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

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ABSTRACT

IR (ischaemia/reperfusion) injury of the intestine occurs commonly during abdominal surgery. We have previously shown that PDTC (pyrrolidine dithiocarbamate), an HO-1 (haem oxygenase-1) donor, improves intestinal microvascular perfusion. In the present study, we have investigated the effects of PDTC on the intestinal microcirculation following IR (ischaemia/reperfusion) injury of the intestine. Male Sprague–Dawley rats (n = 72) were randomly assigned to four groups (n = 18/group): (i) sham-operated group, who underwent laparotomy without induction of IR of the intestine; (ii) IR group, who were subjected to 30 min of superior mesenteric artery occlusion and 2 h of reperfusion; (iii) PDTC + IR group, who received PDTC prior to IR; and (iv) ZnPP group, who received the HO-1 inhibitor ZnPP (zinc protoporphyrin) followed by procedures as in group (iii). The ileum was evaluated for changes in tissue cytochrome c oxidase redox status, RBC (red blood cell) dynamics and leucocyte–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 compared with the IR and ZnPP groups. PDTC also decreased the leucocyte–endothelial interactions (P < 0.05 compared with the IR and ZnPP groups). PDTC induced the expression of HO-1, whereas ZnPP abolished this effect.

INTRODUCTION


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

Pharmacological preconditioning based on enhancing the production or activity of endogenous protective

Key words: haem oxygenase, intestine, ischaemia/reperfusion injury, microcirculation, multiple organ failure, pyrrolidine dithiocarbamate (PDTC).

Abbreviations: Cyt Ox, cytochrome oxidase c; HO, haem oxygenase; HR, heart rate; IR, ischaemia/reperfusion; MABP, mean arterial blood pressure; MPI, mucosal perfusion index; NIRS, near-infrared spectroscopy; OH•, hydroxyl radical; PDTC, pyrrolidine dithiocarbamate; RBC, red blood cell; Sato2, arterial oxygen saturation; SMA, superior mesenteric artery; ZnPP, zinc protoporphyrin.

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molecules is an effective therapeutic intervention to attenuate IR injury. Among such agents, PDTC (pyrroolidine dithiocarbamate) 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 HO (haem oxygenase)-1, which also confers cytoprotection against oxidative stress [10]. HO is the rate-limiting enzyme in the conversion of haem into CO (carbon monoxide), biliverdin (which is rapidly converted into bilirubin) and free iron (Fe$^{2+}$) [11]. Three isoforms of HO have been identified so far: 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, and exerts four broad major beneficial functions: (i) antioxidant effects; (ii) maintenance of the microcirculation; (iii) anti-apoptosis; and (iv) anti-inflammatory effects [11]. The antioxidant function relies on haem degradation, production of bilirubin [12] and the formation of ferritin via Fe$^{2+}$ [13]. The production of CO with its vasodilatory and antiplatelet 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 [14] that the administration of PDTC improves intestinal microvascular perfusion.

The present study investigated the effects of PDTC on the intestinal villous microcirculation, including tissue oxygenation, mucosal perfusion and leucocyte 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 250–300 g were used. Rats were kept in a temperature-controlled environment with 12 h light/dark cycle and were allowed tap water and standard rat chow pellets ad libitum.

Animals were anaesthetized using isoflurane (Baxter) and allowed to breathe spontaneously via concentric masks connected to an oxygen regulator. Body temperature was maintained at 36–38°C using a heating pad (Harvard Apparatus) and monitored with a rectal temperature probe. $\text{Sao}_2$ (arterial oxygen saturation) and HR (heart rate) were monitored continuously with a pulse oximeter (Biox 3740; Ohmeda). The left carotid artery and the right jugular vein were cannulated with polyethylene catheters (0.76 mm and 0.40 mm inner diameters; Portex) for monitoring of MAP (mean arterial blood pressure) and administering normal saline (1 ml·h$^{-1}$·100 g$^{-1}$ of body weight) to compensate for intraoperative fluid evaporation. All animals had an intravenous bolus of heparin (20 units/kg of body weight) to prevent potential thrombus formation in the ischaemic segment of the intestine due to haemostasis.

