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Abstract: Acute myocardial infarction (AMI), which is characterized by severe and fatal arrhythmias resultant from cardiac ischemia/reperfusion (CIR), is the leading cause of morbidity and mortality in the world. Considering that epiisopiloturin (EPI), an imidazole alkaloid found in the leaves of *Pilocarpus microphyllus* produded anti-inflammatory and antioxidant effects in an animal model of peritonitis, paw edema and peritonitis in mice, we decided to investigate the potential cardioprotective activity of EPI in animal model of CIR. Then, adult rats submitted to CIR pretreated with EPI 10 mg/kg (EPI10+CIR group) or 15 mg/kg (EPI15+CIR group) were compared to control animals submitted to CIR treated with saline solution 0.9% (CIR group). To evaluate the incidence of ventricular arrhythmias (VA), atrioventricular block (AVB) and lethality (LET) induced by CIR in these rats, the ECG analysis was performed. Serum levels of cardiac damage markers, total creatine kinase (CK) and CK-MB, were measured. The results showed that the AVB incidence was reduced from 80% to 30%, and LET incidence was reduced from 70% to 20%, compared to CIR group. Serum levels of LDH, total CK and, CK-MB was similar in all groups studied. These results indicate that the treatment with EPI (15 mg/kg, IV) before CIR was effective to produce cardioprotective effects. Thus, the use of EPI could be a promisse strategy to reduce the incidence of severe and fatal arrhythmias associated with AMI.

Keywords: Cardiac Diseases; Cardiac Ischemia-Reperfusion; Myocardial Infarction; Pharmacological Cardioprotection; Epiisopiloloturine

1. Introduction

Cardiovascular diseases (CVD) will account for more than 26 million annual deaths worldwide by 2023, making them the leading cause of death in both industrialized and underdeveloped countries. Among these illnesses, coronary artery disease (CAD) and acute myocardial infarction (AMI) in particular are the leading causes of morbidity and mortality globally [1,2,3]. One of the potentially fatal coronary-related illnesses closely linked to sudden cardiac death, which affects around three million individuals worldwide each year [4,5], is AMI. The main cause of irreversible myocardial damage in AMI is oxygen deprivation in cardiac cells, which can impair diastolic and systolic function and put the patient at risk for severe and deadly cardiac arrhythmias [4,5,6]. Even though AMI can cause several major issues for cardiac function, there are presently limited pharmacological options available for its therapy.

The fastest possible coronary blood flow restoration following ischemia (reperfusion) is essential for treating AMI [4,5,6,7]. The prognosis improves with earlier treatment (less than 6 hours from the onset of symptoms). Reperfusion of the myocardium submitted to ischemia is the primary form of AMI treatment, but this process can result in severe cardiac dysfunctions, primarily because of abrupt oxygen entry and severe ionic deregulation in cardiac cells caused by cardiac ischemia and reperfusion (CIR) process. These dysfunctions can then cause lethal cardiac arrhythmias that are directly related to deregulation of intracellular Ca^{2+} homeostasis in cardiac cells [6,7,8,9,10,11,12].

In order to evaluate potential cardioprotective substances, we decided to study one of the most significant genera in Brazilian flora is *Pilocarpus*, sometimes called "jaborandi," which has native species in the northeast and north of the nation. Because it contains significant amounts of the imidazole alkaloid pilocarpine, which is used to treat glaucoma, stimulate the lachrymal and sweat glands, and manage xerostomia, *Pilocarpus microphyllus Stapf* (Rutaceae) is the most well-known representative of the jaborandi group economically *Pilocarpus microphyllus* has been used to identify pilocarpine as well as additional imidazole alkaloids including isopilosine, epiisopilosine, and epiisopiloturine (EPI) [13,14,15,16,17]. Important published studies have demonstrated that EPI has several pharmacological actions, among which we highlight the antiparasitic, anti-inflammatory and antioxidant effects [18,19,10,21,22,23,24]

In fact, nothing is known about the pharmacological activities of the epiisopiloturine on the cardiovascular system, which is why we decided to evaluate the potential cardioprotective and antiarrhythmic activity of EPI, in the present study we used a classical animal model of CIR to evaluate the effects of treatment with EPI on the incidence of arrhythmias (VA and AVB) and lethality (LET), as well as serum concentrations of cardiac injury biomarkers lactate dehydrogenase (LDH), creatine kinase (CK), and CK-MB in rats submitted to CIR.

