



HAL
open science

Pyrrolidine dithiocarbamate protects small bowel from warm ischemia/reperfusion injury of the intestine: the role of heme oxygenase

Ismail H Mallick, Marc C Winslet, Alexander M Seifalian

► **To cite this version:**

Ismail H Mallick, Marc C Winslet, Alexander M Seifalian. Pyrrolidine dithiocarbamate protects small bowel from warm ischemia/reperfusion injury of the intestine: the role of heme oxygenase. *Clinical Science*, 2006, 111 (6), pp.373-380. 10.1042/CS20060119 . hal-00479326

HAL Id: hal-00479326

<https://hal.science/hal-00479326>

Submitted on 30 Apr 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Pyrrolidine dithiocarbamate protects small bowel from warm ischemia reperfusion injury of the intestine: the role of heme oxygenase

Ismail H. MALLICK^{1,2}, Marc C. WINSLET^{1,2}, Alexander M. SEIFALIAN^{1*}

¹GI & Hepatobiliary Research Unit, Academic Division of Surgical and Interventional Sciences,
University College London, London, United Kingdom.

²Department of Surgery, Royal Free Hampstead NHS Trust Hospital, London, United Kingdom.

***Correspondence to:**

Professor Alexander M. Seifalian

Academic Division of Surgical and Interventional Sciences

University College London

Rowland Hill Street, London NW3 2PF, United Kingdom

Tel: ++ 44 20 7830 2901 (direct) Fax: ++ 44 20 7472 6444

Email: a.seifalian@medsch.ucl.ac.uk

Abstract

Ischemia reperfusion (IR) injury of the intestine occurs commonly during abdominal surgery. We have previously shown that pyrrolidine dithiocarbamate (PDTC), a heme oxygenase-1 (HO-1) donor improves intestinal microvascular perfusion. In this study we investigate the effects of PDTC on the intestinal microcirculation following ischemia reperfusion (IR) injury of the intestine. 72 Male Sprague-Dawley rats were randomly assigned to four groups (n=18/group): 1) sham group underwent laparotomy without induction of IR of the intestine, 2) IR group, subjected to superior mesenteric artery occlusion consisting of 30 minutes and 2 hours of reperfusion, 3) PDTC+IR group, received PDTC prior to IR and 4) Zn-PP group, received HO-1 inhibitor Zinc Protoporphyrin (ZnPP) followed by procedures as in group C. The ileum was evaluated for changes in tissue cytochrome oxidase redox status, red blood cell (RBC) dynamics and leukocyte-endothelial interactions. The expression of HO-1 in the ileal tissue was examined at the end of the reperfusion. PDTC significantly improved the intestinal tissue oxygenation, mucosal perfusion index and RBC velocity as compared to IR and ZnPP groups. PDTC also decreased the leukocyte-endothelial interactions ($p < 0.05$ vs. IR and ZnPP). PDTC induced the expression of HO-1 whereas ZnPP abolished this effect.

KEY WORDS: ischemia, reperfusion injury, intestine, microcirculation, pyrrolidine, multiple organ failure.

INTRODUCTION

Intestinal ischemia reperfusion (IR) injury is associated with a high morbidity and mortality in surgical patients. It is encountered in various clinical conditions such as hypovolemic shock, [1] strangulation-obstruction, [2] cardiovascular surgery, [3] abdominal aortic surgery [4] and small bowel transplantation. [5]

The gut mucosa is extremely sensitive to IR injury, and even short periods of ischemia can induce considerable tissue damage, in addition to which the induction of other organs, particularly to the lungs and liver, is commonly a confounding factor. [6] Pulmonary tissue damage and acute respiratory distress syndrome (ARDS) are common features after intestinal IR and is associated with a high morbidity and mortality in critically ill surgical patients. [7] The pathophysiology of IR injury is still unclear. Microcirculatory failure showing impairment of capillary perfusion, activation and adhesion of leukocytes, derangement of tissue oxygenation and eventual disruption of the mucosal barrier are the crucial hallmarks of IR injury in the mucosal villi. [8]