Laparotomy was carried out through a midline incision. The SMA (superior mesenteric artery) was identified and occluded with a non-traumatic vessel clamp to induce ischaemia. 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 four study groups ($n = 18$/group). In the sham group (group 1), the SMA was identified with no vascular occlusion and animals received a single dose of 0.9% normal saline intramuscularly (0.2 ml/100 g of body weight). In the IR group (group 2), the SMA was occluded for 30 min, followed by a 2 h period of reperfusion. In the PDTC + IR group (group 3), animals received a single dose of PDTC (10 mg/100 g of body weight; Sigma) dissolved in 0.9% normal saline subcutaneously 30 min before IR [15]. In the ZnPP + PDTC + IR group, animals received a single dose of ZnPP (zinc protoporphyrin; 150 µg/100 g of body weight; Sigma), an HO inhibitor, subcutaneously 30 min before PDTC administration. ZnPP was dissolved in 0.2 mol/l NaOH and diluted in 0.9% normal saline [9].

In each group, animals were divided further into three subgroups ($n = 6$ in each) for the evaluation of tissue oxygenation, RBC (red blood cell) dynamics and the interaction of leucocytes with the endothelium.

**Measurement of intestinal tissue oxygenation**

Cyt Ox (cytochrome oxidase c) redox status, which is indicative of the intestinal intracellular oxygenation and reflects mitochondrial function [16], was measured continuously by using NIRS (near-infrared spectroscopy; NIRO-500™; Hamamatsu Photonics). 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 absorbance 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. An NIRS-adapted algorithm was used to continuously measure changes in intestinal Cyt Ox concentration (in µmol/l). We have shown previously [16] that NIRS is an effective technique to monitor intestinal tissue oxygenation. NIRS was
optically initialized to zero at the start of the experiment (baseline) and the changes were monitored continuously online (in μmol/l) during the entire experiment.

**In vivo fluorescence microscopy**

Animals were placed on the stage of a Nikon custom-built microscope with an integrated heating system where the temperature was maintained at 37°C. The whole setup was placed on a pneumatic vibration isolation workstation (Newport) to minimize vibration. The mucosal surface was exposed in a segment of exteriorized ileum by making a 30 mm incision along the antimesenteric 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 antimesenteric incision for the exposure of the mucosa was made in a relatively viable segment of the ileum which was associated with an area of reflow in order to study the effects of IR injury and PDTC. 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 W mercury arc lamp for epi-illumination fluorescence light microscopy. The microscope was equipped with water-immersion 10× and 40× objectives. Images were acquired continuously using a charge-coupled device camera (JVC), digitized by a video-frame grabber and then passed through to the central processing unit of a computer image workstation, where they were analysed using Lucia image analysis software (version 4.80, Laboratory Imaging; Nikon).

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

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

Another six animals received Rhodamine 6G (0.2 ml of a 0.01% solution; Sigma) in order to evaluate the leucocyte–endothelial interactions in the mucosal villi. Rhodamine 6G is a vital dye that stains the leucocytes avidly and accumulates in the mitochondria [19].

The microscopy technique did 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 h.

**MPI**

Villi (n = 15–20) 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 s. Only villi without any perfusion were assigned to the ‘absent’ group. MPI was calculated by the formula:

\[
\text{MPI} = \frac{N_g + 0.5N_p}{N_t}
\]

where Ng represents the number of well-perfused villi, \(N_p\) the number of poorly perfused villi, and \(N_t\) the total number of villi analysed.

**Capillary RBC velocity**

RBC velocity was measured by using the 40× objective lens. A total of five capillaries per villus were chosen, and the velocity was determined by off line frame-to-frame analysis, as described previously [20].

**Quantification of leucocyte adhesion**

The number of leucocytes adhering to the endothelium of up to four separate villi in two separate areas was determined by online analysis. A leucocyte was considered adherent if it did not move or detach from the endothelial lining within an observation period of up to 30 s [20]. Results are expressed as the number of adherent leucocytes per length of villus at each time point. Leucocytes adherent within the blood vessel were often difficult to distinguish from those that had migrated to the interstitial space, but were still in close proximity to the capillaries. Hence both the groups of stationary leucocytes were regarded as adherent.

