

Sildenafil citrate protects skeletal muscle of ischemia-reperfusion injury. Immunohistochemical study in rat model¹

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ABSTRACT

PURPOSE: To investigate the effect of sildenafil citrate (SC) on skeletal muscle ischemia-reperfusion (IR) injury in rats.

METHODS: Adult male Wistar rats were randomized into three groups: vehicle-treated control (CTG), sildenafil citrate-treated (SCG), and sham group (SG). CTG and SCG had femoral artery occluded for 6 hours. Saline or 1 mg/kg of SC was given 5.5 hours after occlusion. SG had a similar procedure without artery occlusion. Soleus muscle samples were acquired 4 or 24h after the reperfusion. Immunohistochemistry caspase-3 analysis was used to estimate apoptosis using the apoptotic ratio (computed as positive/negative cells). Wilcoxon rank-sum or Kruskal-Wallis tests were used to assess differences among groups.

RESULTS: Eighteen animals were included in the 4h reperfusion groups and 21 animals in the 24h reperfusion groups. The mean apoptotic ratio was 0.18±0.1 for the total cohort; 0.14±0.06 for the 4h reperfusion groups and 0.19±0.08 for the 24h groups (p<0.05). The SCG had lower caspase-3 ratio compared to the control groups at the 24h reperfusion time point (p<0.05).

CONCLUSION: Sildenafil citrate administration after the onset of the ischemic injury reduces IR-induced cellular damage in skeletal muscle in this rat hindlimb ischemia model.

Key words: Reperfusion Injury. Muscle, Skeletal. Phosphodiesterase 5 Inhibitors. Caspase 3. Rats.

Introduction

Ischemia-reperfusion injury (IRI) in skeletal muscle has complex and multifactorial mechanisms. Reestablishment of blood flow is essential to salvage ischemic tissues, but may also lead to cellular damage beyond that caused by the initial ischemia. Numerous reactive oxygen species (ROS) are brought along when oxygenated blood flow re-enters ischemic tissues during reperfusion, leading to additional tissue injury. Moreover, inflammatory mediators are also generated, aggravating the local cell death^{1,2}.

As mechanisms of cell death, both necrosis and apoptosis can be induced by IRI. Necrosis used to be considered the solo mechanism in ischemic conditions, but current evidence show apoptosis as the major contributor to cell death due to IRI^{3,4}. Quantifying and mapping apoptosis indicates the extension of IRI³. For the quantification of apoptosis, the assessment of caspase-3 expression by immunohistochemistry staining is a validated and standardized method⁵.

Hindlimb ischemia due to arterial occlusion is a common condition in clinical practice⁶. Skeletal muscles are the predominant tissue damaged in this situation, with high vulnerability to cell death due to ischemia. Typically, severe injury in skeletal musculature starts after a 2-hours ischemic interval^{1,7}. However, irreversible muscle cell damage seems to start after three hours of ischemia and to be nearly complete after a 6- hours interval⁸.

Sildenafil citrate (SC) is a phosphodiesterase-5 inhibitor able to increase nitric oxide release, improving endothelial function⁹. Promising results have been shown for the use of SC in animal models of myocardial IRI, as well as inducing protective effects in the kidney^{10,11}. However, the role of SC as a protective agent during IRI in skeletal muscles is still unknown.

The aim of this study is to determine whether pharmacologic postconditioning with SC is effective to reduce IRI in skeletal muscle, using an experimental model of rodent limb ischemia.

Methods

We included 42 male Wistar albino rats in the study, but three were excluded after the surgical procedure due to research protocol violation. The animals were kept under controlled environmental conditions of temperature, noise, and a cycle of twelve hours for light and dark, with free access to water and adequate chow for the species. The investigational protocol was approved by the Institutional Animal Care Committee, and

the research was performed in accordance with the guidelines of the Brazilian legislation for scientific use of animals (Law n° 11.794/08).

