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EFFECT OF DEFEROXAMINE AND L-ARGININE TREATMENT ON LIPID PEROXIDATION IN AN INTESTINAL ISCHAEMIA-REPERFUSION MODEL IN RATS

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This study investigated lipid peroxidation (LPO) changes during intestinal ischaemia-reperfusion with and without deferoxamine or L-arginine treatment. White Wistar rats were allotted into four groups as follows: sham-operated (Group SOP), ischaemia-reperfusion only (Group I/R), I/R with deferoxamine (Group D) or L-arginine (Group A) treatment. Concentration of thiobarbituric acid reactive substances (TBARS), overall concentration of malondialdehyde and 4-hydroxy-alkenals (LPO586), activities of superoxide dismutase (SOD) and glutathione peroxidase (GPX) of the jejunal homogenates were determined. The same analytes except LPO586 were assayed in RBC haemolysates. Measurements of ferric reducing ability (FRAP), total antioxidant status (TAS) and nitric oxide (NO) concentrations of plasma samples were also completed. The only significant change observed in the SOP group was an increased SOD activity after the ischaemic period. In the I/R group significant increase of intestinal LPO586 concentration was observed during hypoxia that was followed by similar changes in intestinal and RBC TBARS and plasma FRAP values upon reperfusion. In Group D the intestinal TBARS and LPO586 concentrations were significantly lower while FRAP and NO concentrations were significantly higher compared to the I/R group. At the same time RBC TBARS concentration and GPX activity significantly decreased within Group D. In Group A the intestinal LPO586 concentration was significantly lower than in the I/R group whilst RBC TBARS concentration showed a similar pattern. Plasma FRAP and NO concentration showed similar changes to those seen in Group D. It is concluded that I/R increased the LPO in the intestinal tissue and altered some parameters of plasma and RBCs, too. Deferoxamine treatment prevented these effects, while the usefulness of L-arginine remained doubtful.

Key words: Lipid peroxidation, intestine, erythrocyte, nitric oxide, deferoxamine

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Intestinal ischaemia-reperfusion (I/R) induces a series of processes that lead to severe gut wall injury finally resulting in tissue necrosis and shock. The role of free radicals in these situations was investigated from several aspects. There are many pathways involved that can generate free radicals in the I/R. In the small intestine the xanthine oxidase mechanism is one of the most important sources of oxygen free radicals while in the colon aldehyde oxidases are thought to play a similar role (Granger et al., 1981; McCord and Roy, 1982; Parks et al., 1983). Free radical-caused injury forces endothelial cells to release platelet activating factor and various kinds of leukotrienes that trigger neutrophilic granulocyte migration towards the damaged mucosa (Granger et al., 1989; Suzuki et al., 1989; Zimmermann et al., 1990; Kubes et al., 1990). Neutrophils via their myeloperoxidase activity contribute to the production of free radicals (Otamiri et al., 1988). Intestinal I/R can also damage the function of distant organs like the lung and heart via the release of different mediators such as tumour necrosis factor, platelet activating factor, leukotrienes and prostaglandins (Caty et al., 1990; Horton and White, 1991).

There were many trials to prevent the deleterious effects of I/R with different drugs including enzyme inhibitors, free radical scavengers, antioxidant enzymes, anti-inflammatory compounds, anti-neutrophil agents and metal chelators. Deferoxamine is known as the most prominent representative of the latter group. It was used successfully to prevent I/R injury (i.e. increased mucosal permeability) in a cat model and improved survival rates after experimentally induced gastric dilation-volvulus in dogs (Hernandez et al., 1987; Lantz et al., 1992). Furthermore, if deferoxamine was added to cardioplegia solution, 93% of the left ventricular contractility of the isolated perfused rat hearts was preserved (Ely et al., 1992).

