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A Simple and Efficient Methodology for the Study of Cardioprotective Drugs in Animal Model of Cardiac Ischemia-Reperfusion

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Abstract

To study cardioprotective drugs, we developed a simple and efficient methodology to evaluate effects of drugs on cardiac electrical activity using electrocardiogram (ECG) in rats submitted to cardiac ischemia-reperfusion (I/R). Using adult male Wistar rats (14 - 16-week-old) anesthetized (urethane 1.25 g/kg, i.p.) and kept under mechanical ventilation, we used surgical procedures to induce cardiac I/R by means mechanical occlusion of left anterior descendent coronary artery for 10 min (ischemia) with silk suture (tourniquet) followed by its removal to allow coronary recirculation (reperfusion). To evaluate the effects of surgical process, a group of rats (SHAM-operated) was submitted to surgical procedures previously described, but without coronary occlusion. To evaluate cardiac electrical activity in rats submitted to cardiac I/R and SHAM-operated, the ECG system was coupled to animal body to determine the incidence of ventricular arrhythmia (VA), atrio-ventricular blockade (AVB) and lethality (LET). To evaluate injury biomarkers production in rats submitted to cardiac I/R and SHAM-operated, serum concentrations of creatine kinase fraction (MB/CK-MB) and troponin I were determined by biochemical techniques. Using the methodology proposed in this work, we observed that VA, AVB and LET incidence was significantly higher in cardiac I/R group (85%, 79% and 70%, respectively) than in SHAM-operated group (0%, 0% and 0%, respectively). Serum levels of CK-MB and troponin I were also significantly higher in cardiac I/R (1,850 ± 222 U/L and 0.031 ± 0.009 ng/mL, respectively) compared to SHAM-operated group (808 ± 72 U/L and 0.200 ± 0.027 ng/mL). To evaluate efficiency of methodology proposed in this work to study the effect of cardioprotective drugs, the effects of the L-type Ca^{2+} channel blocker (CCB) nifedipine (30 mg/kg, intravenously - IV) in rats submitted to cardiac I/R were studied. The treatment with nifedipine (before ischemia) significantly reduced the incidence of VA (from 85% to 28%), AVB (from 79% to 14%), and LET (from 70% to 14%). These results indicate that the methodology described in the present work is simple, and efficient, to evaluate cardiac functional and biochemical alterations induced by cardiac I/R, and also the study of cardioprotective drugs in rats. This methodology could contribute to the development of new pharmacological cardioprotective strategies for to treatment of ischemic cardiac diseases in humans, such as myocardial infarction.

Keywords: Cardioprotective drugs; Cardiac ischemia/reperfusion; Myocardial injury

Introduction

Recent studies suggest that ischemic cardiac diseases can cause 26 million of deaths per year in worldwide after 2030 [1,2]. Among the ischemic cardiac diseases, acute myocardial infarction (AMI) represents the most common cause of morbidity and mortality worldwide [2,3]. The coronary reperfusion therapy constitutes the major therapeutic strategy to salvage the myocardium from tissue injury following prolonged ischemia in AMI patients [3-5]. However, the beneficial effects of this therapy are compromised by myocardial injuries caused during coronary reperfusion that subsequently lead to cardiac dysfunctions, including arrhythmias [5-7].

Experimental studies showed that cardiac arrhythmias caused by myocardial ischemia and reperfusion (I/R) process are resultant of bioenergetic and electrochemical disbalance caused mainly by reduction of ATP production by mitochondria, and cytosolic Ca^{2+} overload in cardiomyocytes [8,9]. This Ca^{2+} overload is strongly influenced by increase of Ca^{2+} influx through L-type voltage-activated Ca^{2+} channels (VACC) caused by persistent membrane depolarization of cardiomyocytes during cardiac I/R [8,9]. In addition, cytosolic Ca^{2+} overload promotes Ca^{2+} accumulation in the mitochondrial matrix mediated by increase of Ca^{2+} influx by mitochondrial uniporter, leading to mitochondrial bioenergetic collapse, and excessive production of free radical which compromises the structure and function of mitochondria, and other cytoplasmic organelles [8,9]. These cellular mechanisms importantly contribute to generation of arrhythmias, and death in AMI patients [8-10].

