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Original

Study of a cardiac ischemia model induced by oxygen deprivation in physiological solution

Estudo de um modelo de isquemia cardíaca induzida por privação de oxigênio em solução fisiológica

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ABSTRACT

This study aimed to assess the effect of the oxygen deprivation variant in inducing I/R injury in isolated rat hearts using histological and biochemical analyses. The hearts of the control group were perfused with oxygenated Krebs-Henseleit (KH) solution for 120 min, whereas the I/R group developed ischemia with KH solution without oxygen for 30 min. Our histological data provided evidence that the protocol used induced ischemia (Control= 6.20±1.43 vs I/R= 51.07±5.21% of the infarct area). Superoxide dismutase activity did not show significant differences between groups, while catalase exhibited a lower activity in the I/R group (37.56±4.67 U/mg protein) compared to the control group (55.37±4.13 U/mg protein). Lipid peroxidation was doubled in the I/R group (I/R= 4.21±0.49 nmol/mg; Control= 2.24±0.33 nmol/mg protein). A non-significant reduction of the mitochondrial complex I activity was shown in ischemic heart (Control= 1822±352.4 vs I/R= 1584±313.4 nmol·min^{-1}· mg^{-1}protein) and significant reduction in complex II (Control= 2326±156.6 vs I/R= 1512±245.8 nmol·min^{-1}· mg^{-1} protein). We suggest that the methodology described here is an excellent protocol, in which severe ischemia is associated with stress and mitochondrial damage.

Keywords: ischemia-reperfusion, superoxide dismutase, catalase, lipid peroxidation, cardiac damage

RESUMO

Este estudo teve como objetivo avaliar o efeito da variante de privação de oxigênio na indução de lesão por isquemia/reperfusão (I/R) em corações isolados de ratos, por meio de análises histológicas e bioquímicas. Os corações do grupo controle foram perfundidos com solução de Krebs-Henseleit (KH) oxigenada por 120 minutos, enquanto o grupo I/R foi submetido à isquemia com solução KH sem oxigênio por 30 minutos. Nossos dados histológicos forneceram evidências de que o protocolo utilizado induziu isquemia (Controle = 6,20±1,43 vs I/R = 51,07±5,21% da área de infarto). A atividade da superóxido dismutase não apresentou diferenças significativas entre os grupos, enquanto a catalase exibiu menor atividade no grupo I/R (37,56±4,67 U/mg de proteína) em comparação ao grupo controle (55,37±4,13 U/mg de proteína). A peroxidação lipídica foi duplicada no grupo I/R (I/R = 4,21±0,49 nmol/mg; Controle = 2,24±0,33 nmol/mg de proteína). Observou-se redução não significativa da atividade do complexo I mitocondrial no coração isquêmico (Controle = 1822±352,4 vs I/R = 1584±313,4 nmol·min^{-1}·mg^{-1} de proteína) e redução significativa no complexo II (Controle = 2326±156,6 vs I/R = 1512±245,8 nmol·min^{-1}·mg^{-1} de proteína).

Sugerimos que a metodologia aqui descrita constitui um excelente protocolo, no qual a isquemia severa está associada ao estresse e ao dano mitocondrial..

Palavras-chave: isquemia-reperfusão, superóxido dismutase, catalase, peroxidação lipídica, dano cardíaco

INTRODUCTION

Ischemia/reperfusion (I/R) is a deleterious event associated with numerous pathologies, of which myocardial infarction (MI) is one of the most important (1). Two consecutive events have been described in I/R that can trigger metabolic imbalances that lead to cell damage and death. The first event, ischemic injury, occurs due to the interruption of tissue perfusion, whereby damage is determined by the intensity and duration of blood supply restriction (2). During this phase, hypoxia generated by the interruption of blood flow leads to dysfunction of the electron transport chain, decreased ATP production, induction of anaerobic metabolism, and decreased production of antioxidant agents (3). Hypoxia-induced injury is enhanced by tissue reperfusion, where reoxygenation in the ischemic zone brings together pathological mechanisms such as oxidative stress (4), mitochondrial dysfunction (5) and deleterious inflammatory responses (6).

