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ISCHEMIC PRECONDITIONING REDUCES INTESTINAL EPITHELIAL APOPTOSIS IN RATS

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ABSTRACT—Recent experimental studies have described protective effect of ischemic preconditioning (IPC) on ischemia–reperfusion (I/R) injury of the intestine. We hypothesize that to reach a new point of view on the effect of IPC in intestinal barrier function, the relationship between I/R-induced mucosal injury and apoptosis must first be clarified. The present study was undertaken to investigate the role of IPC on intestinal apoptosis and probable contributions of bcl-2 expression to this process. We also investigated the effect of intestinal IPC on ileal malondyaldihyde levels. Forty-four male Wistar rats were randomized into four groups each consisting of 11 rats: sham-operated control, I/R group (30 min of superior mesenteric artery occlusion), IPC-I/R group (10 min of temporary artery occlusion prior before an ischemic insult of 30 min), and IPC alone group (10 min of preconditioning). Twenty-four hours later, ileum samples were obtained. Ileal malondyaldihyde levels were increased in the I/R group (31.9 ± 18.8 vs. 106.8 ± 39.8) but not in the IPC alone and IPC-I/R groups (38.1 ± 13.6 and 44.7 ± 12.7; $P < 0.01$). The number of apoptotic cells was significantly lower in IPC-I/R group than that of I/R group, and these findings were further supported by DNA laddering and M30 findings. Diminished bcl-2 expression observed in the ileal specimens of I/R group was prevented by IPC. Our results indicate that IPC may provide a protective effect on ileal epithelium and that this effect is probably the result of a significant increase in the expression of bcl-2 after the insult. The reversal of apoptosis by IPC might help preserving the vitality of intestinal structures that have a critical function, cessation of which often leads to multiorgan dysfunction syndrome.

KEYWORDS—Ischemia-reperfusion, ischemic preconditioning, apoptosis, bcl-2, DNA laddering, multiorgan dysfunction syndrome, lipid peroxidation

INTRODUCTION

Organ dysfunction is the principal determinant of outcome in critical illness states. Although apoptosis is known to be a homeostatically regulated process that normally occurs in healthy organisms, the role of apoptosis in multiorgan dysfunction syndrome (MODS) has been described but is a relatively new concept (1). The pathophysiological conditions related to the onset of MODS, such as ischemia–reperfusion (I/R) and/or septic states, have been demonstrated to effect the rates of apoptosis in parenchymal cells (1, 2). Intestinal epithelial apoptosis appears to be the key phenomenon in gut barrier function, and it may trigger a vicious cycle from bacterial translocation to MODS (1–4).

Reactive oxygen species (ROS) and reactive nitrogen species have been recognized as common mediators or modulators in not only I/R-induced injury but also in apoptosis (1). Antioxidants can offer protection against ROS-mediated injury, as shown in various cell types (5, 6). Bcl-2, which is a constitutive protein that functions as an antioxidant, prevents apoptotic cell death from a wide array of noxious stimuli, including hypoxia, ionizing irradiation, and calcium ionospheres (7, 8).

Because of the original observation that repeated brief episodes of I/R could trigger potent protective responses, ischemic preconditioning (IPC) has become a major focus of studies in myocardial and intestinal protection from I/R injury (9–11). In studies associated with myocardium, downregulation of bcl-2 and upregulation of apoptosis reported during I/R-induced injury in addition to the role of IPC in reducing apoptosis by upregulating the antideath gene bcl-2 were previously demonstrated (12, 13). In concordance with these results, in a recent study from our laboratory, we demonstrated that IPC prevented intestinal mucosal integrity and bacterial translocation associated with I/R (14). However, there is little knowledge about the effect of IPC on intestinal apoptosis in I/R phenomena. We hypothesized that IPC prevented the expected injury with the same duration of I/R and that lipid peroxidation and ileal epithelial apoptosis were the responsible mechanisms in resultant injury. The present study was therefore designed to investigate the relation between IPC, and bcl-2 expression, DNA laddering, and apoptosis in the intestine. Furthermore, the effect of intestinal IPC on ileal malondyaldihyde (MDA) levels was also investigated.

MATERIALS AND METHODS

The experiments described in this manuscript were performed in adherence to the National Institutes of Health Guidelines on the Care and Use of Laboratory Animals, and approval of the Ethic Committee of Mersin University School of Medicine was obtained before the study was undertaken. Fourty-four male Wistar rats (250–300 g) were used in the present study, and as a standard protocol, all the rats were housed in a quiet nonstressful environment for 1 week before study. However, to eliminate cyclic estrogen interference that might be expected in a female rat, only male animals were used.

