Ischemic pre and postconditioning in skeletal muscle injury produced by ischemia and reperfusion in rats[1]

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ABSTRACT

PURPOSE: To investigate the protective effects of ischemic pre and postconditioning, as well as the association of both methods, in skeletal muscle injury produced by ischemia and reperfusion in rats.

METHODS: An experimental study was designed using 40 Wistar rats divided in four groups (n=10): Control – rats submitted to ischemia for 240 minutes (min) and reperfusion for 60 min; Ischemic preconditioning (Pre) – animals submitted to three cycles of clamping and releasing the aorta for five min before being submitted to the ischemia/reperfusion procedure; Ischemic postconditioning (Post) – rats submitted to three cycles of clamping and releasing the aorta for one min after the 240-minute ischemic phase; Ischemic pre and postconditioning (Pre-post) – animals submitted to the same procedures of Pre and Post groups. Skeletal muscle injury was evaluated by measuring serum levels of aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and creatine phosphokinase (CPK); and muscular levels of malondialdehyde (MDA) and glycogen.

RESULTS: AST levels were significantly higher in Pre and Pre-post groups ($P<.01$). There were no differences in LDH and CPK levels. Muscular MDA levels were similar. Glycogen levels were significantly higher in Pre and Pre-post groups ($P<.01$).

CONCLUSIONS: Both preconditioning and its association with postconditioning had a protective effect by avoiding glycogen depletion in skeletal muscle in rats submitted to ischemia and reperfusion. Association of pre and postconditioning did not show advantage compared to preconditioning alone. Postconditioning alone did not show protective effect.

Key words: Ischemic Preconditioning. Ischemic Postconditioning. Ischemia. Reperfusion. Muscle, Skeletal. Models, Animal. Glycogen. Rats.

Introduction

Tissular injury after ischemia and reperfusion has been intensively studied in the last years[1-3]. Ischemic preconditioning, which consists on producing short periods of ischemia followed by reperfusion before a sustained ischemic period, exerts a protective effect against ischemia and reperfusion injury in many animal species[4]. In humans, this effect is described in the heart[5], brain[6], kidneys[7], liver[8] and skeletal muscle[9]. Ischemic postconditioning, which consists on producing short periods of ischemia and reperfusion just before sustained reperfusion, also produced protective effects in rats[10], rabbits[11], pigs[12] and humans[13].

The optimal protocol for applying ischemic pre and postconditioning aiming maximal muscular tissue protection is yet undetermined. Motivated by this issue, we employed an experimental model of partial ischemia in rats that simulate the clinical condition found in acute arterial occlusion in humans to test ischemic pre and postconditioning.

The purpose of this study was to analyze the protective effects of ischemic pre and postconditioning, as well as the association of both methods, in skeletal muscle injury produced by ischemia and reperfusion in rats.

Methods

All protocols were approved in accordance with the Animal Experimentation Ethics Committee at University of Sao Paulo – Ribeirao Preto, Brazil. Forty male Wistar rats (University of Sao Paulo, Ribeirao Preto, Brazil), weighing 250 to 450 g and clinically healthy were used for this study. Animals were maintained in a room controlled for temperature and light and were provided food and water *ad libitum*. Animal care complied with the Principles of Laboratory Animal Care (formulated by the National Society for Medical Research) and the Council for International Organization of Medical Sciences (CIOMS) ethical code for animal experimentation[14].

Ischemia and reperfusion model

Anesthesia was induced with intraperitonial sodium thiopental (50mg/Kg body weight). Ischemia and reperfusion injury was induced by clamping the infra-renal aorta, following the surgical procedure described in detail elsewhere[15]. Abdominal aorta was exposed through a midline incision and occluded with an atraumatic vascular clamp just below the renal arteries, after full heparinization. After 240 minutes (min) of aortic occlusion (ischemic phase), the clamp was removed for more 60 min (reperfusion phase). The wound was closed with interrupted cotton 3-0 between the phases. At the end of the experiment, blood samples were collected from the inferior vena cava and a biopsy from the right hindlimb gastrocnemius muscle was performed.

Experimental design

Animals were randomly divided in four groups (n=10): Control – rats submitted to ischemia for 240 min and reperfusion for 60 min; Ischemic preconditioning (Pre) – animals submitted to three cycles of clamping the aorta for five min and releasing for more five min before being submitted to the ischemia/reperfusion procedure; Ischemic postconditioning (Post) – rats submitted to three cycles of clamping the aorta for one min and releasing for more one minute after the 240-minute ischemic phase; Ischemic pre and postconditioning (Pre-post) – animals submitted to three cycles of clamping the aorta for five min and releasing for more five minutes before the 240-minute ischemic phase and also submitted to three cycles of clamping the aorta for one minute and releasing for one more min after the 240-minute ischemic phase. Figure 1 represents the experimental protocol.