Groups and surgical procedure

The animals were randomized to one of three groups: 1) vehicle-treated sham group (SG); 2) vehicle-treated control group (CTG); and 3) sildenafil citrate-treated group (SCG). The rats were anesthetised with ketamine hydrochloride (75mg/kg) and xylazine (10mg/kg) intraperitoneally and kept spontaneously breathing in room air. Tramadol hydrochloride (2mg/kg) IM was given immediately before the surgical procedure. For the SCG and CTG, the femoral artery was dissected and then clamped with a vascular microclamp just below the bifurcation of the deep femoral artery (Figure 1). Ischemia was confirmed by the presence of clinical signs of ischemia (cutaneous cyanosis and pallor, reduced temperature compared to the contralateral limb) as well as by the disappearance of arterial pulsatility distal to vascular clamp. In SG, a similar surgical procedure was performed without femoral artery occlusion. The animals were placed in individual cages under heat of red-hot lamps up to the recuperation of the anesthesia and with free access to food and water. The SCG then received 1mg/kg of SC in saline by gavage 5.5h after the onset of ischemia, while the CTG received only saline by gavage at 5.5h. In SG, the animals received saline by gavage 5.5h after the surgical procedure. Six hours after clamping, the blood flow was re-established in the femoral artery by removing the clamp. The reperfusion was confirmed by the disappearance of clinical parameters of ischemia. The animals were placed in individual cages under heat during the reperfusion period. After 4h or 24h (according to group protocol), the soleus muscle was excised and euthanasia was then performed.

Tissue processing and analysis

The soleus muscle was fixed in 10% buffered formalin for 24 hours, dehydrated, and then embedded in paraffin. Sections (6 mm) of the central part of all samples were stained for caspase-3 expression. The immunohistochemical processing was performed using the primary antibody-cleaved anticaspase-3, anti-mouse (Dako™ A/S, Denmark 492), revealing DAB (3, 3-diaminobenzidine) Sigma Co-USAD5632 and obtaining the expression of the caspase-3 staining brown. The quantification of caspase-3 expression was performed in a blinded fashion. Ten images of each animal slide were captured, using an

optical microscope at 10x 40x (400x) magnification (Nikon™, Inc.) connected to a workstation. The apoptotic ratio (AR) was determined by quantifying the number of caspase-3 positive myocyte nuclei divided by the number of caspase-3 negative myocyte nuclei in each image (Figure 1). To assess reproducibility, a slide was randomly selected from each animal and independently analyzed by a second reader.

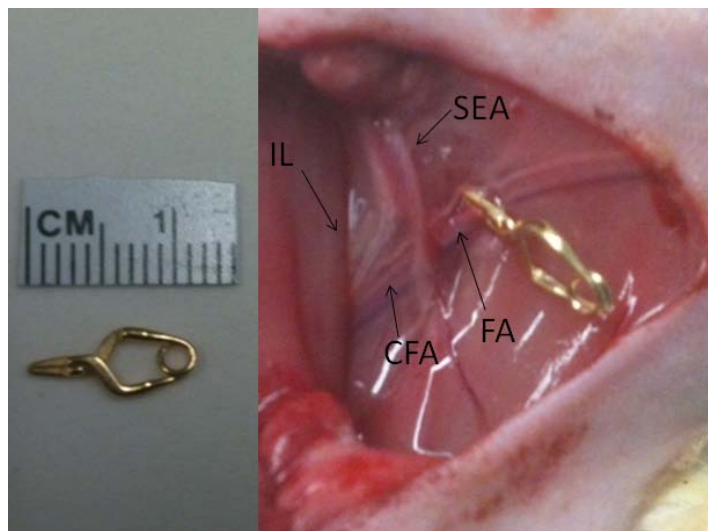


FIGURE 1 - Vascular microclamp and surgical femoral artery occlusion. **IL** – inguinal ligament; **CFA** – common femoral artery; **SEA** – superficial epigastric artery; **FA** – femoral artery.

Statistical analysis

Statistical analysis was performed using STATA version 11.2. Difference between groups was tested using the Wilcoxon rank-sum test when two groups were involved or using the Kruskal-Wallis test when more than two groups were examined. The protective effect of sildenafil citrate was assessed by comparing mean values of AR among groups (SG, SCG and CTG) in total and according to the reperfusion time. The reproducibility analysis was performed using intraclass correlation coefficient and Bland-Altman plots. The level of significance for rejecting the null hypothesis was 5% ($p < 0.05$).

Results

A total of 39 animals were included, with the mean weight of 350 ± 20 g. Of those, 18 animals underwent 4h of reperfusion (six animals in each of three groups: SG, CTG and SCG) and 21 animals underwent 24h of reperfusion (seven rats in each of three groups: SG, CTG and CSG).

Figures 2, 3 and 4 show photomicrographs of the

immunohistochemical expression characteristics of cleaved caspase-3 in animals from CTG, SCG and SG after 24h of reperfusion.

