Postconditioning attenuates acute intestinal ischemia–reperfusion injury

Ilker Sengul a,*, Demet Sengul b, Osman Guler c, Adnan Hasanoglu c, Mustafa Kemal Urhan c, Ahmet Sukru Taner c, Jakob Vinten-Johansen d

a Department of General Surgery, Giresun University Faculty of Medicine, Giresun, Turkey
b Department of Pathology, Giresun University Faculty of Medicine, Giresun, Turkey
c Department of 1st General Surgery, Ankara Education and Research Hospital, Ankara, Turkey
d Department of Surgery (Cardiothoracic), Cardiothoracic Research Laboratory of Emory University, Carlyle Fraser Heart Center, Atlanta, GA, USA

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Abstract The aim of this study was to test the hypothesis that postconditioning (POC) would reduce the detrimental effects of the acute intestinal ischemia–reperfusion (I/R) compared to those of the abrupt onset of reperfusion. POC has a protective effect on intestinal I/R injury by inhibiting events in the early minutes of reperfusion in rats. Twenty-four Wistar–Albino rats were subjected to the occlusion of superior mesenteric artery for 30 minutes, then reperfused for 120 minutes, and randomized to the four different modalities of POC: (1) control (no intervention); (2) POC-3 (three cycles of 10 seconds of reperfusion-reocclusion, 1 minute total intervention); (3) POC-6 (six cycles of 10 seconds of reperfusion-reocclusion, 2 minutes total intervention); and (4) sham operation (laparotomy only). The arterial blood samples [0.3 mL total creatine kinase (CK) and 0.6 mL malondialdehyde (MDA)] and the intestinal mucosal MDA were collected from each after reperfusion. POC, especially POC-6, was effective in attenuating postischemic pathology by decreasing the intestinal tissue MDA levels, serum total CK activity, inflammation, and total histopathological injury scores. POC exerted a protective effect on the intestinal mucosa by reducing the mesenteric oxidant generation, lipid peroxidation, and neutrophil accumulation. The six-cycle algorithm demonstrated the best protection.

* Corresponding author. Giresun Universitesi Tip Fakultesi Dekanligi, Dekan Yardimcisi, Genel Cerrahi Anabilim Dali Kurucu Baskani, Nizamiye Yerleskesi, 28100 Giresun, Turkey.
E-mail addresses: dr.ilker52@mynet.com, ilker.sengul@giresun.edu.tr (I. Sengul).

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Introduction

Murry and colleagues [1] introduced the concept of ischemic preconditioning (IPC) in which repetitive brief episodes of ischemia render the myocardium more resistant to a subsequent prolonged ischemic insult that causes irreversible injury. In recent years, the Vinten–Johansen Laboratory reported in the canine coronary artery occlusion–reperfusion model that a similar regimen of brief episodes of ischemia carried out just after, instead of just before, the prolonged (index) ischemia reduced infarct size, coronary artery endothelial dysfunction, and neutrophil accumulation in the area at risk. This strategy, termed postconditioning (POC), was subsequently found to reduce infarct size comparably to that of IPC. While POC was initially studied in the heart [2], it has also been reported to protect the tissue following ischemia in the liver [3], brain [4], kidney [5], skeletal muscle [6], and skin flaps [7]. Ferencz and colleagues [8] reported that POC decreased infarct size comparably to that of IPC. While POC was initially studied in the heart [2], it has also been reported to protect the tissue following ischemia in the liver [3], brain [4], kidney [5], skeletal muscle [6], and skin flaps [7]. Ferencz and colleagues [8] reported that POC decreased infarct size comparably to that of IPC. While POC was initially studied in the heart [2], it has also been reported to protect the tissue following ischemia in the liver [3], brain [4], kidney [5], skeletal muscle [6], and skin flaps [7]. Ferencz and colleagues [8] reported that POC decreased infarct size comparably to that of IPC.

