

# Pentoxifylline Protects the Small Intestine After Severe Ischemia and Reperfusion

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## Abstract

**Objectives:** Pentoxifylline, a methylxanthine derivative with significant hemorheologic properties, is used for claudication in patients with peripheral vascular disease, and experimentally for ischemic injury to organs because of its antioxidant and anti-inflammatory effects. We used a rat model of severe small intestinal ischemia and reperfusion to determine the ability of pentoxifylline in improving survival, molecular response, and pathological protection.

**Materials and Methods:** We used 6 groups of male Wistar rats (n=25 each). The superior mesenteric artery was occluded for 120 minutes. Laboratory and tissue studies were done on 5 animals, 1 hour after reperfusion, and animal survival was assessed at 7 days. There were 2 control groups that received normal saline, either before ischemia or during reperfusion. The 4 treated groups received pentoxifylline 1 or 10 mg/kg at the same times mentioned above. Laboratory studies included measuring serum lactic acid dehydrogenase, tumor necrosis factor- $\alpha$ , interleukin-1 $\beta$ , and interleukin-6. Intestinal tissue malondialdehyde and myeloperoxidase in small intestine tissue also were measured. Histology and laser vascular blood flow at baseline and reperfusion were obtained, and survival was determined 7 days after ischemia.

**Results:** A significant survival benefit in the animals treated with 10 mg/kg of pentoxifylline at reperfusion was noted. This coincided with a reduction in biochemical markers of cell damage—specifically, serum lactic acid dehydrogenase, and tissue malondialdehyde, ischemia, and reperfusion. Additionally, we saw decreased levels of tumor necrosis factor- $\alpha$ , interleukin-1 $\beta$ , and interleukin-6. Improved postreperfusion blood flow shown by laser Doppler technology also was seen in the treated groups. Histologically, we observed less neutrophil infiltration in the intestine of ischemic-treated rats. Also seen in the control animals were increased necrotic lesions in the microvilli with a higher presence of lysozyme in the Paneth cells. Survival was significantly better at 7 days (70% vs 40%) when we compared the pentoxifylline group treated at reperfusion (10 mg/kg) to the ischemic controls.

**Conclusions:** Pentoxifylline had a significant protective effect on severely ischemic bowel when administered during reperfusion at a dosage of 10 mg/kg. Better survival, improved histology, and molecular response should urge consideration of the consideration of applying these findings in some general surgery and transplant conditions.

**Key words:** *Pentoxifylline, Small intestine, Ischemia and reperfusion*

## Introduction

Ischemia and reperfusion (I/R) is a pathological phenomenon commonly associated with stroke, myocardial infarction, transplant, and trauma, as well as the ischemia occurring in individual organs.<sup>1-21</sup> Because the lack of blood supply is a critical cause of ischemic injury, restoring blood flow to the ischemic tissue can prevent tissue necrosis and regain organ function.<sup>1,2</sup> In addition, and as a consequence, many

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molecular markers have been identified as key contributors to I/R.<sup>8,10,12-17,19,20</sup>

Pentoxifylline (PTX) a methylxanthine hemorheologic compound that reduces blood viscosity and improves erythrocyte deformability, began its use in Europe for intermittent claudication in 1972.<sup>22</sup> Other demonstrated effects (eg, vasodilatation and inhibition of neutrophil adhesion) prompted its potential interest as a beneficial compound for various I/R conditions.<sup>23</sup> In this regard, in the late 1980s Waxman<sup>24</sup> and Coccia,<sup>25</sup> and a few years later, Flynn<sup>26</sup> and Sakio<sup>27</sup> and associates, among others, recognized the positive blood flow effects of PTX in hemorrhagic conditions. Mustafa in 1995<sup>28</sup> used PTX in a typical rat small bowel ischemic model as an anti-ischemic drug. Others followed.<sup>29-32</sup> The discovery that PTX had an anti-TNF- $\alpha$  effect a few years earlier, stimulated its application into organ ischemia.<sup>33</sup>

This study sought to define the role of PTX in severe intestinal ischemia when all clinical (survival), laboratory (molecular), and pathological parameters were evaluated together in a well-accepted model of small intestinal ischemia. In addition, this work studied the time and dosage of PTX administration in small intestine ischemic conditions.

## Materials and Methods

### Experimental design

One hundred fifty male Wistar rats, weighing 240-250 g (Harlan Laboratories, Spain) were used in this study. Animals were housed in a light-controlled room with a 12-hour inverse light/dark cycle.