2. Results

2.1. Incidence of VA, AVB and LET induced by CIR

Before CIR protocol (stabilization periods for 15 min), no cardiac arrhythmias were detected in all animals studied. But, during CIR protocol, VA and AVB incidence in different experimental groups was detected and measured. Figure 1 shows that the VA, AVB and LET incidence in CIR group was 90%, 70%, and 70%, respectively.
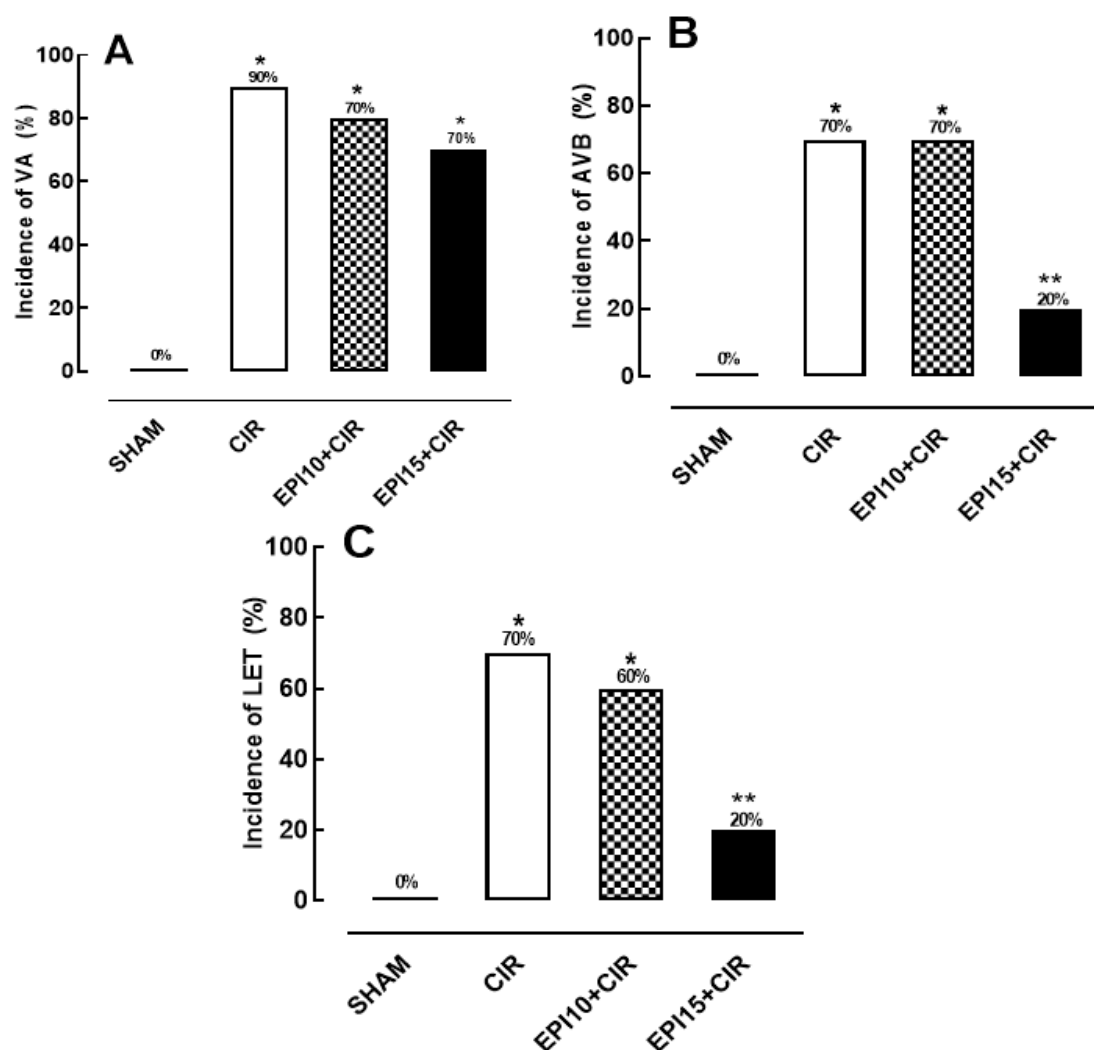
Figure 1. Histograms representing the (A) Incidence of ventricular arrhythmias (AV), (B) Atrioventricular block (AVB) and (C) Lethality (LET) in the SHAM, CIR, EPI10+CIR (EPI 10 mg/kg), and EPI10+CIR (EPI 15mg/kg) groups. Results were expressed as mean and statistical analysis was performed using Fisher's exact test (*p < 0.05). The incidence of VA, AVB and LET were significantly reduced in all groups of animals treated with EPI15mg/kg submitted to CIR when compared to the CIR. *p < 0.05 statistically different when compared to the SHAM group. **p < 0.05 statistically different when compared to CIR group.