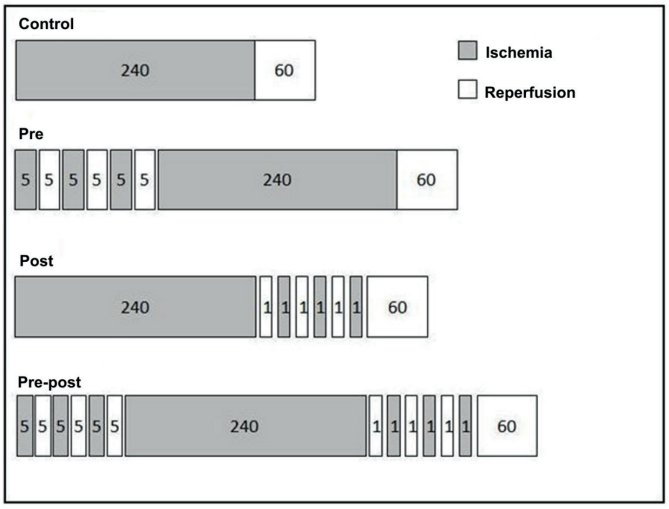

FIGURE 1 - Graphic representation of ischemia and reperfusion times in different groups. Pre – Ischemic preconditioning; Post - Ischemic postconditioning; Pre-post - Ischemic pre and postconditioning. Numbers represent time in minutes.

Skeletal muscle injury was evaluated by measuring serum levels of released cytoplasmic enzymes (aspartate aminotransferase – AST, lactate dehydrogenase - LDH and total creatine phosphokinase -CPK); and tissular levels of a cell membrane degradation product (malondialdehyde - MDA) and muscular energetic storage (muscular glycogen).

Measurement of serum and muscular markers

Blood samples were used for determination of serum levels of AST, LDH and CPK. All determinations were performed with appropriate spectrophotometric essays. Results were expressed in Units/L.

Muscular samples were used for determination of muscular MDA and glycogen levels. For MDA determinations, muscular samples were homogenized, centrifuged (3000×g, 10 min, 4°C) and the supernatant was submitted to a spectrophotometric essay (Lipid Peroxidation Assay Kit – Calbiochem, San Diego, CA). Results were expressed in µM. For glycogen determinations, muscular samples were treated with KOH 30%. Levels of glycogen were measured by spectrophotometry using the anthrone method. Results were expressed in mg%.

Statistical analysis

Data were expressed as the mean ± the standard deviation. Statistical comparisons among groups were done with Kruskal–Wallis one-way analysis of variance with Bonferroni post test. A *P* value of less than .05 was considered statistically significant.

Results

Serum AST, LDH and CPK levels

AST levels were significantly higher in Pre and Pre-post groups, compared with control group ($P<.01$). Post group AST levels were similar to control group (Figure 2).

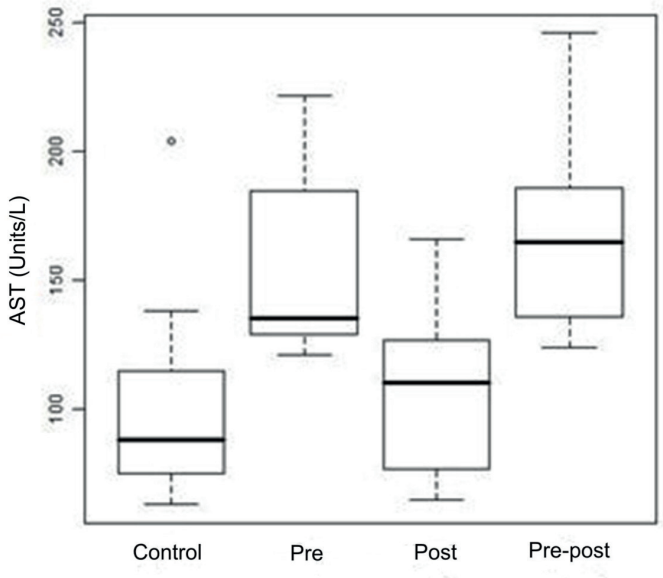

FIGURE 2 - Serum levels of aspartate aminotransferase (AST) at the end of experiment in different groups. Pre – Ischemic preconditioning; Post - Ischemic postconditioning; Pre-post - Ischemic pre and postconditioning; (—) mean; (□) confidence interval; (‡) standard deviation.