The efficacy of POC as a reperfusion therapy was based on the underlying hypothesis that significant reperfusion pathology occurs during the early minutes of reperfusion. Studies by Yang et al. [10] and Kin et al. [11] suggested that endogenous mechanisms are set in motion within the first few minutes of reperfusion that attenuate the early components of reperfusion injury. Early events of reperfusion injury are not independent of one another, but form an array that also triggers later events such as increased capillary permeability, no reflow, apoptosis, and necrosis.

Although all the protective mechanisms of POC are still not known, those that have been identified include delayed opening of the mitochondrial permeability transition pore (mPTP) [12], activation of mitochondrial adenosine triphosphate (ATP)-sensitive potassium (mKATP) channels [13], prevention of mitochondrial peroxide production and glutathione depletion [14], activation of components of the reperfusion injury salvage kinase pathway [15,16], activation of the nitric oxide—guanylate cyclase and protein kinase G pathway [17], and inhibition of reactive oxygen species production and intracellular excessive calcium accumulation [18]. Of these many targets, attenuation of reactive oxygen species may be a major mechanism because reduced oxidant generation would attenuate direct tissue injury as well as remove a major stimulus that opens the mPTP at reperfusion.

Although POC has been demonstrated to protect many organ types, the optimal algorithm is not known. Some studies suggest that the number of POC cycles is more important than their duration, but this is controversial (reviewed in Ref. [19]). The question of the optimal algorithm has not been investigated in the intestinal tract undergoing ischemia and reperfusion. Specifically, it is not known whether increasing the number of cycles increases the degree of protection in the intestinal tract. In the current study, we tested the hypothesis that increasing the number of POC cycles after the intestinal ischemia–reperfusion (I/R) against the ischemic injury in the rat would reduce postischemic injury.

Materials and methods

Animal care

All the animals received humane care in compliance with "The Guide for the Care of Use of Laboratory Animals" published by the National Institute of Health (NIH Publication No. 85-23, revised 1996), as well as with Turkish laws on animal experimentation. The present study received the previous approval of the ethics committee of Ankara Education and Research Hospital.

Surgical preparation

Twenty-four male and female Wistar–Albino rats weighing 200–250 g were obtained from the Animal Laboratory of Ankara Education and Research Hospital. The male and female rats were equally distributed among the experimental groups. The animals were anesthetized with an intramuscular injection of ketamine (50 mg/kg) and xylazine (7 mg/kg), and ventilated with the room air. The agents S-ketamine and xylazine were chosen because they do not exert a preconditioning-like effect by activation of the mKATP channel [20]. The rats were placed in a supine position on a heating pad to maintain the body temperature between 37°C and 38°C. To induce intestinal ischemia, a laparotomy was performed under the sterile conditions, and the superior mesenteric artery was occluded with an atraumatic arterial clamp. The abdominal operative zone was covered with a sterile saline-soaked gauze and a plastic cover to minimize dehydration of exposed tissues during the experiment.

Experimental protocol

In all rats, the proximal superior mesenteric artery was occluded for 30 minutes and then reperfused by loosening the atraumatic arterial clamp for 120 minutes. The rats were randomly assigned to one of four groups based on the intervention (n = 6 in each group) (Fig. 1): (1) control—there was no intervention either prior to or after superior mesenteric artery occlusion; (2) POC-3—immediately at the onset of reperfusion, reflow was initiated with 10 seconds of full mesenteric flow (reperfusion), followed by 10 seconds of reocclusion (ischemia), repeated for a total of three cycles (1 minute total intervention); (3) POC-6—the reflow–occlusion period described above was repeated for six cycles (2 minutes

![Figure 1](https://example.com/figure1.png)

**Figure 1.** A schematic diagram showing the POC protocols in the various groups. I = ischemia; POC = postconditioning; POC-3 = three cycles of postconditioning; POC-6 = six cycles of postconditioning; R = reperfusion.
total intervention); and (4) sham—laparotomy only with the observation time equal to the total experimental time.

