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Remote ischemic conditioning temporarily improves antioxidant defense

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ABSTRACT

Background: Remote ischemic conditioning (RIC) is the most promising surgical approach to mitigate ischemia and reperfusion (IR) injury. It consists in performing brief cycles of IR in tissues other than those exposed to ischemia. The underlying mechanisms of the induced protection are barely understood, so we evaluated if RIC works enhancing the antioxidant defense of the liver and kidney before IR injury.

Materials and methods: Twenty-one Wistar rats were assigned into three groups as follows: sham, same surgical procedure as in the remaining groups was performed, but no RIC was carried out. RIC 10, RIC was performed, and no abdominal organ ischemia was induced. After 10 min of the end of the RIC protocol, the liver and kidney were harvested. RIC 60, similar procedure as performed in RIC 10, but the liver and the kidney were harvested 60 min. RIC consisted of three cycles of 5-min left hind limb ischemia followed by 5-min left hind limb perfusion, lasting 30 min in total. Samples were used to measure tissue total antioxidant capacity.

Results: RIC protocol increased both liver (1.064 ± 0.26 mM/L) and kidney (1.310 ± 0.17 mM/L) antioxidant capacity after 10 min when compared with sham (liver, 0.759 ± 0.10 mM/L and kidney, 1.08 ± 0.15 mM/L). Sixty minutes after the RIC protocol, no enhancement on liver (0.687 ± 0.13 mM/L) or kidney (1.09 ± 0.15 mM/L) antioxidant capacity was detected.

Conclusions: RIC works through temporary and short-term enhancement of liver and kidney cells antioxidant defenses to avoid the deleterious consequences of a future IR injury.

1. Introduction

The syndrome of ischemia and reperfusion (IR) contributes to the morbidity and mortality in several clinical situations, such as liver and kidney transplantation. Mechanisms for induced tissue injury mainly occur because of reactive oxygen species (ROS) formation when tissue perfusion is restored, which can lead to lethal cell injury. Currently, there is not a drug regimen that can satisfactorily avoid the deleterious consequences of IR [1,2].

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The only action that can stop the ischemic cascade is reestablishment of oxygen inflow as early as possible [3]. In addition to early reperfusion, "tissue conditioning" by a series of alternating intervals of brief episodes of IR is currently the most promising surgical approach to limit cell damage caused by prolonged ischemia [4–7].

Tissue conditioning techniques can be applied locally, before (preconditioning) or after (postconditioning) a major ischemic period. These techniques have been demonstrated to significantly minimize the injury secondary to IR syndrome in several organs [8–12]. However, a shortcoming of both local preconditioning and postconditioning is the prolongation in operative time and the necessity of direct access to the occluded artery, being hard or even impossible to perform in a setting of endovascular or pharmacologic revascularization [13].

Tissue conditioning by brief cycles of IR was discovered to be feasible in tissues other than those exposed to ischemia. This concept has been called remote ischemic conditioning (RIC) and was first used by McClanahan *et al.* [14] (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 ischemic preconditioning [14] and remote ischemic postconditioning [15] were developed. Those techniques are minimally invasive and could be applied in several clinical settings, such as liver and kidney transplantation; however, these techniques also have the shortcoming of increase in the operative time [13].

Schmidt *et al.* demonstrated an even more practical technique. They applied a tourniquet to a porcine limb to produce alternating periods of occlusion and reperfusion while the myocardium was under ischemia. This technique was called remote ischemic perconditioning [16], and it has been demonstrated to be protective against the IR syndrome in various animal models [17–19].

Obviously, the best clinical approach to treatment is the use of noninvasive or minimally invasive techniques because the most important goal is to decrease both frequency of complications and the technical risks [13]. Oxman *et al.* [20] reported the first noninvasive technique, carrying out conditioning cycles on the lower limb by the use of an elastic tourniquet. Such noninvasive model was proved to be feasible in liver and kidney RIC protocols [18,19].

There is scarce evidence as to how brief cycles of IR on a distant organ are able to provide organ protection against injury. It is proposed that the short IR cycles in the remote tissue would release humoral factors [13], such as adenosine [21], bradykinin [22], opioids [23], and endocannabinoids [24]. Those humoral factors and the direct stimuli are sensed by the remote organ innervation [25], and there would be a systemic response modulated through the parasympathetic nervous system [26] leading to the propagation of an effector signal to the target organ that is under ischemia [27].

The effector signal would activate specific receptors in cell membranes, which trigger the well-known participant in the mechanism of the different conditioning strategies, the so-called survivor activating factor enhancement (SAFE) pathway, in which signal transducer and activator of transcription proteins would lead to protection against IR injury. It has been also demonstrated that the activation of another pathways, such Rho-kinases and other certain prosurvival kinase elements of the reperfusion injury salvage kinase (RISK) cascade [28].

Mitochondrial protection seems to represent the final common path elements for both the RISK and SAFE pathways. However, the intracellular mechanism underlying such protection remains unknown [13].

