Effects of Trapidil on Intestinal Mucosal Barrier Function and Bacterial Translocation After Intestinal Ischemia and Reperfusion in an Experimental Rat Model

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ABSTRACT

Background: Intestinal ischemia and reperfusion may be the primary triggers of mucosal barrier impairment, cytokine expression, and bacterial translocation (BT). Trapidil is a phosphodiesterase and platelet-derived growth factor inhibitor that reduces lipid peroxidation and inhibits the production of cytokines.

Objective: The goal of this study was to assess whether trapidil might protect the intestinal epithelial barrier by inhibiting lipid peroxidation and proinflammatory cytokines by testing the effect of trapidil on intestinal barrier function in an experimental ischemia/reperfusion (I/R) rat model.

Methods: Trapidil was used in a rat model of intestinal barrier dysfunction caused by intestinal ischemia for 40 minutes followed by reperfusion for 12 hours. To do this, the rats were randomized to 1 of 4 treatment groups, as follows: (1) sham surgery and saline administration (1 mL IV) (Sham group); (2) sham surgery and trapidil administration (8 mg/kg IV) (Sham+T group); (3) I/R and saline administration (1 mL IV) (I/R group); and (4) I/R and trapidil administration (8 mg/kg IV) (I/R+T group). Intestinal barrier function was assessed by histopathologic examination, blood malondialdehyde (MDA) level, and BT.

Results: The I/R+T group showed significantly less incidence of BT compared with the I/R group in the liver and reduced median colony count of translocated bacteria in mesenteric lymph nodes, liver, spleen, and peritoneum compared with the I/R group. Furthermore, the mean blood MDA level demonstrated that lipid peroxidation was significantly decreased in the I/R+T group compared with the I/R group. Histopathologic findings revealed that trapidil

administration before reperfusion preserved intestinal mucosal integrity and inhibited the infiltration of inflammatory cells into the intestines.

Conclusions: In this experimental study, a correlation seemed to exist between intestinal barrier dysfunction and BT. Intestinal barrier dysfunction may allow a large amount of bacteria to pass from the gut to distant organs. Trapidil treatment may inhibit BT by preserving intestinal barrier by inhibiting thromboxane A₂, lipid peroxidation, proinflammatory cytokines, and stimulated prostacyclin. Future dose- and time-dependent studies will be helpful in revealing the effects of trapidil on BT. (*Curr Ther Res Clin Exp.* 2003;64:355–366) Copyright © 2003 Excerpta Medica, Inc.

Key words: bacterial translocation, ischemia/reperfusion, mucosal barrier dysfunction, trapidil.

INTRODUCTION

The immunologic cascade resulting in the sepsis response can be initiated by tissue injury, ischemia/reperfusion (I/R) injury, gram-positive organisms, and fungi, as well as gram-negative organisms and their constituent endotoxins. In multiple trauma or hemorrhagic shock, direct or secondary I/R injury also may lead to an increase in microorganisms and exotoxins released from the gut. The host response to these microbial products, or to the trauma and the I/R injury itself, is rapid activation of the innate immune response and the release of a variety of humoral mediators, including glucocorticoids, catecholamines, and proximal proinflammatory cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-1, and IL-6.¹

The small intestine is sensitive to ischemic insult, which can cause intestinal mucosal barrier dysfunction and cytokine expression, resulting in bacterial translocation (BT).² A previous study³ has shown that reperfusion may significantly exacerbate ischemic injuries of the small intestine. Clinically, BT often is associated with hemorrhage, other shock states, and the need for a liver transplant. The mechanisms responsible for BT are not precisely known, but changes in the intestinal microflora, damage to intestinal mucosa, and impaired host immune defense are considered the major contributing factors.²

Trapidil is a phosphodiesterase and platelet-derived growth factor inhibitor that acts via vasodilation to inhibit platelet aggregation; facilitate prostacyclin biosynthesis; reduce lipid peroxidation^{4,5}; and inhibit the production of TNF- α , IL-6, and IL-12 by inhibiting the CD40/CD40L pathway of monocytes and macrophages.⁶ Recent studies have shown that trapidil may reduce I/R injury in peripheral nerves⁷ and the small intestine.⁸ To assess whether trapidil might protect the mucosal epithelial barrier by inhibiting lipid peroxidation and proinflammatory cytokines, we tested the effect of trapidil on intestinal mucosal epithelial barrier function in an experimental I/R rat model.