Experimental protocol

After fasting overnight, all animals were anesthetized with intramuscular ketamine 80 mg kg^{-1} and xylazine 7 mg kg^{-1} and were placed in supine position on a

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heating pad for maintenance of body temperature between 36 and 37°C. They were randomly divided into four groups. In the first group, which served as sham-operated control (C, n = 11), rats were subjected only to laparotomy. In the second group, which served as I/R group (I/R, n = 11), laparotomy was performed, and the superior mesenteric artery was occluded by an atraumatic microvascular clamp for 30 min followed by a reperfusion period of 24 h. In the third group, which served as IPC-I/R (IPC-I/R, n = 11), rats were subjected to IPC with 10 min of ischemia and 10 min of reperfusion immediately before the ischemic insult of 30 min followed by a reperfusion period of 24 h (14, 15). In the fourth group, rats were subjected to 10 min of ischemia followed by a reperfusion period of 24 h; this group was the IPC-alone group (n = 11). Abdomen was closed with 3/0 silk suture in all of the rats.

Twenty-four hours later, rats were reanesthetized with intramuscular ketamine of 80 mg kg^{-1} and xylazine 7 mg kg^{-1} at the time of sacrifice. Because the ileum was found to be more sensitive to I/R injury than the proximal segments (16), for the evaluation of I/R-induced intestinal apoptosis, three samples of 2.5 cm in length each were collected from ileal part of the small intestine for biochemical, histopathological, and immunohistochemical assessment.

Thiobarbituric acid assay for intestinal MDA concentrations—One of the segments of ileum was removed and homogenized with cold 1.15% KCl to make a 10% homogenate at the end of experiment. An assay for tissue MDA concentration as an index of lipid peroxidation was performed according to the method of Yagi (17). Values expressed as nmol per gram wet tissue weight.

Evaluation of apoptosis by light microscopy, immunohistochemically, and DNA agarose gel electrophoresis—In this study, three independent methods were used to detect apoptosis: 1) conventional light microscopy, 2) immunohistochemical staining for cytokeratin 18, and 3) DNA agarose gel electrophoresis.

Conventional light microscopy—In conventional light microscopy, one of the samples of ileum was fixed in 10% formaldehyde. The tissues had been dehydrated and embedded in paraffin. Sections were cut in a microtome and adhered to glass slides with polylysine. Hematoxylin and eosin-stained specimens were examined by the author (L.C.) blinded to sample identity that identified apoptosis based on characteristic cellular morphologic changes, including cell shrinkage with compacted/condensed nuclei (pyknosis) and/or nuclear fragmentation (karyorrhexis). Apoptotic cells in the crypt epithelium were counted in 10 random fields with a magnification of ×200, and the average number of these cells were calculated.

Immunohistochemical staining for cytokeratin 18—In immunohistochemical staining for cytokeratin 18, a neo-epitope in cytokeratin 18 (CK18) becomes available at an early caspase cleavage event during apoptosis and is not detectable in vital epithelial cell and necrotic cells (18). Immunohistochemistry for M30 CytoDeath (monoclonal antibody, dilution 1:50, Roche, Mannheim, Germany) was performed using a combination streptavidin–biotin–peroxidase method and microwave antigen retrieval on formalin-fixed, paraffin-embedded tissues according to the manufacturers' protocols. Omitting the primary antibody was considered the negative control. The positive control was infiltrative ductal carcinoma of the breast for M30. The slides were evaluated in a blinded fashion by the author (L.C.), and the positively stained cells were counted with the same technique as mentioned above used for the hematoxylin and eosin-stained specimens.

DNA agarose gel electrophoresis—In DNA agarose gel electrophoresis, one of the segments of ileum was weighted, and 10 mg of tissue samples was separated. The tissues were then incubated with an equal volume of binding/lysis buffer. After incubation, the lysed samples were poured into a filter tube containing glass fiber fleece. The DNA was separated from unbound lysate components by using centrifugation. The abound DNA samples were washed twice. The purified DNA samples were eluted from the filter tube and collected by centrifugation. The eluted DNA samples were mixed with gel loading buffer. The samples were applied to a 1% agarose gel that contained ethidium bromide. The gel were run in TBE (Tris-borate EDTA) buffer at 75 V for 90 min at room temperature. The gel was then placed on a UV light box to visualize the DNA ladder pattern (Apoptotic DNA ladder kit, Cat. no 1 835 246, Roche Diagnostics, Mannheim, Germany).