2.2. Effects of the EPI on the incidence of VA, AVB and LET induced by CIR

Figure 1 shows that the VA, AVB and LET incidence in EPI10+CIR group was not different of CIR group, indicating that the pretreatment with EPI 10 mg/kg (IV) did not reduce the VA, AVB and LET incidence induced by CIR. However, these incidences were significantly reduced in EPI15+CIR group compared to CIR group, indicating that the pretreatment with EPI 15 mg/kg (IV) was effective to reduce cardiac arrhythmia (VA and AVB) and LET. In EPI15+CIR group, the AVB incidence was reduced from 80% to 30%, and LET incidence was reduced from 70% to 20%, compared to CIR group (See figure 1).

2.3. Effects of the EPI on the biomarker's levels of cardiac injury total creatine kinase and CK-M in animals submitted to CIR

The increase in serum levels of lactate dehydrogenase (LDH) does not exclusively refer to the occurrence of AMI, but has been used as one of the biochemical markers whose increase in serum levels may be related to AMI, in 92-95% of patients, for Therefore, we decided to measure serum LDH concentrations. In NWR animals, in the SHAM group the plasma LDH concentration was (1600 ± 97 U/L), in the CIR+SS group the plasma LDH concentration was (1930 ± 176 U/L) and in the EPI group 15 mg/kg plasma LDH concentration was (1435 ± 146 U/L) (Figure 2).
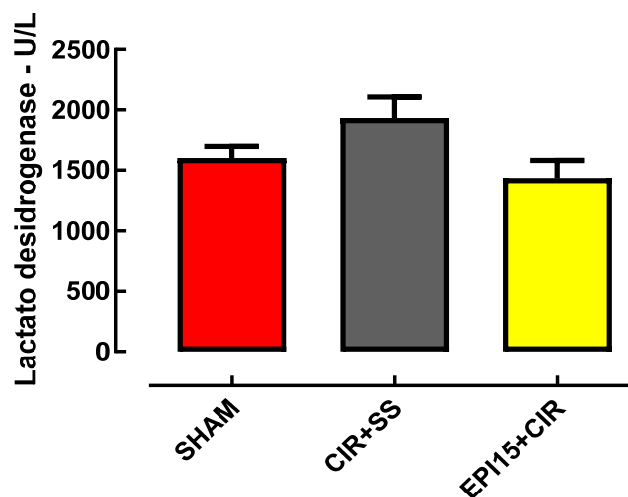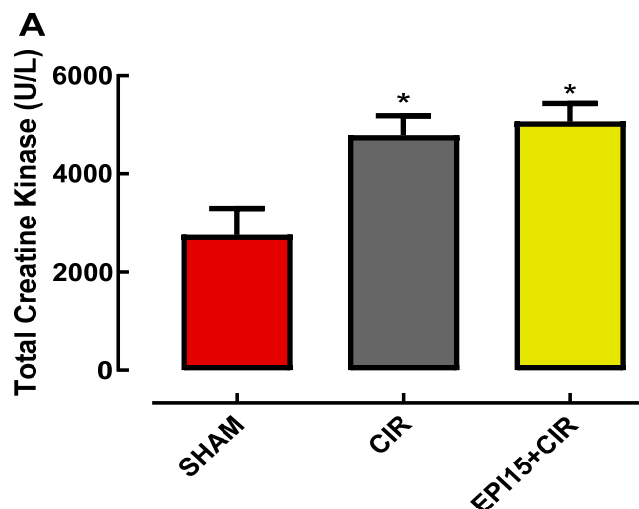

