Decreased hepatosplanchnic antioxidant uptake during hepatic ischaemia/reperfusion in patients undergoing liver resection

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ABSTRACT

Oxidative stress mediates cell injury during ischaemia/reperfusion. On the other hand, experimental findings suggest that ROS (reactive oxygen species) induce processes leading to ischaemic preconditioning. The extent and source of oxidative stress and its effect on antioxidant status in the human liver during intermittent ischaemia and reperfusion remains ill-defined. Therefore the aim of the present study was to investigate the occurrence of oxidative stress in humans undergoing liver resection. Liver biopsies, and arterial and hepatic venous blood samples were taken from ten patients undergoing hepatectomy with an intermittent Pringle manoeuvre. Plasma MDA (malondialdehyde) and hepatic GSSG levels were measured as markers of oxidative stress and plasma uric acid as a marker of xanthine oxidase activity. In addition, changes in hepatosplanchnic consumption of plasma antioxidants and hepatic levels of carotenoids and glutathione (GSH) were measured. After ischaemia, hepatosplanchnic release of MDA and increased hepatic GSSG levels were found. This was accompanied by the release of uric acid, reflecting xanthine oxidase activity. During reperfusion, ongoing oxidative stress was observed by further increases in hepatic GSSG content and hepatosplanchnic MDA release. Uric acid release was minimal during reperfusion. A gradual decrease in plasma antioxidant capacity and net hepatosplanchnic antioxidant uptake was observed upon prolonged cumulative ischaemia. Oxidative stress occurs during hepatic ischaemia in man mainly due to xanthine oxidase activity. Interestingly, the gradual decline in plasma antioxidant capacity and net hepatosplanchnic antioxidant uptake during prolonged cumulative ischaemia, preserved both hydrophilic and lipophilic hepatic antioxidant levels. Decreasing plasma levels and net hepatosplanchnic uptake of plasma antioxidants may warrant antioxidant supplementation, although it should be clarified to what extent limitation of oxidative stress compromises ROS-dependent pathways of ischaemic preconditioning.

Key words: antioxidant, ischaemia/reperfusion, liver, oxidative stress, Pringle manoeuvre, reactive oxygen species (ROS).
Abbreviations: ABTS, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid); BHT, butylated hydroxytoluene; CI, confidence interval; MDA, malondialdehyde; ROS, reactive oxygen species; TBA, thiobarbituric acid; TEAC, trolox equivalent antioxidant capacity.
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INTRODUCTION

Minimization of blood loss during liver resection is essential to limit complications. To diminish blood loss during liver resection, hepatic inflow occlusion or the ‘Pringle manoeuvre’ was introduced by J. H. Pringle in 1908 [1]. However, the Pringle manoeuvre leads to hepatic ischaemia/reperfusion injury. Ischaemia is characterized by ATP depletion and necrotic cell death [2], with subsequent reperfusion aggravating cell injury. An important factor in this process is the formation of ROS (reactive oxygen species) immediately after reperfusion [3,4]. This leads to the peroxidation of membrane phospholipids [5] and oxidative damage to intracellular proteins [4].Classically, the generation of ROS has been ascribed to an increased conversion of xanthine dehydrogenase into xanthine oxidase during ischaemia [6], but concerns about the validity of this concept have been raised [3,7]. Attention has moved to the mitochondrial respiratory chain as the principal source of ROS during ischaemia and reperfusion [3,8], although the hypothesis that xanthine oxidase activity is an important source of ROS formation during hepatic ischaemia/reperfusion is still debated [9].

ROS oxidize glutathione (GSH) to GSSG [10], which renders cells susceptible to cell death [11–13]. Endogenous and exogenous antioxidants offer protection against the action of ROS, consequently antioxidants may become depleted during excessive oxidative stress. Most results on hepatic ischaemia/reperfusion damage are derived from animal studies, which generally rely on prolonged continuous ischaemia to render maximal effects. The severity of these models complicates immediate translation of these results to human clinical practice. Although ROS formation is generally associated with cell death, as outlined above, findings suggest that ROS are important signalling molecules that activate transcription factors, such as HIF-1α (hypoxia-inducible factor-1α), which mediate HSP (heat-shock protein) expression and other processes leading to ischaemic preconditioning [14].