Pharmacologic preconditioning based on enhancing the production or activity of endogenous protective molecules is an effective therapeutic intervention to attenuate IR injury. Among such agents, pyrrolidine dithiocarbamate (PDTC) has a variety of biochemical activities, such as redox state alternation, chelation of heavy metals and enzyme inhibition. [9] PDTC is one of the most effective inducers of heme oxygenase-1 (HO-1), which also confers cytoprotection against oxidative stress. [10] HO is the rate-limiting enzyme in the conversion of heme into carbon monoxide (CO), biliverdin (which is rapidly converted to bilirubin) and free iron (Fe^{2+}). [11] Three isoforms of HO have so far been identified: inducible HO-1; constitutively expressed HO-2 and HO-3 which is related to HO-2, but is less well characterized. [11] The HO-1 system is thought to play a crucial role in the maintenance of antioxidant and oxidant homeostasis during cellular injury. The HO-1 system exerts four major beneficial effects broadly: (a) antioxidant function; (b) maintenance of the microcirculation; (c) anti-apoptosis and (d) anti-inflammatory function. [11] The antioxidant function relies on heme degradation, production of bilirubin [12] and the formation of ferritin via Fe^{2+} . [13] The production of CO with its vasodilatory and anti-platelet properties maintains the microcirculation and may be involved in anti-apoptotic and cell arrest mechanisms. The HO system exerts anti-

inflammatory effects via modulation of endothelial adhesion molecules. Previously, we have demonstrated that the administration of PDTC improved the intestinal microvascular perfusion. [14]

The present study investigated the effects of PDTC on the intestinal villous microcirculation including tissue oxygenation, mucosal perfusion and leukocyte adhesion after intestinal IR and to evaluate the role of HO-1.

METHODS

Animal Model

Animal care and experimental protocols were performed in accordance with the UK Government Guidance in the Operation of the Animals (Scientific Procedures) Act 1986. Male Sprague-Dawley rats weighing 250- 300g were used in this study. Rats were kept in temperature controlled environment with 12 hours light- dark cycle and allowed tap water and standard rat chow pellets ad libitum.

Animals were anesthetized using isoflurane (Baxter, Norfolk, UK) and allowed to breathe spontaneously via concentric mask connected to an oxygen regulator. The animal's body temperature was maintained at 36-38° C using a heating pad (Harvard apparatus Ltd., Kent, UK) and monitored with a rectal temperature probe. The arterial oxygen saturation (SaO₂) and heart rate (HR) were continuously monitored with a pulse oximeter (Ohmeda Biox 3740 pulse oximeter, Ohmeda Louisville, Colorado, USA). The left carotid artery and the right jugular vein were cannulated with polyethylene catheters (0.76-mm and 0.40-mm inner diameter, Portex, Kent, UK) for monitoring of mean arterial blood pressure (MABP) and administering normal saline (1 ml/100g body weight/hr) to compensate for intra-operative fluid evaporation. All animals had a bolus of heparin (20U/kg, i.v.) to prevent potential thrombus formation in the ischemic segment of the intestine due to haemostasis.

Laparotomy was carried out through a midline incision. The superior mesenteric artery (SMA) was identified and occluded with a non-traumatic vessel clamp to induce ischemia. Reperfusion started when the clamp was released. At the end of the experiment the animals were killed by exsanguination.

Experimental Protocol

Rats were randomly allocated to 4 study groups (n=18/group).

Group 1. Sham laparotomy, the SMA was identified with no vascular occlusion and animals received a single dose (0.2ml/100g) of 0.9% normal saline subcutaneously.

Group 2. IR, the SMA was occluded for 30 min, followed by a 2 hour period of reperfusion.

Group 3. PDTC+IR. Animals received a single dose of PDTC (10mg/100g, Sigma Chemical Co, St Louis, Mo, USA) dissolved in 0.9% normal saline subcutaneously 30 min before IR. [15]

Group 4. (ZnPP+ PDTC+ IR). Animals received a single dose (150µg/100g) of a HO inhibitor zinc protoporphyrin (ZnPP) (Sigma Chemical Co, St Louis, Mo, USA) subcutaneously 30 min before PDTC administration. ZnPP was dissolved in 0.2 mol/L sodium hydroxide and diluted in 0.9% normal saline. [9]

In each group, animals were further divided into three subgroups (n=6 each) for the evaluations of tissue oxygenation, RBC dynamics and the interactions of leukocyte to the endothelium.

Measurement of intestinal tissue oxygenation

Cytochrome oxidase c redox status (Cyt Ox) which is an indicative of the intestinal intracellular oxygenation and reflects mitochondrial function [16] was measured continuously by using near Infrared spectroscopy (NIRS) (NIRO-500™; Hamamatsu Photonics, Hamamatsu, Japan). NIRS produces near infrared light at four wavelengths, which is transmitted in sequential pulses via a bundle of optical fibres to the intestine. Photons emerging from the intestine are collected by a second bundle of optical fibres and are detected by a photomultiplier tube. The difference between transmitted and received light intensity at each wavelength was used to determine the optical density changes at each wavelength. The optical fibre bundles (probes) were placed on the surface of the intestine with a 5-mm separation. A flexible probe holder was used to ensure satisfactory contact with the intestinal surface and a fixed interprobe spacing. A NIRS adapted algorithm was used to continuously measure changes intestinal Cyt Ox concentration in micromoles per litre (µmol/L). We have previously shown that NIRS is an effective technique to monitor intestinal tissue oxygenation. [16] NIRS was optically

initialized to zero at the start of the experiment (baseline) and the changes were monitored continuously on-line in $\mu\text{mol/L}$ during entire experiment.