Figure 2. Serum concentration of Lactate dehydrogenase (LDH) in the SHAM, CIR and EPI 15mg/kg groups. The results were expressed as mean ± standard error of the mean and analysis of variance (ANOVA) was applied, followed by Tukey's post-test (*p<0.05). SHAM= sham-operated group; CIR= group treated with saline and subjected to cardiac ischemia and reperfusion; EPI 15mg/kg = group treated with epiisopiloturine and then subjected to cardiac ischemia and reperfusion.

The increase in plasma concentrations of the enzyme total creatine kinase (TCK) has been used as an indicator of cardiac injury, observed in patients with AMI, which is why we decided to evaluate serum levels in NWR animals subjected to our experimental model. In NWR animals, the plasma concentration of CKT in the SHAM group (2,757 ± 532 U/L), in the CIR group (4,815 ± 431 U/L) and in the EPI 15 mg/kg (EPI15+CIR) group (5,063 ± 375 U/L) (Figure 2A). In Figure 2B, we can observe that the serum concentrations of CK-MB in the CIR (2,688 ± 210 U/L, n = 4) and EPI15+CIR (2,511 ± 193 mg/dL, n = 4) groups did not differ statistically from each other, however, the serum CK-MB concentrations of these two groups were statistically higher when compared to the SHAM group (914 ± 118 U/L, n = 4).
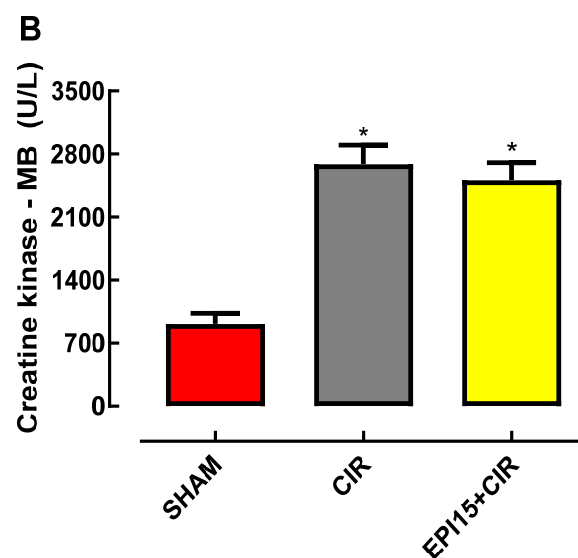
B

Figure 3. Histograms representing the serum concentrations of Total Creatine Kinase (A) and Creatine Kinase -MB (B) in the SHAM, CIR, and EPI15+CIR groups. The results were expressed as mean ± standard error of the mean and analysis of variance (ANOVA) was applied, followed by Tukey's post-test (*p<0.05). SHAM= sham-operated group; CIR= group treated with saline and subjected to cardiac ischemia and reperfusion; EPI 15mg/kg = group treated with epiisopiloturine and then subjected to cardiac ischemia and reperfusion. *p < 0.05 statistically different when compared to the SHAM group.

3. Discussion

In the present work, we showed that pretreatment of animals submitted to CIR with EPI 15 mg/kg (IV) was able to reduce the AVB incidence due to important antiarrhythmic effect, and then reducing the LET incidence (see figure 1). It is worth highlighting that this is the first experimental study that showed consistent evidence on the cardioprotective effects of EPI, an imidazole alkaloid isolated from *Pilocarpus microphyllus.*

Regarding serum LDH levels measured in the different groups studied, our results demonstrated that there was no statistical difference between the groups studied, that is, SHAM, CIR and EPI15+CIR (Figure 2). Therefore, our results demonstrate that the CIR protocol used in this study caused a statistically significant increase when compared to the plasma concentration of CKT in the SHAM group. The same occurred with EPI+CIR when compared to the SHAM group. Thus, we can observe that the treatment of NWR rats with EPI 15 mg/kg was not able to reverse the increase in serum CKT value observed in the CIR group (Figure 1).