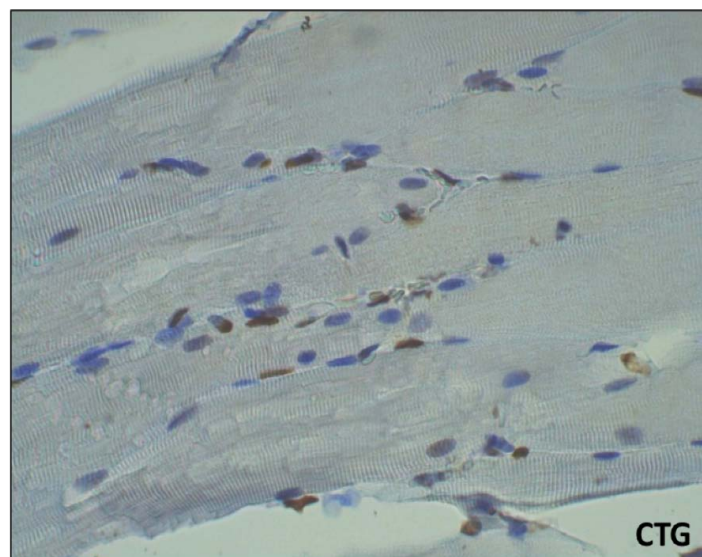


FIGURE 2 - Photomicrograph of a vehicle-treated control group (CTG) animal after 24 hours of reperfusion for immunohistochemical evaluation of caspase-3 expression in brown staining (arrows). Extensive apoptotic nuclei observed in 40x10 magnification.

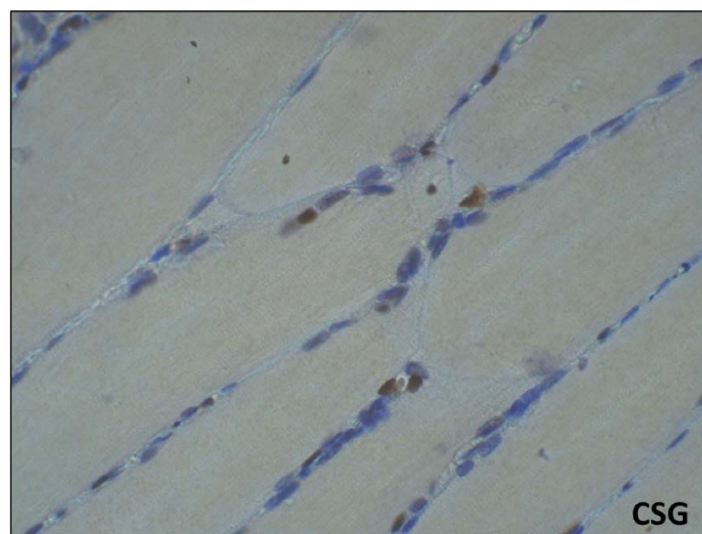


FIGURE 3 - Photomicrograph of a sildenafil citrate group (CSG) animal after 24 hours of reperfusion for immunohistochemical evaluation of caspase-3 expression in brown staining (arrows). Apoptotic nuclei present to a lesser extent (40x10 magnification).

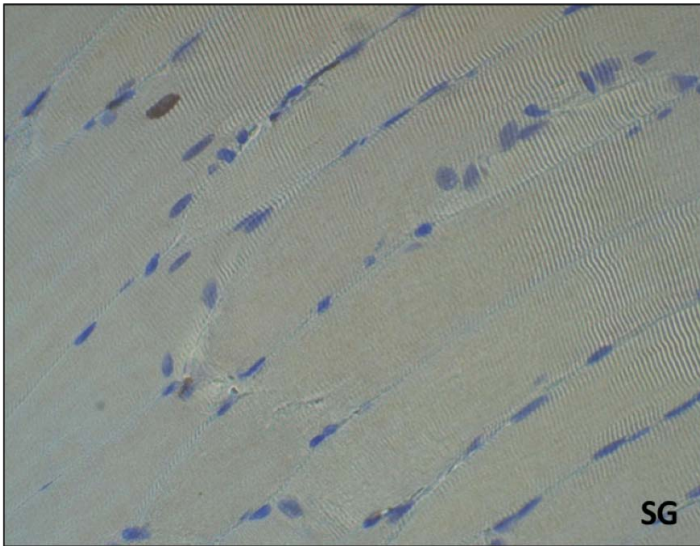


FIGURE 4 - Photomicrograph of an animal of vehicle-treated sham group (SG) after 24 hours of reperfusion for immunohistochemical evaluation of caspase-3 expression in brown staining (arrows). Presence of few stained cells in the upper left quadrant (40x10 magnification).