In vivo fluorescence microscopy

The animals were placed on the stage of a Nikon custom built microscope (Nikon, Japan) with an integrated heating system where the temperature was maintained at 37°C . The whole set up was placed on a pneumatic vibration isolation workstation (Newport, USA) to minimise vibration. The mucosal surface was exposed in a segment of exteriorized ileum by making a 30 mm incision along the anti-mesenteric border using an electric microcautery. Following the period of reperfusion, there was macroscopic evidence of patchy necrosis along the length of the small bowel. Microscopically, the areas of necroses were associated with total stasis of blood flow. This patchy response is consistent with previous studies. [1;17] Because of this patchy response, the anti-mesenteric incision for the exposure of the mucosa was made in a relatively viable segment of the ileum which was associated with area of reflow in order to study the effects of IR injury and PDTTC. The small bowel was placed on to a specially designed plastic stage and a cover slip was then sited on to the mucosa. The microscope was illuminated with a 100 watt mercury arc lamp for epi-illumination fluorescence light microscopy. The microscope was equipped with water immersion 10x and 40x objectives. Images were acquired continuously using a charge-coupled device camera (CCD, JVC, Japan) and digitised by a video-frame grabber and then passed through to the central processing unit of a computer image workstation where they were analyzed using Lucia image analysis software (Version 4.80, Laboratory Imaging, Nikon UK).

During the experiments, the animal's abdomen was covered with a plastic wrap (Saran wrap®, Dow Chemical, Michigan, USA) to prevent fluid evaporation.

Following 30 min of ischemia or at equivalent time point in the sham group, at random 6 animals in each group received fluorescein isothiocyanate (FITC) (Sigma, UK) labelled RBC (0.5 ml) as a bolus injection into the jugular vein to assess microvascular perfusion in the villi, namely mucosal perfusion index (MPI) and RBC velocity. FITC labelled RBC was prepared according to a modification of the method described previously. [18]

Another 6 animals received rhodamine 6G (0.2mL of 0.01%; Sigma UK) in order to evaluate the leukocyte-endothelial interactions in the mucosal villi. Rhodamine 6G is a vital dye that stains the leukocytes avidly and accumulates in the mitochondria. [19]

The microscopy technique does not allow the entire mucosa to be kept under observation; hence two areas were pre-selected in each animal, at a distance from each other for detailed surveillance. Recordings were made from these areas every 15 min after the administration of the fluorochromes for a period of 2 hours.

Mucosal Perfusion Index

15- 20 villi were allocated to three grades of perfusion (absent, irregular or well perfused) according to the method described previously. [20] A villus was considered to be 'poorly perfused' when at least 3-4 capillaries were not perfused over more than 20 seconds. Only villi without any perfusion were assigned to the 'absent' group. The mucosal perfusion index (*MPI*) was calculated by the formula:

$$MPI = (Ng + 0.5Np) / Nt$$

where *Ng* represents the number of well perfused villi, *Np* the number of poorly perfused villi and *Nt* the total number of villi analyzed.

Capillary RBC Velocity

RBC velocity was measured by the utilisation of the 40x lens. 5 capillaries per villus were chosen and the velocity was determined by off line frame-to-frame analysis as described previously. [20]

Quantification of Leukocyte Adhesion

The number of leukocytes adherent to the endothelium of up to four separate villi in two separate areas was determined by online analysis. A leukocyte was considered adherent if it does not move or detach from the endothelial lining within an observation period of up to 30 seconds. [20] Data are expressed as the number of adherent leukocytes per length of villus at each time point. Leukocytes adherent within the blood vessel were often difficult to distinguish from those that had migrated to the

interstitial space, but were still close proximity to the capillaries. Hence, both the groups of stationary leukocytes were regarded as adherent.