Despite continuous improvements in AMI treatment, a high percentage of patients die suddenly in the early hours before arriving at the hospital [10]. Most of these early deaths are due complex ventricular arrhythmias (VA) and atrio-ventricular blockade (AVB) [11,12]. Surprisingly, there is still lack of knowledge about the exact time of these early malignant arrhythmias and their cellular, and molecular, mechanisms. Due to involvement of intracellular Ca^{2+} overload in cardiac arrhythmias caused by myocardial I/R, the use of drugs to attenuate this Ca^{2+} overload represents an alternative pharmacological strategy to treatment of ischemic cardiac diseases in humans, including AMI.

Thus, we developed a simple and efficient methodology to evaluate effects of drugs on cardiac electrical activity using electrocardiogram (ECG) in rats submitted to cardiac I/R. By means ischemia produced by mechanical occlusion of left anterior descendent coronary artery with silk suture (tourniquet) followed by its removal to allow coronary reperfusion, this methodology mimics in laboratory cardiac alterations detected in AMI patients, including the increment of VA, AVB, and lethality (LET). In addition, this methodology also allows the correlation of these functional cardiac parameters with cardiac injury biomarkers produced in response to I/R by means of determination of serum concentration of CK-MB and troponin I.

Methodology

Animals

Adult male Wistar rats (14-16-week-old) weighting between 300 to 340 g were randomized into 4 groups: SHAM-operated (n=33), cardiac I/R (n=33), nifedipine 1 mg/kg + cardiac I/R (n=8) and nifedipine 30 mg/kg + cardiac I/R (n=7). Animals were maintained under standard conditions of nutrition, hydration, temperature, light and humidity, and in accordance to normalization approved by Ethical Committee of the EPM/UNIFESP (#1130/11 and #0065/12).

Protocol of cardiac ischemia and reperfusion (I/R)

In the present work, it was used a similar method to induce cardiac I/R previously described in other study published by our lab [13]. All rats were anesthetized with urethane (1.25 g/kg), and fixed in the supine position. After intubation (Jelco 14G, USA), rats were mechanically ventilated with room air with volume of 10 mL/kg (70 breaths/min) using a mechanic ventilator (Insight EFF 312, Brazil), and the chest was opened with a left thoracotomy. After thoracotomy, the heart was gently exteriorized using pressure on the abdomen and a surgical tourniquet (4/0 braided silk suture attached to a 10 mm micropoint reverse-cutting needle, Ethicon K-890H, USA) was placed around the left anterior descending coronary artery, approximately 2 mm from its origin. In rats of cardiac I/R, the two ends of nylon yarn were passed inside a cylindrical tube of polypropylene to perform a surgical tourniquet. After stabilization for 15 min, this tourniquet was tied for mechanical occlusion of coronary artery (ischemia). This procedure was used to induce ischemia showed in Figure 1, it was not performed in rats of SHAM-operated group. After 10 min of ischemia, the tourniquet was removed to allow coronary recirculation (reperfusion) (Figure 1).

The cardiac electrical activity in SHAM-operated, and cardiac I/R groups, was monitored by ECG system. ECG analysis was performed during ischemia (10 min) and reperfusion (75 min). The ECG was recorded using a biopotential amplifier by means of needle electrodes placed subcutaneously on the limbs. Successful surgical obstruction of the coronary artery was validated by ECG alterations (increase in R wave and ST segment) caused by ischemia. Typical ECG records in rats submitted to cardiac I/R was showed in Figure 2. The effects of two doses of L-type CCB nifedipine (1 mg/kg and 30 mg/kg, IV administrated before ischemia) were studied in rats submitted to cardiac I/R.