Immunohistochemical analysis for bcl-2—Immunohistochemistry for bcl-2 (mouse antihuman monoclonal antibody; dilution 1:50; LabVision Corporation, Fremont, CA) was performed using a combination streptavidin–biotin–peroxidase method and microwave antigen retrieval on formalin-fixed, paraffin-embedded tissues according to the manufacturers' protocols. For the negative control, the primary antibody was omitted. Tonsil was used as positive control for bcl-2 staining. Sections were examined by the author (L.C.) blinded as to the sample identity. Immunohistochemical evaluation was performed only in the epithelial component. The positive cells were counted in 100 adjacent epithelial cells and repeated in three high-power fields, and the total number of positive cells was expressed as a percentage of 300.

Statistical analysis

Biochemical values are given as mean ± SD values. One-way ANOVA followed by Student–Newman–Keuls test was used to evaluate the statistical significance of the differences of ileal MDA values. Hematoxylin and eosin- and M30-stained apoptotic cell counts and bcl-2-stained cells counts were analyzed using paired samples *t* test. *P* values less than 0.05 were considered significant.

RESULTS

All animals survived the experimental protocol. Ileal MDA levels were significantly increased in I/R group when compared with sham-operated control and IPC alone groups (106.8 ± 39.8 vs. 31.9 ± 18.8 and 38.1 ± 13.6; $P < 0.01$), whereas IPC prevented the expected increase in ileal MDA levels (44.7 ± 12.7). Lipid peroxidation assay results are shown in Figure 1.

Although conventional light microscopy with hematoxylin and eosin-stained specimens is one of the least sensitive methods for detection of apoptosis, it is highly specific if characteristic morphologic changes are observed (4). Morphological evaluation of hematoxylin-stained sections from I/R group showed extensive apoptosis ($P = 0.011$); however, in the IPC-I/R group. the number of apoptotic cells was reduced significantly ($P = 0.041$; Fig. 2A and B). However, the immunohistochemical examination with M30 demonstrated that the number of apoptotic cells in sham-operated control, IPC-alone, and IPC-I/R groups were significantly lower than the I/R group ($P = 0.007$, $P = 0.019$, and $P = 0.020$, respectively). No significant statistical difference between sham-operated control, IPC-alone, and IPC-I/R groups was observed (Fig. 3A and B).

DNA agarose gel electrophoresis is highly specific for the apoptosis (19). DNA agarose gel electrophoresis was performed on 33 ileal specimens. In the sham-operated control group, no specimen had DNA laddering. In the I/R group, six of 11 specimens were found positive for DNA laddering, whereas in the IPC-I/R and IPC alone groups, the ratio of positive specimens decreased to 2:11 and 1:11, respectively, in DNA agarose gel electrophoresis (Fig. 4).

Bcl-2-stained cell counts of villus epithelium for all four groups are given in Figure 5. Bcl-2-stained cell counts in the sham-operated control group were significantly lower in the I/R group ($P = 0.010$). Bcl-2 staining in the IPC-alone and IPC-I/R groups remained at similar levels as in sham-operated control but were significantly different from I/R group ($P = 0.019$ and $P = 0.033$, respectively; Fig. 6 A–D).

DISCUSSION

To reach a new point of view on the effect of IPC in intestinal barrier function, the relationship between I/R-induced mucosal injury and apoptosis must be clarified. We herein demonstrated that intestinal IPC reduces I/R-induced DNA

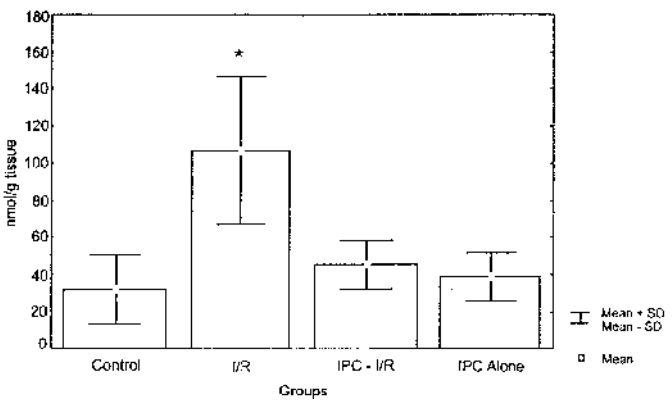

FIG. 1. **Tissue MDA levels.** Data expressed as mean ±SD nmol/g tissue. *$P < 0.01$.