Heme Oxygenase Activity Assay

HO activity in ileal microsomal fractions was measured using a spectrophotometric assay of bilirubin production according to the method described previously. [21] Briefly, tissue microsomes were added to the following mixture: MgCl₂ (2 mM) phosphate-buffered saline (100 mM, pH 7.4; Sigma, UK), rat liver cytosol as a source of biliverdin reductase (3 mg total protein), hemin (10 μM), glucose-6-phosphate (2 mM), glucose-6-phosphate dehydrogenase (0.2 U) and NADPH (0.8 mM). The reaction was conducted in the dark for 30 min at 37°C and terminated by the addition of chloroform. All the above chemicals were purchased from Sigma, UK. The amount of extracted bilirubin was calculated by the difference in absorption between 464 and 530 nm and an extinction coefficient of 40 mM⁻¹cm⁻¹ was used for bilirubin. The total protein content of the samples was determined using a colorimetric assay according to the manufacturer's instructions (Bio-Rad, UK) and bovine gamma globulin was used as a standard.

Western blotting for HO-1

HO-1 expression in ileal tissue was analyzed by the Western immunoblotting. 50μg of total protein from each sample was loaded on a 12% precast SDS-PAGE gel (Bio-Rad, Hercules, CA, USA) and electrophoresed in 1× Tris buffer. The protein was transferred to nitrocellulose membrane and probed with a rabbit polyclonal anti-HO-1 (1:1000 dilution in Tris-buffered saline, pH 7.4) antibody (Stressgen, Victoria, BC, Canada) for 2 hr at room temperature. The non-specific binding of antibodies was blocked with 3% non-fat dried milk in PBS. The blot was incubated with anti-rabbit horseradish peroxidase secondary antibody for 1 h. The secondary reaction was visualized according to the ECL Western blotting detection system (Amersham, Piscataway, NJ, USA). Autoradiograms were analyzed by image-analysis software (Optimas 6.1, Media Cybernetics, Silver Spring, MD, USA).

Histological Investigation

At the end of the reperfusion period, samples of ileum and lungs were removed, fixed in 10% neutral buffered formalin and embedded in paraffin; 4 μ m thick sections were cut using a microtome and mounted on slides for hematoxylin and eosin staining. Assessment of ileal and lung injury was performed with light microscopy without knowledge of study groups. The ileum was assessed for injury by a scoring system devised by Chiu *et al.* [22]

Statistical Analysis

All data are expressed as mean \pm standard error of mean (SEM) values. ANOVA and Bonferroni adjustment for multiple comparisons was used for statistical analysis. Unpaired Student's *t* test was used for comparison between two groups. $P < 0.050$ was considered statistically significant.

RESULTS

Systemic Hemodynamic Parameters

There were no significant changes in HR and SaO₂ throughout the experiment in all the four experimental groups ($p > 0.1$). In sham group, the MABP did not change significantly throughout the experiment ($p > 0.05$). The administration of PDTC or ZnPP did not affect the HR, SaO₂ or MABP. However, clamping of the SMA was associated with a significant increase in MABP during the period of ischemia as shown previously. [16] Reperfusion restored MABP to pre-ischemic values and remained stable during the reperfusion period in all the four groups.

Changes in cytochrome oxidase redox status

Cyt Ox did not change significantly during the experiment in the sham group (*Fig. 1*). IR was associated with a significant decrease in Cyt Ox at the end of the reperfusion ($p < 0.001$ vs. sham). The administration of PDTC increased Cyt Ox levels at the end of the reperfusion. ($p < 0.01$ vs. IR), whereas ZnPP administration reversed the effect of PDTC, showing decreased levels of the Cyt Ox ($p < 0.05$ vs. ZnPP).

Mucosal Perfusion Index

A homogenous microvascular perfusion with almost all villi being well perfused throughout the experiment was observed in sham operated animals. The mean MPI at the end of 2 hr reperfusion was 0.70 ± 0.04 (*Fig. 2*).

In IR injury of the intestine, the mucosal perfusion was significantly decreased with many villi showing complete stasis. The mean MPI in the IR group at the end of 2 hr reperfusion was 0.40 ± 0.03 ($p < 0.001$ vs. sham). In PDTC group, there was a significant increase in the mucosal perfusion compared to IR and the MPI was 0.68 ± 0.04 at the end of 2 hr reperfusion ($p < 0.05$ vs. IR; $p > 0.05$ vs. sham). There was complete absence of areas of non-perfused villi (stasis). ZnPP was associated with decreased mucosal perfusion index of 0.33 ± 0.04 ($p < 0.05$ vs. PDTC).

Capillary RBC Velocity

Within the sham operated group the RBC velocity did not differ significantly during the experiment. On the contrary, in the IR group it decreased significantly compared with sham (0.25 ± 0.02 mm/s vs. 0.60 ± 0.07 mm/s, $p < 0.001$) at 15 min after reperfusion. PDTC increased the RBC velocity to 0.71 ± 0.10 mm/s during the first 15 min of reperfusion and reached a final value of 0.80 ± 0.23 mm/s at the end of the 2 hr reperfusion ($p < 0.05$ vs. IR). The administration of ZnPP was associated with decreased RBC velocity of 0.27 ± 0.04 mm/s at the end of the 2 hr reperfusion ($p < 0.05$ vs. PDTC) (*Fig. 3*).