Some studies have suggested that EPI is able to reduce the extracellular Ca^{2+} influx in neutrophils in a sustained manner, and then significantly reducing the cellular collapse generated by the increment of intracellular Ca^{2+} concentration ($[Ca^{+2}]i$. In fact, compounds that have the imidazole ring in their chemical structure, such as antifungal drugs belonging to the azoles class itraconazole, miconazole and ketoconazole, can inhibits the Ca^{2+} influx through blocking plasma membrane Ca^{2+} channels, as well as inhibit the displacement of intracellular Ca^{2+} into the cytosol of mammalian cells [18,19,10,21,22,23,24]. In the cardiovascular system, these cellular actions of EPI could generate cardioprotective effects due to pharmacological modulation of Ca^{2+}/cAMP/ADO signaling in cardiac cells. Our studies [6,7,10] and other relevant studies have demonstrated that the attenuation of cytosolic Ca^{2+} overload produced by blocking of Ca^{2+} influx mediated by L-type Ca^{2+} channel (LTCC) and/or stimulation of cardiac A_1-adenosine receptors (A_1R) produced by endogenous adenosine (ADO) and others agonists of A_1R constitute a common and effective pharmacological strategy used to abolish the cardiac arrhythmias in various clinical situations, and especially in cardiac surgery [8,9,10,25,26,27,28,29]. These EPI actions could prevent contractile dysfunctions of cardiac cells due

to attenuation of cytosolic Ca^{2+} overload and consequent reduction of arrhythmias incidence due to reduction of the probability of excitation-contraction coupling (CECC) mismatch.

In addition to its role in CECC, LTCC-mediated Ca^{2+} influx in cardiac cells modulates cAMP production by AC isoforms 5 (AC5) and 6 (AC6) [8,9,10], and pharmacological block of Ca^{2+} via LTCC increases production and efflux of intracellular cAMP [8,9,10,30,31]. Biochemical analyses of membrane preparations in overexpression systems have been used to establish the paradigm for Ca^{2+}-mediated inhibition of AC5 and AC6 in the sub micromolar range [8,9,10,29,30,31]. In the extracellular medium, cAMP is transformed into ADO that can stimulate A_1R located in the plasma membrane of cardiac cells to finely regulate cardiac function [8,9,10]. A_1R stimulation with ADO is a common and effective strategy used to abolish the cardiac arrhythmias in various clinical situations, and especially in cardiac surgery [8,9,10,29,30,31,32,33,34,35,36,37,38,39,40]. We have proposed that this pharmacological modulation of Ca^{2+}/cAMP/ADO signaling in cardiac cells by means the attenuation of Ca^{2+} influx via LTCC combined with an increase of activation of A_1R by ADO generated by increment of extracellular transport of cAMP may be effective to prevent sudden mortality in individuals with AMI due to severe arrhythmias brought on by cardiac collapse [41,42,43,44].

Furthermore, recent published studies also suggest that EPI is capable of blocking the phosphorylation of the p65 subunit of NF-KB and, therefore, inhibits its translocation to the cell nucleus, where this dimeric protein composed of two subunits (p50 and p65) acts by promoting modulation of gene expression and protein synthesis and, consequently, its action on gene regulation [21]. Without doubt, this inhibitory effect on NF-KB is of great importance considering that it is a fundamental step regarding the role that NF-KB plays in the inflammatory process, as it is from this process that this molecule regulates the genes responsible for the expression of pro-inflammatory cytokines (TNF-a and IL-6), adhesion molecules, chemokines, growth factors, cyclooxygenase-2, inducible nitric oxide synthase [21,22,23,24,25,26]. In addition, the pretreatment with EPI significantly reduced the levels of nitrite produced by cultured microglial cells treated with LPS, which demonstrates the anti-inflammatory effect of EPI, which, at least in part, it is related to the ability of EPI to promote inhibition of intracellular pathways responsible for the pathophysiology of inflammation, such as NF-κB and MAPKs. Furthermore, EPI was also able to inhibit the synthesis of the enzyme inducible nitric oxide synthase (iNOS), significantly reducing the production of inflammatory cytokines such as IL-1 β, IL-6 and TNF-α when compared to the group of microglial cells treated with LPS [21,22,23,24,25,26].