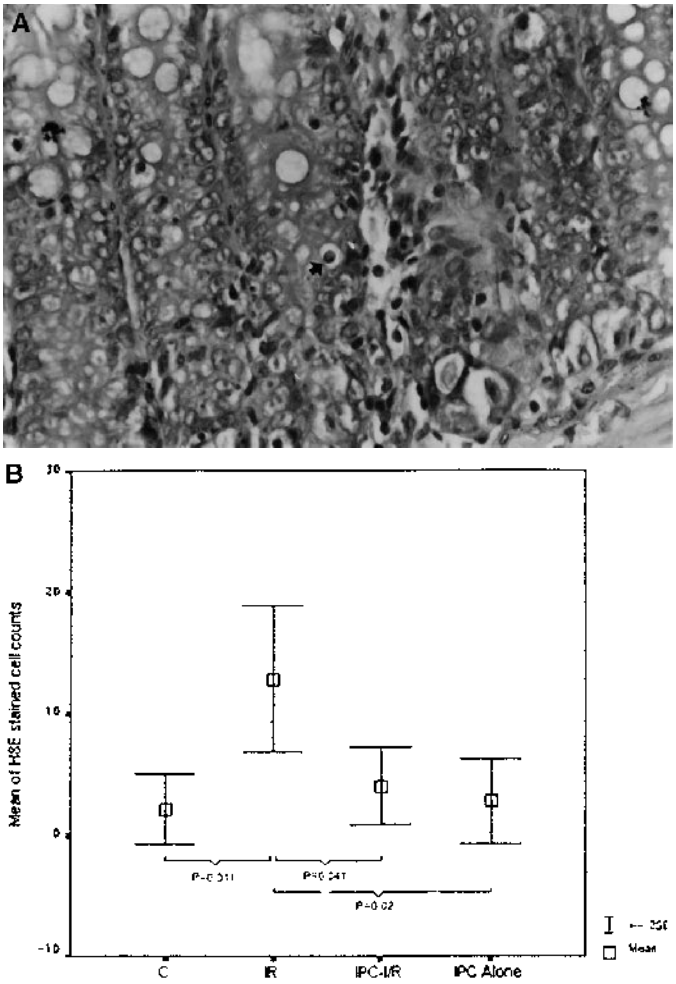

FIG. 2. **(A) Photomicrograph of hematoxylin and eosin-stained sections of the small intestine showing a representative apoptotic cell (arrow) ×400 and (B) hematoxylin and eosin-stained apoptotic cell counts in all groups.** I/R resulted in increased number of apoptotic cells compared with sham-operated control and IPC alone group. IPC-I/R reduced the number of apoptotic cells.