There is increasing evidence that supports that nitric oxide (NO) and its derivatives play an important role in the pathogenesis of intestinal I/R. Nitric oxide is a small lipophilic molecule continuously produced by a constitutive enzyme (constitutive NO synthase, cNOS) expressed in many cells in the intestine, and it plays an important role in the regulation of epithelial permeability (Kubes, 1992). Administration of NO donor (C87-3754) attenuated endothelial dysfunction and improved short-term survival of experimental cats after intestinal I/R (Carey et al., 1992). If rats were given L-arginine (0.5 g/kg body weight *per os*) prior to the establishment of I/R, the process of reparation and cell proliferation was more pronounced and levels of polyamines and cGMP also increased. These effects were antagonised by simultaneous administration of NO synthase inhibitor NG-nitroarginine-methyl-ester (Raul et al., 1995). If cats undergoing intestinal I/R were given NO synthase inhibitor (N-nitro L-arginine methyl ester) the blood to lumen clearance of Cr^{51}-EDTA and I^{125}-albumin was significantly higher than in the control ones. Furthermore, L-arginine treatment was able to attenuate these changes (Kubes, 1993). On the other hand, NO can take part in

different biochemical reactions especially with superoxide to form the harmful peroxynitrite radical (Beckman et al., 1990). There are also numerous studies that are focused on the effects of NO, NO donors, NO synthesis inhibitors, and peroxynitrite in cardiac, lung and brain I/R, but their results are quite contradictory about the effects of these compounds.

It was hypothesised that LPO changes in the intestines undergoing I/R could be reflected in plasma and red blood cell parameters as well. Evaluation of the effects of iron chelation with deferoxamine and NO synthesis enhancement by L-arginine on the aforementioned processes was also aimed.

Materials and methods

Experimental design

Altogether 56, 3-month-old female White Wistar rats were used in this study. Before the experiment the animals were weighed and allotted into 4 groups as follows:

In the experimental ischaemia reperfusion group (Group I/R, n = 14) the animals were anaesthetised by intraperitoneal injection of sodium pentobarbital (50 mg/kg bw). The jugular vein was accessed surgically and blood samples were collected into heparinised collecting tubes. Afterwards, midline laparotomy was performed and the cranial mesenteric artery was ligated using atraumatic surgical clamps. After 30 min, 1 ml of physiological saline solution (Salsol A infusion, Baxter, Toronto, Canada) was injected into the jugular vein of the animals as the vehicle of the active ingredients used in the treated groups. Another 15 min later a blood sample was taken from the jugular vein again and full-thickness specimens were harvested from the ischaemic jejunum from 7 animals. In the other half of the animals in this group, blood flow was restored in the cranial mesenteric artery, and blood sampling was repeated and jejunal sampling was completed after 45 min. After collection of the intestinal samples the animals were euthanatised by an overdose of sodium pentobarbital.

In the L-arginine- (Group A, n = 14) and deferoxamine- (Group D, n = 14) treated groups the animals were prepared by the same procedure as the ones in Group I/R, except that L-arginine (300 mg/kg bw, dissolved in physiological saline solution) or deferoxamine (50 mg/kg bw, dissolved in physiological saline solution) was injected to the animals 15 min prior to reperfusion. Both chemicals were supplied by Sigma-Aldrich Ltd. (Budapest, Hungary).

Sham-operated group (Group SOP, n = 14). Four rats were anaesthetised and jejunal samples were collected immediately after anaesthesia to serve as negative controls for the operated groups. Samples were collected from the rest of the animals according to the same scheme as in Group I/R, except no ligature of the cranial mesenteric artery was performed.

The experiment was approved by the Local Supervising Committee for the Use and Welfare of Experimental Animals at the Faculty of Veterinary Science, Szent István University, Budapest (25-9/2000).

Sample handling

After sampling, blood samples were immediately centrifuged at 3000 rpm for 5 min, plasma was removed and red blood cell haemolysates (1:9 v/v with redistilled water) were prepared. Jejunal specimens were flushed with physiological saline solution then approximately half gram was homogenised with 4.5 ml of physiological saline solution. All samples were frozen and stored at –20 °C to await the biochemical analysis for maximum 5 days.