**HO 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 a mixture containing 2 mmol/l MgCl₂, 100 mmol/l phosphate-buffered saline (pH 7.4) (Sigma), 3 mg of rat liver cytosol as a source of biliverdin reductase, 10 μmol/l hemin, 2 mmol/l glucose 6-phosphate, 0.2 unit glucose-6-phosphate dehydrogenase and 0.8 mmol/l NADPH. The reaction was conducted in the dark for 30 min at 37°C and terminated by the addition of chloroform. All of the above chemicals were purchased from Sigma. The amount of extracted bilirubin was calculated as the difference in absorption between 464 and 530 nm and a molar absorbance coefficient of 40 mM⁻¹·cm⁻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 Laboratories), with bovine γ-globulin used as a standard.
Western blotting for HO-1

HO-1 expression in ileal tissue was analysed by Western immunoblotting. Total protein (50 µg) from each sample was loaded on to a precast SDS/12 % (w/v) acrylamide gel (Bio-Rad Laboratories) and electro-phoresed in 1 x Tris buffer. The protein was transferred on to a nitrocellulose membrane and probed with a rabbit polyclonal anti-(HO-1) antibody [1:1000 dilution in Tris-buffered saline (pH 7.4); Stressgen] for 2 h at room temperature. The non-specific binding of antibodies was blocked with 3 % (w/v) non-fat dried milk/PBS. The blot was incubated with anti-(rabbit Ig)–horseradish peroxidase secondary antibody for 1 h. The secondary reaction was visualized using the ECL® Western blotting detection system (Amersham Biosciences). Autoradiograms were analysed by image-analysis software (Optimas 6.1; Media Cybernetics).

Histological investigation

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

Statistical analysis

All data are means ± S.E.M. 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 < 0.05 was considered statistically significant.

RESULTS

Systemic haemodynamic parameters

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

Changes in Cyt Ox redox status

Cyt Ox did not change significantly during the experiment in any of the four groups (P > 0.1). In the sham group, MABP did not change significantly throughout the experiment (P > 0.05). The administration of PDTC or ZnPP did not affect HR, SaO2 or MABP; however, clamping the SMA was associated with a significant increase in MABP during the period of ischaemia, as shown previously [16]. Reperfusion restored MABP to pre-ischaemic values and remained stable during the reperfusion period in all of the four groups.

Changes in MPI

A homogenous microvascular perfusion with almost all villi being well perfused throughout the experiment was observed in the sham group (Figure 2).

After IR injury of the intestine, mucosal perfusion was significantly decreased (P < 0.001 compared with the sham group), with many villi showing complete stasis. In the PDTC + IR group, there was a significant increase (P < 0.05) in mucosal perfusion compared with the IR group (P > 0.05 compared with the sham group). There was a complete absence of areas of non-perfused villi (stasis). ZnPP was associated with a decrease in MPI (P < 0.05 compared with the PDTC + IR group; Figure 2).
Role of HO in protecting the small bowel from ischaemia/reperfusion injury

**Figure 3** Capillary RBC velocity during the period of reperfusion in the four groups
Results are means ± S.E.M. of six animals from each group. *P < 0.05 and
**P < 0.01 compared with the IR group; #P < 0.05 and ##P < 0.01 compared with the PDTC + IR group; †P < 0.05 and ††P < 0.001 compared with the sham group.

**Capillary RBC velocity**
In the sham group, RBC velocity did not differ significantly during the experiment (Figure 3). On the contrary, in the IR group, it decreased significantly compared with sham group at 15 min after reperfusion (0.25 ± 0.02 compared with 0.60 ± 0.07 mm/s respectively; P < 0.001). PDTC increased 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 2 h of reperfusion (P < 0.05 compared with the IR group). The administration of ZnPP was associated with a decrease in RBC velocity of 0.27 ± 0.04 mm/s at the end of 2 h of reperfusion (P < 0.05 compared with the PDTC + IR group; Figure 3).

**Leucocyte adherence**
In the sham group, only the occasional leucocyte was 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 leucocyte adhesion in the endothelium (P < 0.01 compared with the sham group; Figure 4). It was observed that adherent leucocytes frequently plugged the capillaries, leading to reduced or no flow within the villus microcirculation. PDTC treatment led to a significant decrease in the adhesion of leucocytes to the endothelium (P < 0.05 compared with the IR group). ZnPP administration led to an increase in the adhesion of leucocytes at the end of 2 h of reperfusion (P < 0.05 compared with the PDTC + IR group).

**HO activity**
Figure 5 shows the mean ileal HO activity at the end of 2 h of reperfusion in all of the four experimental groups. IR induced a significant increase (P < 0.001) in mean HO activity compared with the sham group. PDTC treatment led to a significantly greater than 2-fold increase in HO activity compared with the IR group (P < 0.001). The mean HO activity in the group receiving ZnPP was significantly lower (P < 0.001) than in the PTDC + IR group (Figure 5).