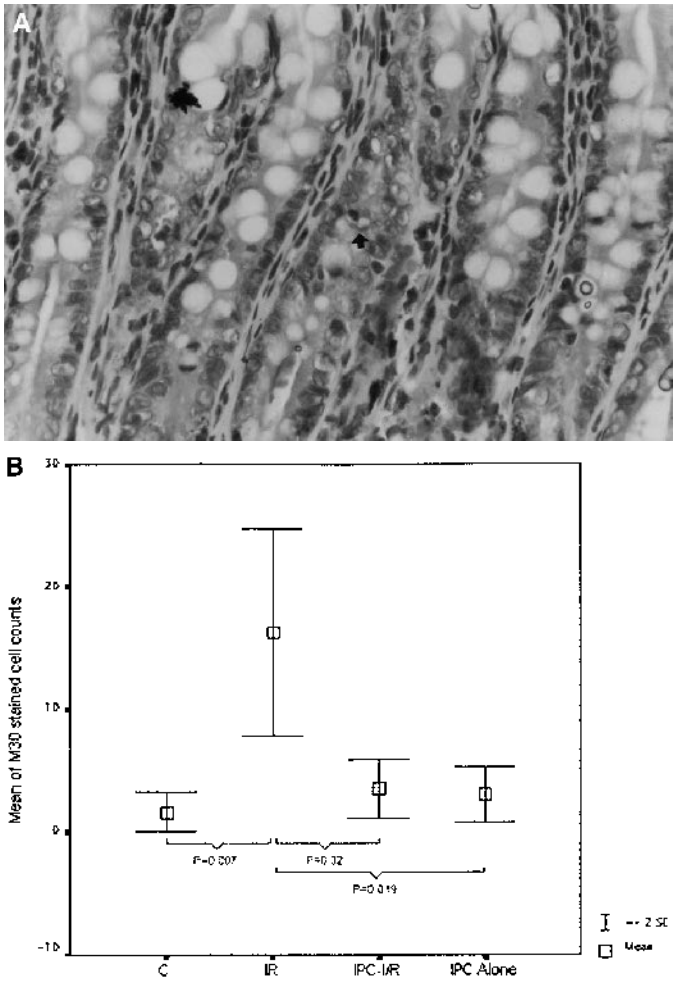

FIG. 3. **(A) Photomicrograph of immunohistochemically stained with M30 sections showing a representative apoptotic cell (arrow) ×400 and (B) M30-stained apoptotic cell counts in all groups.** I/R resulted in increased number of apoptotic cells compared with sham-operated control and IPC alone group. IPC-I/R reduced the number of apoptotic cells.

laddering and apoptosis in ileum by a mechanism that might be initiated by prevention of lipid peroxidation as shown by the marked suppression of increased MDA levels. We also demonstrated that intestinal IPC upregulates bcl-2 expression in the rat small intestine.

ROS and/or reactive nitrogen species can cause significant cell death by apoptosis that increases dramatically in the intestinal mucosa after I/R, as shown in rats (2, 20). Additionally, oxidative stress in intestinal preconditioning is reduced as a consequence of the diminished conversion of xanthine dehydrogenase to xanthine oxidase (15). Similar to the effect of oxidant–antioxidant equilibrium, the equilibrium variations between apoptotic and anti-apoptotic genes also play a role in the I/R injury. Coopersmith et al. (21) reported that forced expression of bcl-2 suppresses I/R-induced apoptosis in intestinal epithelium. Furthermore, the prolonged survival in septic mice with overexpression of an antioxidant Bcl-2 gene highlights the interaction between apoptotic and anti-apoptotic genes in apoptosis (22, 23). In this study, although IPC increased bcl-2 expression in the intestinal epithelium and prevented lipid peroxidation, it also caused a reduction in DNA laddering and apoptosis triggered by I/R. Although the support function of IPC for the antioxidant system by upregulation of antioxidant enzymes and upregulation of bcl-2 has been shown in cardiac tissues (13), the prevention of DNA laddering and increase of bcl-2 expression in intestinal epithelium by IPC is a totally new finding.

Oxido-inflammatory cascade-induced ileal injury is characterized by enhanced lipid peroxidation and morphological injury in ileal mucosa (24–26). The prevention of lipid peroxidation and thus preservation of intestinal mucosa with IPC are confirmed in this study as demonstrated by the prevention of increase in MDA values. Vieira et al. (27) have also shown that apoptosis by lipid peroxidation products, such as 4-hydroxynonenal, can also be inhibited by bcl-2, which may be considered as a supporting finding for our results. However, the documented capability of IPC to inhibit postischemic P-selectin and NFκB expression in the intestine may be the very initial step of the polymorphonuclear neutrophil adhesion, ROS production, and finally lipid peroxidation cascade, which leads to intestinal injury (28, 29). We believe that our data related to the lipid peroxidation along with DNA laddering may be an important protective factor against apoptotic intestinal injury.

