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Ischemic Preconditioning Protects Intestine From Prolonged Ischemia

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ABSTRACT

Ischemic preconditioning (IP), obtained by exposure to brief periods of vascular occlusion, improves organ tolerance to prolonged ischemia. The aim of this study was to evaluate the effects of IP on intestinal morphology. Forty rats were subjected to sham surgery ($n = 20$, group I) or intestinal preconditioning ($n = 20$, group II) with a cycle of brief ischemia/reperfusion (10-minute occlusion of superior mesenteric artery [SMA], followed by 10-minute reperfusion) before prolonged ischemia produced by SMA occlusion (45 minutes). Five animals in each group were sacrificed 2, 12, 24, and 48 hours after reperfusion. Intestinal samples were processed for light and electron microscopy. A TUNEL assay was performed to detect apoptosis. Statistical analysis used Student *t* test and Kaplan-Meier survival curves. The overall mortality for the sham-operated group was 15%, while no animals of group II died (NS). Histological evaluation showed early detachment of epithelial cells from villous stroma accompanied by marked congestion and edema. Successive morphological changes were represented by leukocyte infiltration, focal necrosis, and marked villus denudation or loss. Group II animals showed significantly reduced inflammatory infiltrates in the lamina propria and a greater villus height compared to group I. The maximum number of apoptotic nuclei was observed in both groups, Following 2 hours of reperfusion group II animals showed significantly, greater apoptosis at 2 and 12 hours after reperfusion ($P < .05$). Electron microscopy showed severe mitochondrial and basement membrane damage. The findings from this study confirm that IP preconditioning attenuates morphological alterations that are invariably present after prolonged ischemia and reperfusion.

ISCHEMIC PRECONDITIONING (IP) is defined as one or more brief periods of ischemia with intermittent reperfusion and has been described to improve organ tolerance to subsequent, prolonged ischemia.[1] The beneficial effect was commonly studied in the heart, but has been confirmed in brain, lung, liver, and skeletal muscle models of ischemia/reperfusion (I/R) injury.[2–5] Although not as extensively studied as in the heart, there is recent evidence that IP may also protect the small intestine against deleterious effects of protracted ischemia.[6–8] The aim of this study was to evaluate the effects of IP on intestinal structural/ultrastructural morphology after prolonged ischemia.

MATERIAL AND METHODS

The study was performed using male ACI rats weighing 250 to 300 that were maintained in accordance with the Animal Committee Guidelines for use and care. Animals were fasted for 12 hours before surgery, at which time they were randomly allocated to two experimental groups, and anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally). Forty rats were first subjected to either sham surgery ($n = 20$, group I) or intestinal preconditioning ($n = 20$, group II) using a cycle of 10-minute occlusion of superior mesenteric artery (SMA), followed by 10 minutes of reperfusion. Thereafter prolonged ischemia was achieved by SMA occlusion for 45 minutes. Five animals of each group were euthanized at 2, 12, 24, and 48 hours after reperfusion. Intestinal samples were processed for light and electron microscopy studies.

Light Microscopy

Wedges of 1 to 2 cm from the ileum, were collected, carefully cannulated, and gently flushed with normal saline solution. Follow-

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360 Park Avenue South, New York, NY 10010-1710

0041-1345/04/$–see front matter
doi:10.1016/j.transproceed.2004.01.078

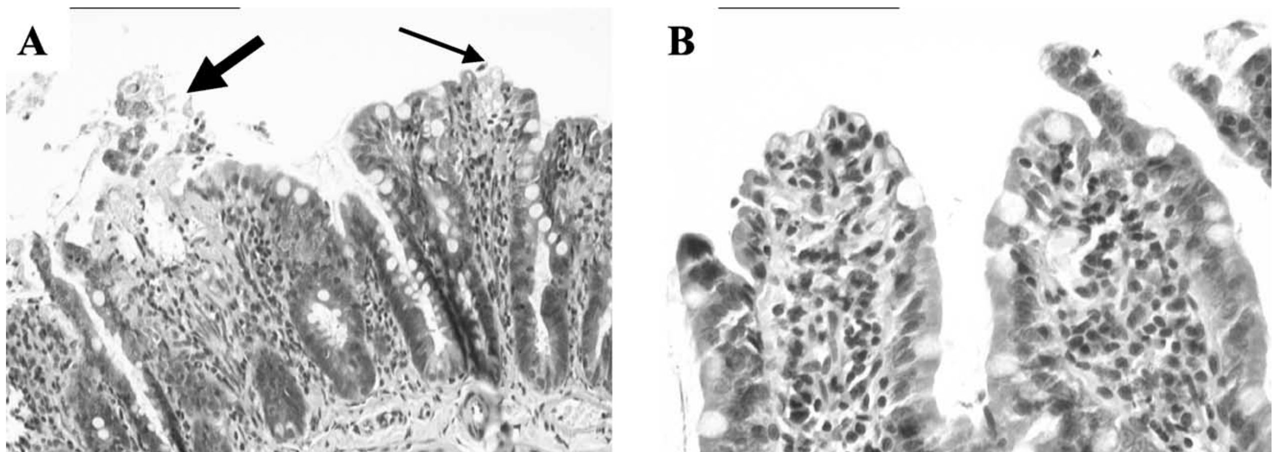

Fig 1. Histologic lesions of the rat small bowel (E&E): **(A)** Control animal at 2 hours after reperfusion (200×). Extended superficial ulceration of the mucosa (thin arrow) and focal area of ulceration with exposed lamina propria (large arrow); **(B)** IP treated animal at 2 hours after reperfusion (400×). Denudated villus with preservation of villi architecture. Slightly increased cellularity of lamina propria can be observed.