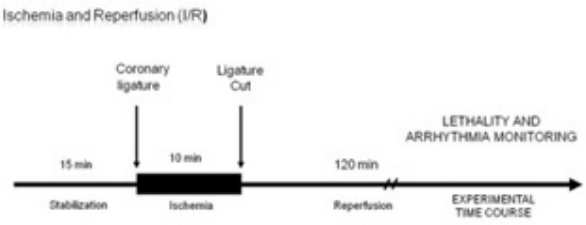

Figure 1: Illustration of summarized experimental protocol used in all groups.

The body temperature was maintained at 37.5°C with a heated operating platform and appropriate heating lamps, and was evaluated routinely via a rectal thermometer. All procedures used in this study were approved by Ethical Committee of the EPM/UNIFESP (#1130/11 and #0065/12).

ECG analysis

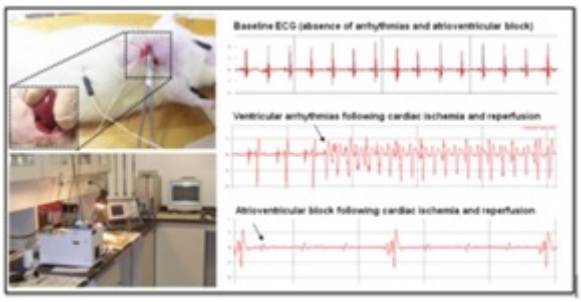

Figure 2: Typical ECG records showing ventricular arrythmias (VA) and atrioventricular block (AVB) in rats submitted to cardiac I/R.

The ECG was monitored and recorded from the beginning of the stabilization period using a commercial acquisition system (AqDados 7.02; Lynx Tecnologia Ltda., Brazil). The recorded raw data was evaluated by a specialized and trained cardiologist using the commercial software included in the acquisition system (AqDAnalysis 7, Lynx Tecnologia Ltda). Heart rates as well as incidence of VA, AVB, and LET in response to cardiac I/R were evaluated by means of ECG analysis. To simplify the presentation of the results, ventricular fibrillation, torsades de pointes, and ventricular tachycardia parameters were considered only as VA. ECG measurements were evaluated according to Lambeth conventions [14,15]. Typical ECG records showing VA and AVB in rats submitted to cardiac I/R were presented in Figure 2.

The duration of P wave was manually measured as the time from the beginning of the upstroke of the P wave until its return to the

isoelectric baseline. QRS duration was measured from the beginning of the Q wave to the peak amplitude of the downward deflection of the S wave. PR interval was measured from the beginning of the upstroke of the P wave until the maximal amplitude of the R wave. RR interval was measured as the time between consecutive R wave peaks. QT interval was measured from the beginning of the Q wave until the T wave returned to the isoelectric baseline. Typical ECG records obtained in rats submitted to cardiac I/R and SHAM-operated were showed in Figure 3.

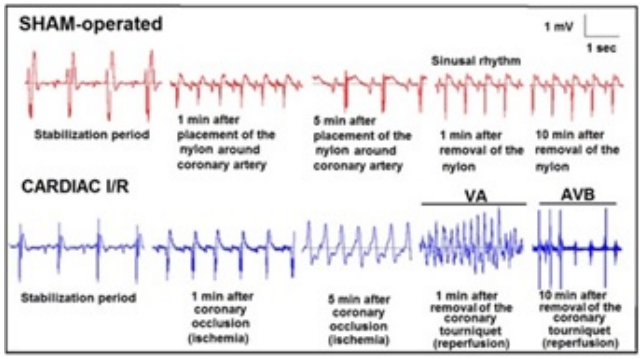

Figure 3: Comparisons of ECG analysis obtained in rats submitted to cardiac I/R and SHAM-operated.

Measurement of biomarkers of cardiac injury

After cardiac I/R protocol, the blood samples (3-4 mL) were collected with the use of a scalp from the abdominal aorta and placed in siliconized tubes. After 10 min at room temperature, blood sample was centrifugated (centrifuge Force® Heal - model Neofuge 15R, Hong Kong, China), 2,500 rpm at 5°C for 40 min. The supernatant was separated and placed storage at -20°C for subsequent enzymatic analysis. The enzymatic activities of creatine kinase fraction MB (CK-MB) and troponin I in serum were determined by kinetic UV test, measured at 340 nm using a Kit produced by LIFE Biotechnology (Belo Horizonte, Brazil).