One hundred twenty rats, were divided into 6 groups (n = 20) and used to study survival rates. Five more rats were used in each group for biochemical and histopathologic analyses. Throughout the process, we followed the standards of animal welfare in the Health's Guide of the Care and Use of Laboratory Animals and the European Union regulations on the matter. The experiments were approved by the Research Committee of the University General Hospital of Valencia. Even though the 3R principle was taken into account, because of the chi-square method, which is used to obtain significant differences when working with qualitative values is stringent, it was not possible to reduce the number of animals used in the survival study.

After superior mesenteric artery occlusion and reperfusion, laboratory and pathological samples were

recovered. Laser Doppler flow studies and histology were analyzed. Finally, at 7 days, survival was evaluated and compared among all groups.

### Surgical technique

All animals had a 12-hour fast before surgery, but were allowed to drink water ad libitum. The rats were anesthetized with ether for induction, and sodium pentobarbital intraperitoneally (30 mg/kg) for maintenance. Under aseptic conditions, the abdomen was carefully cleaned with Betadine and alcohol. A midline laparotomy was performed. The superior mesenteric artery was visualized, and clamped for 120 minutes. After that, the clamp was removed to begin reperfusion. Reperfusion was done for 1 hour before obtaining blood to determine the inflammatory markers such as lactic acid dehydrogenase (LDH), tissue malondialdehyde (MDA), and myeloperoxidase (MPO). Laser Doppler flow studies and histology were done at the same time. Survival was done in 20 animals per group, for 7 days, and the results were compared statistically.

### Experimental groups

Six groups of 25 animals, each, were used and divided according to treatment received, including the amount of, and time of administration of PTX. Controls received only normal saline (NS). (Twenty animals for survival studies and 5 for biochemical and histopathologic analyses). The groups were as follows:

C<sub>1</sub> Control: Twenty rats 0.4 cm<sup>3</sup> NS IV given 60 minutes before intestinal ischemia

C<sub>2</sub> Control: Twenty rats 0.4 cm<sup>3</sup> NS IV given at reperfusion

P<sub>1</sub> Treated: Twenty rats 0.4 cm<sup>3</sup> NS IV 1 mg/kg PTX 60 minutes before intestinal ischemia

P<sub>2</sub> Treated: Twenty rats 0.4 cm<sup>3</sup> NS IV with 1 mg/kg PTX at reperfusion

P<sub>3</sub> Treated: Twenty rats 0.4 cm<sup>3</sup> NS IV with 10 mg/kg PTX 60 minutes before intestinal ischemia

P<sub>4</sub> Treated: Twenty rats 0.4 cm<sup>3</sup> NS IV with 10 mg/kg PTX at reperfusion

### Laboratory tests

The laboratory studies included serum LDH, MDA, and MPO. Also, tissue levels of small intestine were used to determine TNF- $\alpha$ , IL-1 $\beta$ , and IL-6. Five animals per group were used for the laboratory analyses. One hour after reperfusion, plasma and

tissue specimens were taken from all the groups. All samples were analyzed blindly.

When assessing the biological markers and histopathology, an intestinal portion (which had undergone ischemia) was prepared by washing the vascular lumen with a cold saline infusion at low pressure. In 60 cm<sup>3</sup> of cold saline, we flushed the intestinal portion to remove all cellular components from the vascular system and microvasculature. The intestinal lumen also was washed with a 30 cm<sup>3</sup> bolus of room temperature saline. Extracting the intestinal portion was performed quickly to minimize any metabolic processes. All tissues and samples were kept at low temperature and were prepared for laboratory determinations.

#### **Measurement of serum enzymes**

Lactate dehydrogenase was measured to assess the damage to the intestinal tissue. The levels were determined using a Hitachi 717 autoanalyzer (Hitachi Ltd, Tokyo, Japan), measuring the rate of decrease of nicotinamide adenine dinucleotide, directly proportional to the LDH activity in serum. Sample are expressed as U/L.

#### **Assay of lipid peroxidation in intestinal tissue**

To determine the lipid peroxidation in the intestinal wall, we measured levels of MDA, which is an end product of lipid metabolism and peroxidation. The intestine was rapidly excised 60 minutes after reperfusion, and flushed with ice-cold 0.9% NaCl via the vascular system before homogenization. Homogenates were prepared in a ratio of 1 g of wet tissue to 9 mL of 0.9% NaCl with a homogenizer (Thomas Scientific, Swedesboro, NJ, USA). The content of MDA in the homogenate was determined using a colorimetric reaction with thiobarbituric acid, as described by Bieri and associates<sup>34</sup> and revised by Ohkawa and associates.<sup>35</sup> The protein concentration was calculated according to the Lowry method.<sup>34-36</sup> The results are expressed as nmol/mg protein.

