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Combined remote ischemic perconditioning and local postconditioning on liver ischemia–reperfusion injury

Felipe Lobato da Silva Costa, MS,[a,*] *Vitor Nagai Yamaki, MS,*[a] *Thiago Barbosa Gonçalves, MS,*[a] *João Vitor Baia Coelho, MS,*[a] *Sandro Percário, PhD,*[b] *and Marcus Vinicius Henriques Brito, PhD*[a]

[a] *Experimental Surgery Laboratory, Department of Operatory Technique, Para State University, Brazil*
[b] *Oxidative Stress Laboratory, Department of Pharmacology, University of Pará, Brazil*

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ABSTRACT

Background: Remote ischemic perconditioning (rPER) is the newest technique described to mitigate ischemia and reperfusion (IR) injury. Local postconditioning (POS) is also an effective technique for this purpose. It is uncertain if adding local POS to rPER provides superior liver protection, so we tested this hypothesis.

Materials and methods: Twenty five Wistar rats were assigned into five groups: sham, IR, POS, rPER, and rPER + POS. Animals were subjected to liver ischemia for 60 min. POS consisted of four cycles of 5-min liver perfusion followed by 5-min liver ischemia (40 min total) after the major ischemic period. rPER consisted of four cycles of 5-min hindlimb ischemia followed by 5 min hindlimb perfusion contemporaneously to major liver ischemic period, during its last 40 min. After 2 h, median and left lobes were harvested for malondialdehyde and Trolox equivalent antioxidant capacity (TEAC) measurement, and blood for the measurement of serum transaminases.

Results: All tissue conditioning techniques were able to reduce transaminases serum levels, having no differences among them. All tissue conditioning techniques were able to reduce hepatic tissue MDA level; however, only rPER + POS had higher values than SHAM. All tissue conditioning techniques also enhanced TEAC; however, only POS had lower TEAC than SHAM.

Conclusions: rPER appears as the most promising technique to avoid IR injury. This technique reduced oxidative stress of cell membranes and lowered transaminases serum level. There was no additive protection when POS and rPER were held together.

1. Introduction

Reperfusion following temporary tissue ischemia has been identified as an important mechanism contributing to tissue injury [1]. Furthermore, the clinical syndrome of ischemia and reperfusion (IR) is associated with deleterious consequences for several organs. This syndrome contributes to morbidity and mortality in a wide range of pathologies such as myocardial infarction, ischemic stroke, sleep apnea, and circulatory arrest, being a major problem in a setting of liver transplantation [2–4].

Mechanisms for IR-induced tissue injury include intracellular processes such as failure of the Na^+/K^+ ion pump,

* *Corresponding author*. Experimental Surgery Laboratory, Department of Operatory Technique, Para State University, Brazil, Rua dos Pariquis 1880, apt 1401, 66033-590 Belém-PA, Brazil. Tel.: +55 91 84277530; fax: +55 91 32691717.
E-mail address: felipe.lobato@live.com (F.L.daS. Costa).
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increase in intracellular Ca^{2+} concentration, reactive oxygen species (ROS) formation, and associated inflammatory response. ROS formation can cause lipid peroxidation of cell membranes, leading to malondialdehyde (MDA) formation and depleted tissue antioxidant capacity. ROS can also cause proteins and DNA damage, and eventually, cell death [4,5].

The most critical factor that determines the severity of tissue damage caused by IR appears to be the duration of the ischemia [5]. In addition to early reperfusion, "tissue conditioning" by a series of alternating intervals of brief episodes of IR is currently the most promising approach to limit tissue damage caused by prolonged ischemia [6–9].

Tissue conditioning was first used in 1986 by Murry *et al.* [10], who demonstrated the concept of ischemic preconditioning (PRE) in the heart of a dog by performing short cycles of IR before a major period of cardiac ischemia. Since then, this technique has been applied successfully in many clinical situations such as kidney and liver transplantation [6,11].

In 2003, Zhao *et al.* [12] developed the concept of ischemic postconditioning (POS), which is very similar to PRE, but consists of short cycles of reperfusion and ischemia before the free reperfusion of a tissue that has been under ischemia. The efficiency of POS has been demonstrated in several tissues and has been found to be similarly effective as PRE [13,14].

Tissue conditioning by cycles of IR can also be applied to tissues other than those exposed to ischemia. This concept has been called remote ischemic conditioning, and was first used by McClanahan *et al.* [15] (1993) who showed that a short period of renal ischemia provides protection to the myocardium from IR injury. Based on this study, the concepts of remote PRE [16] and remote ischemic postconditioning [17] were developed.

