Methylene Blue Protects Liver Oxidative Capacity after Gut Ischaemia—Reperfusion in the Rat

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WHAT THIS PAPER ADDS?
This experimental study is the first to our knowledge to show that early impairment of liver mitochondrial respiration after gut ischaemia/reperfusion (IR) is related to blood lactate levels and that methylene blue (MB) injection restores liver mitochondria oxidative capacity after gut IR. This observation could have clinical implications in situations such as gut IR during vascular surgery. If injected before vascular unclamping, MB might protect liver mitochondria function and minimise the incidence of postoperative multiple organ dysfunction. MB is already used in many indications (e.g., methaemoglobinaemia) and has been proposed as treatment in vasoplegic shock during sepsis, cardiopulmonary bypass surgery and liver transplantation.

Objectives: Mesenteric ischaemia/reperfusion (IR) may lead to liver mitochondrial dysfunction and multiple organ failure. We determined whether gut IR induces early impairment of liver mitochondrial oxidative activity and whether methylene blue (MB) might afford protection.

Design: Controlled animal study.

Materials and methods: Rats were randomised into three groups: controls (n = 18), gut IR group (mesenteric ischaemia (60 min)/reperfusion (60 min)) (n = 18) and gut IR + MB group (15 mg kg⁻¹ MB intra-peritoneally) (n = 16). Study parameters were: serum liver function markers, blood lactate, standard histology and DNA fragmentation (apoptosis) on intestinal and liver tissue, maximal oxidative capacity of liver mitochondria (state 3) and activity of complexes II, III and IV of the respiratory chain measured using a Clark oxygen electrode.

Results: Gut IR increased lactate dehydrogenase (+982%), aspartate and alanine aminotransferases (+43% and +74%, respectively) and lactate levels (+271%). It induced segmental loss of intestinal villi and cryptic apoptosis. It reduced liver state 3 respiration by 30% from 50.1 ± 3 to 35.2 ± 3.5 µM O₂ min⁻¹ g⁻¹ (P < 0.01) and the activity of complexes II, III and IV of the mitochondrial respiratory chain. Early impairment of liver mitochondrial respiration was related to blood lactate levels (r² = 0.45). MB restored liver mitochondrial function.

Conclusions: MB protected against gut IR-induced liver mitochondria dysfunction.

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Mortality and morbidity rates from acute mesenteric ischaemia following impairment of mesenteric circulation are significant and have changed little over the past decade.¹⁻³ Gut reperfusion leads to the production of numerous mediators such as reactive oxygen metabolites, pro-inflammatory cytokines and nitric monoxide and also activates multiple enzymes.⁴ Interactions between polymorphonuclear neutrophils and endothelial cells would be instrumental in inducing secondary, inflammatory injury to distant organs. This would be the leading cause of death in critically ill patients.⁵

The gut—liver axis is thought to play a key role in the deleterious effects induced by gut ischaemia—reperfusion
Anaesthesia was induced and maintained with IP ketamine. Experimental procedures
reperfusion.

Although gut IR is not characterised by early histological liver lesions, a subcellular approach might nevertheless throw light on a role of the liver in the deleterious effects induced by gut IR. The study of liver mitochondria, in particular, might be useful as mitochondria are the main energy source in cells and convert nutrients into energy through cellular respiration. Impaired mitochondrial function is strongly involved in IR physiopathology.

Methylene blue (MB) is a candidate substance for reducing the effects of gut IR on remote organs such as the liver. It is an inexpensive drug used to treat diseases such as methaemoglobinemia and Alzheimer’s and has been proposed for use in vasopлегic shock during sepsis, cardiopulmonary bypass surgery and liver transplantation. The only histological study of MB action on the intestine after gut IR has shown no alleviation of IR-related intestinal lesions. However, MB is known to enhance key biochemical pathways in mitochondria, and cycling between oxidised and reduced MB forms might block oxidant production. The aim of this study was to determine, first, whether gut IR induces early impairment of liver mitochondrial function and, second, whether MB might reduce such impairment.