All the previous studies applied the RIC in a setting of IR of a target organ. There are no studies that evaluated the effects of RIC in a target organ that has not been under ischemia. Thus, we analyzed the variations in liver and kidney antioxidant capacities promoted by RIC applied in the hind limb.

2. Materials and methods

2.1. Animals

Twenty-one (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 environment; water and the food were provided *ad libitum*. The research followed the rules of the Brazilian Law for Animal Care (Law: 11.794/08) that is based on NIH guidelines and followed the rules of the 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 three groups ($n = 7$ for each):

1. The sham group (sham): In this group, the same surgical procedure as in the remaining groups was performed, but no remote ischemic conditioning was carried out.
2. The RIC group—10 min (RIC 10): In this group, the RIC was performed, and no other organ ischemia was induced. After 10 min of the end of the RIC protocol, the liver and kidney were harvested for biochemical analysis.
3. The RIC group—60 min (RIC 60): In this group, the RIC was performed, and no other organ ischemia was induced. After 60 min of the end of the RIC protocol, the liver and kidney were harvested for biochemical analysis.

2.3. Surgical procedures

All surgical procedures were performed in anesthesia (ketamine hydrochloride and xylazine hydrochloride 70 and 10 mg/kg, respectively, intraperitoneal). The RIC consisted of three cycles of 5-min left hind limb ischemia followed by 5-min left hind limb perfusion, lasting 30 min in total. Hind limb ischemia was achieved using an elastic rubber band tied around the thigh of the left leg.

Ten or 60 min after the RIC protocols, the animals were subjected to a median laparotomy, and the liver and kidney were exposed and harvested for biochemical analysis.

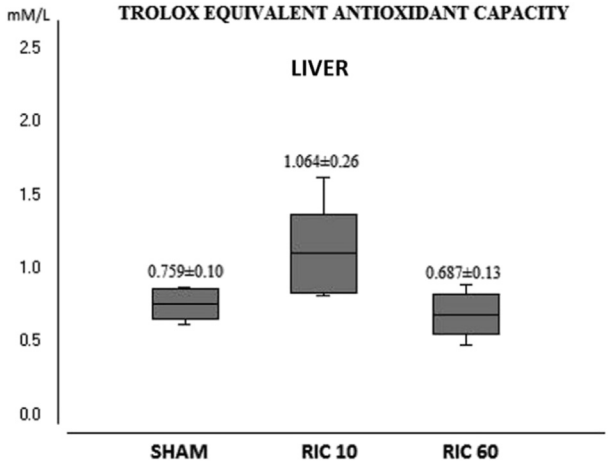

Fig. 1 – Liver antioxidant capacity among groups. Mean and standard deviation. Analysis of variance-Tukey; $P < 0.05$, RIC 10 *versus* sham and RIC 60.

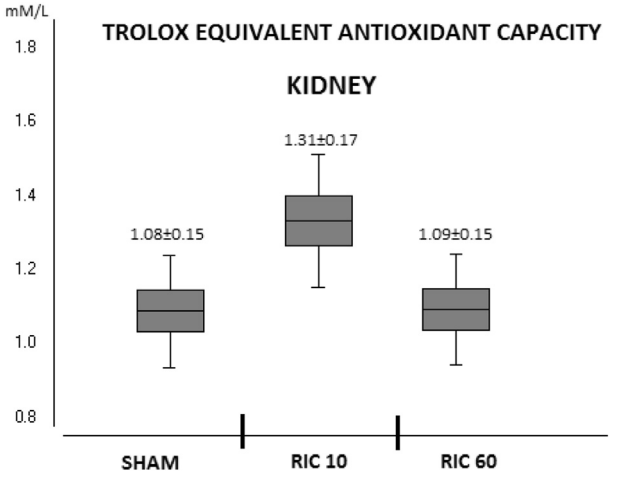

Fig. 2 – Kidney antioxidant capacity among groups. Mean and standard deviation. Analysis of variance-Bonferroni; $P < 0.05$, RIC 10 *versus* sham and RIC 60.

Subsequently, the animals were euthanized by lethal anesthetic doses.

2.4. *Laboratory parameters*

2.4.1. *Trolox equivalent antioxidant capacity*

Trolox equivalent antioxidant capacity measures the antioxidant capacity of biological samples, as compared with the standard, Trolox, water soluble synthetic analog of vitamin E.

The liver tissue samples were kept frozen (−76°C) in a KCl-buffered solution, until laboratory analysis. The samples were taken to an ultrasonic cell disruptor, for breaking all lipid membranes and the formation of a tissue homogenate, which permits the measurement of all intracellular antioxidant substances.

Trolox equivalent antioxidant capacity is a colorimetric technique based on the reaction of ABTS (2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid, diammonium) and potassium persulfate ($K_2S_2O_8$) directly yielding the free radical cation $ABTS^+$, chromophore of green/blue color with maximum absorbance at wavelengths 645, 734, and 815 nm. The addition of antioxidants to this preformed radical $ABTS^+$ reduces it to ABTS, to the extent of antioxidants content in the added substance, in a time- and concentration-dependent manner.

