Remote post-conditioning and allopurinol reduce ischemia-reperfusion injury in an infra-renal ischemia model

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Abstract

Background: The aim of this study was to evaluate the effects of the antioxidant allopurinol and ischemic post-conditioning on the deleterious effects of ischemia followed by reperfusion (I/R) in a standardized model of ischemia involving infra-renal aortic occlusion in rats.

Methods: The animals were randomly divided into five groups: (A) animals not subjected to ischemia; (B) animals subjected to 2 h of ischemia and reperfusion only once; (C) animals given an allopurinol dose by gavage, then subjected to 2 h of ischemia and reperfusion only once; (D) animals subjected to 2 h of ischemia and post-conditioning and (E) animals that received allopurinol, then subjected to 2 h of ischemia and post-conditioning. The blood samples and small intestine segments were harvested for analysis after 3 days.

Results: The protective effects of the use of allopurinol and ischemic post-conditioning were observed by measuring aspartate aminotransferase, alanine aminotransferase and lactate levels. The benefits of post-conditioning were evident from the total antioxidant capacity and creatinine levels, but these could not ascertain any positive effects of allopurinol. The histological analysis of mesentery revealed that both methods were effective in minimizing the harmful effects of the ischemia and reperfusion process.

Conclusion: Individual protocols significantly reduced I/R systemic injuries, but no additional protection was observed when the two strategies were combined.

Keywords: allopurinol, antioxidant, ischemia, pharmacological preconditioning, post-conditioning, reperfusion

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Introduction

Ischemia is defined as the loss of blood supply due to a reduction in the arterial flow within a tissue, in which the supply of metabolic substrates, including glucose, is impaired.1 Reperfusion is the term used to define the re-establishment of flow after a period of ischemia and is a logical step in dealing with any situation of ischemia. However, it is responsible for major lesions in the cells of the ischemic organ and may aggravate the ischemic cellular damage, paradoxically leading to systemic complications such as adult respiratory distress syndrome, renal and liver dysfunction, and occasionally, to failure of multiple organs and systems.1–3 These complications occur in organs distant to those that had ischemia and reperfusion, because of the different mediators released into systemic blood circulation, namely leukocytes, proinflammatory cytokines, adhesins and reactive oxygen species.1,4 Clinical and experimental research based on the involved pathophysiological mechanisms, carried out mostly using rats, have proposed the use of different substances with the purpose of avoiding or minimizing the deleterious effects secondary to ischemia/reperfusion (I/R). However, the majority of these have not presented satisfactory results to justify their use in clinical practice.
A possible role of free oxygen radicals in the pathogenesis of tissue lesions after reperfusion has been studied, comparing them with the use of pharmacological agents to prevent this phenomenon.⁴–⁶ The importance of the xanthine oxidase enzyme in the genesis of reactive oxygen species has been recognized in I/R.⁷ Therefore, allopurinol, a xanthine oxidase inhibitor and a free radical scavenger, has therapeutic potential for the reduction of cellular damage secondary to ischemia followed by reperfusion and consequently for its repercussions,⁴,⁸ and its activity is tested here as a pharmacological preconditioning strategy. Other approaches such as ischemic post-conditioning (IPoC), which involves some cycles of ischemia and reperfusion interspersed after the main ischemic period, have been applied successfully with excellence in organs such as intestine, myocardium and liver.⁵ Ischemia followed by reperfusion is a strategy used in reconstructive vascular surgeries restore the flow in the ischemic extremities, which can occur with certain postoperative complications, such as reperfusion syndrome, rhabdomyolysis, renal failure and gangrene, among others. Few studies have investigated ischemia of infra-renal aorta followed by IPoC performed in the same artery or this approach combined with a pharmacological preconditioning. Therefore, the objective of this study was to use IPoC approaches and treatment with allopurinol as an attempt to reduce I/R-induced systemic lesions using a lower limb ischemia model in rats. Individual and combined protocols were tested.

### Material and methods

#### Animals

The experimental procedures and protocols used in this investigation were approved by the Ethics Committee of the State University of Ponta Grossa, Brazil (UEPG, number 29/2015). All procedures adhered to the guidelines published by the National Institute of Health and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. Housing conditions and husbandry practices were identical across the control and the experimental groups.