The apoptotic ratio (AR) was expressed by mean AR (\pm standard deviation). It was 0.18 ± 0.1 for the total sample, being 0.14 ± 0.06 for the animals that underwent 4h of reperfusion and 0.19 ± 0.08 for those after 24h of reperfusion ($p < 0.05$) (Figure 5).

In both 4h and 24h reperfusion groups, the highest AR values were found in the CTG, followed by SCG and SG. Differences among groups were non-significant in animals with 4h of reperfusion, but statistical significance was seen when comparing groups of animals that underwent 24h of reperfusion (Figure 6).

In SG, no statistical significance was found comparing AR values of animals that underwent 4h to those after 24h of reperfusion ($p = 0.4$). However, animals after 24h of reperfusion had statistically significant higher AR values compared to those that underwent 4h of reperfusion ($p = 0.04$) in CTG and SCG.

The inter-reader reproducibility for the AR measurement showed a very strong correlation between readers, with intraclass-correlation coefficient of 0.97 (95% confidence interval: 0.96, 0.99). The Bland-Altman plots showed a mean difference of 0.003 ± 0.06 and 95% limits of agreement from -0.12 to 0.12 (Figure 7).

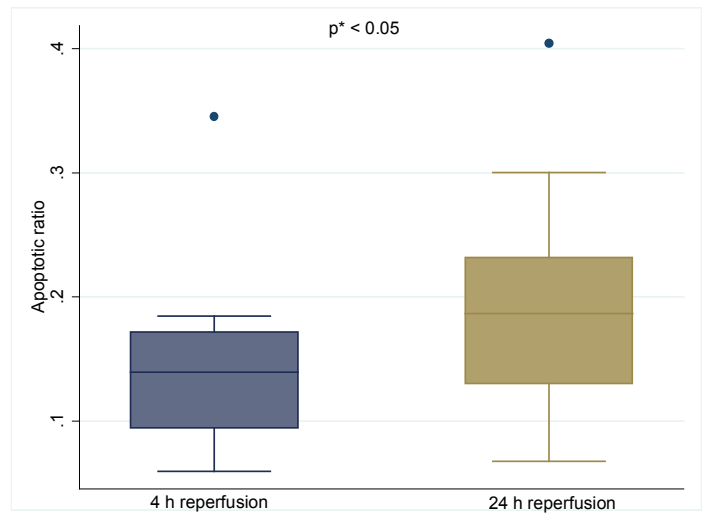


FIGURE 5 - Box-and-whisker plot showing the distribution of the apoptotic ratio in accordance with the time of reperfusion for all animals: 4h ($n = 18$) or 24h ($n = 21$). *p value refers to comparison between the averages of groups of 4h and 24h of reperfusion, using the Wilcoxon test.

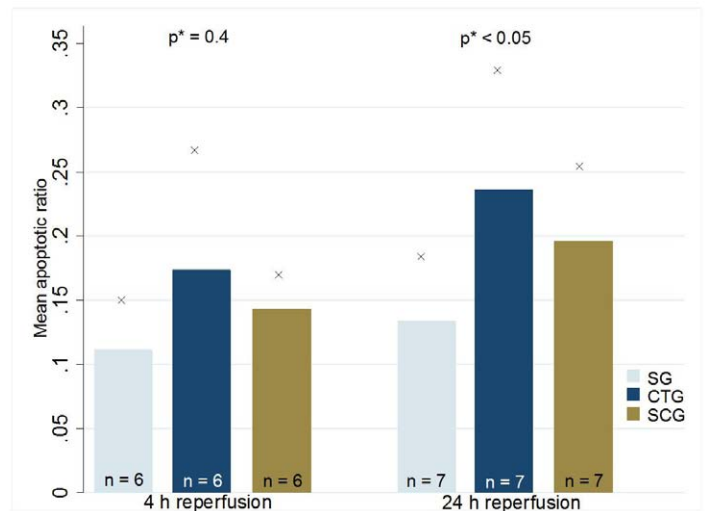


FIGURE 6 - Distribution for apoptotic ratio among groups, according to the reperfusion time. SG – sham group; CTG – vehicle treated control group; SCG – sildenafil citrate group. In the graph, X refers to the value of standard deviation. *p value.