#### **Assay of myeloperoxidase in intestinal tissue**

Myeloperoxidase was measured as a biochemical quantitative marker of neutrophil presence in the small bowel, 1 hour after reperfusion. After intestinal ischemia, a portion of the small intestine was removed, frozen immediately, and stored at -70°C until we assayed MPO.

Small bowel specimens were placed in a 50 mmol potassium phosphate buffer with 0.5% hexadecyltrimethylammonium bromide; then, samples were homogenated and centrifuged for MPO activity according to a modified technique of Renlund and associates.<sup>37</sup> The change in absorbance at 460 nm was measured by spectrophotometer (Gilford Instrument Laboratories, Oberlin, OH, USA) using o-Dianisidine dihydrochloride. The assay was expressed in units of absorbance at 460 nm.<sup>37,38</sup> Enzyme activity was calculated as units/g of tissue.

#### **Blood sample collection**

Multiple blood samples were obtained by venipuncture at baseline (0 min), at 60 minutes after reperfusion to measure plasma levels of TNF- $\alpha$ , IL1- $\beta$ , and IL-6. Plasma samples were obtained by centrifugation of whole blood and stored at -80°C until the assay.

#### **Measurement of plasma levels of tumor necrosis factor- $\alpha$ , interleukin-1 $\beta$ , and interleukin-6**

The concentrations of tumor TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 were determined using a quantitative sandwich enzyme-linked immunosorbent assay kits (R & D Systems Inc, Minneapolis, MN, USA) in plasma samples.<sup>15,39</sup>

#### **Laser Doppler flowmetry test**

Vascular flow after ischemia and subsequent reperfusion is a variable that can indicate the vasomotor behavior of an organ at different stages of an experiment. To quantify blood flow, we used a laser Doppler flowmeter Periflux (Perimed AB) with a multifiber probe PF308. Calibration software and equipment was supplied by the PF5001 drive. Values are expressed in units of infusion UP<sup>40-41</sup> as a relative flow response.

#### **Histologic studies—light microscopy**

Five samples of intestinal tissue for each group were studied to evaluate the histologic response. Intestinal specimens for light microscopy were fixed in 10% formalin and embedded in paraffin. Five- $\mu$ m sections were made and were stained with hematoxylin and eosin for histologic examination. Visual analyses were performed on all samples.

Samples from intestinal tissue were used to study the infiltration of polymorphonuclear cells. These were stained using the Naphthol AS-D Chloroacetate

(Specific Esterase) kit (Sigma-Aldrich Corp. St. Louis, MO, USA)<sup>18</sup> to investigate accumulation of polymorphonuclear cells in the small bowel after reperfusion. Polymorphonuclear cells were identified by positive staining and morphology.

To observe histochemical changes produced by I/R and the consequences of treatment with PTX, we used 2 markers for the inflammatory processes: lysozyme and myeloperoxidase. To visualize the lysozyme, we used the primary antibody A-099 (Dako A/S, Glostrup, Denmark), and for secondary antibodies, we used EnVision (Flex Dako K8024) as a visualization system. Similarly, for myeloperoxidase, we used the primary antibody IR-511 and the same system of detection. We followed the protocols described by Mörsky<sup>42</sup> and Pinkus.<sup>43</sup>

### Statistical analyses

All results values are expressed as the means  $\pm$  SEM or SD. To analyze quantitative variables of the experimental groups, we used the analysis of variance (ANOVA) test to determine existence of linear relations between them. To determine significant differences, we performed a multiple comparison of the Fisher exact test. To compare survival rates and statistical significance, we use the chi-square method. Differences were considered statistically significant at a value for  $P < .05$ . Statistical analyses were performed with SPSS software (SPSS: An IBM Company, version 18.0.1, IBM Corporation, Armonk, NY, USA).

## Results

### Survival after ischemia

Survival was assessed at 7 days after the ischemic episode in control and treated groups ( $n=20$ /group). As indicated in Table 1, controls had a 40% survival rate whereas the treated groups ranged from 55% to 70% at 7 days. An ANOVA test is a highly stringent test method of obtaining statistical differences, and although we obtained a higher rate of survival in all groups treated with PTX, the only significant differences ( $P = .028$ ) between the control group and the treated group were in group P4 = 10 mg/kg of PTX administered at reperfusion.

