in comparison with controls (Table 1). There was no significant difference in hepatocellular injury as indicated by the serum enzyme levels between 30 and 45 min ischaemia, while enzyme levels were significantly higher with 60 min ischaemia than 30 or 45 min ($p < 0.05$ for groups B and C vs. group D).

Bile flow

In group A, bile flow was $4.2 \pm 0.8 \mu\text{l} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ liver wet weight which did not change significantly through the experiment. There was no significant difference in the baseline bile values between the ischaemia groups and controls (Table 2). During

ischaemia there was almost no bile flow. After reperfusion incomplete recovery of bile flow occurred and it remained significantly less than controls (group A) in all I/R groups (Table 2). There was no significant difference in bile volumes between 30 min (group B) and 45 min ischaemia (group C) but the volume was significantly reduced following 60 min ischaemia in comparison with 30 or 45 min ($p < 0.05$ for groups B and C vs. group D).

Hepatic microcirculation

There was no significant difference in the baseline HM in the experimental groups (Table 3). During lobar ischaemia, HM was significantly reduced in all I/R groups. There was no significant difference between the groups (Table 3). During reperfusion a significant reduction of the HM occurred in all groups in comparison with controls (Table 3). There was no significant difference between 30 min (group B) and 45 min ischaemia (group C), while there was a significant difference between these groups and 60 min ischaemia (group D) ($p < 0.01$ for groups B and C vs. group D).

Hepatic tissue oxygenation

Fig. 1 shows typical examples of hepatic tissue oxygenation changes during and after reperfusion in the experimental groups. With ischaemia, there was an instantaneous significant reduction in HbO₂ and Cyt Ox, which was maximal 5–10 min

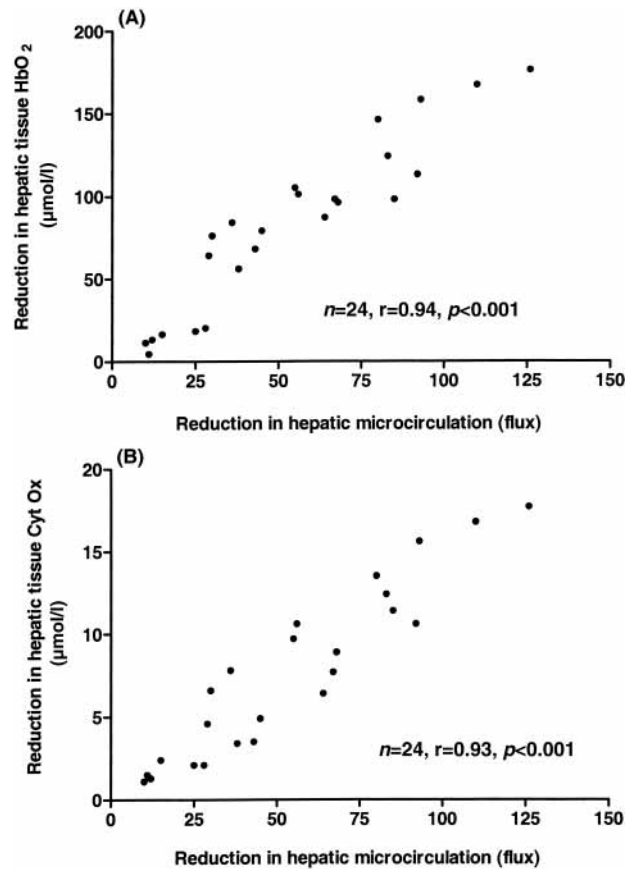


Fig. 2. Correlation between hepatic tissue (A) HbO₂ and (B) Cyt Ox and hepatic microcirculation in the controls and 60 min after reperfusion following 30 min, 45 min, and 60 min of ischaemia. Each point represents the change at the end the reperfusion period in one animal.

Table 4. Hepatic tissue oxygenation (µmol/l) at the end of ischaemia

	Group A	Group B	Group C	Group D
HbO ₂	-4.26±2.4	-223.2±33.9*	-240.5±50.3*	-358.7±39.1*
Hb	4.60±5.1	181.7±24.2*	189.2±42.4*	280.2±30.7*
Cyt Ox	-1.87±1.12	-9.82±2.62**	-11.67±2.27**	-24.53±5.34**

Values are mean±SD of 6 animals in each group. The experimental groups are: controls (group A), 30 min (group B), 45 min (group C) and 60 min (group D) ischaemia. * $p < 0.01$ and ** $p < 0.05$ vs. group A (Student's *t*-test). HbO₂: oxyhaemoglobin. Hb: deoxyhaemoglobin. Cyt Ox: cytochrome oxidase.