Oxidative stress is the most common mechanism associated with I/R injury, in which an imbalance prevails between normal oxidant-scavenging enzyme systems and the intracellular production of reactive oxygen species (ROS) (3,7). In the reperfusion phase, ROS production increases as a consequence of increased oxygen delivery, which induces increased mitochondrial ROS release, giving rise to the regenerative cycle of mitochondrial ROS formation and release (ROS-induced ROS release) (8,9) (Figure 1).

An isolated perfused heart model, also known as the Langendorff model, has been used to reproduce I/R injury. However, despite numerous publications, it is difficult to find descriptions of methodological derivations that lead to the reproduction of I/R injuries. At the beginning we considered performing experiments using the ischemia model by suppression of perfusion, but the results obtained showed massive and irreversible ischemia. (data not shown). Therefore, we implemented the oxygen-deprived perfusion ischemia model (10) and the present work was aim to describe the effectiveness of this protocol to induce infarction which might be confirmed through histological and biochemical studies. By incorporating a variant of perfusing nutrient solution deprived of oxygen instead of suspending perfusion, we also had to determine both the antioxidant and mitochondrial status, related to histological data of the tissue subjected to I/R (11).

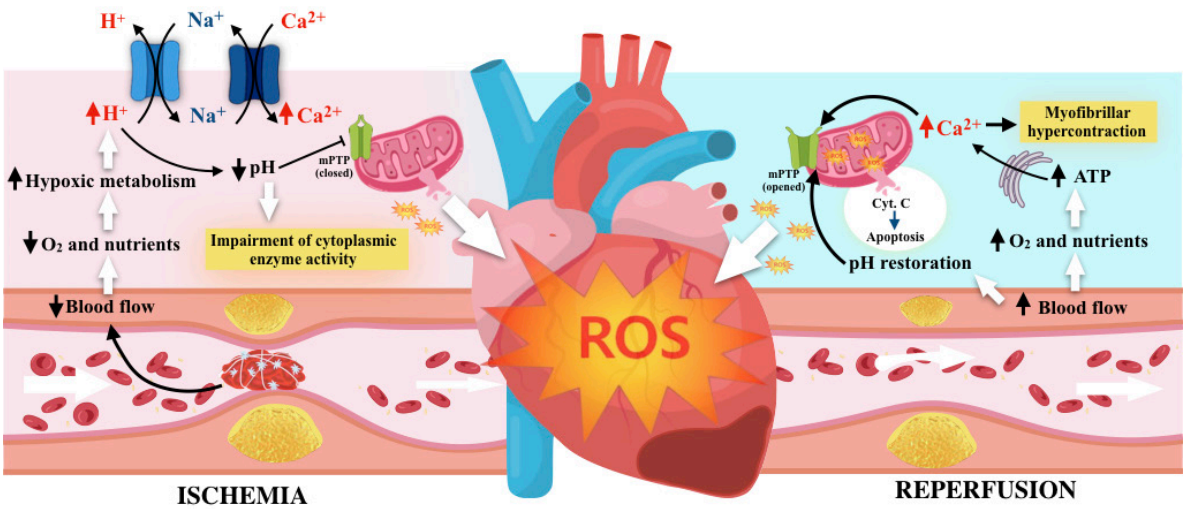

Figure 1. Diagram of the mechanism of myocardial damage induced by I/R and its relationship with the results obtained in the isolated heart model perfused with nutrient solution devoid of oxygen. Acute myocardial ischemia is characterized by an increase in anaerobic metabolism. Under this energy deficit, the Na^+/K^+ ATPase pump

in the cell membrane and the Ca^{2+}-ATPase in the endoplasmic reticulum become dysfunctional. Failure to activate the Na^+/K^+ ATPase pump leads to sodium retention inside the cells, which in turn decreases the activity of the sodium-hydrogen exchanger pumps (Na^+-H^+ pumps). This event leads to the accumulation of hydrogen, which lowers the pH and consequently causes deterioration in intracellular enzymatic activity. With reperfusion, tissue oxygen is restored, which promotes an increase in the production of reactive oxygen species (ROS), combined with a deficit of cellular antioxidants, to induce reperfusion injury in ischemic tissue. ROS induces local inflammatory responses, which together lead to apoptosis due to damage to cellular structures.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Male Sprague-Dawley rats (200–300 g) were purchased from the Instituto de Investigaciones Cientificas de Alta Tecnologia (INDICASAT). The animals were housed in the animal laboratory of the Centro de Investigaciones Psicofarmacologicas (CIPFAR) under appropriate conditions, determined by 12-hour light-dark cycles, a temperature of 22 °C ± 2 °C, and humidity (60%). During the acclimatization period, the rats received water and standard rodent pellets (LabDiet ®) ad libitum.