### Serum lactic acid dehydrogenase

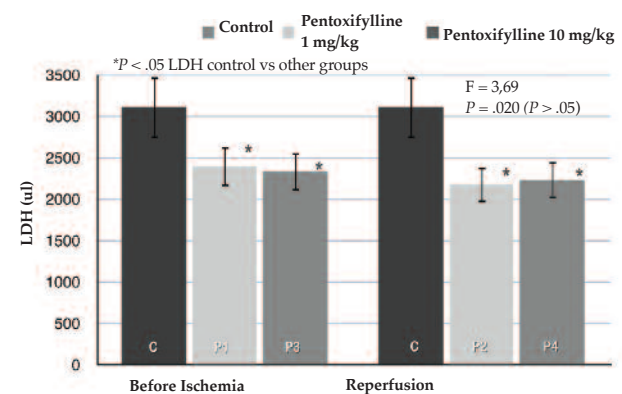
Serum lactic acid dehydrogenase values, as a marker for small intestinal damage, indicated significant

differences ( $P < .05$ ) between the control group and all PTX-treated groups (Figure 1).

**Table 1.** Survival of Control and Pentoxifylline-Treated Groups at 7 Days

| Groups                                      | Survival Rate at 7 Days | No. of Live Animals |
|---------------------------------------------|-------------------------|---------------------|
| Control C1 (1 h before)                     | 40%                     | 8                   |
| Control C2 (at reperfusion)                 | 40%                     | 8                   |
| P1 Pentoxifylline 1 mg/kg (1 h before)      | 55%                     | 11                  |
| P2 Pentoxifylline 1 mg/kg (at reperfusion)  | 65%                     | 13                  |
| P3 Pentoxifylline 10 mg/kg (1 h before)     | 65%                     | 13                  |
| P4 Pentoxifylline 10 mg/kg (at reperfusion) | 70%*                    | 14                  |

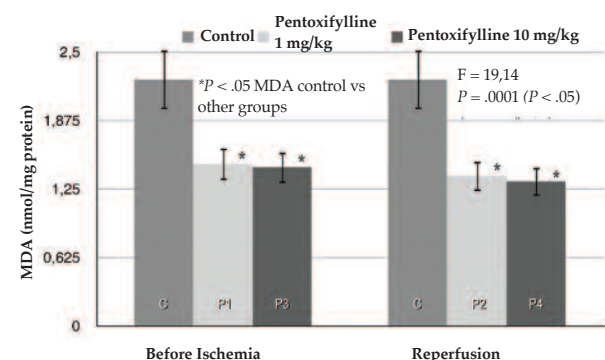
**Figure 1.** Histomorphometric Evaluation of Fully Allogeneic C57BL/6 Aortic Grafts



### Tissue malondialdehyde

Small intestinal MDA is a good indicator of lipid peroxidation and subsequently, of ischemic damage. Comparing results among the control and treated groups seemed to indicate that PTX significantly diminished lipid peroxidation and therefore, ischemic injury (Figure 2). The best group was the one that received PTX at reperfusion at a dose of 10 mg/kg ( $P < .0001$ ).

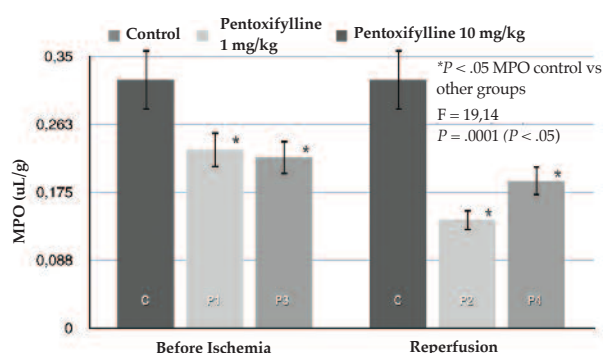
**Figure 2.** Tissue Malondialdehyde (MDA) Levels and Comparative Analysis of Control and Pentoxifylline-Treated Groups



### Tissue myeloperoxidase

Neutrophil presence was measured by MPO levels in the intestinal tissue obtained from all groups. All PTX-treated groups showed significantly lower results than did controls (Figure 3). Infiltration or presence of neutrophils was less evident when PTX was used to treat ischemic bowels. The control group had a mean  $\pm$  SD level of  $0.32 \pm 0.03$  U/g results for the different treatment groups were as follows: P1 =  $0.23 \pm 0.04$ ; P2 =  $0.14 \pm 0.03$ ; P3 =  $0.22 \pm 0.02$ , and P4 =  $0.19 \pm 0.02$  (Figure 3).