Table 5. Hepatic tissue oxygenation (µmol/l) after reperfusion

	Group A	Group B	Group C	Group D
HbO ₂	-14.0±5.7	-80.5±17.1*	-91.3±16.7*	-144.8±29.2*
Hb	9.4±4.5	59.2±22.8*	68.33±18.0*	103.7±20.1*
Cyt Ox	-1.75±0.52	-6.98±2.69**	-7.13±3.11**	-14.56±4.52**

Values are mean±SD of 6 animals in each group. The experimental groups are: controls (group A), 30 min (group B), 45 min (group C) and 60 min (group D) ischaemia. * $p < 0.01$ and ** $p < 0.05$ vs. group A (Student's *t*-test). HbO₂: oxyhaemoglobin. Hb: deoxyhaemoglobin. Cyt Ox: cytochrome oxidase.

after induction of ischaemia. Thereafter, these parameters did not change significantly until the end of ischaemia (Table 4 and Fig. 1). Hb changed in a similar pattern but in the opposite direction (Table 4 and Fig. 1). After reperfusion, the changes in tissue oxygenation occurred in two phases. Firstly, there was a significant increase in HbO₂ and Cyt Ox. However, these parameters did not return to the levels before ischaemia. After 20–30 min of reperfusion, these parameters then underwent progressive reduction to the end of the reperfusion period (Table 5 and Fig. 1). The change in Hb followed a similar pattern but in the opposite direction (Table 5 and Fig. 1).

For all the tissue oxygenation changes, there was no significant difference between 30 and 45 min ischaemia, while there was a significant change between these groups and 60 min ischaemia ($p < 0.05$ for groups B and C vs. group D).