This can be measured spectrophotometrically by observing the change in the absorbance read at 734 nm over a given time interval. Thus, the extent of discoloration is the rate of inhibition of the radical cation $ABTS^+$ and is determined as the total antioxidant activity of the sample which is then calculated through its relationship to the reactivity of Trolox, under the same conditions, and the final results expressed as micromoles per liter [29].

2.5. *Statistics*

The software, BioEstat 5.4 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

The RIC protocol increased both liver (Fig. 1) (1.064 ± 0.26 mM/L) and kidney (Fig. 2) (1.310 ± 0.17 mM/L) antioxidant capacity after 10 min when compared with sham (liver: 0.759 ± 0.10 mM/L and kidney: 1.08 ± 0.15 mM/L). In the RIC 60 group, no enhancement on liver (0.687 ± 0.13 mM/L) or kidney (1.09 ± 0.15 mM/L) antioxidant capacity was detected after 60 min of the RIC protocol.

4. Discussion

RIC is a novel technique that has been showing promising results to diminish the deleterious consequences of IR injury. It has been demonstrated to protect both the liver and kidney in experimental settings in mice, rats, and rabbits [7,19,30,31].

As RIC is a minimally invasive and low-cost technique, it has been gaining augmented importance; and despite being a relatively recent technique with barely understood mechanisms, quickly reached the clinical trials. Currently, it is been evaluated in large clinical trials, such as the Remote Ischemic Preconditioning in Abdominal Organ Transplantation trial, and in another trials of major liver surgeries [32]. A small trial with 16 patients demonstrated pronounced protection of RIC during liver resection [31].

The rapid translation of the RIC without understanding its underlying mechanisms can lead to the misapplication of this promising technique, which could even be lost in translation.

Our results showed that the short cycles of IR applied to the hind limb and led to an enhancement in both liver and antioxidant capacity 10 min after the RIC protocol. This is the first demonstration that RIC prepares a tissue that is not under ischemia, to face a future oxidative burst.

Previous experimental studies demonstrated that when RIC protocol is done in a setting of liver or kidney IR, there is less cell damage by the ROS formed during reperfusion [7,19]. However, it was unknown if RIC acted avoiding ROS formation or increasing cell antioxidant defense. Our study confirmed that RIC increases liver and kidney cells antioxidant defenses, and the protection can be detected as soon as 10 min after the RIC protocol.

To verify if such enhancement in the antioxidant defense is a sustained response, we measured liver and kidney antioxidant capacity 60 min after the RIC protocol. Our data showed no improvement 60 min after the RIC protocol, which demonstrates that RIC only temporarily increases liver and kidney antioxidant capacities.

A new effector mechanism is highlighted by such finding. The humoral factors [13], tissue innervation [25], and the activation of the parasympathetic nervous system [26] would activate the hepatocytes to face a probable future IR injury. Through the activation of the RISK and SAFE pathways [28], the liver and kidney cells would increase their antioxidant defenses. However, such provision to face an ischemic event is temporary, and the antioxidant substances disappear even in the absence of an oxidative stress.

Several substances could be the antioxidant compounds enhanced by the RIC protocol, as for example large carbonic chains, cations, or enantiomers. The discovery of the implicated substance would be of great importance to the better understanding of the RIC mechanisms and further better employment of this promising technique.

Our experimental results are of pronounced importance for the RIC clinical translation. Because the RIC-induced protection is a transitory and short-term event, it produces a short window of opportunity after performing the RIC protocol in the liver graft donor (model adopted by the Remote Ischemic Preconditioning in Abdominal Organ Transplantation trial), because the graft would have its antioxidant defense enhanced for <60 min, and that time would be not sufficient to perform the transplantation procedure and restore organ perfusion.

Given the short-term protection time would be not beneficial to perform prophylactic cycles of remote ischemic preconditioning in the graft receiver while waiting for the surgery. Moreover, the remote ischemic perconditioning applied on the graft receiver, during the surgery, seems to be the most promising technique that might have a positive result in a setting of liver transplantation.

5. Conclusions

RIC works in the liver and kidney through the temporary and short-term enhancement of liver and kidney cells antioxidant defenses to avoid the deleterious consequences of a future IR injury.

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The authors declare no conflict of interest.

Authors' contributions: F.L.S.D.C., R.K.C.T., V.N.Y., A.M.F.D.S., A.L.V., S.P., and M.V.H.B. contributed to the conception and design. F.L.S.D.C., R.K.C.T., V.N.Y., A.M.F.D.S., and A.L.V. did the analysis and interpretation, data collection, and writing of the article. S.P. and M.V.H.B. did the critical revision of the article and obtained the funding.

Disclosure

All authors declare no financial and personal relationships with other people or organizations.

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