Schmidt *et al.* [18] (2007) also applied remote ischemic conditioning in the context of myocardial ischemia. They applied a tourniquet to a porcine limb to produce alternating periods of occlusion and reperfusion while the myocardium was under ischemia. This technique is called remote ischemic perconditioning (rPER) and it has been demonstrated to protect the brain, kidney, myocardium, and liver from the IR syndrome in various animal models [19–22].

Mechanisms underlying rPER protective effects are barely understood [23]. Moreover, underlying mechanisms involved in the protective effects of remote and local tissue conditioning techniques might be linked, working through the activation of reperfusion injury salvage kinase pathway. On the other hand, studies demonstrated the importance of an alternative pathway in remote tissue conditioning techniques, where parasympathetic response plays a critical role, and there is activation of the survivor activating factor enhancement [24].

Recent studies found that POS associated with PRE provides synergistic protection against hepatic IR-induced injury [6]; however, there is limited clinical applicability for PRE in a context of unexpected ischemia. Moreover, little data exist on the efficacy of rPER, which could be easily applied in the context of unexpected temporary hepatic ischemia or liver transplantation, and it is uncertain if adding POS to rPER provides superior hepatic protection compared with rPER alone. Thus, we tested the hypothesis that the combination of rPER and POS provides superior hepatic protection compared with rPER alone in a well-established rat model of hepatic reperfusion injury.

2. Materials and methods

2.1. Animals

Twenty-five (12–15 wk) male Wistar rats, weighing 270–300 g, were used in this study. The animals were kept in a vivarium of the Experimental Surgery Laboratory at the Pará State University (Brazil) with a controlled temperature, light, humidity and noise; water and the food were provided *ad libitum*. The research followed the rules of Brazilian National Law for Animal Care (Law: 11.794/08) that is based on National Institutes for Health guidelines, and followed the rules of Council for International Organization of Medical Sciences ethical code for animal experimentation. The project was previously approved by the Animal use and care committee at the Para State University.

2.2. Experimental protocol

The animals were randomly assigned into the following five groups ($n = 5$ for each group):

1. The SHAM group: In this group, the same surgical procedure as in the remaining groups was performed, but no liver ischemia was induced.
2. The IR group: In this group, liver ischemia was induced for 60 min followed by reperfusion without any form of conditioning.
3. The local ischemic POS group: Here, 60 min of hepatic ischemia was followed by 40 min of autologous POS (four cycles of 5-min hepatic perfusion were followed by 5 min of hepatic ischemia).
4. The rPER group: In this group, liver ischemia was simultaneously accompanied by remote ischemic conditioning. rPER consisted of four cycles of 5-min hindlimb ischemia followed by 5-min hindlimb perfusion, starting 20 min after the beginning of the ischemia and lasting 40 min until the end of the ischemic phase. Hindlimb ischemia was achieved using an elastic rubber band tied around the thigh of the left leg, following a model successfully adopted by Yamaki *et al.* [25].
5. The rPER group + local POS group (rPER + POS): Here, liver ischemia was simultaneously accompanied by rPER in the left hindlimb followed by autologous ischemic POS (four cycles of 5-min hepatic perfusion followed by 5-min hepatic ischemia).

2.3. Surgical procedures

All surgical procedures were performed in anesthesia (ketamine hydrochloride and xylazine hydrochloride 60 mg/kg and 6 mg/kg, respectively, injected intraperitoneally). Through a transverse laparotomy, hepatic lobes were exposed and the left hepatic artery was occluded by microsurgical clamp application, leading to left and median lobe liver ischemia.

After the liver ischemia and conditioning protocols, the animals remained under surgical anesthesia, allowing 2 h of liver reperfusion and postoperatory procedures. Then, blood sample was obtained via puncture of the abdominal vena cava and the left and median lobes were harvested for biochemical analysis. Subsequently, the animals were euthanized by lethal anesthetic doses.

2.4. *Laboratory parameters*

2.4.1. *Aspartate and alanine aminotransferases*

Blood samples were immediately sent to the laboratory for analysis. Aspartate and alanine aminotransferases (AST and ALT) serum levels were measured on Selectra-E auto analyzer. The left and median lobes were harvested for malondialdehyde and Trolox equivalent antioxidant capacity measurements.