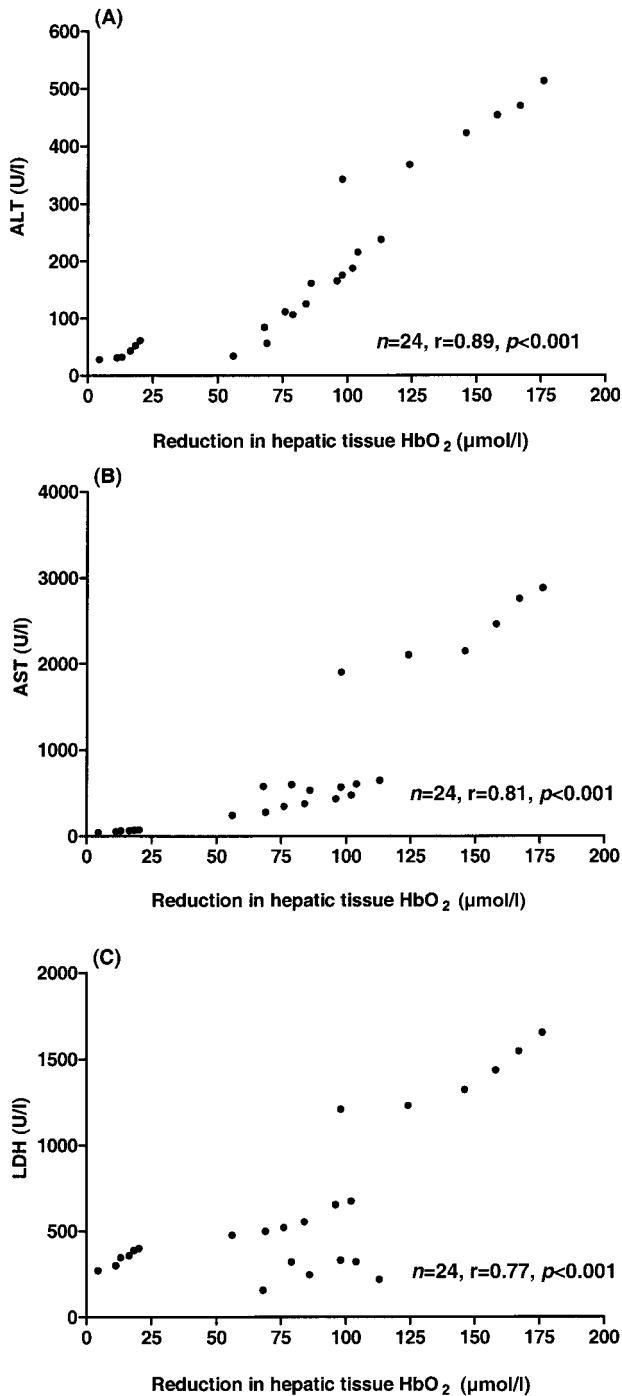


Fig. 3. Correlation between hepatic tissue HbO₂ and the serum enzymes (A) ALT, (B) AST, and (C) LDH in the controls and 60 min after reperfusion following 30 min, 45 min, and 60 min of ischaemia. Each point represents the change at the end of the reperfusion period in one animal.

Correlation of HbO₂ with HM, liver function tests, and bile volume

After reperfusion HbO₂ correlated significantly with HM changes ($r=0.94$, $p<0.001$; Fig. 2A).

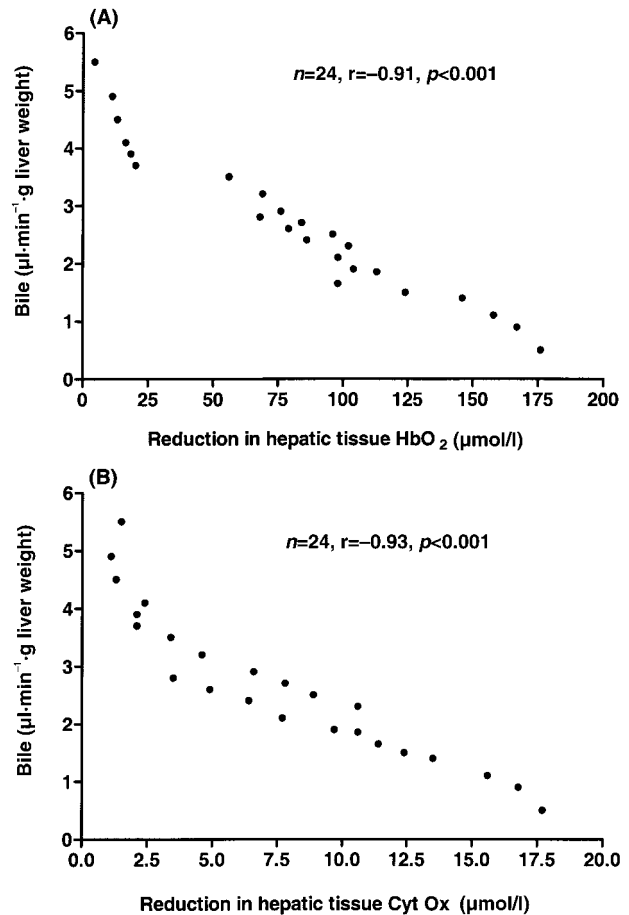


Fig. 4. Correlation between hepatic tissue (A) HbO₂ and (B) Cyt Ox and the bile volume in the controls and 60 min after reperfusion following 30 min, 45 min, and 60 min of ischaemia. Each point represents the change at the end of the reperfusion period in one animal.

HbO₂ correlated significantly with serum ALT, AST, and LDH ($r=0.89$, 0.81 , and 0.77 ; respectively, $p<0.001$) (Fig. 3). Also, a significant negative correlation was shown between HbO₂ and the bile volume ($r=0.91$, $p<0.001$) (Fig. 4A).

Correlation of Cyt Ox with HM, liver function tests, and bile volume

After reperfusion Cyt Ox correlated significantly with HM ($r=0.93$, $p<0.001$; Fig. 2B). Cyt Ox also correlated significantly with serum ALT, AST, and LDH ($r=0.97$, 0.90 , and 0.85 ; respectively, $p<0.001$) (Fig. 5). Also, a significant negative correlation was observed between Cyt Ox and the bile volume ($r=0.93$, $p<0.001$) (Fig. 4B).