Analytical procedures

Glutathione peroxidase (GPX) and superoxide dismutase (SOD) activities in the erythrocytes and intestine were determined using commercially available kits (Ransel, and Ransod kits, respectively; manufactured by Randox, Cork, Ireland). The concentration of thiobarbituric acid reactive substances (TBARS) in RBCs and intestinal homogenates were measured according to Placer et al. (1966). Overall concentration of lipid peroxidation end products malondialdehyde and 4-hydroxy-nonenal in the jejunal samples was assessed by the LPO-586 kit (Oxis Int. Inc.: Bioxytech® LPO-586 Cat. No.: 21012D). The assay is based on the reaction of a chromogenic reagent N-methyl-2-phenylindole with malondialdehyde and 4-hydroxyalkenals at 45 °C. Plasma nitric oxide (NO) concentration was measured via the detection of its metabolites nitrite and nitrate (reduced to nitrite with nitrate reductase) with the Griess-Ilosvay reagent according to Grisham et al. (1996). The ferric reducing ability of plasma (FRAP) was assessed as described by Benzie and Strain (1996). Most parameters (except plasma FRAP and NO) are given per gram protein content of the samples. Total protein concentration was determined spectrophotometrically with the biuret reagent (purchased from Diagnosztikum Ltd., Budapest, Hungary). All other chemicals for antioxidant parameters were supplied by Sigma-Aldrich Ltd. (Budapest, Hungary).

Statistical analysis of the laboratory data was completed by the help of Microsoft Excel 5.0 and the Statgraphics 6.0 (Manugistics Inc. 2115, Rockville, MD) software programs. Data of the intestinal samples and differences of results between each sampling time of plasma and RBC indices (between groups) were compared by two-sample Student's *t*-tests, while plasma and RBC results were checked by paired *t*-tests (within groups). A P value less than 0.05 was considered significant.

Results

There was no significant change of intestinal TBARS concentration in Groups SOP and D, whereas about 2–3 times higher levels ($P < 0.05$) were seen in Groups I/R and A from the 45th to the 90th minute of the experiment (i.e. during reperfusion; 414 ± 172 to 941 ± 312 and 378 ± 90 to 1252 ± 415 nmol/g protein, respectively). Furthermore, intestinal TBARS concentration was significantly higher after reperfusion in these two groups than in Groups SOP and D (941 ± 312 and 1252 ± 415 nmol/g protein compared to 433 ± 117 and 270 ± 50 nmol/g protein, respectively; Fig. 1).

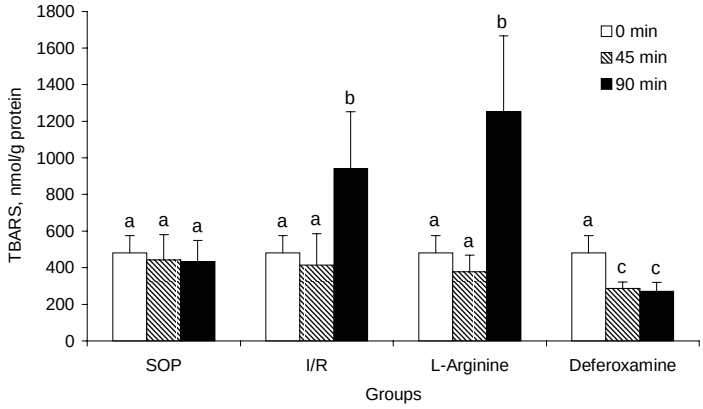

Fig. 1. Intestinal TBARS concentration (nmol/g protein). Average ± SD. Columns marked with different letters indicate significant difference ($P < 0.05$) of results; 0 min = basal value before the ligature of the cranial mesenteric artery; 45 min = 45 minutes after the ligature of the cranial mesenteric artery; 90 min = 45 minutes after reperfusion of the cranial mesenteric artery

Overall concentrations of intestinal malondialdehyde and 4-hydroxyalkenals (LPO586) are shown in Fig. 2. No changes were revealed in Groups SOP, A and D; however, 77–87% higher LPO586 concentration was observed in Group I/R after 45 minutes of ischaemia, staying approximately at the same level after reperfusion as well (98 ± 29 vs. 177 ± 43 and 187 ± 64 nmol/g protein, respectively; $P < 0.05$). Furthermore, post-reperfusion values in Group I/R were significantly higher than the corresponding ones in any other group (Fig. 2).