Okada et al. [45] demonstrated through an animal model of intestinal ischemia and reperfusion (IRI) that there is a direct relationship between increases in malondialdehyde (MDA), reactive oxygen species (ROS) and TNF-alpha and cardiac injuries, as the study demonstrated that the MDA content and TNF-alpha quantified from hearts obtained from animals belonging to the Sham group (sham-operated) was statistically lower when compared with hearts from animals in the control group, that is, animals treated with 0.9% saline solution and subjected to IRI. These results corroborate that the antioxidant and anti-inflammatory effects of EPI may be responsible for the decrease in AVB and LET in animals treated with EPI and subsequently subjected to the CIR protocol.

Pre-treatment with EPI was able to significantly reduce the production of IL-1β when compared to the values obtained from the BV-2 cell line not stimulated with LPS. EPI in the production of IL-10, an anti-inflammatory cytokine. Cells exposed to LPS reduced the production of IL-10 by approximately five times when compared to unstimulated BV-2 cells. Without a doubt, such effects probably participated in the antiarrhythmic and cardioprotective effects demonstrated in this study, as all these actions are important and relevant from the point of view of the pathophysiology of CIR.

Thus, we have proposed that pharmacological modulation of Ca^{2+}/cAMP/ADO signaling in cardiac cells could be a promising therapeutic strategy to reduce the incidence of severe and fatal arrhythmias caused by AMI in humans. The present work supports the idea that EPI, an imidazole alkaloid isolated from *Pilocarpus microphyllus,* could produce cardioprotective effects due its action on the Ca^{2+}/cAMP/ADO signaling in cardiac cells, , just as EPI can also promote cardioprotective effects through both decreasing the production of the pro-inflammatory cytokine IL-1β and

increasing the production of the anti-inflammatory cytokine IL-10 and thus being a promising cardioprotective agent in the treatment of AMI.

4. Materials and Methods

4.1. Animals

Male Wistar rats (12- to 16-week-old) weighting between 280 and 320 g, were kept at $21 \pm 2°C$ with 12:12-h light/dark cycle and were given food and water *ad libitum*. All experimental protocols used in this study were approved by the Ethics Committee of the Escola Paulista de Medicina – Universidade Federal de São Paulo (UNIFESP #9447210317 and #7323080822).

4.2. Cardiac ischemia and reperfusion (CIR) induction

In order to reproduce in laboratory an animal model of AMI, rats were submitted to surgical procedures in accordance with the methodology previously reported by our research group [7,8,9,10,11]. The rats were first given intraperitoneal xylazine (10 mg/kg), ketamine (100 mg/kg), and tramadol (2mg/kg) to induce unconsciousness. Rats were anesthetized, and a Jelco 14G (USA) was used for intubation. An Insight model EFF 312 mechanical ventilator (Insight Equipamentos Cientificos, Brazil) was used for mechanical ventilation [7,8,9,10,11]. After the patient was stabilized for fifteen minutes, a thoracotomy was performed to insert a vascular tourniquet (4/0 braided silk suture coupled to a 10-mm micropoint reverse cutting needle, Ethicon K-890H, USA) around the left anterior descending coronary artery to induce ischemia. After 10 minutes of myocardial ischemia, the tourniquet was removed to enable for 75 minutes of coronary.

4.3. Assessment of cardiac activity during CIR

Using a methodology previously described by our research group [7–12], the cardiac activity during CIR was evaluated by electrocardiogram (ECG) analysis. This high-resolution methodology was used in other studies to evaluate the cardioprotective effect of calcium channel blockers and other drugs on the incidence of cardiac arrhythmias (VA and AVB) and lethality (LET) resultant from CIR [7,8,9,10,11]. Initially, the ECG was recorded for 15 minutes prior to the stabilization phase and for 10 minutes during the ischemia and reperfusion protocols, which lasted for 75 minutes each [7,8,9,10,11]. The ECG was recorded using needle electrodes that were subcutaneously placed into the limbs and a biopotential amplifier. The use of ECG alterations (increase in R wave and ST segment) resulting from CIR was employed to verify the effective coronary artery.