A total of 35 adult female rats (*Rattus norvegicus*, Wistar albino), weighing 250–300 g, were supplied by the UEPG and Pontifical University Catholic of Paraná animal houses and housed in cages under standard conditions (12 h:12 h light-dark cycle, constant temperature of 21°C ± 2°C, and humidity of 55% ± 2%). The rats had free access to tap water and standard rodent chow at all times. The rats were cared for on a daily basis and monitored for pain and distress, between and after the procedures, using a general distress scoring sheet.

The approval for this experiment included a protocol for early euthanasia of animals who exhibited signs of severe illness and consisted of the following endpoints: decreased food and water intake, constant piloerection, signs of infections, bleeding from wounds, and weight loss over 20%. The rats were allowed to acclimate for at least 1 week prior to the surgical procedures.

#### Experimental design

As summarized in Table 1, animals were randomized into five groups (6–7 animals per group): (1) group A (sham): animals that had not undergone any ischemia (laparotomy and dissection of the infra-renal abdominal aorta were performed, followed by closure after 2 h); (2) group B (single I/R): animals underwent the surgical procedure with 2 h of ischemia and removal of the clamp for reperfusion only once; (3) group C (allopurinol/ischemia/single reperfusion): animals received a dose of 100 mg/kg allopurinol (as a suspension at a final volume of 0.5 ml) by gavage, 1 h before the surgical procedure, then underwent 2 h of ischemia and removal of the clamp for reperfusion only once; (4) group D (ischemia/IPoC): animals underwent the surgical procedure, with 2 h of ischemia and reperfusion preceded by ischemic post-conditioning for three cycles of

### Table 1. Groups, number of animals, and treatments.

| Groups | N  | Ischemic period (h) | Allopurinol (100 mg/kg) | IPoC |
|--------|----|---------------------|------------------------|------|
| A      | 7  | 0                   | No                     | No   |
| B      | 6  | 2                   | No                     | No   |
| C      | 7  | 2                   | Yes                    | No   |
| D      | 6  | 2                   | No                     | Yes  |
| E      | 6  | 2                   | Yes                    | Yes  |

IPoC, ischemic post-conditioning.
ischemia and reperfusion for 2 min each; (5) group E (allopurinol/ischemia/IPoC): animals received 100 mg/kg of allopurinol by gavage 1 h prior to the surgical procedure and then underwent 2 h of I/R preceded by IPoC for three cycles of I/R for 2 min each.

**Surgical procedures**

Wistar female albino rats were initially anesthetized by intraperitoneal administration of ketamine and xylazine at 75 mg/kg and 10 mg/kg, respectively. Maintenance of anesthesia during the surgical procedure was performed by administering 1/2 or 1/3 of the first dose intramuscularly. Isotonic saline was administered subcutaneously (4 ml in total) to keep the animals properly hydrated during surgery.

The rats were placed on a heating pad to maintain a stable body temperature. After anesthesia, animals were subjected to wide trichotomy of the abdomen, fixed in dorsal decubitus and antisepsis was done with iodinated alcohol. A xiphoid medium incision laparotomy was then performed by opening all planes of the abdominal wall and exposing the abdominal cavity. The intestinal loops were traced out of the peritoneal cavity and protected with sterile gauze moistened with isotonic saline solution. The posterior parietal peritoneum was dissected, and the vena cava was separated from the infra-renal aorta.

Groups B, C, D, and E underwent aortic occlusion for 2 h, with a double loop of a nontraumatic vessel clamp. During this period, the intestinal loops were collected into the abdominal cavity and moistened with sterile gauze moistened with isotonic saline solution. The posterior parietal peritoneum was dissected, and the vena cava was separated from the infra-renal aorta.

The rats were re-anesthetized after 72 h, and blood samples were collected from their carotid arteries. Animals were sacrificed by exsanguination and the abdomen was opened for removal of the small intestines, which were identified and prepared for histological analyses.

**Biochemical investigation**

Blood samples taken immediately before sacrifice were placed in dry tubes, allowed to clot, and the serum was separated by centrifugation and stored at −80°C until further study. The serum levels of D-lactate, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and creatinine were obtained using automated equipment (Cobas integra 400 plus, Roche, Basel, Switzerland).