There were no differences in LDH (Figure 3) and CPK (Figure 4) levels among the four groups.

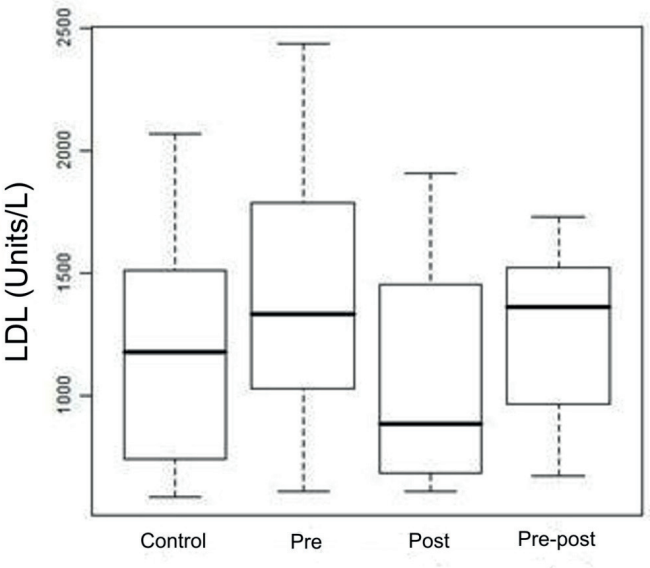

FIGURE 3 - Serum levels of aspartate lactate dehydrogenase (LDH) at the end of experiment in different groups. Pre – Ischemic preconditioning; Post - Ischemic postconditioning; Pre-post - Ischemic pre and postconditioning; (—) mean; (□) confidence interval; (‡) standard deviation.

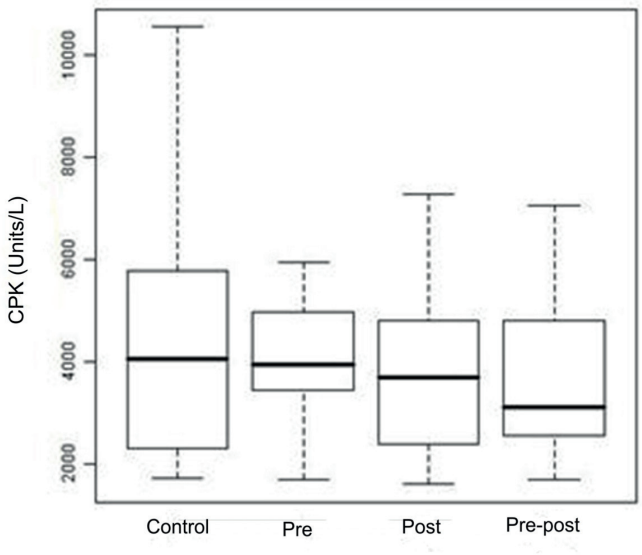

FIGURE 4 - Serum levels of total creatine phosphokinase (CPK) at the end of experiment in different groups. Pre – Ischemic preconditioning; Post - Ischemic postconditioning; Pre-post - Ischemic pre and postconditioning; (—) mean; (□) confidence interval; (‡) standard deviation.

Muscular MDA and glycogen levels

MDA levels were not different in the four groups (Figure 5).

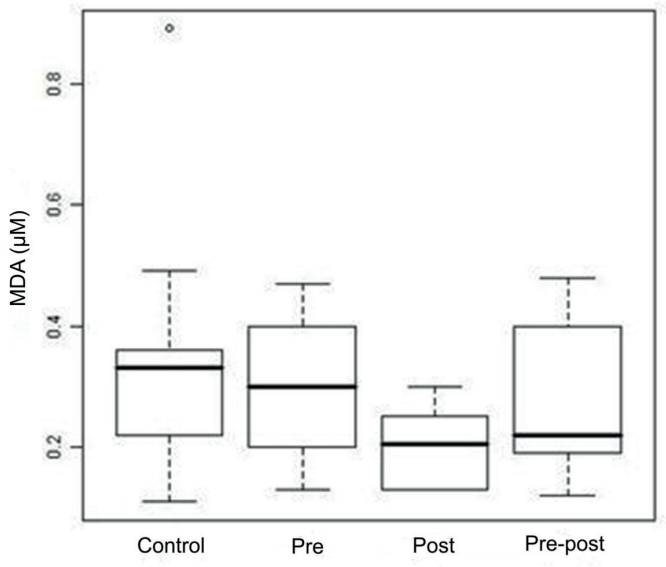

FIGURE 5 - Right hindlimb gastrocnemius tissular levels of malondialdehyde (MDA) at the end of experiment in different groups. Pre – Ischemic preconditioning; Post - Ischemic postconditioning; Pre-post - Ischemic pre and postconditioning; (—) mean; (□) confidence interval; (‡) standard deviation.