Discussion

This study involved a rabbit model of lobar I/R. Blood flow to the median and left lateral lobes was

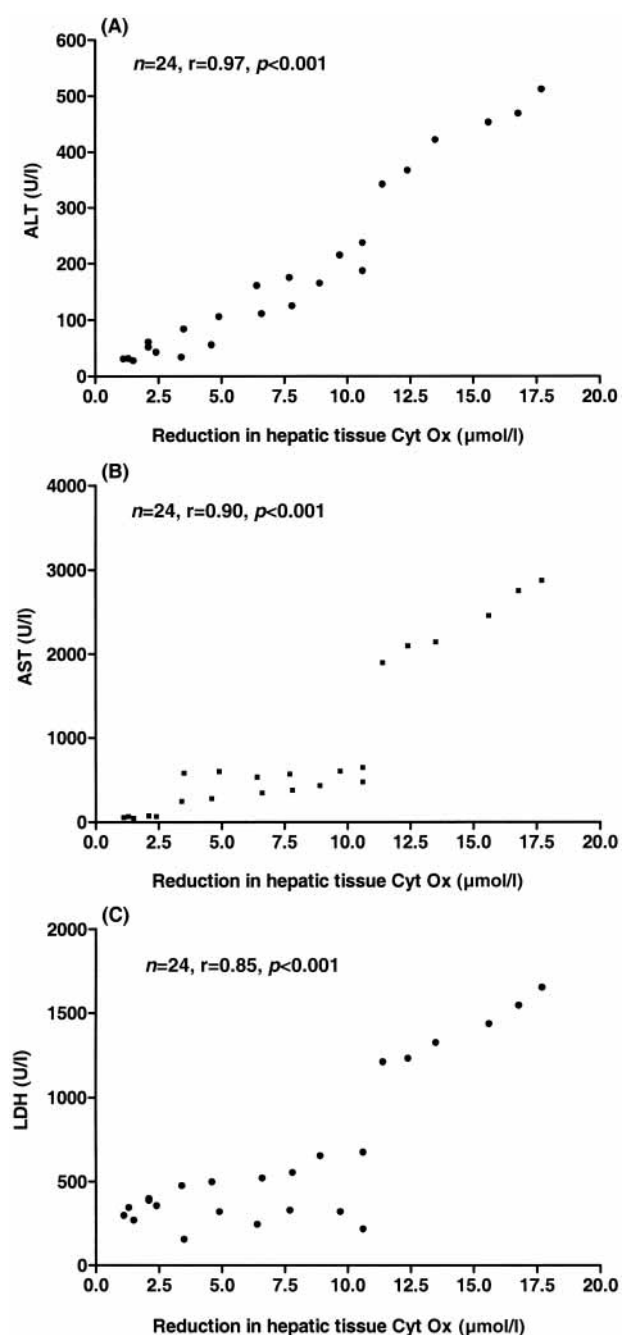


Fig. 5. Correlation between hepatic tissue Cyt Ox and the serum enzymes (A) ALT, (B) AST, and (C) LDH in the controls and 60 min after reperfusion following 30 min, 45 min, and 60 min of ischaemia. Each point represents the change at the end of the reperfusion period in one animal.

interrupted, inducing partial complete hepatic ischaemia while maintaining normal blood flow to the right and caudate lobes. This maintains splanchnic blood flow and prevents portal vein stasis and intestinal venous congestion, which results in portal bacteraemia with subsequent haemodynamic instability (9, 10).

The systemic haemodynamic parameters were not significantly changed with this lobar I/R model, which excludes any systemic influence on the extent of the liver injury.

I/R caused an increase in the serum enzyme levels (AST, ALT, and LDH) that correlated with the ischaemia time. This increase in serum enzyme levels is due to rupture of the plasma membrane with leakage of the cellular enzymes into the circulation (12). The enzyme levels are known to relate to the degree of cell injury (12).

In this study, bile flow was used as an index of I/R injury and to reflect changes in cellular ATP level (13). Reduction of the bile flow occurred with I/R injury and correlated with the ischaemia time. This reduction of bile flow could be due to decreased hepatic tissue blood flow and oxygenation (14), reduction of cellular ATP level (as bile production involves ATP-consuming steps (13)) or occlusion of the bile canaliculi by cell swelling (15).