MATERIALS AND METHODS

Adult male Wistar rats weighing 240 to 260 g were used. The animals were acclimatized for 1 week to our laboratory conditions before experimental manipulation. They had free access to standard laboratory chow and water and were exposed to a cycle of 12 hours of light and 12 hours of darkness. All procedures were reviewed and approved by the Committee for Institutional Animal Care and Usage of Mersin University (Mersin, Turkey).

The number of rats used in this study was determined by the Committee for Institutional Animal Care and Usage of Mersin University, which attempted to restrict the use of the laboratory animals to the minimum number required for statistical analyses. According to the committee, 6 rats would be needed in each arm to detect an expected BT rate of 50% with an α error of 5% and a β error of 20%. However, considering the possibility of death during the experiment, the sample size was set at 10 rats in each arm to provide appropriate statistical power.

Before the surgery, the rats were placed in 1 cage and were then arbitrarily assigned to 1 of 4 groups. All groups had a reperfusion period of 12 hours. The 4 groups were treated as follows: (1) sham surgery and saline administration (1 mL IV) (Sham group); (2) sham surgery and trapidil* administration (8 mg/kg IV) (Sham+T group); (3) I/R and saline administration (1 mL IV) (I/R group); and (4) I/R and trapidil administration (8 mg/kg IV) (I/R+T group). The dose of trapidil was identical to the dose used successfully in earlier studies^{7,8} and was administered just prior to clamp removal.

Under sterile conditions, rats were anesthetized with ketamine hydrochloride[†] 50 mg/kg and xylazine[‡] 5 mg/kg IM. After the abdomen was shaved and prepared with povidone-iodine, a long (5 cm) midline laparotomy was performed and the superior mesenteric artery (SMA) was identified after deflecting the loops of intestine to the left with sterile, moist gauze swabs. The SMA then was separated from its accompanying lymphatic trunk and temporarily occluded by a bulldog clamp for 40 minutes at the origin from the aorta. Immediate balancing of the small intestine and cecum verified that the blood supply to these intestinal segments had been stopped. The intestine was covered with sterile gauze pads soaked with saline at 37°C. After 40 minutes of intestinal ischemia, the bulldog clamp was removed from the SMA, and, after verification of the return of the blood supply to the gut, the laparotomy was closed with 2-layer suturing and the rats were allowed to awaken. This procedure was followed by a 12-hour reperfusion period. During the reperfusion period, the rats were allowed to freely take standard laboratory chow and water. The sham surgery consisted of separation of the SMA without clamping for 40 minutes,

*Trademark: Rocornal® (UCB Pharma AG, Brussels, Belgium).

†Trademark: Ketalar® (Eczacıbasi-Warner Lambert, Istanbul, Turkey).

‡Trademark: Rompun® (Bayer, Leverkusen, Germany).

followed by a 12-hour sham reperfusion period.⁹ Body temperature was maintained at 37°C using a heating pad and a heating lamp. In addition, 10 mL of Ringer's lactate solution was given subcutaneously to prevent dehydration.

At the end of the 12-hour reperfusion period, the animals were euthanized and the following assessments were performed.