Only a few studies are currently available concerning the occurrence and extent of oxidative stress during short-term intermittent warm hepatic ischaemia and reperfusion in clinical practice. Therefore the aim of the present study was to investigate the occurrence of oxidative stress in humans undergoing liver resection with an intermittent Pringle manoeuvre. Emphasis was placed on sources of ROS formation and on the changes in exogenous and endogenous antioxidant status.

MATERIALS AND METHODS

Patients

Ten patients (six male and four female) undergoing partial hepatectomy for secondary liver malignancies [colorectal cancer (n = 9) and insulinoma (n = 1)] were studied after an overnight fast. All patients had normal liver function as assessed by pre-operative laboratory parameters, and were on their standard diets in the pre-study period.

Patient characteristics are summarized in Table 1.

![Table 1: Characteristics of the patients enrolled in the study (n = 10)](https://example.com/table1.png)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Median (range)</th>
<th>Reference value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/female (n)</td>
<td>6/4*</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>58 (52–71)</td>
<td></td>
</tr>
<tr>
<td>Aspartate aminotransferase (IU/l)</td>
<td>21 (6–35)</td>
<td>&lt; 35</td>
</tr>
<tr>
<td>Bilirubin (μmol/l)</td>
<td>11.8 (7.3–17.6)</td>
<td>2.0–17</td>
</tr>
<tr>
<td>Prothrombin time (s)</td>
<td>10.4 (9.8–11.9)</td>
<td>9.9–11.5</td>
</tr>
<tr>
<td>Creatinine (μmol/l)</td>
<td>80 (67–116)</td>
<td>53–110</td>
</tr>
<tr>
<td>Urea (mmol/l)</td>
<td>4.5 (2.3–6.6)</td>
<td>3.0–7.0</td>
</tr>
</tbody>
</table>

The protocol has been reviewed and approved by the Medical Ethical Committee of the University Hospital Maastricht, and all subjects provided written informed consent. The study adhered to the Declaration of Helsinki.

Anaesthesia and the surgical procedure

All patients had radial artery and central venous catheters inserted pre-operatively to monitor arterial and central venous BP (blood pressure) as part of standard anaesthetic care. Surgical procedures were performed as described previously [15]. Liver surgery was performed using a Cavitron Ultrasonic Surgical Aspirator (Valleylab; Tyco Healthcare) and electrocautery or ligation of vessels as appropriate.

Intermittent Pringle manoeuvre

During liver transection, an intermittent Pringle manoeuvre was applied by tightening a rubber tube around the entire hepatoduodenal ligament (total Pringle manoeuvre) or selectively around the left or right portal vein and hepatic artery, as considered appropriate for the intended resection (selective Pringle manoeuvre, n = 4). At least two cycles of 15 min of occlusion of the portal vein and hepatic artery and 5 min of reperfusion were required to complete hepatic transection.

Sample collection

Using the same approach we have described previously [16], liver biopsies and blood samples were taken prior to and following ischaemia and after reperfusion, according to the schedule shown in Figure 1. Liver tissue samples (approx. 200 mg) were excised with a scalpel from non-tumorous regions of the liver, distant from the transection plane. Simultaneously, blood was drawn from the arterial line and from a hepatic vein that was draining the (post-)ischaemic part of the liver, allowing the measurement...
of arteriovenous differences across the hepatosplanchnic bed. Post-ischaemia blood samples were taken immediately after hepatic blood flow was restored.

Sample processing
Blood was collected into EDTA-containing tubes (Vacutainer; Becton Dickinson). All blood samples were immediately put on ice, and kept on ice during sample preparation. Plasma was obtained by centrifugation (800 g for 10 min at 4 °C) and stored at −80 °C until analysis. Liver biopsies were divided in two pieces and rapidly frozen in liquid nitrogen. Prior to analyses, liver biopsies were homogenized in MilliQ water (150 mg of tissue/ml).

Laboratory analyses
Chemicals
ABTS [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)], uric acid and BHT (butylated hydroxytoluene) were obtained from Sigma. ABAP [2,2′-azinobis(2-amidinopropane)dihydrochloride] was obtained from Brunschwig Chemicals. All chemicals were of analytical purity.