ing fixation by immersion in 10% buffered formaldehyde solution, paraffin embedding, and staining with hematoxylin and eosin. Histopathological grading was performed according to criteria established by Chiu et al.[9] Briefly, mucosal damage was graded from 0 to 5, normal to severe, according to the criteria: grade 0, normal mucosa; grade 1, subepithelial Gruenhage's space at the tip of the villus, often with capillary congestion; grade 2, extension of the space with moderate epithelial lifting; grade 3, massive epithelial lifting with a few denuded villi; grade 4, denuded villi with exposed lamina propria and dilated capillaries, possibly with increased cellularity of lamina propria; grade 5, disintegration of the lamina propria, ulceration, and hemorrhage.

Electron Microscopy

Wedges of 3 to 4 mm in length were harvested from the ileum, longitudinally cut, and immersed in 2.5% phosphate buffer glutaraldheyde solution (24 h/4°C). Specimens were washed in phosphate buffer solution, postfixed in 1% OsO_4 for 2 hours at 4°C, dehydrated in ethanol and propylene oxide, and embedded in Epon 812 for 48 hours. Semi-thin sections (1 μm thick) were stained with toluidine blue. Ultra-thin sections cut with a diamond knife, were stained with uranyl acetatelead citrate for transmission electron microscopy.

TUNEL Assay

Four-micrometer thick sections were collected on poly-L-lysine-coated glass slides. The nuclear DNA fragmentation of the apoptotic cell was labeled in situ by the TUNEL assay. After treatments with xylene and rehydration with progressively decreasing alcohol concentrations followed by phosphate-buffered saline (PBS), each section was treated with 20 μg/mL proteinase K (Sigma) in 0.1 mol/L Tris/HCL buffer (pH 7.4) for 15 minutes. After rinsing with PBS, endogenous peroxidase activity was blocked with 3% H_2O_2 for 5 minutes. After rinsing with PBS, they were incubated with 0.5 U/μL terminal deoxynucleotidyl transferase (Boerhinger Mannheim, Germany) and 0.05 nmol/μL biotinylated deoxyuridine triphosphate in terminal deoxynucleotidyl transferase buffer (Boerhinger Mannheim, Germany) for 60 minutes in a humidified chamber at 37°C. Each slide was then observed with a microscope to check the staining quality before image acquisition. For each animal, five sections were analyzed by counting apoptotic bodies in five randomly chosen fields.

Data are expressed as means values ± standard deviation (SD). Statistical analysis was performed using Student *t* test and Kaplan-Meier survival curves. Significant differences were assumed when $P < .05$.

RESULTS

The overall mortality for group I animals (IR without ischemic preconditioning) was 15% with all animals dead within 24 hours. No animals in group II (preconditioning + IR) died (P = NS). Histologic evaluation showed early detachment of epithelial cells from the villous stroma accompanied by marked edema and congestion in both groups. Successive morphological changes included marked villus denudation or loss, focal necrosis, and leukocyte infiltration. However, according to the histological scoring scheme, group I animals showed a higher histology score than group II animals, indicative of a quantitatively and qualitatively increased tissue injury. In fact, group I animals showed marked villous disintegration with focal hemorrhage and extensive exposure of the lamina propria (grade 4 and 5 injury). Although group II animals, revealed focal massive epithelial lifting and occasional areas of denuded villi (grade 2 and 3 injury), when compared to group I animals, they demonstrated preserved villus height and mucosal architecture (Figs 1 and 2). Lamina propria inflammatory infiltrates which were present in both groups beginning at 24 hours, were significantly reduced among group II animals.