**Figure 3.** Tissue Myeloperoxidase (MPO) Levels and Comparative Analysis of Control and Pentoxifylline-Treated Groups



**Table 2.** Inflammatory Cytokine Levels in Control and Pentoxifylline-Treated Animals

| Groups<br>*P < .05 Survival vs<br>Other Groups | TNF- $\alpha$ (pg/mL) | IL1- $\beta$ (pg/mL) | IL-6 (pg/mL)       |
|------------------------------------------------|-----------------------|----------------------|--------------------|
| Control (mean $\pm$ SEM)                       | 960 $\pm$ 32.7        | 81.3 $\pm$ 13.2      | 1740.5 $\pm$ 73.2  |
| P1 Pentoxifylline 1 mg/kg<br>(1 h before)      | 740 $\pm$ 27.5*       | 56.22 $\pm$ 10.4*    | 249.64 $\pm$ 18.9* |
| P2 Pentoxifylline 1 mg/kg<br>(at reperfusion)  | 450 $\pm$ 19.6*       | 42.90 $\pm$ 8.5*     | 194.94 $\pm$ 17.2* |
| P3 Pentoxifylline 10 mg/kg<br>(1 h before)     | 630 $\pm$ 25.3*       | 48.96 $\pm$ 8.9*     | 59.01 $\pm$ 8.9*   |
| P4 Pentoxifylline 10 mg/kg<br>(at reperfusion) | 355 $\pm$ 14.8*       | 35.86 $\pm$ 6.4*     | 105.48 $\pm$ 10.4* |

### Tumor necrosis factor- $\alpha$ cytokines

Serum levels of several inflammatory cytokines (eg, TNF- $\alpha$ , IL-1 $\beta$ , and IL-6) were measured in the control and PTX-treated groups. Those serum cytokines levels were significantly lower in the PTX-treated groups when compared with the saline controls given at reperfusion. The best group was the PTX-treated at 10 mg/kg given at reperfusion even though all groups showed a marked difference (Table 2).

### Laser Doppler flowmetry

Small bowel blood flow was measured several times during the experiment, using laser Doppler; the main

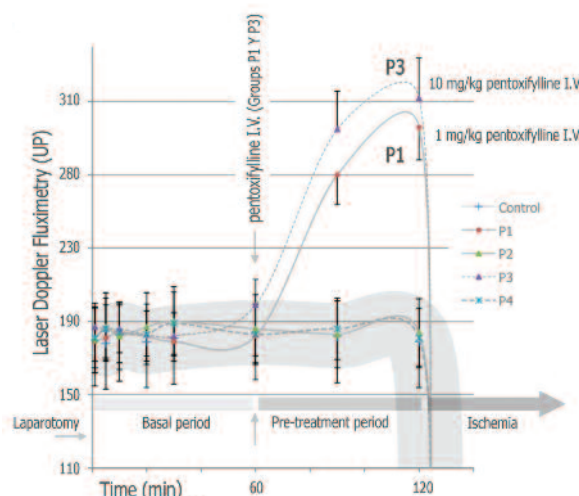
results are shown in Table 3. Analyzed flow values were taken 3 times during the experiment: (1) at basal flow before ischemia, (2) flow during ischemia, and (3) during the flow after reperfusion.

**Table 3.** Basal Flow Values During Ischemia and After Reperfusion

| Groups<br>*P < .05 Survival vs<br>Other Groups | Basal           | Ischemia       | Reperfusion      |
|------------------------------------------------|-----------------|----------------|------------------|
| Statistics                                     | Mean $\pm$ SEM  | Mean $\pm$ SEM | Mean $\pm$ SEM   |
| Control                                        | 180 $\pm$ 7.2   | 44 $\pm$ 1.22  | 70.4 $\pm$ 6.8   |
| P1 Pentoxifylline 1 mg/kg<br>(1 h before)      | 239 $\pm$ 10.7* | 43 $\pm$ 2.2   | 74 $\pm$ 7.6     |
| P2 Pentoxifylline 1 mg/kg<br>(at reperfusion)  | 184 $\pm$ 12.3  | 42 $\pm$ 2.8   | 124.8 $\pm$ 6.8* |
| P3 Pentoxifylline 10 mg/kg<br>(1 h before)     | 272 $\pm$ 13.7* | 45 $\pm$ 2.16  | 74.8 $\pm$ 7     |
| P4 Pentoxifylline 10 mg/kg<br>(at reperfusion) | 181 $\pm$ 14.6  | 40 $\pm$ 3.5   | 130.6 $\pm$ 5.3* |
| Perfusion units (PU)                           | PU              | PU             | PU               |