MDA (malonaldehyde) assay
The assay is based on the formation of a coloured adduct of MDA-like breakdown products of lipids with 2-TBA (thiobarbituric acid) [17,18]. Plasma (100 μl) was added to 1 ml of reagent [containing 12 mmol/l TBA, 0.32 mol/l o-phosphoric acid, 0.68 mmol/l BHT and 0.01 % EDTA], and the mixture was incubated for 1 h at 100 °C in a water bath. After cooling, the TBA product was extracted with 500 μl of butanol. A portion (30 μl) of the butanol layer was injected on to an HPLC system (Agilent) equipped with a fluorescence detector, set at an excitation wavelength of 532 nm and emission wavelength of 553 nm, and a Nucleosil C18 column (150 mm × 3.2 mm; particle size, 5 μm; Supelco). Samples were eluted with 35 % (v/v) methanol containing 0.05 % trifluoric acid. A calibration curve was constructed using MDA bis(diethylacetal) as a standard.

Uric acid assay
Uric acid, the reaction product of xanthine oxidase activity, was determined using an HPLC method as described by Lux et al. [19].

Plasma antioxidant capacity assay
Plasma antioxidant capacity was assayed using the method described by Fischer et al. [20]. Briefly, 950 μl of ABTS** radical solution was incubated for 1 min at 37 °C, thereafter 50 μl of deproteinized plasma was added. After incubation for 5 min, the absorption at 734 nm was measured. The decrease in absorption after 5 min relative to the blank (buffer alone) was related to that of trolox calibrators. The resulting value is expressed as TEAC (trolox equivalent antioxidant capacity). As TEAC is partly determined by the uric acid concentration, the raw data from this assay were corrected for the uric acid content of the sample (plasma antioxidant capacity = TEAC − uric acid concentration).

Hepatic GSH and GSSG content
GSH and GSSG concentrations were measured in the supernatant of homogenized liver biopsies, using the method described by Anderson [21]. Total protein concentrations in the homogenates were measured using the BCA (bicinchoninic acid) method as described by Smith et al. [22]. The final concentrations of GSH and GSSG were expressed per mg of protein.

Hepatic levels of carotenoids and α-tocopherol
Analysis of the concentrations of the different carotenoids and tocopherols in the supernatant of homogenized liver biopsies was performed as described by Broekmans et al. [23]. Liver biopsies were homogenized in MilliQ water (150 mg of tissue/ml). Final concentrations of carotenoids and α-tocopherol were expressed per mg of protein.

Statistics
Values are expressed as means ± S.E.M. Overall changes in plasma and tissue levels during the experiment were assessed by one-way ANOVA with Bonferroni post-testing comparing values at each time point with baseline values.

For arteriovenous gradients, a theoretical time point with an arteriovenous gradient of zero was included in the ANOVA, and Bonferroni post-testing was performed for values at each time point compared with baseline as well as compared with this theoretical zero gradient. This approach is in fact a modification of the one-sample Student’s t test that calculates whether the mean of a study population significantly differs from a theoretical value [24] and is frequently used in organ balance studies [25]. This approach allows the simultaneous calculation of the difference in arteriovenous gradients at multiple time points compared with both baseline and zero using
Figure 2 Systemic MDA levels (a) and the hepatosplanchnic gradients of MDA (b) in ten patients undergoing liver resection with intermittent Pringle manoeuvre
(a) No significant changes in systemic plasma levels were observed. (b) The assessment of arteriovenous MDA concentration gradients revealed a significant increase in hepatosplanchnic lipid peroxidation during an intermittent Pringle manoeuvre, particularly after the second period of 15 min of ischaemia. ∗P < 0.05 compared with baseline; #P < 0.05 compared with zero time (one-way ANOVA with Bonferroni’s post-test for multiple comparisons). isch, ischaemia; rep, reperfusion.

Figure 3 Systemic levels of the xanthine oxidase reaction product uric acid (a) and the hepatosplanchnic gradients of uric acid (b) in ten patients undergoing liver resection with intermittent Pringle manoeuvre
(a) Systemic uric acid levels increased after 15 min of hepatic ischaemia. (b) At baseline uric acid release was not significantly different from zero. Arteriovenous concentration gradients revealed significant changes in hepatosplanchnic uric acid release after hepatic ischaemia. ∗P < 0.05 compared with baseline; #P < 0.05 compared with zero time (one-way ANOVA with Bonferroni’s post-test for multiple comparisons). isch, ischaemia; rep, reperfusion.