**Total antioxidant capacity**

Total antioxidant capacity (TAC) was measured in serum. Blood samples obtained were centrifuged at 1500 × g for 10 min. The samples were run immediately or stored at −80°C. The major advantage of this test is to measure the antioxidant capacity of all antioxidants in a biological sample and not just the antioxidant capacity of a single compound.

TAC is based on the inhibition of the absorbance of the radical cation of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate; ABTS), which has a characteristic long-wavelength absorption spectrum showing maximum absorbances at 660, 734, and 820 nm as determined by testing antioxidants.9 ABTS was dissolved in water at a concentration of 7 mM. The ABTS radical cation (ABTS.+\(^{-}\)) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. For the study, the ABTS.+\(^{-}\) solution was diluted with phosphate buffer saline, pH 7.4 (PBS), to an absorbance of 0.70 (±0.02) at 734 nm and equilibrated at 30°C. After addition of 2.0 ml of diluted ABTS.+\(^{-}\) solution to 20 μl of biological sample, or Trolox standard, the reaction mixture was incubated for 6 min in a glass cuvette at 30°C.

The decrease in absorbance at 734 nm was determined exactly at 6 min after initial mixing for all samples. The absorbance of ABTS.+\(^{-}\) without the sample, (i.e. the control), was measured daily. All measurements were performed in four repetitions. The result was expressed as a percentage of the captured radical.
Histological analysis

Segments of the small intestine of animals of all groups were collected 72 h after the surgical procedures and fixed in ALFAC (85% ethanol 80%, 5% glacial acetic acid, 10% formalin) for 16 h, processed with conventional histological techniques and stained with hematoxylin and eosin dyes.

The sections were analyzed, photographed, and classified according to the degree of tissue injury, as reported by Chiu and colleagues.10 All samples to be independently analyzed, were distributed among the examiners with no knowledge of the treatment group, in a randomly selected manner.

Morphological changes in the mucosa were graded into six scores, where grade 0 meant normal mucosa and grade I to V meant increasing damage to the surface epithelium. The pathognomonic lesion for grade I was the development of subepithelial space at the tip of the villus. This space expanded more in grade II, where epithelial lifting was also seen. In grade III, there was destruction of the free portions of the villi, dilated capillaries and massive epithelial lifting down the sides of the villus, whereas in grade IV the villus was denuded with necrotic material. Grade V was characterized by disintegration of the lamina propria, hemorrhage and ulceration in the mucosa, absence of glandular structure, and only amorphous material present on the submucosal surface.

Statistical analysis

The sample was calculated based on a pilot study with groups containing a sample N of three animals. A significance level of α = 0.05 was used for the five experimental groups. Calculations were performed based on the mean and standard deviations, establishing the power of the test at 80%. All calculations were performed using the G*Power 3.1 program.11 The results were presented as mean ± standard error of the mean (SEM), for the biochemical tests and estimation of TAC and median ± SEM for Chiu’s score in the small intestine. Normality tests were applied. One-way analysis of variance (ANOVA) was used if these tests were passed, and the Tukey test was used for pairwise multiple comparison procedures. Kruskal–Wallis one-way ANOVA on multiple comparisons versus the ischemia group (group B; Dunn method) were used for statistical analysis when the normality test or equal variance test were not passed. The significance level accepted for the tests was p < 0.05. All tests were performed using GraphPad Prism statistical software (version 5.01, San Diego, California, USA).

Results

Post-conditioning increases TAC

The TAC was measured as the ability of the serum obtained from animals in all groups to reduce ABTS.−. Serum TAC levels were lower in rats of group B (single reperfusion) and C (ischemic for 2 h and allopurinol treatment). A significant increase in TAC levels was observed in groups D and E as compared with group B. No additional or synergistic effect was observed when both strategies were applied (group E; Table 2).