Glycogen levels were significantly higher in Pre and Pre-post groups, compared with control group (P<.01). Post group presented increased glycogen levels, not significantly different form control groups (Figure 6).

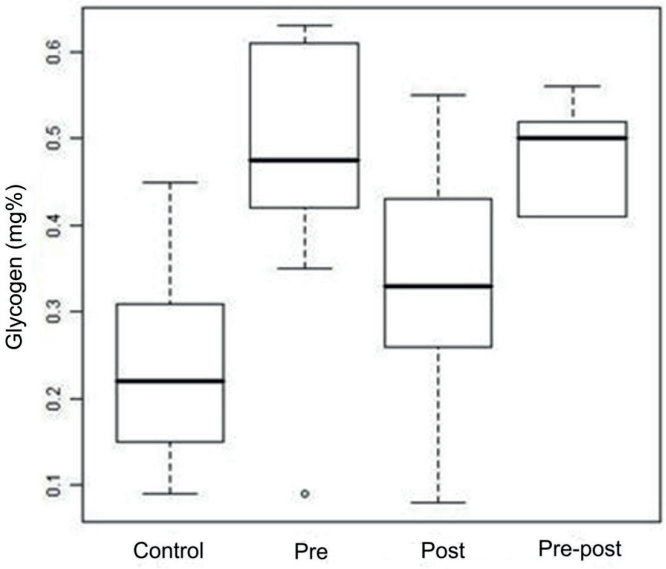

FIGURE 6 - Right hindlimb gastrocnemius tissular levels of glycogen at the end of experiment in different groups. Pre – Ischemic preconditioning; Post - Ischemic postconditioning; Pre-post - Ischemic pre and postconditioning; (—) mean; (□) confidence interval; (‡) standard deviation.

Discussion

The protective effect of ischemic preconditioning in skeletal muscle was first demonstrated in a swine model, in which three cycles of ten minutes of ischemia and reperfusion was followed by four hours of ischemia[16]. Several experimental protocols were later developed aiming to maximize the protective effect. The most studied preconditioning protocols used sequential clamping times of five to ten minutes, and one to five cycles of ischemia and reperfusion. Pang *et al.*[9], using preconditioning of three cycles of ischemia and reperfusion with ten minutes of clamping, followed by four hours of ischemia and 90 minutes of reperfusion, obtained preserved muscular levels of phosphocreatine and adenosine triphosphate and low serum lactate levels. Mattei *et al*.[17] investigated the efficiency of different preconditioning protocols in skeletal muscle in rats, comparing three cycles of 2.5, five and ten minutes. The best protective effects were found using three cycles of five minutes. Efficacy of preconditioning seems to be less dependent on clamping duration than on the number of cycles of ischemia and reperfusion.

Zhao *et al*.[18] presented the concept of ischemic postconditioning[18], and McAllister *et al*.[12] first tested it in skeletal muscle. The supposed molecular mechanism of tissue protection of ischemic postconditioning involved the inhibition of opening of mitochondrial permeability transition pores (mPTP). Szijártó *et al*.[19] tested ischemic postconditioning in a model of partial ischemia using infra-renal aortic clamping. Rats were submitted to 180 min of ischemia followed by four hours of reperfusion. The postconditioning group was submitted to six cycles of ten seconds of reperfusion and ischemia, just before the beginning of the sustained reperfusion. Postconditioning group had a decreased systemic inflammatory response (TNF-α) and a marked reduction in reperfusion-related organ dysfunctions (lungs and kidneys).

The experimental model employed in the present study differs from most described in the literature. The latter employed muscular flaps, in which a single pedicle was clamped, generating total ischemia. In our model partial ischemia was generated in skeletal muscle, since the collateral pathways were not occluded. Partial ischemia model simulates the clinical condition commonly found in acute arterial occlusion. These conditions are also encountered in arterial surgery, when clamping is necessary.