MATERIALS AND METHODS

Experimental design

The study was performed in adult male Wistar rats (330–380 g) (Depré, Saint Doulchard, France). The rats were housed at a temperature of 22 ± 2 °C, kept on a 12:12-h photoperiod, and provided with food and water ad libitum. Procedures were conducted in accordance with the institutional guidelines for the care and use of laboratory animals and the study was approved by the institutional animal care committee of the University of Strasbourg (CREMEAS authorisation N° AL/03/11/06/09).

Fifty-four rats were randomised into three groups: (i) a gut IR group (n = 18) which underwent gut ischaemia (60 min) and reperfusion (60 min) by superior mesenteric artery (SMA) clamping/unclamping and received 2 ml saline serum intra-peritoneally (IP) 10 min before reperfusion; (ii) a gut IR + MB group (n = 16), treated as the gut IR group but with 15 mg kg−1 MB diluted in saline serum before injection; (iii) a control group (C, n = 18) which underwent anaesthesia and sham surgery and received 2 ml of saline serum at a time-point corresponding to 10 min before reperfusion.

Experimental procedures

Anaesthesia was induced and maintained with IP ketamine (Imalgène 1000°, Merial, France). The rats were placed in a supine position on heating pads to maintain body temperature at 37 °C (rectal temperature probe) and kept under spontaneous ventilation during the experimental procedure. After a midline abdominal incision, the SMA was isolated at its origin and occluded with an atraumatic clamp for 60 min. This was followed by reperfusion for 60 min. Gut IR was confirmed by complete pulse cessation and restoration in the mesenteric arcades and by a change in intestinal colour. Control animals underwent the same protocol but their SMAs were not occluded.

All tests were performed at the end of reperfusion (treated groups) or an equivalent time (controls).

Circulating liver function markers and blood lactate

Blood obtained by abdominal aorta puncture was centrifuged (3500 g for 15 min) to obtain serum. Serum markers of liver insult (lactate dehydrogenase (LDH), aspartate and alanine aminotransferases (AST and ALT)) and markers of cholestasis (bilirubin and alkaline phosphatase (ALP)) were measured on a Synchron IR (Beckman-Coulter, Fullerton, CA, USA) after calibration according to the International Federation of Clinical Chemistry recommendations. Blood lactate levels were measured by a micromethod on a Lactate Pro device (LT1710, Arkray, KGK, Japan).

Standard gut and liver histology and DNA fragmentation (TUNEL) assay

Fragments of the last ileal loop and liver segment VI (eight samples) were placed into a buffered formol solution immediately after harvesting, then processed, embedded in paraffin and sectioned (3–5 μm). A pathologist blinded to the study protocol analysed the sections under a light microscope.

For standard histology, tissue sections were stained with haematoxylin–eosin and intestinal tissue injury was graded according to the Chiu/Park scale: Grade 0, normal mucosa; Grade 1, development of a subepithelial space at the tips of the villi; Grade 2, more extensive subepithelial space than in Grade 1; Grade 3, massive epithelial lifting down the sides of the villi; Grade 4, villi denuded of epithelium; Grade 5, loss of villi; Grade 6, crypt layer injury; Grade 7, transmucosal injury; and Grade 8, transmural infarction.

In the TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) assay, after paraffin removal, the gut and liver tissue sections from eight samples were rehydrated in a series of xylene, ethanol and distilled water baths, rinsed in phosphate buffered saline (PBS) 1×, treated with proteinase K (20 μg ml−1) (SIGMA, France) for 15 min at 37 °C, and then incubated for 1 h at 37 °C with a mixture containing deoxynucleotidyltransferase terminal enzyme (In Situ Cell Death Detection Kit, POD, Roche Diagnostics, Penzberg, Germany). Incorporated fluorescein was detected by anti-fluorescein antibody Fab fragments from sheep, conjugated with horseradish peroxidase (POD) and incubated for 30 min at 37 °C.

Lastly, tissue sections were exposed for 10 min to a peroxidase substrate, rinsed in PBS 1×, and counterstained with Mayer’s Hemalum (Merck, France). Cells with brown-coloured nuclei were considered to be apoptotic.