The only significant change of the intestinal SOD activity was an increase from 270 ± 54 to 531 ± 174 U/g protein, which was found in the SOP group during the first 45 minutes of anaesthesia. Then the values showed about the same levels for the rest of the experiment (Fig. 3). The same tendency was observed in all the other groups.

Intestinal GPX activity showed no significant difference in any group (Fig. 4).

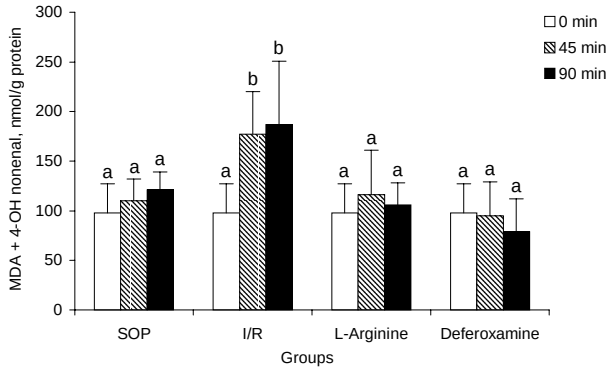

Fig. 2. Intestinal MDA and 4-OH nonenal concentration (nmol/g protein). Average ± SD. Columns marked with different letters indicate significant difference ($P < 0.05$) of results; 0 min = basal value before the ligature of the cranial mesenteric artery; 45 min = 45 minutes after the ligature of the cranial mesenteric artery; 90 min = 45 minutes after reperfusion of the cranial mesenteric artery

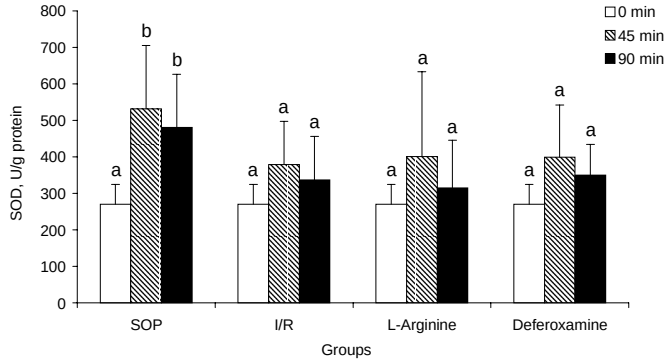

Fig. 3. Intestinal SOD activity. Average ± SD. Columns marked with different letters indicate significant difference ($P < 0.05$) of results; 0 min = basal value before the ligature of the cranial mesenteric artery; 45 min = 45 minutes after the ligature of the cranial mesenteric artery; 90 min = 45 minutes after reperfusion of the cranial mesenteric artery

FRAP values did not change in the SOP group. Significantly higher FRAP concentrations were measured in Groups I/R, A and D in samples taken at 90 minutes of anaesthesia (1.13 ± 0.27, 2.9 ± 1.64 and 2.25 ± 0.85 mmol/l, respectively) than in those harvested at 45 minutes. In addition, FRAP concentration also increased significantly during the first 45 minutes in Group D from 0.70 ± 0.17 to 1.29 ± 0.39 mmol/l (Fig. 5). During ischaemia, FRAP values showed significantly higher increase in Group D compared to Group I/R. By the end of reperfusion the change of FRAP concentration in Group I/R (+0.61 ± 0.18 mmol/l) was significantly higher than in Group SOP (–0.35 ± 0.33 mmol/l) but significantly lower than in Group D (+1.5 ± 0.57 mmol/l). Comparing the changes of FRAP concentration during the whole experimental period only Groups I/R and D differed significantly (0.69 ± 18 vs. 1.95 ± 0.95 mmol/l).