Lipid Peroxide Assay

Blood malondialdehyde (MDA) levels, which are an indication of lipid peroxidation, were determined by the thiobarbituric acid reaction. In this method, barbituric acid interacts with MDA to form a pink compound; the intensity of the pink color indicates the level of lipid peroxidation. The pink compound, 1,1,3,3-tetraethoxy propane (monoaldehyde bis [diethylacetyl]), was used as the primary standard. Blood MDA levels were determined using the method described by Yagi.¹⁰

Processing of Histologic Samples

Serial samples were taken from the ileum and fixed with 10% neutral formalin. Tissues were processed routinely and embedded in paraffin. Specimens stained with hematoxylin and eosin were examined by the pathologist, who was blinded to the source of the slides. The degree of histopathologic changes was graded semiquantitatively using the histologic injury scale previously defined by Chiu et al.¹¹ Histologic mucosal damage was graded using the Chiu Injury Scale¹¹ (from 0 to 5 according to the following criteria): 0 = normal mucosal villi; 1 = development of subepithelial space, usually at the apex of the villi with capillary congestion; 2 = extension of the subepithelial space with moderate lifting of the epithelial layer from the lamina propria; 3 = massive epithelial lifting down the sides of the villi and ulceration at the villous tips; 4 = denuded villi with dilated capillaries and increased cellularity of the lamina propria; and 5 = degradation and disintegration of the lamina propria, hemorrhage, and ulceration.

Microbiologic Analysis

Microbiologic analysis was performed as described by Isenberg.¹² Broths were incubated at 35°C until turbid, and the turbidity was adjusted to match that of a 0.5 McFarland standard (10^8 colony-forming units [CFU]/mL). Using isotonic saline, a 1/100 dilution of the suspension was made to give an adjusted concentration of 10^6 CFU/mL. Subsequent subcultures were performed on blood agar, eosin-methylene blue (EMB) agar, and chocolate agar. All samples were stained by acridine orange using Gram's method. Mesenteric lymph node (MLN), liver, spleen, and peritoneum specimens were placed in 2 mL of brain-heart infusion broth after being weighed and homogenized. These samples also were placed on blood agar and EMB agar. All cultures were incubated under aerobic and anaerobic conditions and were examined at 24 and 48 hours for growth. The identification of bacterial species was performed using standard microbiologic

methods in a blinded manner. Colonization was expressed as CFU per gram of tissue homogenate.

Statistical Analysis

Statistical comparisons were performed using 1-way analysis of variance and the Tukey test for blood MDA levels. These data are expressed as mean (SEM). Differences in histologic grading, incidence of BT, and colony count of translocated bacteria to distant organs were tested using the Kruskal-Wallis test by ranks. Post hoc comparisons between pairs of means were performed using the Wilcoxon rank sum test. These data are expressed as median (25%–75% range). Statistical significance was set at $P < 0.05$.

RESULTS

Forty rats were used. Three rats in the I/R group died during the reperfusion period (1 each in hours 1, 3, and 7 after beginning reperfusion). No cause other than the I/R event was identified for these deaths. Because the rats died at different times and before the 12-hour reperfusion period ended, the data for these animals were not assessed. In the other 3 groups, no rats died within the 12-hour reperfusion period. Consequently, 7 rats in the I/R group and 10 rats in each of the other 3 groups were assessed for each outcome.

The mean (SEM) serum MDA levels in the Sham and Sham+T groups were not statistically significantly different (3.28 [0.10] nmol/mL vs 3.01 [0.08] nmol/mL, respectively). However, 40-minute ischemia and 12-hour reperfusion (I/R group) significantly increased the mean MDA level compared with the Sham group (5.83 [0.19] nmol/mL vs 3.28 [0.10] nmol/mL, respectively; $P < 0.001$) or the Sham+T group (5.83 [0.19] vs 3.01 [0.08] nmol/mL; $P < 0.001$). Trapidil administration before the reperfusion period (I/R+T group) decreased the mean serum MDA level significantly compared with that of the I/R group (3.88 [0.18] vs 5.83 [0.19] nmol/mL; $P < 0.001$) (Figure 1).