Leukocyte Adherence

In sham operated animals only occasional leukocytes were adherent within the endothelial lining of the mucosal villi throughout the experimental period. IR injury of the intestine induced a rapid, sustained and a significant increase in the leukocyte adhesion in the endothelium ($p < 0.01$ versus sham; *Fig. 4*). It was observed that adherent leukocytes were frequently plugging the capillaries leading to reduced or no flow within the villus microcirculation. PDTC led to a significant decrease in the adhesion of the leukocytes to the endothelium ($p < 0.05$ vs. IR). ZnPP administration led to increase in the adhesion of leukocytes at the end of 2 hr reperfusion ($p < 0.05$ vs. PDTC).

Heme Oxygenase Activity

As it shows in *Fig. 5*, depicts the mean ileal HO activity at the end of 2 hr of reperfusion in all the four experimental groups in pmoles bilirubin/ mg/protein/hr. IR led to an increase of mean HO activity as compared to the sham group ($p < 0.001$). PDTC led to more than two-fold increase of HO activity (2062.66 ± 106.11) as compared to IR (842.33 ± 85.12) ($p < 0.001$ PDTC vs. IR). The mean HO activity in the ZnPP group was 94.00 ± 18.41 ($p < 0.001$ vs. PDTC).

Western Blotting for HO-1

PDTC significantly induced increased expression of HO-1 at the end two hours of reperfusion as shown in the western blot results (*Fig. 6*), which was reversed with ZnPP.

Histology

In ileal tissue, the mean histological score was 1.0 ± 0.4 in sham operated animals. IR resulted in an increase in the histological score to 3 ± 0.4 . ZnPP group led to an increase in score to 2.6 ± 0.4 . However, PDTC treatment improved the histological score significantly to 1.5 ± 0.2 ($p < 0.01$, *Fig 7*). In the lung, the alveoli were either collapsed or not distended to the same degree as sham with IR and ZnPP groups (*Fig 8*). The capillaries within the interstitium and, occasionally, within the alveolar spaces were congested with red blood cells and a marked neutrophilic infiltration. In PDTC group, however, alveoli were distended with thin flattened and delicate walls and few neutrophils were observed within the interstitium.

DISCUSSION

Gut IR injury have been proposed to be associated with the development and maintenance of the systemic inflammatory response, a key factor causing multiple organ failure. [23] The small bowel is extremely sensitive to IR injury and even a short episode of ischemia can induce substantial damage. [6] IR injury of the intestine induced severe mucosal damage- a total loss of villi, occasionally accompanied by crypt layer infarction. IR injury of the intestine resulted in decreased intracellular

tissue oxygenation, decreased mucosal perfusion, RBC velocity and increased leukocyte endothelial interactions. Our study has clearly demonstrated that PDTC attenuates these microvascular disturbances in the mucosal villi of small bowel following IR injury. PDTC also attenuated IR induced pulmonary tissue injury. These protective effects were associated with increased expression of HO-1 as the administration of ZnPP, a potent inhibitor of HO-1 abolished the protective effects of PDTC.

Experimental data suggest that IR induced intestinal injury occurs in a biphasic manner; an early phase of injury that develops over the course of 2 h of reperfusion and a later progressive phase that develops at 6 to 24 hrs after reperfusion. [24] A time period of 2 hr of reperfusion was chosen to assess changes in the early phase of reperfusion.

Capillary no-reflow and leukocyte endothelial interaction hallmark IR injury of the intestine. Following IR injury, many cell types may be damaged by oxygen free radicals with RBC one of the most vulnerable. [25] Impaired antioxidant defenses or increased production of oxygen free radicals may disturb the critical balance and result in oxidative damage to the RBC. [26] Injury to RBC is manifested both morphologically and physiologically. [25] Since damaged RBC tend to increase their deformity and aggregate, the RBC velocity may decrease as demonstrated in this study. In addition, RBC lysis may occur, both within the capillary lumen and in the extracellular space, thereby releasing a source of free heme which may further exacerbate IR injury.

