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Effect of postconditioning and atorvastatin in preventing remote intestinal reperfusion injury

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ABSTRACT

Objective: To evaluate the capacity of ischemic postconditioning and atorvastatin in prevent or minimize reperfusion injury in small bowel of rats subjected to ischemia and reperfusion by abdominal aorta clamping.

Methods: 41 Wistar norvegic rats were distributed into 5 groups: ischemia and reperfusion, ischemic postconditioning, postconditioning + statin, statin and Sham. After anesthesia, laparotomy and dissection of the infra-renal abdominal aorta were performed; except the Sham group, all others were subjected to aorta clamping for 70 min (ischemia) and withdrawal of clamp for 70 min (reperfusion). In the IPC and IPC + S groups, four cycles of postconditioning were performed between the phases of ischemia and reperfusion lasting 30 s each. In IPC + S and S groups, 3.4 mg/day of atorvastatin was given for seven days per gavage; 1 cm of the ileum were removed for histological study and the results were subjected to statistical treatment considering significant $p < 0.05$.

Results: The average of intestinal lesion was 2 in the I/R group, 0.66 in the IPC group, 0 in the IPC + S group, 0 in the S group, and 0 in the SHAM group.

Conclusion: The ischemic postconditioning and atorvastatin were capable of minimizing intestinal reperfusion injury, either alone or in combination.

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Efeito do pós-condicionamento e da atorvastatina na prevenção de lesão de reperfusão intestinal remota

Palavras-chave:
Isquemia
Reperfusão
Pós-condicionamento isquêmico
Hidroximetilglutaril-CoA redutase, inibidores da
Intestino delgado

RESUMO

Objetivo: Avaliar a capacidade do pós-condicionamento isquêmico e da atorvastatina para prevenir ou minimizar a lesão de reperfusão no intestino Delgado de ratos submetidos à isquemia e reperfusão por pinçamento de aorta abdominal.

Métodos: 41 ratos noruegueses Wistar foram distribuídos em 5 grupos: isquemia e reperfusão, pós-condicionamento isquêmico, pós-condicionamento + estatina, estatina e simulacro. Depois da anestesia, procedeu-se à laparotomia e dissecação da aorta abdominal infrarrenal; exceto no grupo de simulacro, todos os demais grupos foram submetidos ao pinçamento da aorta durante 70 minutos (isquemia) e à retirada do pinçamento também durante 70 minutos (reperfusão). Nos grupos PCI e PCI + E, foram efetuados quatro ciclos de pós-condicionamento entre as fases de isquemia e de reperfusão, com duração de 30 segundos cada. Nos grupos PCI + E e E, foram administrados 3,4 mg/dia de atorvastatina durante 7 dias por gavagem; procedemos à remoção de 1 cm do íleo para o estudo histológico, e os resultados foram estatisticamente tratados. Consideramos $p < 0,05$ como estatisticamente significativo.

Resultados: As médias para as lesões intestinais foram 2 no grupo I/R, 0,66 no grupo PCI, 0 no grupo PCI + E, 0 no grupo E, e 0 no grupo S.

Conclusão: O procedimento de pós-condicionamento e atorvastatina demonstraram capacidade de minimizar a lesão de reperfusão intestinal, tanto isoladamente como em conjunto.

Introduction

Reperfusion is a fundamental step in the treatment of ischemia. However, clinical and experimental evidence shows that the main events leading to cell and tissue dysfunction are related to reperfusion.[1]

The ischemia and reperfusion (IR) injury is a pathophysiological event common to several diseases of daily clinical practice. The intestine may be the target of IR injury directly, as in mesenteric ischemia, or be reached at a distance, as in cases of shock or reperfusion injury in other organs, such as aortic clamping, used in aneurysm surgeries.[2]

Increasing evidence is emerging that the gut can be affected by remote reperfusion injury, since toxic reactive oxygen species (ROS) act systemically. In surgeries with temporary aortic occlusion, intestinal vascular involvement is a frequent complication, with a multifactorial etiology, including reperfusion injury.[3]

Regardless of where ischemia occurs, when reperfusion happens, there is a systemic impairment to a greater or lesser extent. IR is associated with the production of tumor necrosis factor (TNF). Injury of the intestinal mucosa by IR allows the release of endotoxin into the portal circulation, inducing the production of TNF by liver macrophages. Increased TNF in the systemic circulation is capable of leading to inflammatory lung injury, characterized by the accumulation of neutrophils. This sequence of events was demonstrated by Caty et al.[4] in a model of IR by temporary occlusion of the superior mesenteric artery in rats. After reperfusion, endotoxin levels in portal venous blood and TNF were increased in the systemic circulation. In parallel, there was accumulation of neutrophils in the lungs and increased pulmonary capillary permeability.