The findings of the present study demonstrated that in Pre and Pre-post groups there was an increase in serum AST, but no alterations in CPK levels during reperfusion phase. Castro e Silva Jr. *et al*.[8], studying postconditioning and hepatic ischemia observed similar results. These observations could be explained by the fact

that serum enzyme levels correlate with timing of an ischemic process. During an ischemic injury, CPK usually increases before AST, but also decreases first. Increased CPK levels associated with decreased AST levels indicate recent injury. Persistent increased levels of both enzymes indicate continued injury and decreased CPK levels associated with increased AST levels indicate recovery form an ischemic insult[20]. As preconditioning and also its association with postconditioning promoted a significant increase in AST levels, which was not shared by CPK and LDH, one can say that when blood was collected, skeletal muscle was under recovery from the ischemic injury.

Previous experiments involving hepatic ischemia demonstrated that postconditioning caused a decrease in lipid peroxidation[10]. Lipid peroxidation produces a number of metabolites, whose combination with proteins and DNA results in toxic substances, such as MDA. Most damage caused by lipid peroxidation occurs in cell membrane[10]. Kin *et al*.[21] demonstrated that the protective effect of postconditioning was mediated by endogenous adenosine, through activation of receptors A2A and A3AR. This effect was responsible for a decreased lipidic peroxidation. Sudden reperfusion removes from tissues endogenous protective substances that were generated during ischemia, such as adenosine. With gradual reperfusion, these substances are kept in the tissue, exerting a protective effect[10]. In the present experiment, as MDA levels were similar in all groups, the protective effect of preconditioning, postconditioning and their association in skeletal muscle was not demonstrated.

In the present study, muscular glycogen levels in Pre and Pre-post groups were significantly greater than in the control group. Thus, preconditioning, as well as its association with postconditioning prevented the depletion of glycogen storage caused by ischemic process. These results are similar to those of Weiss *et al*.[22] and Pescador *et al*.[23]. The exact molecular mechanism to explain this phenomenon is yet to be understood.

During hypoxia, anaerobic glycolysis occurs and tissue demands of glucose are increased. In this scenario, maintenance of glycogen storage and also the occurrence of glycogen synthesis seem to be paradoxal. In our study, ischemic preconditioning and its association with postconditioning caused this phenomenon. One possible explanation is the role of hypoxia-inducible factors (HIF). These factors are dependent on genic expression and would stimulate glycogen synthase isoform 1 (GSY-1) during the short hypoxic periods. Glycogen synthesis would replenish cell glycogen storages, which would be utilized in posterior periods. Thus, GYS-1 induction by HIF would consist in a cellular adaptation to hypoxia, in which cells prepare themselves for future oxygen shortage. A better comprehension of this process could explain physiologic adaptations to hypoxia, and also help to develop therapeutic strategies against diseases which hypoxia plays a major role[23].

In current surgical and clinical practice, the protective effect of ischemic preconditioning over skeletal muscle has the potential to be applied in several situations. Good examples are procedures that require increased clamping time. In general surgery, it may be used in complex trauma operations. In vascular surgery, preconditioning may benefit aortic and extremity reconstructive arterial procedures. In plastic surgery, muscular and myocutaneuos flaps[24], and also techniques of replantation, free tissue transfer, and composite tissue allotransplantation[25] may be favored.

Another practical application is that ischemic preconditioning, even in remote sites can protect distant tissues and organs. Remote ischemic preconditioning reduces myocardial injury after major cardiovascular surgery[26]. A trial in patients undergoing abdominal aortic aneurysm repair reported a significant reduction in perioperative myocardial infarctions[27]. However, large-scale trials testing the technique are required in patients undergoing major vascular surgery. Postconditioning has a potential advantage over preconditioning, since it can be performed in the end of the ischemic period. Thus, it could be applied in clinical situations where intervention is performed in the end of the ischemic period, such as trauma surgery.

Both ischemic pre and postconditioning are techniques with great potential of being applied in clinical and surgical practice. However, more studies are required to clarify diverse results published with different animal species and organs. Remote pre and postconditioning need to be more studied, as well as its relation with drugs that could produce the same protective effects.

Conclusions

Both preconditioning and association of pre and postconditioning had a protective effect by avoiding glycogen depletion in skeletal muscle in rats submitted to ischemia and reperfusion. Association of pre and postconditioning did not show advantage compared to preconditioning alone. Postconditioning alone did not show protective effect.

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