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Isolation of mitochondria and mitochondrial respiratory chain complex analysis

Liver mitochondria were isolated on ice from 8 (IR + MB group) or 10 (control and gut IR groups) animals (Fig. 1). Tissue was placed in isolation buffer A containing 70 mM sucrose, 210 mM mannitol, 1 mM ethylene glycol tetra-acetic acid (EGTA) in 50 mM Tris/HCl pH 7.4. The tissue was finely minced with scissors and then homogenised in the same buffer using a Potter Elvejem. The homogenate was centrifuged at 3000 rpm for 3 min at 4 °C and the resultant supernatant at 10,000 rpm for 10 min at 4 °C. The mitochondrial pellet was washed in isolation buffer B containing 70 mM sucrose and 210 mM mannitol in 50 mM Tris/HCl pH 7.4 before being re-suspended in this buffer. Aliquots were then removed for protein measurement by the Bradford method (Biomate 3, Thermo Fisher Scientific, Inc.).

Mitochondrial respiration was measured (oxygen consumption) by methods adapted from a previously described procedure using a Clark oxygen electrode (Strathkelvin Instruments Limited, North Lanarkshire, Scotland) in a 3 ml volume chamber at 25 °C with continuous stirring of the respiratory medium (100 mM KCl, 50 mM Mops, 1 mM EGTA, 5 mM Kpi, 0.1% fatty acid-free bovine serum albumin (BSA) at pH 7.4). Basal oxygen consumption (‘state 4’) was measured after addition of glutamate—malate as substrate. Maximal respiration rate (‘state 3’) was measured after addition of adenosine diphosphate (ADP), which causes a sudden burst of oxygen uptake as the diphosphate (ADP) is converted into the triphosphate (ATP).

The relative contribution of respiratory chain complexes to the global mitochondrial respiratory rate was measured by adding specific blockers and activators. During state 3, electrons flow through each of the complexes I, III and IV. Complex I was blocked with amytal (0.02 mM) and complex II was stimulated with succinate (25 mM). Under these conditions, oxygen consumption reflects the activity of complexes II, III and IV (state 3). Then, the artificial electron donor N,N,N’,N’-tetramethyl-p-phenylenediaminedihydrochloride (TMPD) and ascorbate (0.5 mM), which maintains TMPD in a reduced state, were added. As reduced TMPD donates electrons to cytochrome c, this enabled determination of the activity of cytochrome c oxidase (complex IV) alone (state 3).

Statistical analysis

Results were expressed as means ± standard error of the mean (SEM). Groups were compared by one-way analysis of variance (ANOVA) and the Tukey post hoc test. The relationship between lactate and liver mitochondria parameters was tested by Pearson correlation and regression analysis. p-values <0.05 were considered significant. Statistical analysis was performed using GraphPad Prism software (version 5.03 for Windows) (GraphPad Software, San Diego, CA, USA).

RESULTS

Liver function tests and blood lactate levels

Gut IR induced liver injury as evidenced by a significant increase in LDH (+982%, 235 ± 41 vs. 2308 ± 530 UI l⁻¹; P < 0.05), AST (+43%, 86 ± 10 vs. 199 ± 20 UI l⁻¹; p < 0.05) and ALT (+74%, 43 ± 1 vs. 158 ± 21 UI l⁻¹; P < 0.05) (Fig. 2). Injury was not associated with cholestasis as there was no significant change in conjugated bilirubin (1.4 ± 0.2 vs. 1.9 ± 0.2 µmol l⁻¹) or in ALP (126 ± 22 vs. 132 ± 20 µkat l⁻¹).

![Figure 1. Isolation of liver mitochondria and substrates used to measure respiratory chain activity (modified from Charles et al.33).](image-url)
MB did not impact on the results of these liver function tests.

Blood lactate level was significantly higher in the IR and IR + MB groups (3.3 ± 1 and 3.8 ± 1.9 mmol l⁻¹ respectively) than in controls (1.4 ± 04 mmol l⁻¹) (P < 0.001). The difference in level between the IR and IR + MB groups was not significant (p = 0.58).