The ECG was recorded using a biopotential amplifier and needle electrodes that were subcutaneously implanted into the limbs. The effective blockage of the coronary artery by surgery was verified by the ECG abnormalities (increase in R wave and ST segment) caused by CIR [46,47,48,49]. The body temperature was maintained at 37.5°C using a heated operating table and the appropriate heating lamps, and the temperature was frequently checked with a rectal thermometer [7,8,9,10,11]. ECG data were processed using a computer system composed by AqDados 7.02 hardware (Lynx Tecnologia Ltda, Brazil), and AqDAnalysis 7 software [7,8,9,10,11]. Using this system, we measured heart rates as well as the incidence of VA, AVB, and LET induced by CIR. Ventricular fibrillation, torsades, and atrial fibrillation were all considered VA.

4.4. Biochemical assessment of heart lesions' biomarkers

To measure the serum levels of markers of cardiac injury biomarkers lactate dehydrogenase (LDH), creatine kinase (CK), and CK-MB, it was determined using the methodology described in our previous studies [12]. Rats that made it through the entire 75-minute CIR treatment provided the blood samples. These samples (4–5 mL) collected from the abdominal aorta and keep in siliconized tubes, were centrifuged at 2,500 rpm for 40 minutes at 5 °C. The supernatant was removed and stored at -20°C for enzymatic analysis of CK and CK-MB at 340 nm using a kinetic UV test kit from Vida Biotecnologia, Belo Horizonte, Brazil.

4.5. Pharmacological Treatments

EPI used to carry out this study was obtained from the residue obtained from the extraction of pilocarpine from the leaves of *Pilocarpus microphyllus* [17]. The organic phase was subjected to liquid-liquid extraction, alkalinized with ammonium hydroxide solution to precipitate EPI in neutral form and then the solution was filtered under reduced pressure. Then, to remove all impurities, EPI was subjected to the purification process by high-performance liquid chromatography (HPLC), proving to be pure by HPLC (>95%) and showed data consistent with literature values [17]. We used EPI 10 mg/kg and 15 mg/kg, intravenously, on the incidence of VA, AVB and LET caused by CIR, rats were treated with the EPI intravenously (IV) before of CIR. In the present work, the animals used were divided into the following experimental groups:

1) **SHAM-operated group** (n = 8) - Rats submitted to all CIR procedures, except for both left descending coronary artery tourniquet and myocardial reperfusion and submitted to ECG analysis for determination of the VA, AVB and LET incidence.

2) **CIR group** (n =30) – Rats submitted to CIR pretreated with saline solution (SS) 0.9% (IV), and submitted to ECG analysis for determination of the VA, AVB and LET incidence;

3) **EPI10+CIR group** (n = 10) - Rats submitted to CIR pretreated with EPI (10 mg/kg, IV), and submitted to ECG analysis for determination of the VA, AVB and LET incidence;

4) **EPI15+CIR group** (n = 10) – Rats submitted to CIR pretreated with EPI (15 mg/kg, IV), and submitted to ECG analysis for determination of the VA, AVB and LET incidence.

4.6. Statistical analysis

Using the Prism 8.0 program (GraphPad, USA), data related to VA, AVB, and LET incidences were expressed as percentages and statistically analyzed using Fisher's exact Test [11]. The values of LDH, total CK and CK-MB were measured in terms of serum concentration (U/L) and expressed as mean ± standard error of mean (SEM). The data were submitted to an Analysis of Variance Test using Prism for statistical analysis. The results were considered statistically significant when $p < 0.05$ [11].

5. Conclusions

The results obtained in the present study suggest that treatment with EPI decreased both AVB and LET, in a dose-dependent manner, in rats subjected to the CIR protocol and, therefore, appears to be a potential and promising pharmacotherapeutic strategy to reduce the incidence of serious and fatal arrhythmias caused by ischemic heart diseases, such as AMI in humans.

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