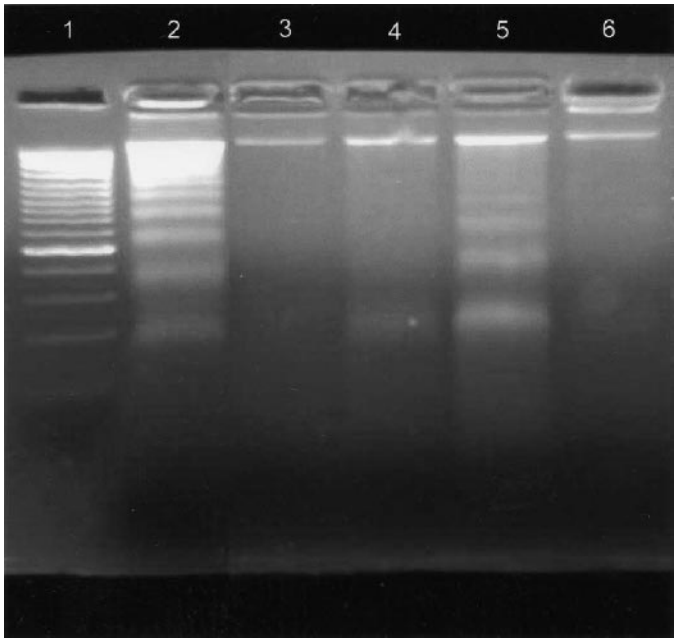

FIG. 4. **Apoptotic DNA ladder assay.** A representative ethidium bromide-stained 2% agarose gel containing DNA ladder assayed with apoptotic DNA Ladder Kit is shown. The samples were loaded in the following manner: marker (lane 1), positive control from the kit (lane 2), control samples (lanes 3, 4), I/R sample (lane 5), and IPC-I/R sample (lane 6). The lane 1 shows a 100-bp DNA size marker.

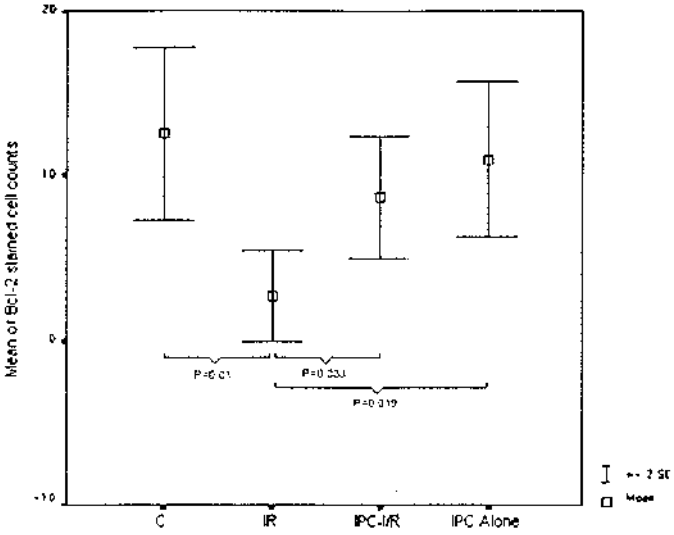

FIG. 5. **Bcl-2-stained cell counts in all groups.** I/R resulted in decreased number of bcl-2-stained cells compared with sham-operated control and IPC alone group. IPC-I/R prevented the bcl-2 staining.

Tissue protective effect of IPC is partly related to its ability to inhibit apoptosis, which is also important for functional recovery of the preconditioned organ. Brocheriou et al. (30) found that improved cardiac function has been correlated with a reduction of cardiomyocyte apoptosis. Because apoptotic cells retain their membrane integrity, they are excluded during