Histopathologic findings showed no evidence of epithelial disruption, and the villi were intact and clearly visible in ileal sections from the Sham and Sham+T groups. But the I/R event resulted in massive destruction (including peeling) of villi, and a large amount of inflammatory cell infiltration into the lamina propria of the small intestine. On the other hand, I/R injury decreased markedly when trapidil was used before reperfusion (I/R+T group). Minimal histologic changes were seen in the I/R+T group. Capillary congestion decreased and sparse inflammatory cell infiltration was observed in this group (data not shown).

The median (25%–75% range) grade of mucosal damage in the I/R group was significantly higher than in the Sham group (4.0 [3.5–4.5] vs 0.0 [0.0–0.0], respectively; $P < 0.001$) and the Sham+T group (4.0 [3.5–4.5] vs 0.0 [0.0–0.0], respectively; $P < 0.001$). The median (25%–75% range) grade in the I/R+T group was higher than that of the Sham group (1.5 [0.0–2.0] vs 0.0 [0.0–0.0],

Figure 1. Mean (SEM) blood malondialdehyde level by groups. T = trapidil; I/R = ischemia/reperfusion. * $P < 0.001$ versus Sham. † $P < 0.001$ versus Sham and $P < 0.001$ versus I/R.

respectively; $P = 0.007$) or the Sham+T group (1.5 [0.0–2.0] vs 0.0 [0.0–0.0], respectively; $P = 0.007$). The median (25%–75% range) grade of mucosal damage in the I/R group was significantly higher than in the group using trapidil just before reperfusion (I/R+T group) (4.0 [3.5–4.5] vs 1.5 [0.0–2.0], respectively; $P = 0.001$) (Figure 2).

The incidence of BT and median (25%–75% range) colony counts of translocated bacteria are shown in Tables I and II, respectively. No significant difference in incidence of rats with BT in the MLN, liver, spleen, and peritoneum was observed between the Sham and Sham+T groups (1 rat [10%] in each group). However, 40-minute ischemia and 12-hour reperfusion caused extensive BT in all rats in the I/R group. Differences in the incidence of rats with BT between the I/R group and the Sham or Sham+T group were statistically significant (100% [7/7] of rats vs 10% [1/10] of rats, respectively; $P = 0.001$ for I/R vs Sham and I/R vs Sham+T). Treatment with trapidil (I/R+T) resulted in markedly decreased

Figure 2. The medians (25%–75% range) of grade of mucosal damage in the small intestine. Chiu Injury Scale¹¹ grades: 0 = normal mucosal villi; 1 = development of subepithelial space, usually at the apex of the villi with capillary congestion; 2 = extension of the subepithelial space with moderate lifting of the epithelial layer from the lamina propria; 3 = massive epithelial lifting down the sides of the villi and ulceration at the villous tip; 4 = denuded villi with dilated capillaries and increased cellularity of the lamina propria; and 5 = degradation and disintegration of the lamina propria, hemorrhage, and ulceration. T = trapidil; I/R = ischemia/reperfusion. * $P < 0.001$ versus Sham and versus Sham+T, and $P = 0.001$ versus I/R+T. † $P = 0.007$ versus Sham.

Table I. Incidence (no. [%]) of bacterial translocation (BT) by study group (N = 37).

Group	MLN	Liver	Spleen	Peritoneum
Sham (n = 10)	1 (10)	1 (10)	1 (10)	1 (10)
Sham+T (n = 10)	1 (10)	1 (10)	1 (10)	1 (10)
I/R (n = 7)*	6 (86)	7 (100)	7 (100)	5 (71)
I/R+T (n = 10)†	1 (10)	2 (20)	1 (10)	2 (20)

MLN = mesenteric lymph node; T = trapidil; I/R = ischemia/reperfusion.

* $P = 0.001$ versus Sham and versus Sham+T for overall incidence of BT.

† $P = 0.005$ versus I/R for overall incidence of BT.

incidence of BT compared with the I/R group (20% [2/10] of rats vs 100% [7/7] of rats, respectively; $P = 0.005$). No statistically significant difference was found for the incidence of BT between the I/R+T group and the Sham or Sham+T group (20% [2/10] of rats vs 10% [1/10] of rats) (Table I).