The results of the present study clearly demonstrate that PDTC maintained capillary perfusion *in vivo*, and indicate that the protective effect of PDTC may be mediated by the action of HO-1, which catalyzes the conversion of heme to CO, biliverdin and free iron. These byproducts of heme degradation were believed to be the effector molecules underlying the potent cytoprotection observed with the HO-1 system. CO and its action on capillary pericytes by causing smooth muscle relaxation and improving the blood flow, may play a major role in this protection. In addition, there is increasing evidence that CO inhibits the aggregation of platelets, [27] which could result in higher red blood cell velocity because of unhindered flow. Hence tissue viability increases because of improved nutritional supply and better elimination of toxic residues of oxidative stress.

Biliverdin is subject to further degradation to bilirubin by the cytosolic enzyme biliverdin reductase. [28] It acts as an antioxidant and is capable of scavenging oxygen free radicals that are

thought to be primarily responsible for the tissue injury. [12] Iron, the last product of heme breakdown, acts as an oxidant like other transition metals and catalyzes the formation of reactive hydroxyl radicals (OH^\bullet) by the Haber-Weiss reaction. Typically the OH^\bullet causes biological damage by stimulating the free chain reaction known as lipid peroxidation, in which OH^\bullet attacks the fatty acid side chains of the membrane phospholipids and causes organelle and cell disruption. [29] Therefore it seems to be of paramount importance to eliminate free iron from the tissue in order to maintain the cellular integrity after the stress event. To enable this process, an additional expression of ferritin, the iron-binding protein, is induced simultaneously by HO. [30]

With IR there was accumulation of leukocytes in the mucosal villous microcirculation, whereas PDTTC attenuated the accumulation of leukocytes thereby leading to anti-inflammatory effects. In IR injury it was observed that adherent leukocytes were frequently plugging the capillaries leading to reduced or no flow within the villus microcirculation. Hayashi *et al.* demonstrated that overexpression of HO-1 in microvascular endothelial cells ameliorates oxidative injury and reduced the leukocyte endothelial interactions. [31] Hence, reduction of the leukocyte endothelial interaction by expression of HO-1 would reduce the plugging of the capillaries and thereby improve microvascular flow.

Mitochondrial dysfunction is one of the most critical events associated with intestinal I/R injury. The release of cytochrome c from mitochondria into cytosol is a critical initiation step in reactive oxygen species-triggered initiated damage. The reduction in Cyt Ox in IR and ZnPP groups reflects severe intracellular hypoxia and mitochondrial dysfunction due to an inability to fulfil the oxygen demand after reperfusion injury. In contrast, PDTTC was associated with an increase in Cyt Ox levels. The increase in Cyt Ox suggests that PDTTC probably preserves the mitochondrial function. This protective function of PDTTC on the intestinal mitochondria probably resulted from improved perfusion as the mitochondria are extremely sensitive to ischaemic changes.

The profound effects on the lungs following IR injury of the intestine may be induced by the activation of neutrophils following translocation of bacterial endotoxins. It is possible that the

increased mucosal permeability following ischemia allows passive diffusion of endotoxin, which is a potent activator of neutrophils and could subsequently lead to remote multiple organ injury. [32]

CONCLUSION

In summary, this study shows that IR injury of the intestine induces rapid microcirculatory breakdown with severe regional and systemic consequences. The administration of PDTC maintained a functioning mucosal microcirculation and markedly attenuated the IR injury. Thereby, PDTC may prove a prophylactic or therapeutic option to support the integrity of the gastrointestinal mucosa and to preserve or restore its barrier function. This may reduce translocation of toxins from the gastrointestinal lumen and consequently reduce the onset or severity of multiple organ failure.

ACKNOWLEDGEMENT

We would like to thank Dr. Wenxuan Yang for his invaluable help and support during the writing of this article. Tragically he died before submission. We also like to thank Miss Saleha Jabbar, MRes student her technical support.

REFERENCES

1. Kong,S.E., Blennerhassett,L.R., Heel,K.A., McCauley,R.D. and Hall,J.C. (1998) Ischaemia-reperfusion injury to the intestine. *Aust.N.Z.J.Surg.* **68**, 554-561.
2. Akcakaya,A., Alimoglu,O., Sahin,M. and Abbasoglu,S.D. (2002) Ischemia-Reperfusion Injury Following Superior Mesenteric Artery Occlusion and Strangulation Obstruction. *J.Surg.Res.* **108**, 39-43.
3. Ghosh,S., Roberts,N., Firmin,R.K., Jameson,J. and Spyt,T.J. (2002) Risk factors for intestinal ischaemia in cardiac surgical patients. *Eur.J.Cardiothorac.Surg.* **21**, 411-416.
4. Reber,P.U., Peter,M., Patel,A.G. et al. (2001) Ischaemia/reperfusion contributes to colonic injury following experimental aortic surgery. *Eur.J.Vasc.Endovasc.Surg.* **21**, 35-39.
5. Kimizuka,K., Nakao,A., Nalesnik,M.A. et al. (2004) Exogenous IL-6 inhibits acute inflammatory responses and prevents ischemia/reperfusion injury after intestinal transplantation. *Am.J.Transplant.* **4**, 482-494.
6. Mallick,I.H., Yang,W., Winslet,M.C. and Seifalian,A.M. (2004) Ischemia-reperfusion injury of the intestine and protective strategies against injury. *Dig.Dis.Sci.* **49**, 1359-1377.