Some techniques for protection against reperfusion injury have already been tried and tested, and among them, ischemic postconditioning (IPC), which consists of one or more short cycles of reperfusion, followed by one or more short cycles of ischemia, immediately after the ischemic phase and before permanent reperfusion occurs. Although IPC has already demonstrated a protective effect in many organs submitted to IR as well as in distance protection,[5] its efficacy in the prevention of remote intestinal lesion is still very early.[6]

Much has been studied about the pathophysiology of reperfusion injury and some mechanisms have already been well evidenced such as the role of free radicals, vascular endothelial dysfunction, and neutrophil-mediated injury.[1] Recently, there has been an increase in interest in statins, drugs known for their anti-dyslipidemic effect, this time due to its pleiotropic effect, which is characterized by anti-inflammatory properties, immunomodulatory, antithrombogenic and endothelial function.[7] Recent experimental studies[8] have shown promising results with the use of statins demonstrating their role in the protection against IR injury, a fact that led us to inquire about its benefits in the face of reperfusion injury, the objective of this study being to evaluate the capacity of IPC and statins in reducing intestinal injury, alone and in combination.

Considering the lack of studies on the protective effect of postconditioning on remote intestinal reperfusion injury, as well as the potential protective effect of statins, which have not yet been tested in a model similar to the one presented

here, it is fundamental that such research be performed. Thus, the objective of this study is to evaluate the capacity of ischemic postconditioning and atorvastatin in prevent or minimize reperfusion injury in small bowel of rats subjected to ischemia and reperfusion by abdominal aorta clamping.

Methods

The study was approved by the Committee of Ethics in Animal Experimentation of the University Anhanguera-Uniderp. A total of 41 Wistar norvergic male rats weighing 250–300 g were collected from the Anhanguera-Uniderp University Animal Hospital. The animals were kept in cages at ambient temperature of approximately 23 °C with 12 h light cycles and received water and feed ad libitum.

The number of animals of each group was made by a sample calculation and based on previous researches of our group, following the ethical principle of using as few animals as possible for research. The animals were distributed in the following groups:

- Ischemia and reperfusion group (I/R): Nine rats were submitted to ischemia for 70 min by aortic clamping, followed by reperfusion of 70 min. The duration of the ischemia and reperfusion phases of 70 min was defined based on previous publications that demonstrated that this time was sufficient to cause remote lesion and, consequently, allow the evaluation of the protective effect of the methods used.[5,6]
- Ischemic postconditioning group (IPC): Nine rats were submitted to the ischemia procedure for 70 min by aortic clamping and reperfusion for 70 min. Between ischemia and reperfusion, four cycles of reperfusion (30 s each) were performed, interspersed with four cycles of ischemia (30 s each).
- Ischemic postconditioning + statin group (IPC + S): Nine rats received a dose of 3.4 mg/day of atorvastatin, one dose per day through the gavage method, for seven days and were submitted to the ischemia procedure for 70 min by aortic clamping and reperfusion for 70 min. Between ischemia and reperfusion, four cycles of reperfusion (30 s each) were performed, interspersed with four cycles of ischemia (30 s each).
- Statin group (S): Nine rats received a dose of 3.4 mg/day of atorvastatin, one dose per day through the gavage method, for seven days, and then subjected to the ischemia procedure for 70 min by aortic clamping and reperfusion for 70 min.
- SHAM group: Five rats submitted to laparotomy, dissection and isolation of infra-renal abdominal aorta artery.

The animals were anesthetized by intraperitoneal injection of a 2:1 solution of Cetamine Hydrochloride (Cetamin®, Syntec, Cotia-Brazil), 50 mg/mL, and Xilazin Hydrochloride (Xilazin®, Syntec, Cotia-Brazil), 20 mg/mL, respectively, at a dose of 0.1 mL/100 g.

After anesthesia, the rats were submitted to median longitudinal laparotomy of approximately four centimeters, exteriorization of the small intestine, identification and dissection of infra-renal abdominal aorta artery.