All experimental procedures described in this research were performed following the instructions included in the "Guide for the Care and Use of Laboratory Animals," described by the Institute of Laboratory Animal Resources (ILAR) of the National Research Council for the ethical handling of biological reagents (12). The protocol was approved by Comite de Bioetica, University of Panama (N° CBUP/029/2020).

CHEMICALS AND REAGENTS

EDTA (Sigma-Aldrich, 99% purity), KCl (Merck; 99% purity), K2HPO4 (Fisher Scientific, 98% purity), KH2HPO4 (Sigma-Aldrich, 99% purity). NaOH (VWR Chemicals, 98% purity), HCl (Sigma-Aldrich, 37% purity), Triton X-100 (molecular grade, Thermo Fisher Scientific, 99% purity), PMSF (molecular grade, RPI, 99% purity), Beta-mercaptoethanol (biotechnology grade, Merck, 99% purity), TBA (analytical grade, Sigma-Aldrich, 98% purity), TCA (Fisher Scientific, 99% purity), Succinate (Sigma-Aldrich, 98% purity), Glutamate, biotechnology grade (Merck, 99% purity), 2,6-Dichlorophenolindophenol, analytical grade (Merck), NAD, biotechnology grade (Thermo Fisher Scientific, 99% purity), Nicotinamide (analytical grade, Sigma-Aldrich, 98% purity), Coomassie Blue G250 (Bio-Rad, 95% purity), Superoxide dismutase CS0009 (Sigma-Aldrich). All other chemicals were of analytical grade and purchased from Sigma-Aldrich.

DEVELOPMENT OF ISCHEMIA/REPERFUSION

For testing, all animals were anesthetized using a pentobarbital/heparin mixture (40 mg/kg / 100 U/kg). We performed a thoracotomy and rapidly removed the heart which was placed in cold modified Krebs-Henseleit (KH) buffer (118 mM NaCl, 4.75 mM KCl, 1.18 mM KH_2PO_4, 1.18 mM $MgSO_4 \cdot 7H_2O$, 5 mM $CaCl_2$, 25 mM $NaHCO_3$, 5 mM glucose and 100 µM sodium octanoate; pH 7.4). A loop of surgical suture thread was placed in the aortic artery to attach the heart to the Langendorff cardiac perfusion system.

To induce I/R injury in isolated rat hearts, we used the method described by Bell et al. (2011) with some modifications (13). Hearts from control animals (n=8) were perfused through the aorta at a constant flow rate of 10.5 ml/min for 2 h with KH solution under physiological conditions (37 °C, bubbled with a carbogenic mixture of 95% O_2 and 5% CO_2). In the group subjected to I/R injury (n=8), we started with 30 min of continuous perfusion using KH solution under the same conditions as described for the control group. At the end of this period, we started the ischemia period, and for 30 min, the hearts were perfused using KH deprived of the carbogenic mixture and then started the reperfusion period for one hour using physiological KH. Finally, the hearts were removed from the system and processed for biochemical and histological analysis. *For biochemical evaluations, cardiac samples were exclusively taken from the left ventricle, given its higher susceptibility to damage during ischemia-reperfusion.*

HISTOPATHOLOGICAL EVALUATION

After perfusion protocols were completed, the hearts were frozen for 10 minutes at -80 °C, and transverse cuts were made above the apex, obtaining segments of approximately 2 mm, including both ventricles. To determinate the infarct size, two segments were treated with 1% triphenyltetrazolium chloride (TTC) for 15-20 minutes at 37 °C and washed out with 10% formaldehyde during 20 min. Images of the slices were obtained with a stereoscope (Carl Zeiss, model Stemi 305) and processed using ImageJ ® software. The other sections were preserved in 10 % formaldehyde for paraffin embedding and stained with hematoxylin and eosin (H&E). The assays were performed in triplicate.