**Western blotting for HO-1**
PDTC significantly increased the expression of HO-1 at the end of 2 h of reperfusion, as shown by Western blotting (Figure 6), and this was reversed with ZnPP.

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Figure 6  HO-1 expression in the small intestine at the end of 2 h of reperfusion
(a) Representative Western blot of HO-1 expression. (b) Densitometric values (means ± S.E.M.) of six animals from each group. PDTC treatment significantly increased the expression of HO-1. **P < 0.001 compared with the IR group.

Figure 7  Comparison of the ileal mucosa histological scores between the four experimental groups
*P < 0.01 compared with the IR group; #P < 0.01 compared with the ZnPP + PDTC + IR group.

**DISCUSSION**

Gut IR injury has 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 ischaemia can induce substantial damage [6]. IR injury of the intestine induces severe mucosal damage, including a total loss of villi and, occasionally, accompanied by crypt layer infarction. IR injury of the intestine also resulted in decreased intracellular tissue oxygenation, mucosal perfusion and RBC velocity, and increased leucocyte–endothelial interactions. Our present study has clearly demonstrated that PDTC attenuates these microvascular disturbances in the mucosal villi of the 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 6–24 h after reperfusion [24]. In the present study, a 2 h of reperfusion was chosen to assess changes in the early phase of reperfusion.

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

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 catalyses the conversion of haem into CO, biliverdin and free iron. These byproducts of haem degradation were believed to be the effector molecules underlying the potent cytoprotection observed with the HO-1 system. CO and its action on
**Role of HO in protecting the small bowel from ischaemia/reperfusion injury**

Figure 8: Representative photomicrographs showing lung histology in (A) sham, (B) IR, (C) PDTC + IR, and (D) ZnPP + PDTC + IR groups.

Collapsed alveoli, thickened interstitial walls and a dense neutrophilic infiltrate were observed in animals from the IR and ZnPP + PDTC + IR groups. However, relatively normal histology, with numerous alveoli and thin alveolar walls, was observed in the PDTC + IR group. Original magnification, ×100.

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 RBC velocity because of unhindered flow. Hence tissue viability increases because of improved nutritional supply and better elimination of toxic residues resulting from 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 haem breakdown, acts as an oxidant like other transition metals and catalyses the formation of reactive OH• (hydroxyl radical) by the Haber–Weiss reaction. Typically OH• cause biological damage by stimulating the free chain reaction known as lipid peroxidation, in which OH• attack the fatty acid side chains of membrane phospholipids and cause organelle and cell disruption [29]. Therefore it seems to be of paramount importance to eliminate free iron from the tissue in order to maintain 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].

During IR, there was an accumulation of leucocytes in the mucosal villous microcirculation, which was attenuated by PDTC, thereby leading to anti-inflammatory effects. In IR injury it was observed that adherent leucocytes frequently plugged the capillaries, leading to reduced or no flow within the villus microcirculation. Hayashi et al. [31] have demonstrated that overexpression of HO-1 in microvascular endothelial cells ameliorates oxidative injury and reduces leucocyte–endothelial interactions. Hence a reduction in leucocyte–endothelial interactions by expression of HO-1 would decrease the plugging of capillaries and thereby improve microvascular flow.

Mitochondrial dysfunction is one of the most critical events associated with intestinal IR injury. The release of cytochrome c from mitochondria into the cytosol is a critical initiation step in ROS (reactive oxygen species)-triggered damage. The reduction in Cyt Ox in IR and ZnPP + PTDC + IR groups reflects severe intracellular hypoxia and mitochondrial dysfunction due to an inability to fulfil the oxygen demand after IR injury. In contrast, PDTC was associated with an increase in Cyt Ox levels. The increase in Cyt Ox suggests that PDTC probably preserves mitochondrial function. This protective function of PDTC on 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 ischaemia allows passive diffusion of endotoxin, which is a potent activator of neutrophils and could subsequently lead to remote multiple organ injury [32].

**Conclusions**

In summary, the present study has shown 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 IR injury. Therefore PDTC may prove a prophylactic or therapeutic option to support the integrity of the gastrointestinal mucosa and 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.

ACKNOWLEDGMENTS

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

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Received 17 May 2006; accepted 2 August 2006