Post-conditioning and allopurinol reduce AST, ALT, and creatinine levels

The severity of systemic reperfusion injury was assessed by serum AST, ALT and creatinine levels. Animals subjected to allopurinol pretreatment and IPOC had significantly lower serum AST and ALT levels than the single reperfusion group (group B) at 72 h after reperfusion (Table 3). Creatinine levels after 72 h of reperfusion in post-conditioning groups (D and E) were significantly lower than that in the single reperfusion group. No statistical difference was observed in the creatinine levels between group C (allopurinol treatment) and group B (single reperfusion). Besides, no additional or synergistic effect was observed

### Table 2. Antioxidant capacity of the different treatment groups.

| Group | ABTS         |
|-------|--------------|
| A     | 48.17 ± 1.07 |
| B     | 41.69 ± 3.26 |
| C     | 44.02 ± 1.94 |
| D     | 51.12 ± 0.73*|
| E     | 50.51 ± 0.90*|

Results of analysis of five replicates expressed as mean ± standard error of mean, using one-way ANOVA and Tukey’s post-hoc tests. *p < 0.05 related to group B (single reperfusion).

ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate; ANOVA, analysis of variance.)
When both the strategies were applied together (group E; Table 3).

**Post-conditioning reduces lactate serum levels**
As shown in Figure 1, a significant increase in lactate dehydrogenase levels was observed in the group subjected to ischemia and reperfusion (group B). When reperfusion was preceded by cycles of ischemia (IPoC, groups D and E), the increase in lactate dehydrogenase level was attenuated. Treatment with allopurinol alone did not induce significant alterations in D-lactate levels (Figure 1, group C).

**Post-conditioning and allopurinol prevent damage in small intestine after I/R**
The histopathological changes in the small intestine after 72 h of reperfusion were assessed by standard hematoxylin and eosin staining. Representative images of small intestines of rats from each group are shown in Figure 2(a). The small intestinal mucosal injury was evaluated using Chiu’s scores. In the sham group A, the intestinal mucosa was normal. Injury scores of group B (single reperfusion) were significantly higher than those of the sham group. Severe edema of mucosal villi and infiltration by necrotic epithelial and inflammatory cells were observed; additionally, the gap between epithelial cells had increased significantly. IPoC and allopurinol pre-treatment reduced the histological damage as compared with that in the I/R group [Figure 2(a)]. The score of small intestinal mucosal pathology is given in Figure 2(b). The rats preconditioned with allopurinol and subjected to IPoC presented significant reduction in Chiu’s scores, as compared with I/R group rats. No additional reduction in Chiu’s score was observed when the two approaches were combined [Figure 2(b), group E].

**Discussion**
In the present study, we showed a new approach of using allopurinol as a pharmacological compound that can alleviate the systemic effects of I/R injury induced by lower limb ischemia through a rat model. Animals subjected to allopurinol pre-treatment, as a pharmacological preconditioning approach, had significantly lower serum AST and ALT levels than the single reperfusion group (group B) 72 h after reperfusion (Table 2). Prevention of liver injury probably occurred by inhibition of systemic free radical formation. Messiha and Abo-Youssef (2015) also showed reductions in the AST and ALT levels in rats that received allopurinol (50 mg/kg/day, 1 h before

| Group | ALT         | AST          | Creatinine |
|-------|-------------|--------------|------------|
| A     | 43 ± 4.68*  | 154.7 ± 29.02* | 0.52 ± 0.017* |
| B     | 91 ± 23.21  | 426.8 ± 180.2 | 0.61 ± 0.01 |
| C     | 43.8 ± 7.06*| 148 ± 31.05*  | 0.54 ± 0.02 |
| D     | 42.4 ± 1.99*| 150.8 ± 22.36 | 0.51 ± 0.014* |
| E     | 44.6 ± 0.81*| 171.8 ± 24.82*| 0.50 ± 0.03* |

Results of analysis of five replicates expressed as mean ± standard error of mean, using one-way ANOVA and Tukey’s post-hoc tests. *p < 0.05 related to group B (single reperfusion). ALT, alanine aminotransferase; ANOVA, analysis of variance; AST, aspartate aminotransferase.
ischemia) and then subjected to hepatic I/R. There was also a marked improvement in histopathological findings. Hypoxanthine was preserved through the blockade of xanthine oxidase enzyme and might provide another possible explanation for the beneficial effects of allopurinol in the preservation of hypoxanthine as a substrate to form adenosine triphosphate (ATP). Allopurinol administration also protected the small intestinal tissue from I/R injury [group C, Figure 2(a) and (b)]. Among the internal organs, the small intestine is probably the most sensitive to I/R injury. Intestinal I/R injury can lead to, or further enhance, oxidative stress during I/R.