Table 1 – Classification of intestinal lesion degrees according to Chiu et al.[9]

Grade of lesion	Changes observed in histology
0	Mucosa without changes
1	Well-constituted villi, without cell lysis or inflammatory process, but with formation of the subepithelial space of Grunhagen
2	Presence of cell lysis, formation of Grunhagen subepithelial space and increased spacing between villi
3	Destruction of the free portion of the villi, presence of dilated capillaries and inflammatory cells
4	Structural destruction of villi, with only a few sketches, consisting of inflammatory cells and necrotic material, with hemorrhage and basal glandular ulceration
5	Destruction of every mucous tunic, no more glandular structure, but only amorphous material deposited on the submucosal screen

In all groups except SHAM, the abdominal aorta was occluded by atraumatic vascular clamp that remained for 70 min (ischemia phase). After clamp placement, the small intestine was repositioned into the abdominal cavity and the surgical wound was closed with continuous suturing of the skin with 4-0 monofilament nylon thread. After the ischemia phase, the abdominal wall was reopened by removal of the suture and in the I/R and S groups the vascular clamp was removed, initiating the reperfusion phase, lasting 70 min. In the IPC and IPC + S groups, preceding the reperfusion phase, the ischemic postconditioning was performed through four cycles of reperfusion (removal of the atraumatic vascular clamp of the abdominal aorta) with duration of 30 s each, interspersed with four cycles of ischemia (occlusion of the abdominal aorta artery by atraumatic vascular clamp), also with duration of 30 s each.

In all groups after started the reperfusion phase, the abdomen was again closed by continuous suturing of the skin with 4-0 monofilament nylon thread until the end of the experiment.

In the SHAM group, only a median longitudinal laparotomy of approximately four centimeters was performed, exteriorization of the small intestine, identification and dissection of infra-renal abdominal aorta artery.

After the reperfusion phase, all animals were resected one centimeter of the ileum, five centimeters proximal to the ileocecal valve, and the specimens were washed with saline solution and placed in 10% formaldehyde solution for histological analysis.

Euthanasia was performed by intraperitoneal administration of a lethal dose of Cetamine + Xylazine hydrochloride (0.4 mL/100 g).

Slides were prepared with the harvested material, which were stained with hematoxylin–eosin and analyzed by optical microscopy by a single observer, without prior knowledge of it on the group belonging to each rat.

The intestinal segments were classified according to the degree of tissue injury according to Chiu et al.[9] (Table 1).

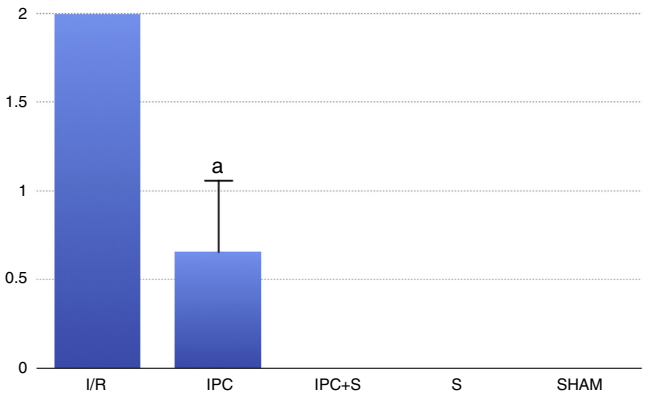

Fig. 1 – Comparison of the degrees of intestinal lesion among the different groups analyzed (Kruskal–Wallis; $p = 0.0006$; "a" $p < 0.05$ in relation to the I/R group).

After the analysis of the data, the results were submitted to statistical treatment, using the non-parametric Kruskal–Wallis test, being considered significant $p < 0.05$.

Results

After the histological analysis of each slide, the mean of the degree of intestinal lesion of each animal was defined, following the method used by other researchers with a study design similar to the one used here. The means of the intestinal lesion grades were 2 in the I/R group, 0.66 in the IPC group, and 0 in the IPC + S, S and SHAM groups (Table 1 and Fig. 1). All groups had a lower degree of tissue injury when compared to the control group with a statistically significant difference ($p < 0.05$ between control and IPC; $p = 0.0006$ between control and IPC + S, S and SHAM).