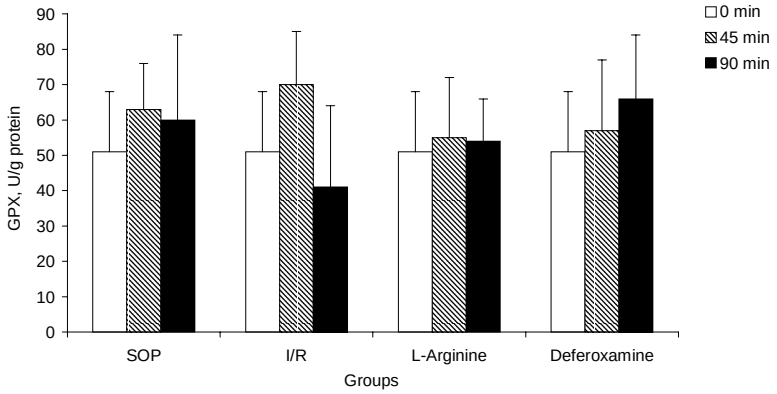

Fig. 4. Intestinal GPX activity. Average ± SD; 0 min = basal value before the ligature of the cranial mesenteric artery; 45 min = 45 minutes after the ligature of the cranial mesenteric artery; 90 min = 45 minutes after reperfusion of the cranial mesenteric artery

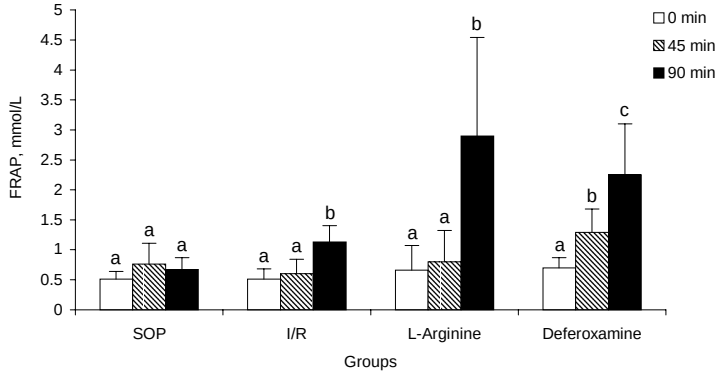

Fig. 5. Plasma FRAP values. Average ± SD. Columns marked with different letters indicate significant difference ($P < 0.05$) of results within groups; 0 min = basal value before the ligature of the cranial mesenteric artery; 45 min = 45 minutes after the ligature of the cranial mesenteric artery; 90 min = 45 minutes after reperfusion of the cranial mesenteric artery

Plasma NO concentration did not show any change between sampling times in Groups SOP and I/R (Fig. 6). However, there was a significant increase upon reperfusion from 18.0 ± 3.1 to 28.9 ± 7.2 µmol/l in Group D. Similarly, differences of NO concentration were significant only between Group I/R and Group D, considering either 45 or 90 minutes sampling.

Erythrocyte TBARS concentration was significantly higher (430 ± 73 nmol/g protein) than the basal value (197 ± 33 nmol/g protein) after 90 minutes in Group I/R; however, no significant change was observed at 45 minutes after ischaemia compared to the basal values of this group. Similar but not so marked elevation was observed in Group A as well (301 ± 26 vs. 258 ± 30 nmol/g protein; $P < 0.05$; Fig. 7).