Allopurinol preconditioning was not able to reduce levels of lactate dehydrogenase and creatinine or increase TAC. Roden and colleagues showed a significant reduction in serum creatinine in rats subjected to renal ischemia–reperfusion pretreated with 100 mg/kg allopurinol, intraperitoneally, divided in two doses, 5 and 1 h before ischemia. Yildirim and colleagues pretreated rats with 50 mg/kg of allopurinol, injected in the iliac vein, 15 min before I/R produced by hepatic pedicle clamping. This approach prevented an increase in lactate levels after 45 min of ischemia. Considering these and most articles in the literature using models of ischemia in rats, our results with allopurinol could be better if a shorter time of ischemia had been applied. We have worked with a very long time period (2 h). Also, studies cited here delivered allopurinol by the parenteral route and not by gavage.

IPoC can specifically be used in ischemia that develops as a result of trauma or stroke, where preconditioning is not possible. Its clinical use was first experimentally shown by Andreka and colleagues. In our results, animals subjected to IPoC (group D) had significantly lower serum AST and ALT levels than those belonging to the single reperfusion group (group B), 72 h after reperfusion (Table 2). Zhang and colleagues measured ALT and AST levels as well as nuclear factor-kappa beta (NF-κB) p65 (generated due to a large amount of free oxygen radicals) in a hepatic direct ischemia model, and found lower levels of ALT and AST, increased superoxide dimutase levels and reduced apoptotic index in the IPoC group in comparison with those in the I/R group. They also observed differences under light and electron microscopy, with a lower degree of congestion of hepatic sinusoids, reduced neutrophilic infiltration, and fewer cells with disruption of nuclear and mitochondrial membranes or degranulation of endoplasmic reticulum in the IPoC group, as compared with those in the I/R group. The authors attributed the protection afforded by post-conditioning to the controlled slow and intermittent oxygenation through the several cycles of ischemia (on/off flow) before permanent reperfusion. Interposing three cycles of reperfusion followed by three cycles of ischemia (lasting 30 s each) between ischemia and prolonged reperfusion, another group of researchers showed attenuated liver injury assessed by serum AST and ALT levels and attenuated histological scores of hepatic lesions in the post-conditioned group. Several other studies also showed similar results.

The IPoC group also showed a discrete reduction in creatinine levels as compared with I/R group (Table 2). Chen and colleagues reported similar results in the renal ischemia using a reperfusion model in Wistar rats. In this study, authors showed a reduction in the expression levels of nuclear factor (NF)-κB, intercellular adhesion molecule (ICAM)-1, interleukin (IL)-6, and tumor necrosis factor (TNF)-alpha, displaying potent anti-inflammatory properties against renal I/R injury.

Previous reports showed that, IPoC inhibits the generation of oxidants and prevents formation of mitochondrial permeability transition pores (mPTP) in I/R injury, which can explain the increase in TAC values and accentuated decrease in lactate dehydrogenase levels, as observed in our IPoC group (Table 1 and Figure 1, respectively). Several other studies have shown an accentuated decrease in D-lactate levels using a post-conditioning approach. Albrecht and colleagues showed a significant reduction in D-lactate levels and in other serum markers of cell damage, by using remote IPoC in a cardiac arrest model in pigs. In another study, Li and colleagues showed that, IPoC protects the heart against reperfusion injury by reducing serum D-lactate levels, inhibiting protein PTEN and activating the PI3K/AKT pathway by lowering cardiomyocyte apoptosis.