Data analysis

The incidence of VA, AVB, and LET was compared by the Fisher's exact test. Statistical analysis of biomarkers of cardiac injury data were evaluated by the "t" Student test. Values were considered significant to $p < 0.05$. Statistical analysis was performed by Prism 5.0 software (GraphPad, USA), and data were expressed as means ± SE.

Results

Figure 3 shows that cardiac arrhythmias were absent during stabilization period in SHAM-operated and I/R groups, but they were observed during ischemia and reperfusion period in cardiac I/R group.

Figure 4 shows significant differences of duration of PR, QRS, QT and RR intervals in rats from I/R group evaluated by ECG. The PR and QT intervals were increased during the ischemia and reperfusion periods. In contrast, QRS interval was reduced in the same periods. The RR interval was unaltered.

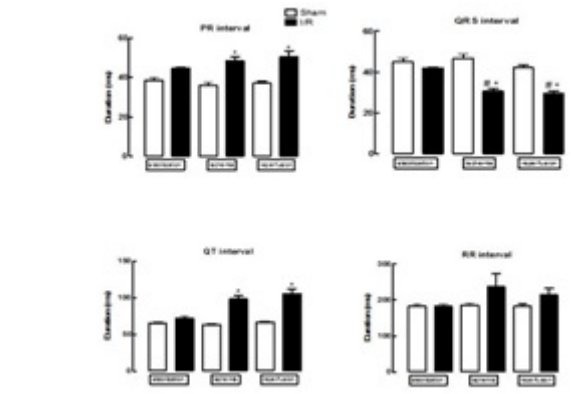

Figure 4: Duration of PR, QRS, QT and RR intervals obtained in rats of cardiac I/R and SHAM-operated groups. *P<0.05, compared to the sham group; #P<0.05, compared to the stabilization period (ANOVA followed by Dunn's Multiple Comparison post hoc test).

Table 1 shows significant differences in the incidence of VA, AVB, and LET among the groups. In SHAM-operated group, the VA, AVB and LET incidence was 0.0%. In I/R group, VA incidence was 85%, the AVB incidence was 79%, and LET was 70%.

Groups	n	VA	AVB	LET
SHAM	33	0/33	0/33	0/33
I/R	33	28/33***	26/33***	23/33***

*** p<0.0005, compared to SHAM-operated group using Fisher's exact test. The "n" represents number of rats studied.

Table 1 Incidence of ventricular arrythmias (VA), atrio-ventricular block (AVB) and lethality (LET) obtained in rats of cardiac I/R and SHAM-operated groups.

Table 2 shows that serum concentration of biomarkers of cardiac injury was higher in I/R group compared to SHAM-operated group. The CK-MB concentration in I/R group was 129% higher compared to SHAM-operated group. Similarly, troponin I concentration in I/R group was 650% higher compared to SHAM-operated group.

Groups	n	CK-MB (U/L)	Troponin (ng/mL)
SHAM	33	808 ± 72	0.031 ± 0.009
I/R	33	1,850 ± 222*	0.200 ± 0.027*

* p<0.05, compared to SHAM-operated group using "t" Student test. The "n" represents number of rats studied.

Table 2 Serum concentrations of CK-MB (U/L) and troponin I (ng/mL) obtained in rats of cardiac I/R and SHAM-operated groups.

Figure 5 shows that treatment of rats with nifedipine 1 mg/kg and 30 mg/kg IV before ischemia significantly reduced incidence of VA from 85% to 28%, AVB from 79% to 14%, and LET from 70% to 14%, in rats submitted to cardiac I/R, indicating cardioprotective action of this L-type CCB.