RESULTS

MDA in plasma
The mean arterial plasma level of MDA was 1.2 ± 0.1 μmol/l. This systemic level did not change significantly during the intermittent Pringle manoeuvre (Figure 2a). In contrast, the hepatosplanchnic MDA gradient had already tended to increase after 15 min ischaemia [95% CI (confidence interval) of the mean increase compared with baseline, −0.10 to 0.26 μmol/l, as determined using Bonferroni’s test], indicating the occurrence of oxidative stress and lipid peroxidation during ischaemia. After a cumulative ischaemia time of 30 min, hepatosplanchnic MDA release tended to remain elevated above baseline values (95% CI of the mean increase compared with baseline, −0.05 to 0.31 μmol/l, as determined using Bonferroni’s test) during the second reperfusion period (Figure 2b).

Hepatosplanchnic uric acid release
Arterial plasma level of uric acid (250 ± 16 μmol/l at baseline) increased significantly after 15 min of hepatic ischaemia (Figure 3a). Hepatosplanchnic uric acid release, indicative of xanthine oxidase activity, was not significantly different from zero at baseline, but increased significantly following both 15 min periods of hepatic ischaemia. Upon reperfusion, hepatosplanchnic uric acid release returned to baseline values (Figure 3b).

Plasma antioxidant capacity during intermittent Pringle manoeuvre
A gradual decrease in plasma antioxidant capacity (TEAC—uric acid concentration) was observed during the experiment (Figure 4a). At baseline, there was a
Figure 4  Systemic levels of plasma antioxidant capacity (a) and hepatosplanchnic net balance of plasma antioxidant capacity (b) in ten patients undergoing liver resection with intermittent Pringle manoeuvre

Plasma antioxidant capacity was determined by TEAC—uric acid concentration, as described in the Materials and methods section. (a) During the study a gradual decline in systemic antioxidant capacity was observed. (b) Hepatosplanchnic net balance of plasma antioxidant capacity showed a gradual decrease in net hepatosplanchnic antioxidant uptake during prolonged cumulative ischaemia/reperfusion. *P < 0.05 compared with baseline, #P < 0.05 compared with zero time (one-way ANOVA with Bonferroni’s post-test for multiple comparisons).

GSH and GSSG during intermittent hepatic ischaemia/reperfusion

Hepatic tissue GSH content increased significantly during hepatic inflow occlusion and returned to baseline values during reperfusion (Figure 5a), but no evidence of GSH depletion was found. In addition, hepatic GSSG content increased significantly after 15 min of ischaemia, but tended to keep increasing after 5 min of reperfusion (95% CI of the mean increase compared with baseline, −0.03 to 0.74 μmol/g of protein, as determined using Bonferroni’s test). Thereafter, a rapid decrease in hepatic GSSG content was found, resulting in a return to baseline values (Figure 5b). The GSSG:GSH ratio, indicative of cellular redox state, tended to increase transiently during the study (Figure 5c).