Our findings revealed that, IPoC and treatment with allopurinol prevented the damage caused to the small intestine after I/R (Figure 2). The lesions of group B (single reperfusion) were significantly higher than those of the allopurinol and IPoC groups. The lesion scores of these groups
Figure 2. Histopathology of small intestinal tissue from the different groups. (a) Hematoxylin and eosin stained sections of rat’s small intestine. Images are representative of 5–6 rats per group. 200× magnification provided. (b) Injury score (median ± SEM) of small intestine. *p < 0.05, related to group B (single reperfusion; Kruskal–Wallis test).
SEM, standard error of the mean.
were also significantly lower than those of group B. Hotter and colleagues showed that a 10 min brief ischemia followed by a 5-min reperfusion decreased intestinal injuries caused by intestinal reperfusion in rats. Santos and colleagues also showed that IPoC exhibits protective effects against intestinal I/R injury.

In this study, the protective effects of allopurinol and IPoC were evaluated upon I/R injury induced by lower limb ischemia. The results show that both strategies were effective in protecting different organs, but IPoC showed better results as evidenced from the values of creatinine, D-lactate and TAC.

In the IPoC protocol, animals were exposed to a long period of ischemia (2 h) and subjected to three cycles of reperfusion at 2 min each. This protocol was standardized for our experiments, and based on data from literature for rats, with promising results. Despite this, some algorithms are being praised for mice and rats with low time cycles, like 30 or 10 s, but many results in the literature are conflicting, since the difference between prolonged and shorter cycles is not observed in some studies.

Allopurinol was applied here as a pharmacological preconditioning approach, was chosen because of its capability of inhibiting systemic free radical formation. A lot of studies have shown the benefits of post-conditioning in different models of ischemia. Post-conditioning allows controlled slow and intermittent oxygenation through the several cycles of ischemia before permanent reperfusion and attenuates multiple triggers of reperfusion injury including oxidants, proinflammatory cytokines, neutrophils, and proapoptotic regulators. Group E combined these two perspectives and could result in an additive effect; however, no additional protection is achieved when these two strategies are combined as compared with the individual treatment strategy.

Limitations
There are some limitations to the current study, including the small number of animals in each group. Besides, most interesting results probably occurred by inhibition of systemic free radical formation, but this study did not identify the species of oxidants can be attenuated.

Conclusion
This study demonstrated that, IPoC could reduce I/R injury of the liver, small intestine, and probably the kidney in simulated infra-renal ischemia using a rat model. Allopurinol pretreatment also presents beneficial effects, but no additional effects were obtained with its combination with the IPoC strategy.

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Conflict of interest statement
The authors declare that there is no conflict of interest.

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References
1. Wu MY, Yang GT, Liao WT, et al. Current mechanistic concepts in ischemia and reperfusion injury. Cell Physiol Biochem 2018; 46: 1650–1667.
2. Kalogeris T, Baines CP, Krenz M, et al. Cell biology of ischemia/reperfusion injury. Int Rev Cell Mol Biol 2012; 298: 229–317.
3. Loerakker S, Oomens CW, Manders E, et al. Ischemia-reperfusion injury in rat skeletal muscle assessed with T2-weighted and dynamic contrast-enhanced MRI. Mag Reson Med 2011; 66: 528–537.
4. Ciz M, Cizova H, Lojek A, et al. Ischemia-reperfusion injury of rat small intestine: the effect of allopurinol dosage. Transplant Proc 2001; 33: 2871–2873.
5. Theodoraki K, Karmaniolou I, Tympa A, et al. Beyond preconditioning: postconditioning as an alternative technique in the prevention of liver ischemia-reperfusion injury. Oxid Med Cell Longev. Epub ahead of print 2 June 2016. DOI: 10.1155/2016/8235921.
6. Granger DN and Kvietys PR. Reperfusion injury and reactive oxygen species: the evolution of a concept. Redox Biol 2015; 6: 524–551.
7. Berry CE and Hare JM. Xanthine oxidoreductase and cardiovascular disease: molecular mechanisms and pathophysiological implications. J Physiol 2004; 555: 589–606.
8. Messiha BA and Abo-Youssef AM. Protective effects of fish oil, allopurinol, and verapamil on hepatic ischemia-reperfusion injury in rats. *J Nat Sci Biol Med* 2015; 6: 351–355.

9. Re R, Pellegrini N, Protegge vA, et al. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic Biol Med* 1999; 26: 1231–1237.

10. Chiu CJ, McArule AH, Brown R, et al. Intestinal mucosal lesion in low-flow states: a morphological, hemodynamic and metabolic reappraisal. *Arch Surg* 1970; 101: 478–483.