2.4.2. *Malondialdehyde*

MDA levels were measured through spectrophotometer analysis after reaction with thiobarituric acid [26]. MDA is formed when cell membranes are damaged by ROS, and high levels can be detected after 1 h after hepatic IR injury; as such, it is assayed *in vivo* as a biomarker of oxidative stress [26].

2.4.3. *Trolox equivalent antioxidant capacity*

Trolox equivalent antioxidant capacity (TEAC) was used to measure the antioxidant capacity of the hepatic tissue, as compared with the standard, Trolox. Tissue antioxidant capacity reduction is directly related to the ROS formation [27].

2.5. *Statistics*

The software, Bioestat 5.0 was used. All data were expressed as means ± standard deviation. Analysis of variance, followed by Tukey *post hoc* test correction, was performed. Statistical significance was assumed at $P < 0.05$.

3. Results

All tissue conditioning techniques were able to reduce transaminases serum levels, and had no difference among them. The IR group had the highest levels compared with all other groups (Fig. 1).

All tissue conditioning techniques were able to reduce the hepatic tissue MDA level. The IR group (215.2 ± 33) had the highest MDA levels compared with all other groups. POS (112.6 ± 47.5), rPER (103.3 ± 43.6), and rPER + POS (131.8 ± 41.7) had similar values; however, POS and rPER showed no difference in MDA levels when compared with SHAM (57.2 ± 27.4) whereas rPER + POS had higher values than SHAM (Fig. 2).

All tissue conditioning techniques enhanced TEAC. The IR group (0.49 ± 0.14) had the lowest TEAC compared with all other groups. POS (0.86 ± 0.12), rPER (0.93 ± 0.13), and rPER + POS (0.91 ± 0.26) had similar values; however, rPER and rPER + POS showed no difference in TEAC when compared with SHAM (1.19 ± 0.14) whereas POS had lower TEAC than SHAM (Fig. 3).

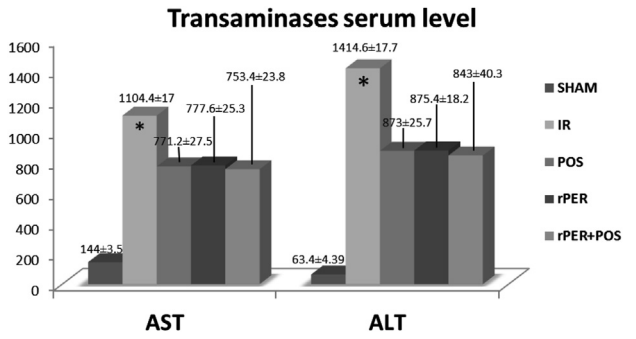

Fig. 1 – AST and ALT serum mean values and standard deviation according to groups. *P < 0.01 analysis of variance.

4. Discussion

rPER is the newest technique described to mitigate IR injury in many tissues, such as myocardium, kidney, brain, and liver [16,20,21,25]. Our study is innovative, evaluating the potential protective effect of rPER alone or rPER combined with POS on liver IR-induced injury.

Transaminases serum level is a good measurement of liver damage. AST is similar to ALT in that both enzymes are associated with liver parenchymal cells. The difference is that ALT is found predominantly in the liver whereas AST is found in the myocardium, the kidneys, the brain, and the red blood cells. As a result, ALT is a more specific indicator of liver damage than AST [28]. Our data for transaminases serum level showed results following the same pattern when both AST and ALT were analyzed, eliciting that minimal nonhepatic trauma was exempted.

All adopted protocols of tissue conditioning techniques were able to reduce AST and ALT serum levels in a very similar manner, demonstrating that rPER and local POS are very effective, and conferred protection against hepatocytes injury secondary to liver IR-induced syndrome (Fig. 1). However,

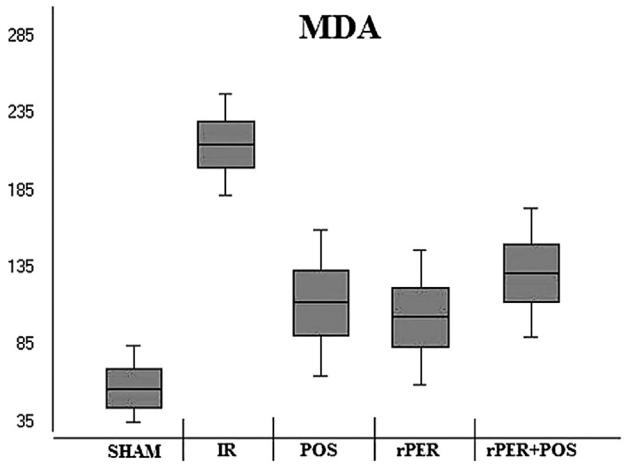

Fig. 2 – Malondialdehyde level in hepatic tissue according to groups. IR *versus* all other groups P < 0.01; SHAM *versus* rPER + POS P < 0.05. Analysis of variance (Tukey).