Histology and DNA fragmentation

The intestine of control rats was normal with no signs of ischaemic injury and presented incidental apoptotic cells which were generally found among the epithelial cells of the villi tips (Fig. 3). Gut IR induced focal injuries ranging in grade from 0 (normal mucosa) to 5 (loss of villi) and an increase in DNA fragmentation in intact mucosal structures, especially in the crypt layer. The extent of focal injury and apoptosis was similar in the IR and IR + MB groups.

The livers of the control and the IR groups showed normal architecture and cellularity on histological examination, with no signs of ischaemia or inflammation in the IR group, and rare (<1%) apoptotic hepatocytes in the DNA fragmentation assay. Histology and DNA fragmentation results did not differ in the IR and IR + MB groups (normal in both).

Liver mitochondrial function

Significant impairment of liver mitochondrial oxidative capacity occurred after gut IR (Table 1, Fig. 4). Basal mitochondrial respiration (state 4) decreased by 28%, maximal oxidative capacity (state 3) by 30%, complex II, III and IV activity (state 3₃+TMPD) by 29%. MB treatment restored all these activities (Table 1, Fig. 4).

Relationship between gut IR injury and liver mitochondrial impairment

To steer clear of any possible interference by MB, the relationship between liver mitochondrial impairment and gut insult, as assessed by blood lactate, was investigated by combining data for the control and IR groups only. Liver mitochondrial respiration was significantly and inversely correlated with lactate level for state 3 (60 ± 5.1 − (0.11 × lactates) µM O₂ min⁻¹ g⁻¹, r² = 0.45, P = 0.001) (Fig. 5), state 3₃+succ (r² = 0.54, P = 0.002) and state 3₃+TMPD (r² = 0.45, P = 0.001).

DISCUSSION

In our study, gut IR was characterised by villi necrosis, crypt apoptosis and increased blood lactate. Gut IR significantly decreased the activity of each complex of the liver mitochondrial respiratory chain but these decreases were counteracted by pharmacological post-conditioning with MB.

Intestinal histology after gut IR revealed typical focal gut mucosal necrosis without transmucosal infarction ranging in grade from 0 (normal) to 5 (loss of villi) within a sample. In addition, as already reported, the mucosal cells of the crypt layer were subject to DNA fragmentation processes. These histology and biology data thus confirm that our gut IR model induced moderate yet significant impairment as can be observed clinically in non-lethal gut IR.

Gut IR increased transaminases and LDH, a marker of ischaemic damage, but did not affect serum bilirubin nor...
Figure 3. Gut histology and apoptosis. Representative small intestines from rats: (A–C) Haematoxylin–eosin stained sections (×10). (A) Controls: normal mucosa (grade 0); (B) IR: loss of villi (grade 5); (C) IR + MB: as in B. (A′–C′) Apoptotic cells in DNA fragmentation assay (see arrows) (×20). (A′) Controls: few apoptotic cells; (B′) IR: apoptosis in all intact villi, particularly in crypt layer; (C′) IR + MB: as in B′.
Means are given with ±SEM. *Tukey post-hoc test.

did induce liver lesions, suggesting early liver insult. About 80% of patients with ischaemic injury have substantially elevated LDH (ALT/LDH ratio <1) yet low serum bilirubin.

Liver subcellular dysfunction may precede histological lesions after gut IR. In our study, gut IR significantly decreased liver maximal oxidative capacity and also impaired the activity of complexes II, III and IV of the liver mitochondrial respiratory chain. These results support data obtained by indirect assessment of mitochondrial function. Poggetti et al. demonstrated gut IR-induced impairment of the liver mitochondrial redox state by using serum acetocetate/3-hydroxybutyrate levels as indicator. Horie et al. found that gut IR caused liver cell hypoxia and mitochondrial impairment by using autofluorescence of a hepatic pyridine nucleotide (reduced nicotinamide adenine dinucleotide phosphate, NADH) as an indicator of mitochondrial O₂ consumption and redox state. Increased lactate may also signal liver mitochondrial dysfunction. In our study, blood lactate was inversely correlated with liver maximal oxidative capacity (r² = 0.45). In humans, splanchnic hypoperfusion is correlated with hepatic cellular damage. As decreased oxidative capacity is generally associated with reduced ATP production and subsequent organ dysfunction, impairment of liver mitochondrial respiration during gut IR might contribute towards a poor prognosis and warrant effective treatment.