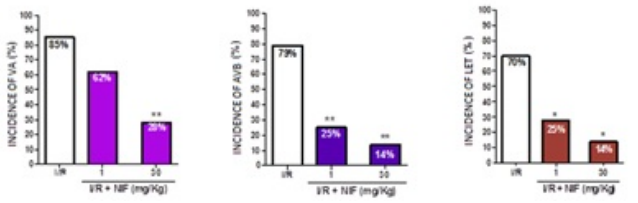

Figure 5: Histograms showing the incidence of ventricular arrhythmia (VA), atrio-ventricular blockade (AVB) and lethality (LET) in rats submitted to cardiac I/R, before (white bars) and after (colored bars) treatment with L-type Ca2+ channel blocker nifedipine (1 mg/kg and 30 mg/kg, EV, before ischemia).

Discussion

Here, we showed that methodology developed by our laboratory to induce cardiac I/R in rats, by means mechanical occlusion of left anterior descendent coronary artery with silk suture followed by removal of this tourniquet to allow coronary reperfusion, is simple and efficient to study cardioprotective drugs. The ECG analysis showed the high VA, AVB and LET incidence in rats submitted to cardiac I/R, mimicked in laboratory cardiac alterations detected in AMI patients [2-4]. This methodology also allowed correlated functional cardiac parameters with cardiac injury biomarkers produced in response to I/R (CK-MB and troponin I), confirming occurrence of myocardial lesions caused by cardiac I/R. Using this methodology, we showed that attenuation of cytosolic Ca^{2+} overload in myocardial cells produced by L-type VACC blockers, such as nifedipine, could be a good alternative to reduce cardiac arrhythmias and myocardial injuries caused by I/R in AMI patients.

It is important to note that AMI represent one of the most prevalent causes of death, and hospitalization, in intensive therapy unity with significative cost to the health system [1,2]. The classical treatment of AMI consists in removing the coronary artery obstruction and reestablishment of coronary flow [16]. However, coronary reperfusion can induce myocardial injury due to increment of free radical production, and cytosolic and mitochondrial Ca^{2+} overload in cardiomyocytes [17]. Several studies showed that reperfusion is the most important cause of arrhythmias and death in hospitalized patients after the treatment to AMI [2-4,18]. Because of this, we decided to develop a methodology *in vivo* using small animals with the scope of mimicking the cardiac I/R injuries, and contribute to development of new pharmacological cardioprotective strategies.

We observed that rats submitted to cardiac I/R presented important changes in the ECG tracing, characterized by occurrence of cardiac arrhythmias. Prolongation of PR and QT intervals, decreasing of the amplitude of the QRS complex and RR variation, were observed during the first minute of ischemia. It is important to note that the surgical procedures in SHAM-operated group did not induce animal death, and the sinus rhythm kept always stable for all study period, indicating that surgical procedures do not interfere with the analysis of cardiac changes during I/R. The cardiac alterations detected in the present study in rats submitted to cardiac I/R were similarly to those detected in AMI patients [2-4].

In rats submitted to cardiac I/R, the VA and AVB were observed from first to sixth minute of ischemia in 73% of animals. In these animals, VA and AVB incidence was 85% and 79%, respectively. In only 6% (2/33) of the rats submitted to cardiac I/R protocol, the arrhythmias resulted in death during ischemia period. However, after the reestablishment of coronary flow by removing of the surgical thread (reperfusion), we observed large variations of the ECG tracing with significant increase of VA and AVB incidence. During reperfusion, some animals had ventricular premature beats followed by ventricular tachyarrhythmias still related to the ischemia. The animals showed no arrhythmias during ischemia, but the arrhythmic events began to occur only during the reperfusion. Interestingly, no deaths were observed in the first four minutes of reperfusion.

The increase of VA and AVB incidence demonstrated by ECG analysis were accompanied by increment of cardiac injury biomarkers. Serum concentration of CK-MB and troponin I was significantly higher (129% and 650%, respectively) in cardiac I/R compared to SHAM-operated group, suggesting that cardiac I/R protocol used in the present work produced myocardial injury. The increase of cardiac injury biomarkers is typically observed in AMI patients hospitalized after myocardial injury.