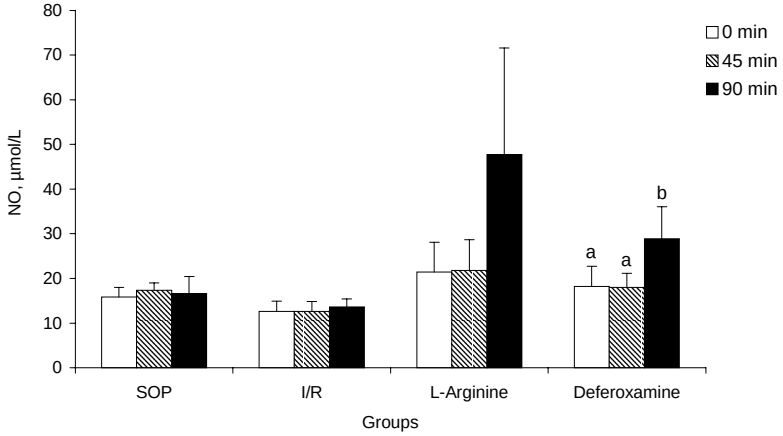

Fig. 6. Plasma NO concentrations. Average ± SD. Columns marked with different letters indicate significant difference ($P < 0.05$) of results within groups; 0 min = basal value before the ligature of the cranial mesenteric artery; 45 min = 45 minutes after the ligature of the cranial mesenteric artery; 90 min = 45 minutes after reperfusion of the cranial mesenteric artery

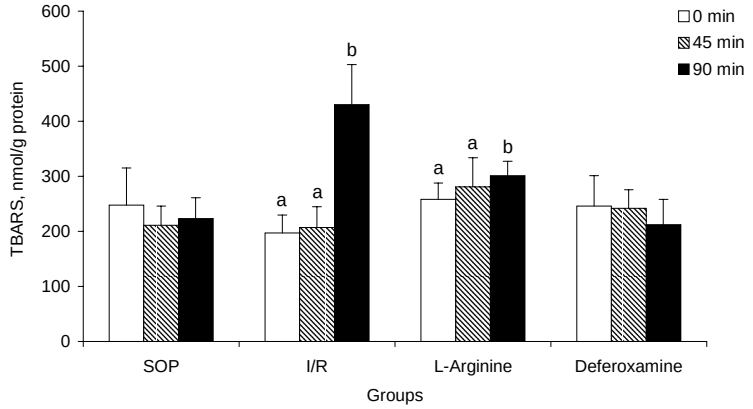

Fig. 7. Erythrocyte TBARS concentrations. Average ± SD. Columns marked with different letters indicate significant difference ($P < 0.05$) of results within groups; 0 min = basal value before the ligature of the cranial mesenteric artery; 45 min = 45 minutes after the ligature of the cranial mesenteric artery; 90 min = 45 minutes after reperfusion of the cranial mesenteric artery

Figure 8 represents that during reperfusion RBC GPX activity decreased from 206 ± 36 to 143 ± 32 U/g protein in Group D. Furthermore, the change of RBC GPX activity was significantly different between Group I/R and Group D (+17 ± 35.2 vs. –68.2 ± 64 U/g protein).

The RBC SOD activity increased significantly during ischaemia from 313 ± 61 to 391 ± 31, then returned to the initial values of 307 ± 94 nmol/g protein in Group I/R. Similar tendency characterised this parameter in Groups A and D as well (Fig. 9).

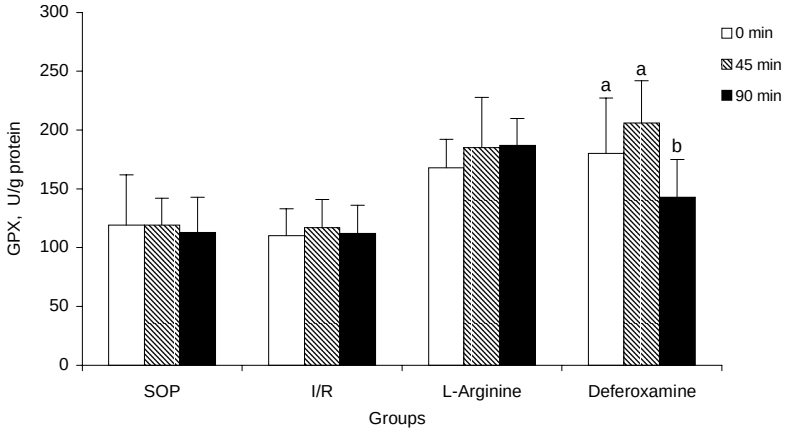

Fig. 8. Erythrocyte GPX activities. Average ± SD. Columns marked with different letters indicate significant difference ($P < 0.05$) of results within groups; 0 min = basal value before the ligature of the cranial mesenteric artery; 45 min = 45 minutes after the ligature of the cranial mesenteric artery; 90 min = 45 minutes after reperfusion of the cranial mesenteric artery