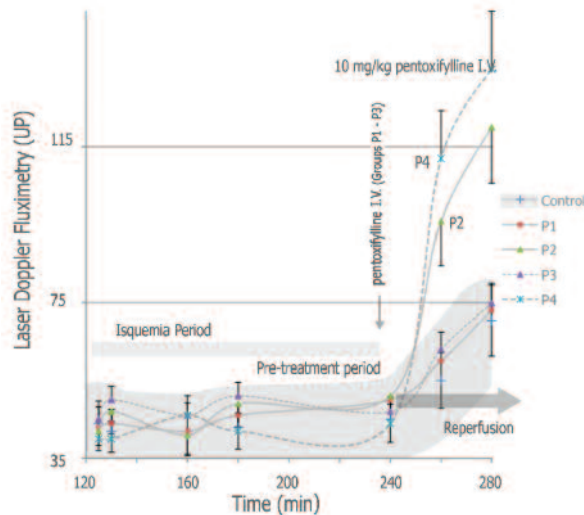
We highlighted the flow values obtained during: (A) The basal blood flow measured in the small bowel wall. This was significantly better in animals in the groups (P1 and P3) that were treated with PTX (1 and 10 mg/kg) 60 minutes before intestinal ischemia, when compared with the controls, which received saline at the same time (Figure 4). There was a statistically significant increase in flow ( $P < .001$ ) at the point of intravenous pretreatment with PTX 1 and 10 mg/kg, in groups P1 and P3, as shown in the Figure 5. These results confirm the immediate vasodilator effect of PTX in the small intestine of the rat in the 2 groups treated with 1 and 10 mg/kg (groups P1 and P3). We might suggest a better starting vascular arrangement to support ischemia as to

**Figure 4.** Absolute Values of Intestinal Blood Flow During Stabilization and After Pentoxifylline Administration



subject all experimental groups. On reperfusion, groups P1 and P3 did not show higher values than the controls, thereby suggesting the vasomotor effect of PTX administered before ischemia did not persist during this time (Figure 5).

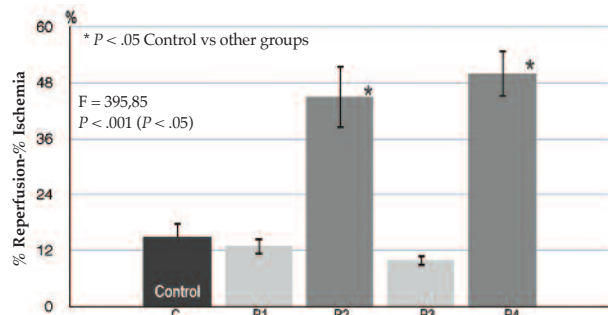
**Figure 5.** Absolute Values of Intestinal Flow Obtained at Different Time Points During Ischemia, Reperfusion, and Pentoxifylline Treatment



(B) During reperfusion (Figure 5), there was a significant increase in flow among animals in groups (P2 and P4) treated with 1 and 10 mg/Kg PTX after reperfusion, which suggests a greater blood supply immediately after removing the clamps used to produce ischemia.

(C) We did the calculations required to determine the percentage of recovery flow at reperfusion (percentage of recovery FLOW = percentage of FLOW REPERFUSION - percentage of FLOW ISCHEMIA) (Figure 6). The ANOVA test showed significant differences ( $P < .0001$ ) between treated and control groups ( $F = 395.85$  for 4 degrees of freedom and  $P = .0001$ ). We performed a Fisher exact test to determine which groups showed significant

**Figure 6.** Percentage Recovery of Vascular Flow in All Groups—Control and Pentoxifylline-Treated



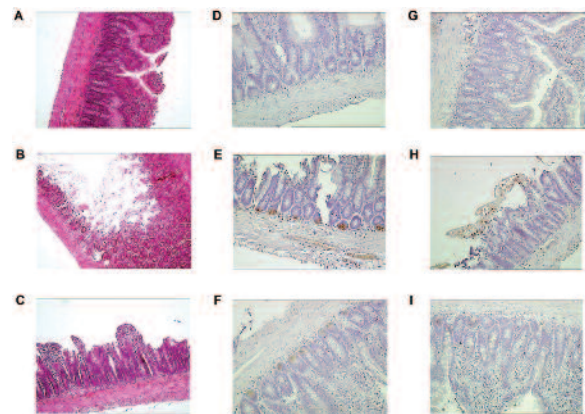
differences. We found significant differences between the controls and groups P2 and P4, which had received PTX after reperfusion. Again, the data suggest a potential beneficial, vasodilation effect of PTX used at reperfusion (Figure 6).