These results show that the high incidence of VA and AVB resultant of cardiac I/R protocol used in the present work is strongly related to high LET incidence. These cardiac arrhythmias have been mainly attributed to alteration of ATP production by mitochondria, and cytosolic Ca^{2+} overload in cardiomyocytes [8,19-22]. The cytosolic Ca^{2+} overload promotes accumulation of Ca^{2+} in mitochondrial matrix mediated by influx of Ca^{2+} through mitochondrial uniporter, leading to bioenergetic mitochondrial collapse, and excessive production of free radical which compromises the structure and function of mitochondria, and other cytoplasmic organelles involved in the regulation of cellular function [8,19,22,23]. These cellular changes caused by I/R have been implicated in occurrence of death in AMI patients [19,22,23]. These deaths were mainly observed between the 5^{th} and the 20^{th} minutes of reperfusion. Changes in ECG observed in the present work have been attributed to abnormalities in the cardiac electrical activity, resultant of the reestablishment of coronary flow, but not due to heart manipulation to remove surgical thread.

During the ischemia, the lower availability of oxygen stimulates the anaerobic metabolism increasing the intracellular accumulation of inorganic phosphate and hydrogen, promoting reduction of intracellular pH, and increasing of change activity Na+-H+ with cytosolic accumulation of Na+. The ATP deficit during ischemia decreases the activity of proteins transporting ions ATP-dependents, like Na+/K+-ATPase (pumper of Na+), Ca^{2+}-ATPase plasmalemal (PMCA) and Sarco-Endoplasmic Reticulum Ca^{2+}-ATPase (SERCA) with accumulation of Na+ and Ca^{2+} in the cytosol [8]. Cytosolic accumulation of these ions during ischemia produces inhibition of Na + and Ca^{2+} transport to extracellular medium, contributing to cytosolic Ca^{2+}overload [24]. This Ca^{2+} overload increases Ca^{2+} influx in mitochondrial matrix, promoting mitochondrial Ca^{2+} overload and ATP production [8]. These cellular alterations caused by myocardial I/R severely compromise cardiac excitation-contraction coupling, contributing to development of cardiac arrhythmias and death in animals [7,8,13,15] and AMI patients [9].

Several studies showed that cardiac arrhythmias are very common after reperfusion in AMI patients [24-28] and increase of cardiac injury biomarkers is typically observed in AMI patients hospitalized after myocardial injury [29-32]. L-type VACC participates of control of cardiac function by finely regulating cytosolic Ca^{2+} concentration [8]. Using the methodology described in this work, we showed that

treatment with L-type VACC blocker nifedipine before ischemia significantly reduced VA, AVB and LET incidence in rats submitted to cardiac I/R. These results suggest that blockade of the L-type VACC could attenuate cytosolic Ca^{2+} overload in myocardial cells, and thus reduces cardiac arrhythmia incidence in AMI patients. This proposition has been supported by other studies [14,33]. Considering the arrhythmogenicity mechanisms in the AMI patients caused by cardiac I/R are mainly related to cytosolic Ca^{2+} overload in cardiomyocytes, this methodology of cardiac alterations related to I/R could be advantageous for studying of molecular mechanisms involved in cardiac arrhythmias, and injuries caused by I/R. In addition, this methodology could be useful to development of new pharmacological cardioprotective strategies to treat ischemic cardiac diseases in humans, including AMI.

Conclusion

The present study showed that methodology developed by our laboratory to induce cardiac I/R in rats, by means mechanical occlusion of left anterior descendent coronary artery with silk suture followed by removal of this tourniquet to allow coronary reperfusion, is simple and efficient to study of cardioprotective drugs. The ECG analysis showed the high VA, AVB and LET incidence, and increase of cardiac injury biomarkers in rats submitted to cardiac I/R, mimicking in laboratory cardiac alterations detected in AMI patients. The use of this methodology, we showed that attenuation of cytosolic Ca^{2+} overload in myocardial cells produced by L-type VACC blockers could represent a good pharmacological cardioprotective strategy to reduce cardiac arrhythmias, and myocardial injuries caused by ischemic cardiac diseases in humans, including AMI.

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