7. Koksoy,C., Kuzu,M.A., Ergun,H., Demirpence,E. and Zulfikaroglu,B. (2000) Intestinal ischemia and reperfusion impairs vasomotor functions of pulmonary vascular bed. *Ann Surg* **231**, 105-111.
8. Kaminski,P.M. and Proctor,K.G. (1989) Attenuation of no-reflow phenomenon, neutrophil activation, and reperfusion injury in intestinal microcirculation by topical adenosine. *Circ.Res.* **65**, 426-435.
9. Tsuchihashi,S., Tamaki,T., Tanaka,M. et al. (2003) Pyrrolidine dithiocarbamate provides protection against hypothermic preservation and transplantation injury in the rat liver: the role of heme oxygenase-1. *Surgery* **133**, 556-567.
10. Hartsfield,C.L., Alam,J. and Choi,A.M. (1998) Transcriptional regulation of the heme oxygenase 1 gene by pyrrolidine dithiocarbamate. *FASEB J.* **12**, 1675-1682.
11. Katori,M., Anselmo,D.M., Busuttill,R.W. and Kupiec-Weglinski,J.W. (2002) A novel strategy against ischemia and reperfusion injury: cytoprotection with heme oxygenase system. *Transpl.Immunol.* **9**, 227-233.
12. Stocker,R., Yamamoto,Y., McDonagh,A.F., Glazer,A.N. and Ames,B.N. (1987) Bilirubin is an antioxidant of possible physiological importance. *Science* **235**, 1043-1046.
13. Balla,G., Jacob,H.S., Balla,J. et al. (1992) Ferritin: a cytoprotective antioxidant strategem of endothelium. *J Biol.Chem.* **267**, 18148-18153.
14. Mallick,I.H., Yang,W.X., Winslet,M.C. and Seifalian,A.M. (2005) Pyrrolidine dithiocarbamate reduces ischemia-reperfusion injury of the small intestine. *World J Gastroenterol.* **11**, 7308-7313.
15. Liu,S.F., Ye,X. and Malik,A.B. (1999) Inhibition of NF-kappaB activation by pyrrolidine dithiocarbamate prevents In vivo expression of proinflammatory genes. *Circulation* **100**, 1330-1337.
16. Mallick,I.H., Yang,W., Winslet,M.C. and Seifalian,A.M. (2005) Ischaemic preconditioning improves microvascular perfusion and oxygenation following reperfusion injury of the intestine. *Br.J Surg* **92**, 1169-1176.
17. Kalia,N., Brown,N.J., Hopkinson,K., Stephenson,T.J., Wood,R.F. and Pockley,A.G. (2002) FK409 inhibits both local and remote organ damage after intestinal ischaemia. *J.Pathol.* **197**, 595-602.
18. Zimmerhackl,B., Parekh,N., Brinkhus,H. and Steinhausen,M. (1983) The use of fluorescent labeled erythrocytes for intravital investigation of flow and local hematocrit in glomerular capillaries in the rat. *Int.J.Microcirc.Clin.Exp.* **2**, 119-129.
19. Dunn,K.W., Sandoval,R.M., Kelly,K.J. et al. (2002) Functional studies of the kidney of living animals using multicolor two-photon microscopy. *Am.J.Physiol Cell Physiol* **283**, C905-C916.
20. Mallick,I.H., Yang,W., Winsletand,M.C. and Seifalian,A.M. (2005) Protective effects of ischemic preconditioning on the intestinal mucosal microcirculation following ischemia-reperfusion of the intestine. *Microcirculation.* **12**, 615-625.
21. Motterlini,R., Foresti,R., Intaglietta,M. and Winslow,R.M. (1996) NO-mediated activation of heme oxygenase: endogenous cytoprotection against oxidative stress to endothelium. *Am.J.Physiol* **270**, H107-H114.