## Histopathologic findings

As expected, histologic analyses of intestinal sections in control animals without ischemia showed no abnormal findings. Severe mucosal changes appeared after I/R. Intestinal tissue showed congestion and necrotic areas that were visible throughout the sections. Necrotic areas were concentrated in the distal region of the microvilli, with progression to the proximal intestinal mucosal region (Figure 7, panel B). After PTX treatment, the tissue remained congested, but the necrotic areas disappeared, and the intestinal structure was similar to that of controls (Figure 7, panel C). At the base of the microvilli, the Paneth cells secrete lysozyme in response to inflammatory damage. Immunohistochemical tests were performed using enzyme-specific antibodies to detect any changes in lysozyme secretion as a result of I/R.

Paneth cells were stained, thus highlighting the specificity of the antibody used. Lysozyme expression increased in specimens after I/R (Figure 7, panel E) compared with controls (Figure 7, panel D). Administering PTX significantly inhibited this increase (Figure 7, panel F). These results coincided with those obtained from immunohistochemical

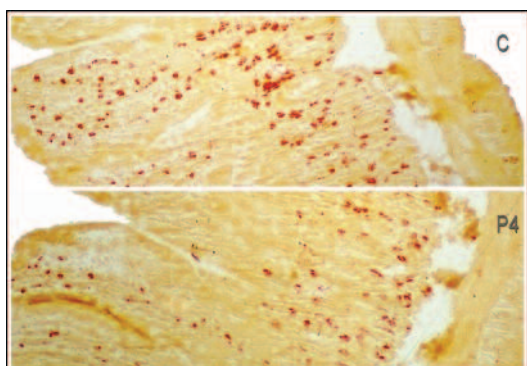
**Figure 7.** General Histopathologic Changes in Control and Pentoxifylline-Treated Groups



Control group without ischemia (A, D, and G), intestinal ischemia without treatment (B, E, and H), and P4 group treated with pentoxifylline on reperfusion (C, F, and I). Hematoxylin-eosin (A, B, and C). Immunohistochemical staining of lysozyme (D, E, and F) and myeloperoxidase (G, H, and I) 5- $\mu$ m sections. (Original magnification  $\times 400$ ; except panels A, D, and E:  $\times 250$ ).

analysis of MPO (Figure 7, panels G, H, and I). The number of MPO-positive cells was significantly higher in animals exposed to I/R (panel H) compared with controls (panel G). The density of MPO-stained cells was higher in regions proximal to the necrotic areas. These stained cells disappeared after PTX treatment (panel I). Fewer naphthol-stained neutrophils were found on the intestinal walls of specimens taken from PTX-treated animals, indicating less leukocyte infiltration, and accordingly, fewer effects of I/R in PTX-treated animals (Figure 8).

**Figure 8.** Neutrophil Infiltration, as Determined by Naphthol Technique in Control and Pentoxifylline-Treated Group



## Discussion

This study demonstrates that PTX can effectively protect against severe ischemic damage in the small intestine in rats. Animals treated with PTX (10 mg/kg at reperfusion) showed a significantly higher survival rate than did control animals ( $P < .05$ ); viability of treated animals was 70%, compared with 40% viability in the control group.

Besides a greater survival rate of animals treated with PTX during reperfusion (group P4) (which would be significant proof that the animals were able to overcome the physiopathological changes produced by intestinal ischemia), other laboratory variables and histopathologic findings confirmed less ischemic damage in the intestine with PTX treatment (better LDH, MDA, and MPO results, and inflammatory cytokine levels). These findings show that PTX can overcome intestinal ischemic injury, because the drug has been shown to be highly protective when administered before ischemic insult, and also, when given at reperfusion, when even better results are obtained.

Pentoxifylline is a multifaceted drug with its effects encompassing red cell deformability as well

as antioxidant, anti-inflammatory and antineutrophil infiltration properties.<sup>22,23</sup> Specifically, PTX down-regulates inflammatory cytokines, such as TNF- $\alpha$ , IL1- $\beta$ , and IL-6. Because of this, many intracellular down-stream molecules directly related to TNF- $\alpha$  or the other inflammatory cytokines, also demonstrate a diminished response to ischemia or inflammation.<sup>44-46</sup>

This is the first study of a small intestinal ischemia used for survival of more than a few hours when evaluating the clinical response of ischemic injured small bowels.<sup>27-32</sup> In fact, survival in this study was extended to 7 days to demonstrate the complete effect of PTX pharmacological treatment on this important clinical endpoint. Only 1 other work by Tireli and associates<sup>4</sup> used 7-day testing, but in a different context, where PTX was used to observe its effect on healing of ischemic small intestine anastomoses. Our model focused specifically on I/R injury.