We confirmed that MB does not alleviate IR-induced intestinal lesions. However, MB can prevent haemodynamic instability, base deficit and lung injury after gut or liver IR. It attenuates liver damage in extrahepatic cholestasis and protects against different types of liver injury.

We observed no decrease in transaminases on MB injection, maybe because we chose a moderate dose (15 mg kg⁻¹) with known benefits and no major adverse effects. MB doses of 2 and 60 mg kg⁻¹ had no beneficial effects in a similar rat model of gut IR whereas 6 and 20 mg kg⁻¹ reduced haemodynamic deterioration and metabolic acidosis. During vasoplegic shock, patients typically receive an intravenous (IV) bolus of 1–2 mg kg⁻¹ MB. A decrease in transaminases might have been missed because reperfusion time was too short (1 h) with regard to transaminase kinetics in hepatic disease which is apparently slow. Alternatively, MB was perhaps just unable to normalise transaminases. It should be stressed, however, that a decrease in aminotransferase levels alone after a marked increase has no prognostic meaning as both resolution and massive hepatic necrosis may show a similar biochemical pattern.

In our study, MB counteracted the deleterious effects of gut IR on liver oxidative capacities and restored cellular energy homeostasis. The underlying mechanisms warrant study. MB might modulate the nitric oxide (NO)/guanylate cyclase pathway and reduce oxidative stress and/or might interact directly with mitochondria. MB passes through both cellular and mitochondrial membranes, accumulates within mitochondria and improves mitochondrial respiration at low concentrations (0.5–2 mM) by shuttling electrons to oxygen in the electron transport chain. MB also counteracts perturbed mitochondrial metabolism induced by mutagens, acts as an alternative electron acceptor in mitochondria and might inhibit the production of superoxide by competing directly with molecular oxygen.

Our study has several limitations. First, we used a mild gut IR model (SMA ischaemia (1 h)/reperfusion (1 h)) simulating certain clinical and surgical situations of gut suffering (vascular surgery, intestinal surgery, shock...) but this model cannot be generalised to all situations. An earlier model using supra-coeliac ischaemia (40 min)/reperfusion (1 h) was associated with higher intestinal injury (lactate 7.1 ± 1.6 mmol l⁻¹). One hour of ischaemia was associated with >50% lethality within the first hour of reperfusion (unpublished data). Our present model induced significant histological injury, apoptosis and an increase in lactate (3.3 ± 1 mmol l⁻¹) but was not lethal, and is moreover the most widely used experimental model.

Second, we used a single MB dose (15 mg kg⁻¹ IP) based on published work. This dose restored 100% of liver mitochondria oxidative capacities and has no known adverse effects. However, the lowest MB dose that protects liver mitochondria needs to be determined. We injected MB 10 min before reperfusion because onset of ischaemia is rarely predictable in clinical practice. MB injection
immediately after ischaemia seemed clinically sound and relevant. Such post-conditioning has been shown to reduce reperfusion-induced injury on occlusion of other vascular beds.\textsuperscript{33}

Third, we measured mitochondrial activity after gut IR in liver only. A multi-organ approach is severely hampered by the need to extract mitochondria from fresh tissue and measure oxygen consumption immediately. However, we used the same protocol as in an earlier study on pulmonary mitochondria.\textsuperscript{34} Gut IR induced a 50% decrease in oxidative capacity in the liver and a 30% decrease in the lung. Finally, we addressed only a small piece of a complex situation.

In conclusion, the liver–gut axis has a key role in the deleterious effects of gut IR. We have demonstrated early impairment of the complexes of the liver mitochondrial respiratory chain after gut IR. MB suppressed this remote impairment. If injected before vascular unclamping in a clinical setting, MB might protect liver mitochondria function and minimise the incidence of postoperative multiple organ failure. Clinical studies on how to best use MB to limit the deleterious effects of gut IR during vascular surgery are thus warranted.

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CONFLICT OF INTEREST

None.

REFERENCES