22. Chiu,C.J., McArdle,A.H., Brown,R., Scott,H.J. and Gurd,F.N. (1970) Intestinal mucosal lesion in low-flow states. I. A morphological, hemodynamic, and metabolic reappraisal. *Arch.Surg.* **101**, 478-483.
23. Pastores,S.M., Katz,D.P. and Kvetan,V. (1996) Splanchnic ischemia and gut mucosal injury in sepsis and the multiple organ dysfunction syndrome. *Am.J.Gastroenterol.* **91**, 1697-1710.
24. Slavikova,H., Lojek,A., Hamar,J. et al. (1998) Total Antioxidant Capacity of Serum Increased in Early but Not Late Period after Intestinal Ischemia in Rats. *Free Radic Biol Med* **25**, 9-18.
25. Chien,S. (1982) Rheology in the microcirculation in normal and low flow states. *Adv.Shock Res.* **8**, 71-80.
26. Claster,S., Chiu,D.T., Quintanilha,A. and Lubin,B. (1984) Neutrophils mediate lipid peroxidation in human red cells. *Blood* **64**, 1079-1084.
27. Brune,B. and Ullrich,V. (1987) Inhibition of platelet aggregation by carbon monoxide is mediated by activation of guanylate cyclase. *Mol.Pharmacol.* **32**, 497-504.
28. Maines,M.D. (1988) Heme oxygenase: function, multiplicity, regulatory mechanisms, and clinical applications. *FASEB J.* **2**, 2557-2568.
29. Halliwell,B. and Gutteridge,J. (1999) *Free Radicals in Biology and Medicine*, Clarendon Press, Oxford
30. Bauer,M. and Bauer,I. (2002) Heme oxygenase-1: redox regulation and role in the hepatic response to oxidative stress. *Antioxid.Redox.Signal.* **4**, 749-758.
31. Hayashi,S., Takamiya,R., Yamaguchi,T. et al. (1999) Induction of heme oxygenase-1 suppresses venular leukocyte adhesion elicited by oxidative stress: role of bilirubin generated by the enzyme. *Circ.Res.* **85**, 663-671.
32. Foulds,S., Cheshire,N.J., Schachter,M., Wolfe,J.H. and Mansfield,A.O. (1997) Endotoxin related early neutrophil activation is associated with outcome after thoracoabdominal aortic aneurysm repair. *Br.J.Surg* **84**, 172-177.

FIGURE LEGENDS

Fig 1. Changes in Cytochrome oxidase redox status levels during ischemia and reperfusion. Values are mean \pm SEM of six animals per group. # $p < 0.01$ PDTC vs. IR; * $p < 0.05$ ZnPP vs. PDTC.

Fig 2. Mucosal perfusion index at the end of 2 hr reperfusion in all the four experimental groups. For assessment of the perfusion pattern all villi were counted and divided into three groups according to quality of perfusion (well, irregular and non-perfused).

Fig 3. Capillary RBC velocity in all the four groups during the period of reperfusion. Results are presented as mean \pm SEM of 6 animals from each group (* $p < 0.05$ PDTC vs. IR; ** $p < 0.01$ PDTC vs. IR; # $p < 0.05$ ZnPP vs. PDTC; ## $p < 0.01$ ZnPP vs. PDTC; † $p < 0.05$ IR vs. Sham; †† $p < 0.001$ IR vs. Sham).

Fig 4. Number of adherent leukocytes in the capillary endothelium in all the four groups. Results are presented as mean \pm SEM of 6 animals from each group. (* $p < 0.05$ PDTC vs. IR; ** $p < 0.001$ PDTC vs. IR; # $p < 0.05$ ZnPP vs. PDTC; ## $p < 0.01$ ZnPP vs. PDTC; † $p < 0.01$ IR vs. Sham; †† $p < 0.001$).

Fig 5. HO activity in the ileum in all the four experimental groups at the end of 2 hr of reperfusion. Results are presented as mean \pm SEM of 6 animals from each group.

Fig 6. HO-1 expression in the small intestine at the end of 2 hour of reperfusion. (a) representative western blot and (b) summary densitometry. Results are presented as mean \pm SEM of 6 animals from each group. PDTC treatment significantly increased the expression of HO-1.

Fig 7. Comparison of histological scores of the ileal mucosa between the four experimental groups (* $p < 0.01$ PDTC vs. IR; # $p < 0.01$ PDTC vs. ZnPP).

Fig 8. Representative photomicrography showing lung histology in (A) sham laparotomy, (B) IR, (C) PDTC+IR and (D) ZnPP+PDTC+IR groups. Collapsed alveoli, thickened interstitial walls, and a dense neutrophilic infiltrate were observed in untreated intestinal IR and ZnPP animals. However, relatively normal histology, with numerous alveoli and thin alveolar walls, was observed in PDTC pretreated IR animal.