**Determination of serum total creatine kinase and serum malondialdehyde activity**

The arterial blood samples [0.3 mL for total creatine kinase (CK) and 0.6 mL for malondialdehyde (MDA)] were collected from each rat after 120 minutes of reperfusion. Plasma was analyzed spectrophotometrically for CK activity at 350 nm absorbance. To measure plasma MDA levels, an index of lipid peroxidation reflecting oxygen free radicals, the method of Hunter et al. [21], was used. The MDA product has a long half-life in the plasma, and therefore its plasma levels are cumulative over time. The plasma was stored at $-70^\circ$C until analyzed. CK activity was expressed as units per liter of the plasma and MDA values as nanomoles per milliliter of the plasma.

**Determination of intestinal mucosal MDA**

The Uchiyama method [22] was used to determine the MDA in the intestinal mucosa. The weight of each sample of intestinal mucosa was measured. The tissue samples were homogenized and cold 1.5% potassium chloride (KCl) was added to achieve a 10% homogenate. Then 3 mL of 1% phosphoric acid and 1 mL of 0.6% thiobarbituric acid were added to 0.5 mL of the homogenate. After boiling and refrigerating for 45 minutes, 4 mL of N-butanol were added, and the samples were centrifuged at 3500 $\times$ for 10 minutes to separate butanol gas. Absorbances of these gases were analyzed spectrophotometrically at 520 and 535 nm, and the differences of absorbances were measured as the MDA levels.

**Histopathologic assessment**

The postischemic intestinal tissue samples were rinsed promptly in a cold saline and immediately fixed for 48 hours in a 10% buffered formalin. The tissues were then embedded in paraffin and sectioned transversely following routine procedures. Serial sections were stained with hematoxylin and eosin, and evaluated by the light microscopy. Photomicrographs were taken at 10 $\times$ /0.25 and 40 $\times$ /0.65 magnification by two separate investigators blinded to the previous interventions. Morphological evaluation was performed according to the Chiu classification [23] (Table 1). [23] The intestinal mucosal injury in each slide was graded on a six-tiered scale as follows: grade 0 = normal mucosa; grade 1 = development of subepithelial (Gruenhagen’s) spaces near the tips of villi with the capillary congestion; grade 2 = extension of the subepithelial spaces with moderate epithelial lifting from the lamina propria; grade 3 = significant epithelial lifting along the length of the villi with a few denuded villus tips; grade 4 = denuded villi with exposed lamina propria and dilated capillaries; and grade 5 = disintegration of the lamina propria, with hemorrhage and ulceration.

**Statistical analysis**

All data were expressed as means ± standard error of means. Differences between the groups were analyzed by Kruskal–Wallis one-way analysis of variance. All the data were analyzed using SPSS for Windows 11.5 and $p < 0.05$ was considered significant.

**Results**

**Plasma total CK activity**

The total plasma CK activity after I/R (as shown in Fig. 2) was significantly greater in the control group than in the sham group ($p < 0.05$, $p = 0.003$). In the POC-3 group, the plasma CK activity did not alter significantly relative to that in the control group. However, plasma CK activity in POC-6 group was significantly reduced relative to the control and