Similar results were obtained for colony counts of translocated bacteria to MLNs, liver, spleen, and peritoneum (Table II). The I/R event resulted in significantly higher median (25%–75% range) colony counts than in the Sham group for all investigated organs: MLN (250 CFU/g [104.0–387.5 CFU/g] vs 0.0 CFU/g [0.0–0.0 CFU/g], respectively; $P < 0.001$); liver (45.0 CFU/g [35.0–49.5 CFU/g] vs 0.0 CFU/g [0.0–0.0 CFU/g], respectively; $P < 0.001$); spleen (72.0 CFU/g [39.5–117.5 CFU/g] vs 0.0 CFU/g [0.0–0.0 CFU/g], respectively; $P = 0.001$); and peritoneum (223.0 CFU/g [47.0–236.5 CFU/g] vs 0.0 CFU/g [0.0–0.0 CFU/g], respectively; $P = 0.012$). Similar results were obtained for the I/R group compared with the Sham+T group, which had the same medians (25%–75% range) as the Sham group. However, colony counts were significantly lower when trapidil was used before the reperfusion period (IR+T group) compared with the I/R group for all investigated organs: MLN (0.0 CFU/g [0.0–0.0 CFU/g] vs 250.0 CFU/g [104.0–387.5 CFU/g]; $P < 0.001$); liver, (0.0 CFU/g [0.0–0.0 CFU/g] vs 45.0 CFU/g [35.0–49.5 CFU/g]; $P < 0.001$); spleen, (0.0 CFU/g [0.0–0.0 CFU/g] vs 72.0 CFU/g [39.5–117.5 CFU/g]; $P < 0.001$); and peritoneum (0.0 CFU/g [0.0–0.0 CFU/g] vs 223.0 CFU/g [47.0–236.5 CFU/g]; $P = 0.007$). No statistically significant

Table II. Median (25%–75% range) bacterial colony counts* by study group (N = 37).

Group	MLN	Liver	Spleen	Peritoneum
Sham (n = 10)				
Median	0.0	0.0	0.0	0.0
Range	0.0–0.0	0.0–0.0	0.0–0.0	0.0–0.0
Sham+T (n = 10)				
Median	0.0	0.0	0.0	0.0
Range	0.0–0.0	0.0–0.0	0.0–0.0	0.0–0.0
I/R (n = 7)				
Median	250.0 [†]	45.0 [†]	72.0 [‡]	223.0 [§]
Range	104.0–387.5	35.0–49.5	39.5–117.5	47.0–236.5
I/R+T (n = 10)				
Median	0.0	0.0	0.0	0.0 [¶]
Range	0.0–0.0	0.0–0.0	0.0–0.0	0.0–0.0

MLN = mesenteric lymph node; T = trapidil; I/R = ischemia/reperfusion.

*Colony number per 1000 colony-forming units per gram.

[†] $P < 0.001$ versus Sham.

[‡] $P = 0.001$ versus Sham.

[§] $P = 0.012$ versus Sham.

^{||} $P < 0.001$ versus I/R group.

[¶] $P = 0.007$ versus I/R group.

differences in colony counts were found between the I/R+T group and the Sham or Sham+T group.

DISCUSSION

In the present study, intestinal I/R injury caused massive destruction of villi, massive inflammatory cell infiltration into the lamina propria of the wall of the small intestine, and markedly increased BT to several organs. The use of trapidil before reperfusion significantly reduced the severity of the intestinal I/R injury and the rate of BT. Furthermore, trapidil exerted its protective effects even when administered after the majority of the ischemic event had occurred.