Carotenoids and α-tocopherol

Lycopene and β-carotene were the most abundant carotenoids in the liver (Table 2). When expressed per
Table 2  Hepatic carotenoids and α-tocopherol content during intermittent hepatic ischaemia/reperfusion
Values are means ± S.E.M.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>First cycle</th>
<th></th>
<th>Second cycle</th>
<th></th>
<th></th>
<th>One-way ANOVA</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>15 min of ischaemia</td>
<td>5 min of reperfusion</td>
<td>15 min of ischaemia</td>
<td>5 min of reperfusion</td>
<td></td>
</tr>
<tr>
<td>β-Carotene (nmol/g of protein)</td>
<td>16.6 ± 2.8</td>
<td>18.2 ± 2.6</td>
<td>15.0 ± 3.3</td>
<td>19.7 ± 5.2</td>
<td>18.1 ± 4.2</td>
<td>0.729</td>
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<tr>
<td>Lycopene (nmol/g of protein)</td>
<td>61.8 ± 20.5</td>
<td>70.2 ± 23.3</td>
<td>62.3 ± 23.5</td>
<td>58.1 ± 20.1</td>
<td>65.6 ± 21.9</td>
<td>0.738</td>
</tr>
<tr>
<td>Lutein (nmol/g of protein)</td>
<td>4.2 ± 0.6</td>
<td>4.7 ± 0.7</td>
<td>4.3 ± 0.6</td>
<td>4.5 ± 0.6</td>
<td>4.5 ± 0.6</td>
<td>0.488</td>
</tr>
<tr>
<td>Zeaxanthin (nmol/g of protein)</td>
<td>1.7 ± 0.3</td>
<td>1.9 ± 0.3</td>
<td>1.7 ± 0.4</td>
<td>1.8 ± 0.4</td>
<td>1.7 ± 0.3</td>
<td>0.532</td>
</tr>
<tr>
<td>Cryptoxanthin (nmol/g of protein)</td>
<td>4.5 ± 0.5</td>
<td>5.2 ± 0.6</td>
<td>4.6 ± 0.7</td>
<td>4.6 ± 0.8</td>
<td>5.1 ± 0.9</td>
<td>0.045</td>
</tr>
<tr>
<td>α-Tocopherol (nmol/g of protein)</td>
<td>0.43 ± 0.04</td>
<td>0.50 ± 0.06</td>
<td>0.48 ± 0.07</td>
<td>0.47 ± 0.08</td>
<td>0.46 ± 0.06</td>
<td>0.379</td>
</tr>
</tbody>
</table>

Table 2

Hepatic carotenoids and α-tocopherol content during intermittent hepatic ischaemia/reperfusion

DISCUSSION

To our knowledge, the present study is the first to meticulously describe the development of oxidative stress during hepatic ischaemia and reperfusion and its effect on antioxidant status in humans. During the Pringle manoeuvre, the hepatoduodenal ligament is temporarily ligated, resulting in total obstruction of both portal venous and hepatic arterial inflow and in an obstruction of biliary outflow. As pointed out in the Introduction, human studies on this subject are scarce and are limited to the description of changes in systemic plasma markers of oxidative stress. In these studies, no changes in MDA and GSH levels could be detected in peripheral blood [28]. In the present study, by measuring transorgan gradients and by taking liver biopsies, however, we have observed a profound effect of ischaemia and reperfusion on hepatic oxidative stress and antioxidant status. No differences were observed between cases where a selective or complete hepatic inflow occlusion was used. In addition, it may be expected that the intermittent hepatic inflow occlusion provided some ischaemic preconditioning during the second period of ischaemia [16]. This may hamper translation of the present findings to situations where a similar cumulative ischaemic period is applied in a continuous fashion. Most probably, prolonged ischaemia exaggerates oxidative stress upon reperfusion [5].

Lipid peroxidation, as shown by MDA release, already occurred during ischaemia within 15 min of hepatic inflow occlusion. Moreover, we simultaneously observed a significant increase in hepatosplanchnic release of uric acid. Uric acid is generated upon oxidation of xanthine by xanthine oxidase, yielding equimolar amounts of uric acid and superoxide anion radicals [6]. These results suggest that oxidative stress and lipid peroxidation occurs within 15 min of hepatic ischaemia in vivo in humans and that xanthine oxidase forms a significant source of ROS during ischaemia. The occurrence of lipid peroxidation and xanthine oxidase activity during ischaemia may appear paradoxical as these processes are considered to depend on the presence of molecular oxygen; however, hypoxic ROS generation has been observed previously in vitro in the heart [29], lungs [30] and murine macrophages [31]. Alternatively, it has been suggested that retrograde perfusion from the hepatic veins sustains some blood and oxygen supply to the liver during the Pringle manoeuvre [32]. In vivo studies in rats have also shown progressive lipid peroxidation during continuous ischaemia for up to 120 min [5]. Hepatosplanchnic MDA and uric acid release ceased within 5 min of reperfusion following the initial 15 min of hepatic ischaemia, suggesting that xanthine oxidase activity during reperfusion is limited. During reperfusion, however, a marked increase in the oxidation of GSH to GSSG was observed, as shown by increasing GSSG levels and an increased GSSG:GSH ratio. These results suggest ongoing or additional oxidative stress which is probably ascribed to ROS-generating systems other than xanthine oxidase, presumably the mitochondrial respiratory chain [3,8].