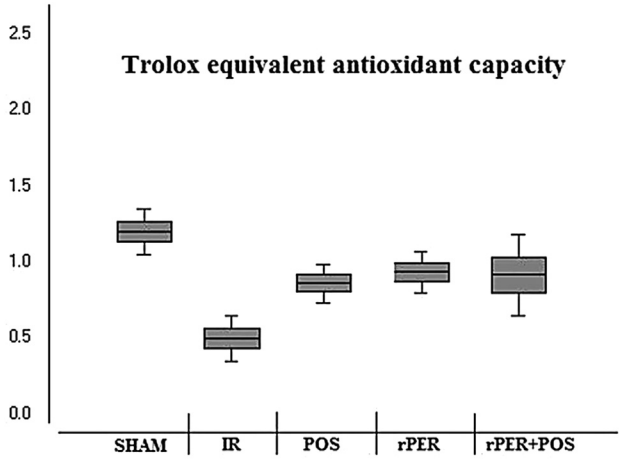

Fig. 3 – Trolox equivalent antioxidant capacity of hepatic tissue according to groups. IR *versus* all other groups $P < 0.01$; SHAM *versus* POS $P < 0.05$. Analysis of variance (Tukey).

when the association of both techniques was held, no additive effect was observed; this fact suggests that maximal protection capacity was already induced by performing either rPER or POS, and no further protection can be achieved by associating the techniques.

Other studies reported that the addition of PRE to POS led to a better AST and ALT outcome than POS or PRE [6] only. This fact demonstrates that it is possible to achieve further protection when adding other tissue techniques to POS. Thus, this indicates that POS cannot induce maximum capacity of hepatic tissue protection.

To measure cell membrane injury due to ROS activity, we measured the hepatic tissue MDA level. Our data presented that all tissue conditioning techniques were able to reduce the oxidative stress satisfactorily. Interestingly, rPER and POS were able to reduce MDA levels to the same found on SHAM, but rPER + POS did not have such result (Fig. 2).

We could clearly detect that there was no additional effects when both techniques were held together. Actually, the association had slightly worse outcome that could be of statistical significance in a larger series. We suggest that the complementary POS IR cycles might lead to additional tissue damage, in opposition to the expected additional protection.

Furthermore, no protection could be achieved after performing rPER, and because previous studies showed that additional protection can be obtained by adding another tissue technique to POS, we inferred that rPER is the technique that can confer the maximum amount of tissue protection.

To measure the formation of ROS, we carried out TEAC of hepatic tissue, which shows how strongly the formation of ROS consumed endogenous antioxidant reserve. We noted that all tissue conditioning techniques were able to avoid the consumption of endogenous antioxidant substances. rPER and rPER + POS enhanced TEAC levels to similar values found on SHAM; however, results found on POS were not similar to those found on SHAM (Fig. 3).

From the TEAC data, we could detect again that when both techniques were held, no further protection was achieved. rPER works better than POS on preventing the general formation of ROS. This fact suggests that rPER is the technique that can confer better tissue protection when compared with POS or the combination of techniques, because they failed in promoting additional protection and in preventing ROS formation, respectively.

rPER appears as the most promising technique to avoid deleterious consequences of hepatic IR-induced injury. No improved outcome was detected when the association of both techniques was held, and ROS formation was not similar to SHAM only when purely POS was held, eliciting that rPER conferred better protection, and the complementary POS IR cycles might lead to additional tissue damage.

We claim that rPER works better than POS in a setting of hepatic IR injury, and there is no benefit in the combination of those techniques.

5. Conclusions

rPER appears as the most promising technique to avoid IR injury. This technique reduced oxidative stress of cell membranes and lowered transaminases serum level. Furthermore, rPER led to the formation of less ROS than POS.

There was no additive protection when POS and rPER were held together.

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Disclosure

The authors reported no proprietary or commercial interest in any product mentioned or concept discussed in the article.

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