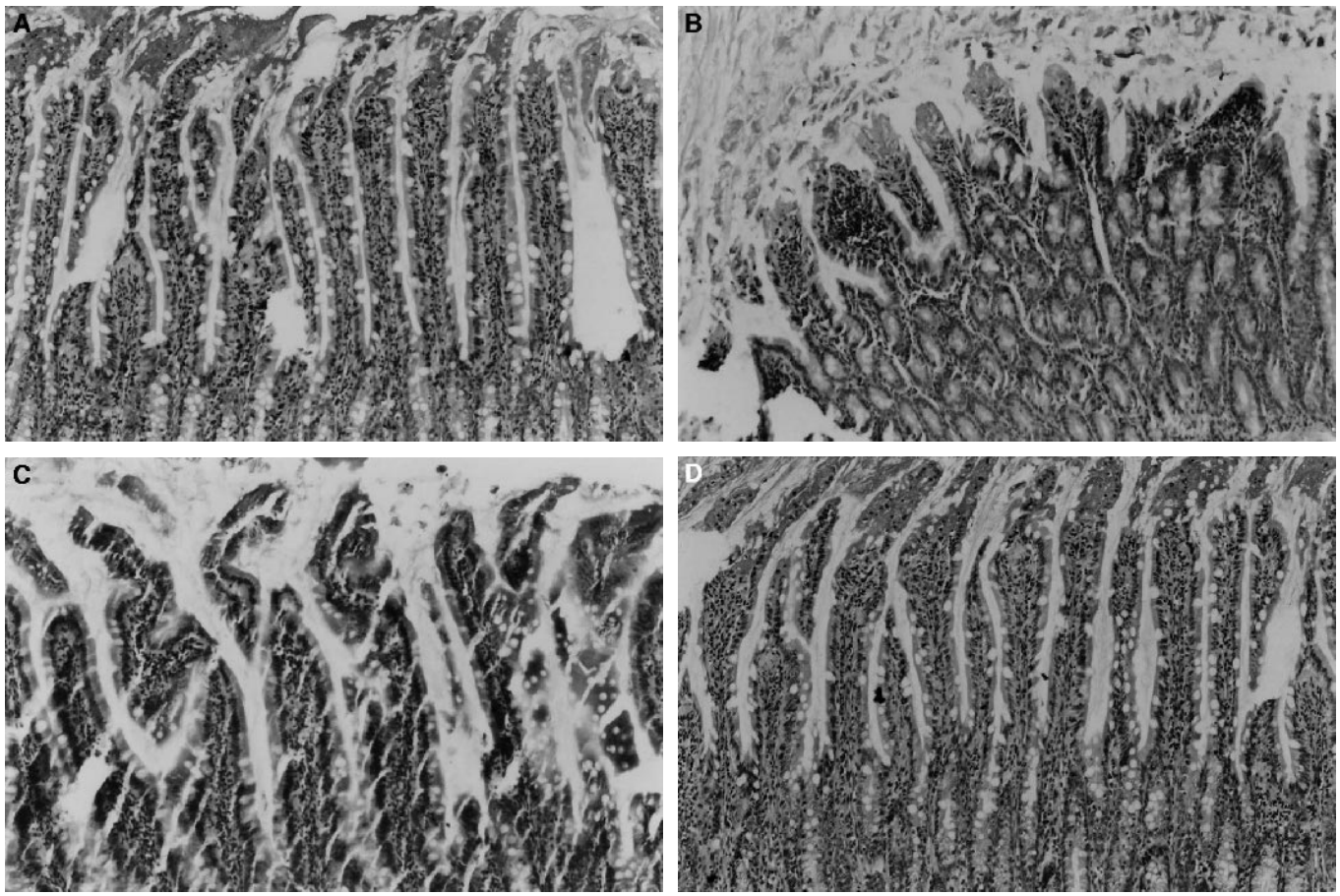

FIG. 6. **Photomicrographs of immunohistochemical staining of bcl-2 in ileal villus epithelium ×200.** (A) Strong staining for bcl-2 in sham-operated control group, (B) Weak staining for bcl-2 and massive epithelial denudation in I/R group, (C) In IPC+I/R group bcl-2 also strongly stained, and (D) IPC alone group showing bcl-2 staining similar to the sham-operated control group.

the routine histopathologic diagnosis of ischemic necrosis. This leads to underestimation of tissue injury unless apoptosis is evaluated by specific methods. The functional importance of apoptosis in the small intestine is an associated increase in bacterial translocation, which may trigger the vicious circle leading to MODS (3). In this study, prevention of intestinal apoptosis is in parallel direction with our previous data demonstrating the prevention of ileal morphology and bacterial translocation by the IPC (14).

There is increasing evidence of anesthetic agent-induced detrimental effects on I/R. The role of ketamine, which we used as an anesthetic agent, was previously investigated in few I/R and IPC studies. Ketamine was found to block K_{ATP} channels, and this influence was found to be most likely enantiomer specific (31). Other anesthetics, such as thiopental and midazolam, in addition to ketamine, were also shown to reduce postischemic adhesion of neutrophils (32). Nevertheless, ketamine may seem as a poor choice of anesthetic in an I/R setting because it has been shown to stimulate the release of endogenous catecholamines, further inducing ischemia of the intestine. Because ketamine was the common denominator for all groups in our study, we were satisfied with the analysis of other parameters. Liu et al. (33) only recently have shown that ketamine/xylazine combination when compared with isoflurane anesthesia did not have any depressant or stimulating effect on gut I/R-induced changes, and Piriou et al. (34) have pointed the pharmacological preconditioning by halogenated anesthetics.

Our results indicate that IPC may provide a protective effect on ileal epithelium and that this effect is probably the result of a significant increase in the expression of bcl-2 after the insult. The reversal of apoptosis by IPC might help preserving the vitality of intestinal structures that have a critical function, cessation of which often leads to multiorgan dysfunction syndrome. In the future, the use of agents causing bcl-2 upregulation against spontaneous I/R attacks as a preservative measure in critically ill patients could be seriously considered.

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