**Table 1** System of histopathological scores of Chiu et al. [23] (Chiu classification).

<table>
<thead>
<tr>
<th>Score</th>
<th>Determination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucosal injury</td>
<td>Normal mucosa</td>
</tr>
<tr>
<td>1</td>
<td>Subepithelial gaps of villi</td>
</tr>
<tr>
<td>2</td>
<td>Moderate separation of epithelium</td>
</tr>
<tr>
<td>3</td>
<td>Intensive separation of epithelium</td>
</tr>
<tr>
<td>4</td>
<td>Evanescence of villi and manifestation of the lamina propria</td>
</tr>
<tr>
<td>5</td>
<td>Breaking into pieces of lamina propria with ulceration</td>
</tr>
<tr>
<td>Inflammation</td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>In lamina propria, locally</td>
</tr>
<tr>
<td>2</td>
<td>In lamina propria, diffusely</td>
</tr>
<tr>
<td>3</td>
<td>Local subendothelial collections</td>
</tr>
<tr>
<td>4</td>
<td>Diffuse subendothelial collections</td>
</tr>
<tr>
<td>5</td>
<td>Massive collections</td>
</tr>
<tr>
<td>Hyperemia/hemorrhage</td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>Dilated capillaries in lamina propria</td>
</tr>
<tr>
<td>2</td>
<td>Local hemorrhage in lamina propria</td>
</tr>
<tr>
<td>3</td>
<td>Diffuse hemorrhage in lamina propria</td>
</tr>
<tr>
<td>4</td>
<td>Subendothelial hemorrhage</td>
</tr>
<tr>
<td>5</td>
<td>Massive hemorrhage</td>
</tr>
</tbody>
</table>
POC-3 groups, but was comparable to that in the sham group. Therefore, the six-cycle algorithm of POC reduced this biomarker of the morphological tissue injury.

**Plasma MDA levels**

Changes in the plasma MDA levels are summarized in Fig. 3A. The plasma MDA levels were significantly greater in the control group subjected to I/R compared to that in the sham group (p < 0.05, p = 0.012). The plasma MDA levels in both POC groups tended to be lower than in the control group, but this was not a significant difference.

**Intestinal tissue MDA levels**

As shown in Fig. 3B, the intestinal tissue MDA levels were significantly elevated in the control group compared to that in the sham group. POC-3 group was not associated with a significant reduction in tissue MDA, but in the POC-6 group the tissue MDA was significantly reduced compared to both control and POC-3 groups.

**Postexperimental histopathological assessment**

Assessment of each slide was performed according to the Chiu classification and differences between the groups were evaluated using the parameters of mucosal injury, inflammation, and hyperemia/hemorrhage (Table 1).

**Mucosal injury scores**

As shown in Fig. 4A, I/R injury was associated with greater mucosal injury scores in the control group than in the sham group. The mucosal injury score was not significantly reduced in the POC-3 group compared to that in the control group. However, the POC-6 group was associated with a significantly lower mucosal injury score compared to the control and POC-3 groups. Fig. 4B–E show the photomicrographs representative of the different scores for the POC-6, control, and sham groups.

**Inflammation scores**

As shown in Fig. 5A, the inflammation scores in the control group were significantly greater compared to all other interventional groups (p < 0.05, p = 0.003). Inflammatory scores were significantly lower in both POC-3 and POC-6 groups than in the control group. Fig. 5B–E show the photomicrographs representative of the different scores for the POC-3, POC-6, and sham groups.

**Hyperemia/hemorrhage scores**

There was no significant difference in the tissue hyperemia/hemorrhage scores among all experimental groups (Fig. 6A). [23] Fig. 6B–D show the photomicrographs representative of the different scores for the POC-6, POC-3, and sham groups.

**Total scores**

As shown in Fig. 7, the average total score in the control group was significantly greater compared to all other interventional groups (p < 0.05, p = 0.004). Both POC-3 and POC-6 algorithms reduced the total injury scores relative to control. There was no significant difference between the POC-3 and POC-6 groups.