Discussion

Ischemia followed by reperfusion may induce apoptosis and an inflammatory response that affects tissue repair, especially the lung. As a result, many have evaluated the impact of IPC on subsequent apoptotic and inflammatory responses. In experimental IR models in rats with 30 min of ischemia and 3 h of reperfusion there was a significant decrease in tissue necrosis with PCI. There is also a decrease in ROS generation and protection of mitochondrial integrity, suggesting that the protective effect of IPC may be the result of a reduction in the inflammatory response. However, few studies have directly assessed the impact of IPC on inflammation. IPC may limit the expression of P-selectin, which is required for neutrophil bearing and its recruitment. In addition, it may reduce the accumulation of neutrophils in the affected region, decrease adhesion to ischemic vascular endothelium, and attenuate the endothelial dysfunction of the involved vessel, events that normally occur in IR.[10]

In the present study intestinal protection was observed with IPC, demonstrating the efficacy of the method against this IR model, which may be justified by the fact that ROS, regardless of where they are produced, when reperfusion occurs are scattered throughout the organism causing the remote reperfusion injury, so much so that in the I/R group it was observed grade two intestinal lesion, that is, moderate. By acting as a moderator of ROS production, IPC causes less local and distant injury.

The study of the reperfusion injury at a distance on the intestine is a relatively new subject, therefore, lacking publications that allow us to further compare with the present results. Turóczi et al.[7] also performed aortic clamping ischemia and evaluated the protective effect of IPC, confirming the results presented here that this method may decrease the degree of intestinal damage. Another study that obtained similar results was that of Yang et al.,[11] in which the authors also applied infra-renal aortic clamping as a method of ischemia and obtained intestinal protection with IPC.

As seen, there are few publications aimed at evaluating the remote intestinal damage against IR, especially using IPC as a protection method. Existing publications point to a promising role in this method, similar to other IR conditions in which IPC provides tissue protection.[12] Despite this, there are few studies that have used IPC in clinical practice, which reinforces the importance of finding a pharmacological method that presents the same or higher efficacy, in a way that is growing interest for safe drugs such as statins and that at least their pleiotropic effect could be useful in such situations.

In the present study intestinal protection was obtained with the use of atorvastatin, at the same intensity as with IPC. As there are no studies with the same design used here, i.e., aortic clamping and atorvastatin use, the comparison with the literature is also impaired. Moreover, since the use of statins for the prevention of reperfusion injury is relatively new, the best route of administration and ideal dose are items to be better clarified in future research. It has been chosen by gavage administration with the intention of simulating what is practiced in humans, that is, the absorption by the gastrointestinal tract, aiming at its clinical applicability.

Statins have been successfully tested for this purpose in several situations. Wu et al.[13] performed renal IR in rats and demonstrated that atorvastatin decreased tissue damage compared to the control group. The same results were obtained by Cusomano et al.[14] in the renal IR of rats using atorvastatin.

Statins also protect other tissues in the course of IR such as heart,[15–17] nervous system[18] and liver.[19] In the lung, the efficacy of statins has also been demonstrated, as demonstrated by Matsuo et al.,[20] however, with a method different from the one used here, since these authors performed IR directly on the pulmonary hilum and were therefore not a protective study of remote reperfusion injury. In addition, these authors used rosuvastatin as a protective drug and not atorvastatin as in the present study.

The mechanism of protection of statins to the situations of IR is due to its pleiotropic effect. By inhibiting the conversion of HMG-CoA to L-mevalonate, statins prevent the synthesis of isoprenoids, which are precursors of cholesterol biosynthesis, which serve as important lipid ligands for the post-translational modification of intracellular proteins, such as small GTPases, Rho, Rac and Ras. This protein isoprenylation allows adequate subcellular localization and intracellular trafficking of proteins, which control various

cellular functions, and the inhibition of these pathways may determine important components of the pleiotropic effects of statins. The Rho pathway is related to oxidative stress, atherosclerosis and elevated blood pressure. Rac pathway signaling is involved in two crucial mechanisms, such as cytoskeletal remodeling and ROS synthesis.[15]

In the development of this project we did not know that the therapeutic methods applied would achieve the results presented here, so that an association group was created (IPC + S) aiming at enhancing tissue protection. However, there was no advantage in the association, since in isolation these therapeutic methods obtained statistically similar mean tissue lesion to the SHAM group, i.e., it would not be possible to have a lower lesion than had already been achieved. Thus, it can be verified that atorvastatin has the capacity to protect the intestine in situations of reperfusion at a distance, at the same intensity as IPC, and it is possible to invest in research that confirms the best method of using these therapies in order to apply them in clinical practice.

Although the method used here is similar to that of other publications, which allowed us to make comparisons of our results, it should be emphasized as a limitation of the study that we use only histological evaluation. Future research may be done using other methods of evaluating local and remote reperfusion injury such as tissue and plasmatic malondialdehyde, which may offer greater value to the results.

In conclusion, ischemic postconditioning and atorvastatin were able to minimize intestinal reperfusion injury, either alone or in combination.

Conflicts of interest

The authors declare no conflicts of interest.

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