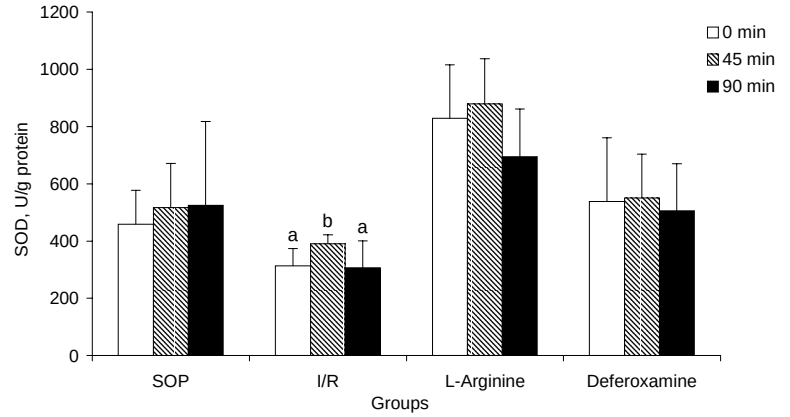

Fig. 9. Erythrocyte SOD activities. Average ± SD. Columns marked with different letters indicate significant difference ($P < 0.05$) of results within groups; 0 min = basal value before the ligature of the cranial mesenteric artery; 45 min = 45 minutes after the ligature of the cranial mesenteric artery; 90 min = 45 minutes after reperfusion of the cranial mesenteric artery

Discussion

The only significant change observed in the SOP group is the slight increase of SOD activity in the first 45-minute period indicating the effect of anaesthesia that was reversed by the much greater damage of enzyme activity caused by I/R in all the other groups. Apart from this anaesthesia and laparotomy themselves had no other effect on LP processes.

Increasing intestinal TBARS and LPO586 concentration indicates that lipid peroxidation occurred in the jejunal specimens during reperfusion in the I/R group. At the same time the elevation of intestinal TBARS concentration was preceded by an increase in the LPO586 concentration during ischaemia in this group (Figs 1 and 2). This incongruity can be explained by the different specificity of the two methods. Many other molecules besides malondialdehyde can react with thiobarbituric acid, interfering with the results obtained by the method applied in this experiment. It is also possible that a large portion of lipid peroxidation end products was masked by protein-bound complexes that, according to the manufacturer, do not react in the LPO586 assay. Similarly, hydrolysis in alkaline conditions is necessary to release malondialdehyde from protein to be reactive in the TBARS method (Matsuki et al., 1991). Malondialdehyde is also recognised as a by-product of enzymatic thromboxane formation which may occur during intestinal I/R, and therefore the malondialdehyde produced by this pathway interferes with LPO-originated molecules using TBARS or LPO586 method.

The situation found in the L-arginine treated group is even more perplexing as the huge TBARS peak is not reflected in the LPO586 results. This may suggest that L-arginine treatment was able to reduce the formation of MDA and 4-OH-nonenal but not other TBA-reactive by-products of LPO. Some data support the latter findings, as L-arginine and nitric oxide donors were found to decrease intestinal epithelial permeability especially within the first two hours of reperfusion (Kubes, 1992; Payne and Kubes, 1993; Kanwar et al., 1994; Kawata et al., 2001). Others have not found beneficial effects of increasing the nitric oxide level (i.e. administration of L-arginine or NO donor molecules) and warn that nitric oxide may form peroxynitrite radical with superoxide anion which can even worsen the reperfusion injury in heart and brain I/R models (Beckman et al., 1990; Schulz and Wambolt, 1995; Lopez et al., 1997). Furthermore, evidence was produced for peroxynitrite formation in intestinal I/R related with constitutive nitric oxide synthase (cNOS) activity in a rat experimental model similar to ours (Cuzzocrea et al., 1998).