**Discussion**

I/R injury is a contributing factor to mortality and morbidity from myocardial infarction, septic shock, and multiorgan failure (MOF). It has been reported that intestinal
hypoperfusion triggers a systemic inflammatory response that leads to MOF [24]. The gastrointestinal tissue is highly sensitive to I/R injury. However, in addition to affecting the gastrointestinal tissue, this injury causes a secondary injury to the remote tissue such as the lung. The gastrointestinal tissue is the target of the oxidant injury and is itself a source of inflammation as enterocytes can generate the proinflammatory cytokines that may lead to enterocyte dysfunction, bacterial translocation [25], and the larger problem of sepsis and its MOF [26]. Hence, protection of the gastrointestinal tract from I/R injury is important. IPC has been reported to reduce postischemic gastrointestinal injury [9,27]. However, it has limited applicability in protection of the gastrointestinal tract as it does in the heart [9] and other organs. However, recently POC has also been reported to attenuate postischemic gastrointestinal injury [8,28–33]. Contrarily, Bretz et al. [34] propounded that POC was ineffective in decreasing I/R injury of the small intestines in a rabbit model of intestinal I/R. For all that, Ferencz et al. [8] reported that POC alleviate oxidative stress and histologic damage of the small intestines during the small bowel autotransplantation. They performed total orthotopic intestinal autotransplantation in 30 white domestic pigs and stored the grafts in cold University of Wisconsin solution for 1, 3, or 6 hours. They found that the group of POC significantly decreased the reperfusion-ended lipid peroxidation, endogenous antioxidant protective systems (glutathione and superoxide dismutase), and

![Figure 4](image-url)

(A) A comparative graph of the histopathological analysis of mucosal injury scores of rats in the groups according to classification of Chiu et al. [23]. (B) Note the subepithelial gaps on the top of mucosal villi (indicated by arrow) (mucosal injury, score 1) in the POC-6 group (hematoxylin and eosin stain; original magnification, 40×). (C) Note the intensive separation of the epithelium (indicated by arrow) (mucosal injury, score 3) in the control group (hematoxylin and eosin stain; original magnification, 20×). (D) Note the evanescence villi and manifestation of the lamina propria (indicated by arrow) (mucosal injury score 4) in the control group (hematoxylin and eosin stain; original magnification, 20×). (E) Note the normal mucosa (mucosal injury, score 0) in the sham group (hematoxylin and eosin stain; original magnification, 5×). POC-6 = six cycles of postconditioning; SE = standard error.
intestinal wall injury. Additionally, it was detected that the tissue injury was increased by the duration of cold preservation (mostly in 6 hours of preservation). They performed the three cycles of ischemia for 30 seconds and reperfusion for 30 seconds.

In the present study, we report the following occurrences: (1) mesenteric I/R was associated with significant injury to the mucosa and submucosal tissues; (2) POC reduced the posts ischemic injury by attenuating the membrane lipid products of oxidant injury and inflammatory processes; (3) the three-cycle algorithm was largely ineffective, but increasing the number of POC cycles to six was effective in reducing posts ischemic injury. These studies suggest that the posts ischemic mesenteric injury can be reduced by six cycles of POC applied at the time of reperfusion, consistent with the reduction of early posts ischemic events reported for the heart [2,19,35].

The tissue-protective effect of POC is exerted by the alternating “units” of reperfusion and reocclusion (ischemia). There is controversy over whether the efficacy of POC is due primarily to the number of cycles, duration of each (reperfusion–reocclusion) period, or total duration of the POC algorithm. However, it is clear that each of the alternating phases contributes to the cardioprotection of

Figure 5. (A) A comparative graph of the histopathological analysis of inflammation scores of rats in the groups according to classification of Chiu et al. [23] (p < 0.05, p = 0.000). Values are group means ± SE. (B) Note the diffuse subendothelial collections (indicated by three arrows) (inflammation, score 4) in the POC-3 group (hematoxylin and eosin stain; original magnification, 20×). (C) Note the massive collections (indicated by arrow) (inflammation, score 5) in the control group (hematoxylin and eosin stain; original magnification, 20×). (D) Note the inflammation in the lamina propria, locally (indicated by arrow) (inflammation, score 1), in the POC-6 group (hematoxylin and eosin stain; original magnification, 20×). (E) A photomicrograph showing no inflammation (inflammation, score 0) in the sham group (hematoxylin and eosin stain; original magnification, 20×). POC-3 = three cycles of postconditioning; POC-6 = six cycles of postconditioning; SE = standard error.
POC. For example, the ischemic phase maintains tissue acidosis [36] part through inhibition of calpain activation [37] and in part by inhibiting the opening of the mPTP [36], the latter event being a potential final common pathway of the tissue protection. Kin et al. [38] reported in a rat model of coronary artery reperfusion that the three cycles of reperfusion occlusion were as effective as six cycles. Yang et al. [10] reported a similar degree of the protection with four and six cycles of reperfusion—occlusion. However, the effective algorithm has not been explored in other tissues, including the mesentery. In the present study, the six-cycle algorithm was more protective than the three-cycle algorithm.