Hepatic sinusoidal perfusion failure secondary to I/R has been shown to be a key factor in the pathogenesis of I/R injury (9, 16). HM changes following I/R were measured by LDF, which has been applied and validated in many studies (17, 18). LDF signal was observed during ischaemia despite total lobar ischaemia. This has been reported in other studies and can be caused by a random wandering motion of residual red blood cells and the influence of breathing movements (17, 18). After reperfusion there was an increase in blood flow in the microcirculation but it did not return to baseline level and was followed by progressive reduction in the microcirculation.

NIRS was used to monitor hepatic tissue oxygenation changes following I/R. This technique has been extensively investigated for monitoring hepatic tissue oxygenation in both small and large animal models. It is reliable and the measurements are reproducible (7, 8). In NIRS readings, measurements of liver tissue oxygenation are directly related to hepatic vein blood oxygenation (19). With ischaemia, there was a reduction in HbO_2 and Cyt Ox, indicating extracellular and intracellular tissue oxygenation, respectively. These changes reflect reduced blood and oxygen supply to the tissue. There was a simultaneous increase in Hb due to tissue extraction of oxygen from haemoglobin.

After reperfusion, two phases were recorded. At first, the return of blood and oxygen increases HbO_2 and Cyt Ox, with concomitant reduction of Hb. None of these parameters returned to the baseline before ischaemia. After about 20–30 min of reperfusion, the second phase occurred with progressive reduction of tissue oxygenation (HbO_2 and Cyt Ox) with simultaneous increase of Hb.

The reduction of flow in the microcirculation

and tissue oxygenation after I/R in this experiment results from sinusoidal perfusion failure after I/R (9, 20).

Several mechanisms may contribute to this impairment of the sinusoidal perfusion, including sinusoidal endothelial cell swelling with luminal narrowing (18), sinusoidal vasoconstriction mediated by altered endothelin/nitric oxide balance (21, 22), and increase expression of adhesion molecules (23).

The biphasic change in the microcirculation and tissue oxygenation observed in this experiment reflects the time between the start of reperfusion and the occurrence of sinusoidal flow impairment with about 50% of the sinusoids showing cessation of blood flow at 18 min (9). This biphasic change after reperfusion has been shown in other studies with rat I/R (4, 24).

A significant correlation was found between the tissue oxygenation parameters (HbO₂ and Cyt Ox) measured by NIRS and the HM measured by LDF, which supports the value of NIRS in monitoring hepatic tissue perfusion. A significant correlation was also observed between tissue oxygenation parameters and indicators of the severity of I/R injury, including serum hepatic enzymes and bile flow. This reinforces the central role of tissue oxygenation in I/R injury and the potential of tissue oxygenation monitoring for assessing the severity of I/R injury.

Cyt Ox is the terminal electron carrier of the mitochondrial respiratory chain that catalyses the reduction of oxygen to H₂O in a four-electron reaction with concomitant synthesis of adenosine triphosphate through the oxidative phosphorylation process (25). In hepatocytes, about 90% of the oxygen taken up is consumed by cytochrome oxidase in the mitochondria (26). Thus, assessment of the redox state of cytochrome oxidase could be used as an indicator of the intracellular oxygen availability and cellular adenosine triphosphate (ATP) level (27, 28). Currently, techniques such as enzymatic assays (29), chromatography (30), and ³¹P NMR (31) are being used to monitor ATP as an indicator of cellular ischaemia/reperfusion injury. These methods are either invasive, requiring tissue biopsy, or involve a complicated procedure and time for processing of data, which make them unsuitable for routine clinical use. Cytochrome oxidase is the terminal enzyme in respiratory chain, which leads to the formation of the ATP. Assessment of changes in cytochrome oxidase reflects the changes in ATP and a significant correlation between these two has been demonstrated (32). The role of Cyt Ox in monitoring I/R injury has not been studied before. In this experiment Cyt Ox measurement correlated with parameters of

hepatocellular injury and bile flow to a higher degree than HbO₂ measurements. Cyt Ox reflects intracellular tissue oxygenation and correlates directly with ATP production, which is even more fundamental to cell membrane integrity and bile production than extracellular oxygenation.

This study shows the potential of NIRS, a non-invasive method, to assess the hepatic tissue intracellular oxygenation and as an indicator of the severity of I/R injury.

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