Erythrocyte TBARS concentration reflects the intestinal events showing that the increased intestinal LPO escalated to RBCs as well, though the amplitude of changes is considerably less than in the intestinal samples (Figs 1 and 7).

The intestinal process did not influence plasma NO level in the I/R group (Fig. 6). This matches the findings of the authors cited above who found no increase in plasma NO level and intestinal inducible nitric oxide synthase (iNOS) expression (Cuzzocrea et al., 1998). In contrast to these, a recent publication reports elevated plasma NO concentration in horses with naturally acquired small intestinal strangulation (Mirza et al., 1999). Other researchers also found increased NO levels during intestinal I/R in wild mice in contrast to iNOS knockout ones (Suzuki et al., 2000). In this trial both L-arginine and deferoxamine administration induced higher plasma NO levels. In case of L-arginine obviously

due to increased substrate supply for NO synthases, while in case deferoxamine probably by preventing iron-catalysed superoxide formation that could have reacted with nitric oxide to form peroxynitrite. This molecule is bound by various proteins as nitrotyrosine thereafter. Deferoxamine also has to be considered to decrease the non-haem iron pool of cells that are thought to bind NO in the form of different complexes (Keberle, 1964; Henry et al., 1991; Reif, 1993). Finally it cannot even be ruled out with hundred percent certainty that NO is released during the decomposition of deferoxamine in the organism. At the same time, in Group D a slight decrease was found in the intestinal TBARS and LPO586 concentrations showing that the applied deferoxamine therapy was able to protect the tissues from I/R triggered LPO (Figs 1 and 2). These findings are well in line with those of Hernandez et al. (1987) who could prevent the development of I/R induced increase in the intestinal vascular permeability by iron-chelation with deferoxamine and apotransferrin in a cat model. Similarly, deferoxamine improved survival rates of dogs with surgically induced gastric dilation-volvulus (Lantz et al., 1992). Like in other groups, erythrocyte TBARS concentration followed a pattern similar to the intestinal changes in Group D indicating that deferoxamine administration had beneficial effect on LPO processes of RBCs (Fig. 7).

Intestinal SOD activity showed significant increase in Group SOP after the first 45-minute period possibly due to the anaesthetic procedure and laparotomy, and was followed by a slight, not significant decrease. Similar tendencies were observed in all the other groups as well as in case of intestinal GPX activity, except that the decrease of GPX activity was reversed by the administration of deferoxamine (Figs 3 and 4).

The SOD activity of erythrocytes followed the changes found in the intestine but no unequivocal tendency was observed in the intestinal GPX activity (Figs 8 and 9).

Plasma FRAP concentrations showed marked elevation after reperfusion in all groups where I/R was accomplished (Fig. 5). This may serve as a footprint of uric acid release from the reperfused intestinal segments as, according to the original publication of Benzie and Strain some 60% of plasma FRAP is given by the uric acid produced by the xanthine oxidase mechanism from hypoxanthine, the intermediary molecule of ATP degradation (Granger et al., 1981; Benzie and Strain, 1996). Unfortunately uric acid was not assayed in this study, but assuming that approximately the same amount of this substance appeared in the circulation in all of these groups, the significantly bigger increase of FRAP values in the treated groups could be explained by improvement of overall plasma antioxidant power due to L-arginine and deferoxamine treatment (Fig. 5). It is interesting that in Group D the elevation of FRAP values was recognised already 15 min after drug administration possibly due to the direct hydroxyl-radical scavenging effect of deferoxamine that had previously been evidenced in other studies (Menasche et al., 1987; Morehouse et al., 1987).

It is concluded that intestinal I/R induced changes in LPO parameters are reflected in the plasma and red blood cell parameters. Deferoxamine treatment was proven to be beneficial in the prevention of I/R induced LPO, but the role of L-arginine and nitric oxide remains controversial and necessitates further investigation.

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