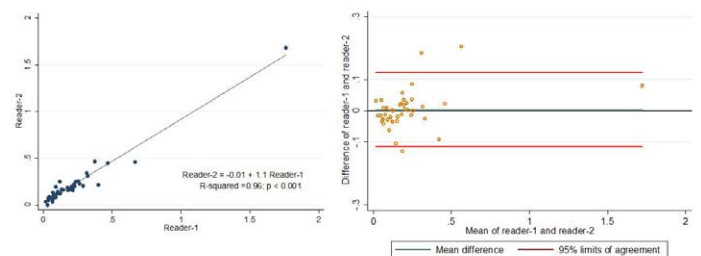


FIGURE 7 - Scatter plots and Bland-Altman plots for the inter-reader reproducibility of apoptotic ratio by caspase-3 expression ($n=39$).

Discussion

To the best of our knowledge, this is the first study assessing the influence of SC in skeletal muscle IRI lesions. SC showed a protective effect for hindlimb skeletal muscle damage due to IRI. These beneficial effects were more pronounced at the late reperfusion (24h) time point, compared to early reperfusion (4h). Additionally, the assessment of apoptotic ratio based on the caspase-3 expression by immunohistochemistry staining had robust precision.

Despite improvements in operative techniques, acute ischemia of the lower limb remains a challenging clinical problem with significant morbidity and mortality. Its incidence may be as high as 13-17 cases per 100.000 people per year and mortality rates may reach 18% in some series¹². Due to the high metabolic demand of skeletal muscle, irreversible damage may occur when perfusion occurs even after brief ischemic intervals^{7,13}. In this regard, the soleus muscle is particularly sensitive to ischemia due to its metabolism¹⁴. Surgical reperfusion is the established therapy early after the onset of ischemic conditions, but long-term ischemia increases additional tissue damage due to IRI.

Quantitative evaluation of apoptosis by caspase-3 expression using immunohistochemistry staining is well established⁵. We assessed the precision of the method and found a strong intra-class correlation, with mean difference between two independent readers close to null and narrow limits of agreement (Figure 4). This robust inter-reader reproducibility for the AR strengthens the reliability of our findings.

In our study, a six-hour ischemia ensured a significant injury to the soleus muscle, as showed by the lower AR found in SG when compared to the other groups (Figure 2). Evidence shows that ischemic morphological lesions are present minutes after reperfusion and increase progressively over time, due to an additional inflammatory response. In fact, cell death due to IRI is more evident after 4 hours of reperfusion, reaching the maximum peak at 24 hours^{14,15}. In early reperfusion stages, most of the injury is associated with the acute release of free radicals. In late reperfusion, cell death factors are mostly related to IRI inflammatory pathways, with increasing importance of a neutrophil response.

SC is a PDE5 inhibitor and its mechanism of action involves increased tissue levels of cGMP, resulting in smooth muscle relaxation and increased vasodilation¹⁶. SC is effective for the treatment of erectile dysfunction and pulmonary hypertension^{17,18}. Moreover, SC has protective effects over IRI in heart, kidney, liver, and brain^{19,20}. However, the literature is focused on the use

of SC before the onset of ischemia, acting in a pharmacological preconditioning. In the clinical setting, the onset of acute ischemia is usually unanticipated, which makes the delivery of a protective agent before the onset of ischemia impossible. We assessed the cytoprotective effect of SC administrated half an hour before the reperfusion, so that they would be present in the blood at the start of the reperfusion period. This nearly simulates the normal human condition when a patient is admitted to an emergency clinic and undergoes a revascularization procedure.

We assessed soleus muscle apoptosis in periods of early reperfusion (4h) and late reperfusion (24h). Animals that received SC had lower AR than those in the control group. Although the full mechanism involved was not explored, our results suggest that SC has a protective effect over IRI of skeletal muscle. In the early reperfusion phase, the mean difference on AR among groups did not show statistical significance. After 24h of reperfusion, AR values were significantly lower for SCG compared to CTG (Figure 3). These findings indicate that SC protects against skeletal muscle damage, especially in injuries related to the late phase of reperfusion. On the other hand, the results found in a late reperfusion phase may be consequence of the SC action in reducing ROS in the early phase.

Some points to be explored in future studies is the use of parenteral SC, an assessment of its effects when applied before ischemia as a preconditioning pharmacological, the effects of SC in remote organ damage after ischemia of skeletal muscle, as well as investigating the molecular mechanisms by which the SC has the cytoprotective effect in IRI in skeletal muscle. Subsequent studies could confirm these findings and translate the knowledge on the use of SC in IRI in the clinical area, making possible advances that bring therapeutic benefit in future medical practice.

Conclusion

In the model of six hours of ischemia in the rat hind limb, sildenafil citrate significantly reduced the apoptotic ratio in rat soleus muscle after 24 hours of reperfusion.

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