After a second period of 15 min of hepatic ischaemia (again leading to significant release of uric acid and MDA), MDA was persistently released during reperfusion, reflecting a sustained lipid peroxidation within the hepatosplanchnic area following a cumulative ischaemic period of 30 min. In contrast, uric acid release rapidly returned to baseline values during reperfusion. Thus our results consistently suggest that xanthine oxidase may particularly be involved in ROS generation during ischaemia. Hepatic GSSG content returned to baseline values during the second cycle of the intermittent Pringle manoeuvre. A possible explanation for this somewhat surprising observation is an increased activity of the enzyme glutathione...
reductase. An increased efflux of GSSG during oxidative stress has been demonstrated in rats [33]; however, hepatocellular GSSG efflux will probably not decrease the total hepatic GSSG content during ischaemia, as the absence of blood and bile flow prohibits the drainage of GSSG accumulated in these extracellular fluids.

Subsequently, we studied the consequences of the intermittent Pringle manoeuvre on some important antioxidants, i.e. GSH, plasma antioxidants and hepatic carotenoids and α-tocopherol. An additionally unexpected finding was the increase in hepatic GSH content during ischaemia. As hepatic GSH content decreased rapidly to normal values during reperfusion, this transient increase in GSH content during inflow occlusion is probably ascribed to the accumulation of constitutively synthesized GSH during inflow occlusion, due to the absence of hepatic blood flow and the consequent absence of washout, which is restored upon restoration of blood and bile flow. Our results provide no evidence for the occurrence of hepatic GSH depletion during the intermittent Pringle manoeuvre. Important lipophilic antioxidants are carotenoids and α-tocopherol. Carotenoids are derived from vegetable intake and are stored intracellularly. Consistent with findings on hepatic (hydrophilic) GSH levels, no time-dependent effects of intermittent Pringle manoeuvre on hepatic levels of lipophilic carotenoids and α-tocopherol were observed. A substantial amount of the body’s antioxidant system is present in plasma as, among others, glutathione, albumin, bilirubin and ascorbic acid [34]. Upon progressive ischaemia, systemic plasma antioxidant capacity gradually declined (approx. 10% after 30 min of ischaemia and 10 min of reperfusion). At baseline, there was a negative hepatoplaschnic balance of plasma antioxidant capacity, indicating hepatoplaschnic uptake of antioxidants from the plasma. Simultaneously with decreasing plasma antioxidant capacity, this net uptake declined and ultimately disappeared throughout the present study. One may speculate that this decreasing hepatoplaschnic net uptake of hydrophilic antioxidants is causally related to the equally diminishing arterial antioxidant concentration.

When considering the effects of ischaemia and reperfusion on ROS formation and antioxidant status, it must be realized that ROS also are important signalling molecules. Consistent with this, previous findings suggest that oxidative stress may lead to a rapid induction of cytoprotective pathways during intermittent Pringle manoeuvre and ischaemic preconditioning [14]. Therefore, in future studies on antioxidant supplementation during liver ischaemia, changes in the induction and expression of cytoprotective mechanisms should be assessed as well as changes in hepatocellular integrity and liver function.

In conclusion, short-term hepatic ischaemia and reperfusion in humans leads to xanthine oxidase activity and lipid peroxidation within 15 min. Prolonged cumulative ischaemia/reperfusion injury leads to sustained oxidative stress, demonstrated by lipid peroxidation, and to systemic antioxidant depletion. Interestingly, the gradual decline observed in plasma antioxidant capacity and net hepatoplaschnic antioxidant uptake during prolonged cumulative ischaemia did preserve both hydrophilic and lipophilic hepatic antioxidant levels. Decreasing plasma levels and net hepatoplaschnic uptake of plasma antioxidants may warrant antioxidant supplementation when cumulative ischaemia time exceeds 30 min, although potential deleterious effects of antioxidant supplementation on ischaemic preconditioning should not be disregarded.

ACKNOWLEDGMENTS

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