POC inhibits a number of the pathophysiological mechanisms leading to cell injury. The data suggest that inhibition of the inflammatory response to the reperfusion is an important mechanism of POC. POC has been reported to inhibit the activation and postischemic dysfunction of the coronary vascular endothelium. In addition, generation of proinflammatory cytokines was reduced by POC [39]. Preliminary studies by Granfeldt et al. [40] suggest that POC inhibits the inflammatory response to the reperfusion mediated by neutrophils. In support of this notion, POC has been reported to inhibit the adherence of neutrophils to the coronary vascular endothelium and the accumulation in the extravascular and intravascular spaces. One of the key triggers of POC, notably adenosine, has potent antiinflammatory and antineutrophil effects. The results reported in the present paper support a similar antiinflammatory mechanism for POC in the intestinal I/R. In addition, MDA data showing a reduction in membrane lipid peroxidation by POC may suggest another mechanism, namely the reduction of the oxidant generation, which has also been reported in the heart. However, whether the
source of oxidants was neutrophils or parenchymal tissue is not known either for the heart or for the other tissues. The plasma MDA levels in both POC modalities tended to be lower than in control, but this was not a significant difference. The mechanisms of POC may not be associated with the oxygen radical reduction in this model. It may be related to the reduction in inflammatory processes, in contrast to the heart. This is supported by the data.

In terms of adverse effects of POC, increased infarct size has been reported for very short durations of ischemia only and none in the models of more prolonged ischemia. Potential adverse event is damage to the endothelium at the site of occlusion—reocclusion. But no clinical adverse events have been reported yet.

Our data showed that six cycles of POC were more effective than three cycles; however, the effects of variation in the duration of these cycles were not investigated in the present study. One mechanism of protection is related to decreasing the MDA levels of intestinal tissue, which is an indirect indicator of the lipid peroxidation, and the decreasing the mucosal injury, inflammation, and total histopathological injury scores. However, no significant group difference was detected in regard to hemorrhage/hyperemia. This may be because the mucosa is more susceptible to the duration of ischemia than the submucosal tissue. For example, the vascular endothelium sustains injury prior to the underlying vascular muscular tissue and myocardium. Temporal progression of vascular injury for the heart may be different from that for the intestines, since the latter is not a highly metabolic tissue.

Therefore, we conclude that increasing the number of cycles of POC from three to six increases its efficacy in reducing the mesenteric oxidant generation, lipid peroxidation, and neutrophil accumulation. Future studies are required that should include other algorithms (different cycles) and longer durations of occlusion to answer the question about the threshold of the phenomenon.

**Summary and Conclusion**

POC is more clinically applicable than IPC when a wide group of patients are thought as potentially treatable. In contradistinction to IPC, POC can be applied during the reperfusion at the point of service for catheter-based reperfusion [41], cardiac surgery [42,43], and organ transplantation, so it may have widespread clinical applications compared to the limited applications of IPC. The results of the present study may give rise to the possible application of POC in fields such as revascularization of the ischemic bowel primarily, bowel resection and reanastomosis, intestinal organ transplantation, or other surgical procedures. Therefore, more advanced, sophisticated, and multicentered experimental and clinical studies are essential to optimize POC for application to the intestinal tract.

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