Only a few studies have addressed the role of PTX in ischemic small intestine and reperfusion.<sup>26-32</sup> A search of the literature using the wording “pentoxifylline in intestinal ischemia” returned only 25 articles between 1992 and the date of this study, of which, only 5 studies were similar to our current work.<sup>28-32</sup> However, when we searched more general wording “pentoxifylline in ischemia,” we found 307 articles. This led us to believe that the interest in using PTX has not been in small intestinal ischemia but rather, on other types of ischemic injury. The mechanisms of protection, however, are similar, and one could learn from the studies in other ischemic organs.

Specifically, our study demonstrated that the anti-inflammatory effect of PTX is perhaps one of the most-prominent ones when analyzing the mechanism by which this drug exerts its protective action in small intestinal ischemia. In this case, not only was the antineutrophil capacity of PTX significant, but more importantly, the response of the inflammatory cytokines of the TNF- $\alpha$  type was diminished also. Our work confirms the anti-TNF- $\alpha$ , anti-IL1 $\beta$  and anti-IL-6 effects observed after using PTX on severe organ ischemia in rats.<sup>11,12,15,20</sup> The TNF- $\alpha$  findings, although not novel from the perspective of the effect of PTX since they were reported by Sullivan and associates in 1988,<sup>33</sup> previously had not been reported after severe intestinal ischemia and PTX treatment, except for the work of Yamamoto.<sup>15</sup> The implications associated with these findings are significant because the above-mentioned

anti-inflammatory molecules are the orchestrators of the inflammatory response in the human or animal ischemic cell.

The improvement in the laser Doppler vascular flow of normal and ischemic intestines indicates a relation between vascular flow and molecular response noted in these animals. Flows correlated better with less inflammatory, physiological, and chemical changes. Pentoxifylline was the main reason for these positive results. Although this is a logic reflection on these findings, they have not been previously published in the context of severe intestinal ischemia in animals. In our study, the best recovery of vascular blood flow was seen in those animals that received PTX at reperfusion. Dosing at 1 mg/kg or 10 mg/kg was not as relevant. The clinical applicability is clear. When using drugs like PTX, the laser Doppler vascular flow could readily separate those small intestinal organs that had viable ischemia and potentially salvageable after severe damage after this pathological injury. Additionally, the timing of drug administration is clinically meaningful. While preischemic drug administration may not always be feasible, medicating with PTX at reperfusion is a realistic option in surgical or clinical practice.

Infiltration of neutrophils in the small intestinal tissue as measured by the Naphthol technique was helpful in defining the response of ischemic organs and determining how neutrophil infiltration was critical in characterizing the ischemic injury. The same was true when measuring the MPO in tissue as marker of neutrophil infiltration and organ response to PTX. The role of neutrophil infiltration as a leading cause of ischemic damage has been known for 2 decades.<sup>47</sup> But use of these markers for small intestinal ischemia and its correlation to survival, inflammatory cytokines, and histopathologic response is relatively new and valuable in assessing tissue viability as indicated by laser flow improvement above.

The other variables used in this study to evaluate ischemic damage such as LDH, MDA, and other general histologic findings referred to previously, do not need an explanation because they are related more to a general pathological response than to a well-defined ischemic insult. However, their enhanced response confirms the current findings and lends support to the theory that ischemic damage not only has a local effect, but a more universal or comprehensive effect.

In short, PTX was significantly protective of severe ischemic small bowel injury, even when used at reperfusion and at levels not previously reported (10 mg/kg). These effects were manifested not only in an improved biochemical and histologic profile, but in a significantly improved survival. Lower doses of PTX (1 mg/kg) also demonstrated a significantly protective effect on inflammation and tissue markers of I/R injury, and a trend toward improved survival (lower dosages showed improvement but did not attain higher survival rates). Specifically, PTX was shown to be highly effective in down-regulating inflammatory cytokines TNF- $\alpha$ , IL1- $\beta$ , and IL-6, decreasing neutrophil infiltration and improving laser Doppler flow in the treated ischemic small intestinal blood vessels. Pentoxifylline demonstrated superior outcomes in severe rat intestinal ischemia that are worth confirming under experimental conditions or even applying to rescue therapy in the clinical arena.

In summary, the significantly higher survival rate in animals treated with PTX during reperfusion seems to indicate that the effect of PTX on physiological mechanisms arising from ischemia leads not only to more favorable results for the analytic variables studied, but also, effectively increases animal survival. And improved outcomes regarding reperfusion flow in PTX-treated animals could be a key indicator that treating transplant patients would significantly improve flow in the transplanted organ and have a beneficial effects at the start of reperfusion.

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