11. Faul F, Erdfelder E, Lang AG, et al. *G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behav Res Methods* 2007; 39: 175–191.

12. McCord JM. Oxygen-derived free radicals in post ischemic tissue injury. *N Engl J Med* 1985; 312: 159–163.

13. Röth E, Hejuel L, Jabemanari M, et al. The role of free radicals in endogenous adaptation and intracellular signals. *Exp Clin Cardiol* 2004; 9: 13–16.

14. Rhoden E, Teloken C, Lucas M, et al. Protective effect of allopurinol in the renal ischemia–reperfusion in uninephrectomized rats. *Gen Pharmacol* 2000; 35: 189–193.

15. Yildirim S, Tok H, Koksal H, et al. Allopurinol plus pentoxifillin in hepatic ischaemia/ reperfusion injury. *Asian J Surg* 2002; 25: 149–153.

16. Andreza G, Vertesaljai M, Santho G, et al. Remote ischemic post conditioning protect the heart during acute myocardial infarction in pigs. *Heart* 2007; 93: 749–752.

17. Zhang WX, Yin W, Zhang L, et al. Preconditioning and postconditioning reduce hepatic ischemia–reperfusion injury in rats. *Hepatobiliary Pancreat Dis Int* 2009; 8: 586–590.

18. Santos CH, Pontes JC, Miji LN, et al. Postconditioning effect in the hepatic ischemia and reperfusion in rats. *Acta Cir Bras* 2010; 25: 163–168.

19. Belghiti J, Noun R, Malafosse R, et al. Continuous versus intermittent portal triad clamping for liver resection. A controlled study. *Am Surg* 1999; 229: 369–375.

20. Chen H, Wang L, Xing BZ, et al. Ischemic postconditioning attenuates inflammation in rats following renal ischemia and reperfusion injury. *Exp Ther Med* 2015; 10: 513–518.

21. Song SQ, Gan HL, Zhang JQ, et al. Post-conditioning through limb ischemia–reperfusion can alleviate lung ischemia-reperfusion injury. *Int J Clin Ep* 2015; 8: 14953–14961.

22. Kin H, Zhao ZQ, Sun HY, et al. Postconditioning attenuates myocardial ischemia-reperfusion injury by inhibiting events in the early minutes of reperfusion. *Cardiov Res* 2004; 62: 74–85.

23. Cohen MV and Downey JM. Signaling pathways and mechanisms of protection in pre and postconditioning: historical perspective and lessons for the future. *Br J Pharmacol* 2015; 172: 1913–1932.

24. Albrecht M, Meybohm P, Broch O, et al. Evaluation of remote ischaemic post-conditioning in a pig model of cardiac arrest: a pilot study. *Resuscitation* 2015; 93: 89–95.

25. Li CM, Shen SW, Wang T, et al. Myocardial ischemic post-conditioning attenuates ischemia reperfusion injury via PTEN/Akt signal pathway. *Int J Exp Clin Med* 2015; 8: 15801–15807.

26. Hotter G, Closa D, Prados M, et al. Intestinal preconditioning is mediated by a transient increase in nitric oxide. *Biochem and Biophys Res Commun* 1996; 222: 27–32.

27. Santos CHM, Pontes JCDV, Gomes OM, et al. Evaluation of ischemic postconditioning effect on mesenteric ischemia treatment. Experimental study in rats. *Rev Bras Cir Cardiovasc* 2009; 24: 150–156.

28. Granfeldt A, Lefer DJ and Vinten-Johansen J. Protective ischaemia in patients: preconditioning and postconditioning. *Cardiov Res* 2009; 83: 234–246.

29. Santos CH, Aydos RD, Nogueira NE, et al. Importance of duration and number of ischemic postconditioning cycles in preventing reperfusion mesenteric injuries. Experimental study in rats. *Acta Cir Bras* 2015; 30: 709–714.

30. Nakamura RK, Santos CH, Miji LN, et al. Very short cycles of postconditioning have no protective effect against reperfusion injury. Experimental study in rats. *Rev Bras Cir Cardiovasc* 2014; 29: 521–526.