Several mechanisms causing intestinal I/R injury contribute to cellular damage and death. Lipid peroxidation caused by free radicals is one of the most important mechanisms of injury.¹³ Intestinal tissue is considered sensitive to I/R injury due to xanthine dehydrogenase activity, which occurs in the villus tips.¹⁴ The release of free arachidonic acid, which is the precursor of prostaglandins and thromboxane A₂ (TXA₂), activates phospholipase, leading to increased prostanoid production, free radical release, and lipid peroxidation.¹⁵ Moreover, in a previous study,¹⁶ light and electron microscopy were used to show that trapidil significantly reduced cellular damage and edema at the injury zone. This finding¹⁶ was explained by the membrane-stabilizing effect of trapidil, which results from inhibition of TXA₂ synthesis. In the present study, blood MDA level, which is considered a reliable indicator of I/R damage due to lipid peroxidation, was measured to determine the severity of the injury. The findings suggest that trapidil administration before reperfusion markedly decreased lipid peroxidation.

Blood MDA levels have been accepted as a good indicator of the severity of I/R injury.^{17,18} In this study, blood MDA level was used to determine the severity of the I/R injury. We found a direct correlation between blood MDA levels and histopathologic injury grade, which is an objective indicator of the status of the mucosal barrier. We also found that the more severe the mucosal injury, the greater the passage of bacteria from the gut to the MLN and other distant organs (eg, liver, spleen, peritoneum). This direct correlation between blood MDA levels, mucosal injury, and incidence of BT demonstrated the reliability of these measurements when taken after an ischemic event and subsequent reperfusion.

Trapidil has been shown to act in part as a phosphodiesterase inhibitor. Trapidil inhibits the adhesion and phagocytosis of neutrophils while preventing migration by increasing intracellular cyclic adenosine monophosphate.¹⁹ Furthermore, 1 study⁶ demonstrated that trapidil inhibits the production of proinflammatory cytokines, such as TNF- α , IL-6, and IL-12, by inhibiting the CD40/CD40L pathway of monocytes and macrophages. Inhibition of the CD40/CD40L pathway may play an important role in preventing BT, multiple organ failure

(MOF), and sepsis, because macrophages, which are present in large numbers in the intestine, have been implicated as a primary source of these mediators and have been described as the “motor” of MOF.²⁰ Trapidil also directly stimulates vasodilation by increasing the release of prostacyclin from the endothelium.²¹ Restoration of blood flow in the postischemic phase may save the intestine and be beneficial in restoring the energy stores and in preventing the anaerobic metabolism that provides the precursor metabolites for xanthine oxidase. However, this restoration of flow could result in development of MOF and death.²²

Neutrophil–endothelial cell interaction is thought to play a central role in the pathogenesis of intestinal barrier failure following I/R and MOF.²³ Circulating neutrophils, platelet activating factor, and some cytokines (eg, IL-1, IL-6) may be involved in the development of intestinal barrier dysfunction.⁹ Leukocyte adherence to the microvascular endothelium appears to be an early and rate-limiting step in the development of I/R injury. Leukocyte adhesion glycoprotein complex (CD11/CD18) is the primary mediator of the adherence of neutrophils to postischemic intestinal microvasculature. TXA₂ induces neutrophils to release free oxygen radicals and mediates diapedesis by regulating CD11/CD18 activity.²⁴ Moreover, the use of thromboxane inhibitors has been shown to attenuate neutrophil hydrogen peroxide production and diapedesis in an experimental model of I/R.²⁵ Because trapidil is a selective inhibitor of TXA₂ secretion and a selective stimulator of prostacyclin secretion from endothelial cells, our strategy to modify eicosanoids to decrease I/R injury seems successful. Changing the balance of the TXA₂:prostacyclin ratio in favor of the latter may also prevent postischemic reduced reflow phenomenon in the intestines.

CONCLUSIONS

In this experimental study, a correlation seemed to exist between intestinal barrier dysfunction and BT. Intestinal barrier dysfunction may allow a large amount of bacteria to pass from the gut to distant organs. Trapidil treatment may inhibit BT by preserving intestinal barrier by inhibiting TXA₂, lipid peroxidation, proinflammatory cytokines, and stimulated prostacyclin. Future dose- and time-dependent studies will be helpful in revealing the effects of trapidil on BT.

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