The count of apoptotic nuclei by a TUNEL assay showed that the maximum number of apoptotic nuclei was observed following 2 hours of reperfusion in both groups. Some apoptotic bodies were shed into the lumen. Significantly increased apoptosis was observed among group II animals

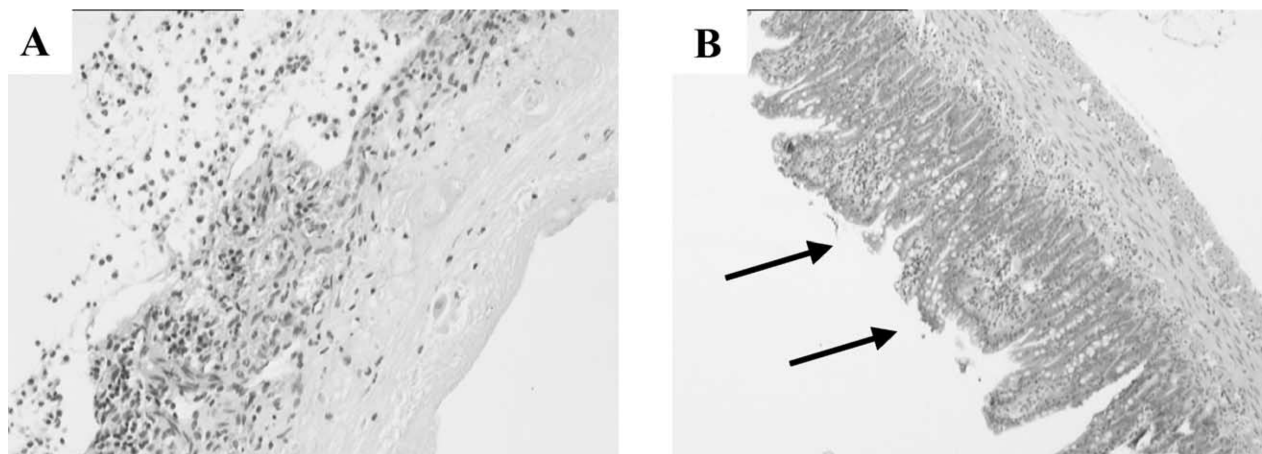

Fig 2. **(A)** Control animal at 24 hours (40×). Extensive ulceration with total loss of mucosal epithelium. **(B)** IP treated animal at 24 hours after reperfusion (40×). Focal areas of villi denudation with little epithelial lifting (arrows).

at 2 and 12 hours after reperfusion (respectively $P < .002$ and $P < .002$) (Table 1). However, at 24 and 48 hours the number of apoptotic bodies was lower than group I animals (NS). Electron microscopy confirmed the damage observed by light microscopy.

CONCLUSIONS

Ischemic preconditioning is a phenomenon in which a brief IR episode confers protection against subsequent prolonged ischemia. Although the role of IP has been widely studied in different organ and tissues, only scant data are available on its role in intestinal IR injury. The present study was undertaken to delineate the effects of IP on intestinal mucosa after IR injury, evaluating structural and ultrastructural morphological changes.

The major finding of this study was that intestinal ischemic preconditioning markedly attenuated the morphological injury that was invariably present after prolonged ischemia and reperfusion. IP abrogated lamina propria disintegration, ulcer formation, and necrosis. Furthermore after IP, cell death seemed to occur mostly by apoptosis. The degree of lamina propria inflammation was significantly reduced.

Table 1. Counts of Apoptotic Nuclei

Groups	Time points (hours) 6*	12*	24	48
Group I (IR)	25 ± 6.6	18.5 ± 5.6	14.8 ± 4.95	12.2 ± 3.8
Group II (preconditioning + IR)	32 ± 5.5	24.2 ± 6.9	11.9 ± 3.9	10.15 ± 2.4

Results are expressed as mean TUNEL positive nuclei/observe field ± SD.
*Significant difference $P < .05$ (group I vs group II).

REFERENCES

1. Murry CE, Jennings RB, Reimer KA: Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 74:1124, 1986
2. Lloris-Carsi JM, Cejalvo D, Toledo-Pereyra LH, et al: Preconditioning: effect upon lesion modulation in warm liver ischemia. Transplant Proc 25:3303, 1993
3. Mounsey RA, Pang CY, Forrest C: Preconditioning: a new technique for improved muscle flap survival. Otolaryngol Head Neck Surg 107:549, 1992
4. Li G, Chen S, Lu E, et al: Protective effects of ischemic preconditioning on lung ischemia reperfusion injury: an in-vivo rabbit study. Thorac Cardiovasc Surg 47:38, 1999
5. Gidday JM, Shah AR, Maceren RG, et al: Nitric oxide mediates cerebral ischemic tolerance in a neonatal rat model of hypoxic preconditioning. J Cereb Blood Flow Metab 19:331, 1999
6. Sola A, Hotter G, Prats N, et al: Modification of oxidative stress in response to intestinal preconditioning. Transplantation 69:767, 2000
7. Sola A, De Oca J, Gonzalez R, et al: Protective effect of ischemic preconditioning on cold preservation and reperfusion injury associated with rat intestinal transplantation. Ann Surg 234:98, 2001
8. McCallion K, Wattanasirichaigoon S, Gardiner KR, et al: Ischemic preconditioning ameliorates ischemia- and reperfusion-induced intestinal epithelial hyperpermeability in rats. Shock 14:429, 2000
9. Chiu CJ, McArdle AH, Brown R, et al: Intestinal mucosal lesion in low flow states. A morphological, hemodynamic and metabolic reappraisal. Arch Surg 4:478, 1970