OXIDATIVE STRESS ASSESSMENT

SUPEROXIDE DISMUTASE DETERMINATION

A kit for the determination of SOD activity, CS009 (Sigma), was used for this assay. We employed a mixture containing 10µL of cardiac homogenate in 0.1 M tris /HCl (pH 7.4) (0.5% triton 100x; 5mM B-ME; 0.1 mg/mL PMSF). The absorbance was read using a spectrophotometer (Epoch, BioTek Instruments, Inc., model M491) at 450 nm for 5 min, and the results were expressed as enzyme activity in U/mL using a standard curve according to the manufacturer's instructions. The assays were performed in triplicate.

CATALASE DETERMINATION

Cardiac tissue (50 mg) was washed with a mixture of KCl (1.15%) and EDTA (0.5 mM) dissolved in 0.9% NaCl. Thereafter, it was homogenized with 0.1 M potassium phosphate buffer (K_2HPO_4 and KH_2PO_4) at pH 7, centrifuged at 15 000 g for 30 min at 4 °C, and the supernatant was collected.

A mixture containing 10µL of homogenate and 2990 µL of 0.063% H_2O_2 in 0.1 M phosphate buffer (pH 7.4) was used for CAT determination. The kinetic method employed was based on the decrease in hydrogen peroxide levels in one minute, using a spectrophotometer (Thermo Scientific, Genesys model) at 240 nm (15). Enzymatic activity was expressed as U/mg of protein. Proteins were determined by the Bradford method (16) using an extinction coefficient of 43.6 M^{-1} cm^{-1}(15). The quantification of enzyme activity was performed in triplicate.

LIPID PEROXIDATION IN CARDIAC TISSUE

To determine lipid peroxidation in cardiac tissue samples, the protocol of Prabhakar (14) was based on the method of Wills (17) with modifications. This assay is based on the determination of thiobarbituric acid-reactive substances (TBARS), which are formed as a product of lipid peroxidation. Malondialdehyde (MDA) is one of several end products formed through the degradation of lipid peroxidation products.

For this assay, 100 µL of homogenate was mixed with 1 mL of TBA-TCA (0.375 and 15%, respectively), and distilled water is added to make up 1.5 mL. The samples were maintained at 95 °C for 20 min and then placed in a cold bath to stop the reaction. The TBA-MDA adduct was extracted with 1.5 mL butanol, and the concentration was determined spectrophotometrically (Thermo Scientific, Genesys model) at 532 nm in an end-point assay. An extinction coefficient of 1.56 x 10^5 M^{-1} cm^{-1} was used for the calculations, and the results were expressed in nmol of TBARS/mg of protein according to the Bradford method (16). Assays were performed in triplicate for each sample.

PROCEDURE FOR MITOCHONDRIAL EVALUATION

PREPARATION OF THE HOMOGENATE

Mitochondrial extraction was performed according to the experimental biochemical technique of Switzer and Garrity with minor modifications (18). We used 1000 mg of cardiac tissue, washed with 1 mM EDTA, and homogenized with 0.02 M potassium phosphate buffer (K_2HPO_4 and KH_2PO_4 /1 mM EDTA) at pH 7.4. The sample was centrifuged at 1 500 g at 4 °C for 30 min. The obtained precipitate was homogenized and centrifuged under the same conditions. The supernatants from the two processes were mixed, and the pH was adjusted to 5.6 with 1 M CH_3-COOH. The supernatant mixture was centrifuged twice, and the resulting pellet was stored at 4 °C until use.

EVALUATION OF MITOCHONDRIAL COMPLEX I AND II

The stored pellet was resuspended in potassium phosphate buffer/EDTA (0.25 M) at a pH of 7.4. Protein levels were determined using the Bradford method (16).

To determine the activity of complex I, the reaction mixture was prepared with 15 µg of protein, 22 µL of NAD^+ (0.01 M), 11 µL of glutamate (0.5 M), 500 µL of 0.3 M potassium phosphate buffer, and 44 µL of 2,6 dichloroindophenol (2,6-DCIP). For mitochondrial complex II, we used 15 µg of protein to which we added 11 µL of succinate (0.5 M), 500 µL of 0.3 M potassium phosphate buffer, and 44 µL of 2,6-DCIP. Both reaction mixtures were prepared using 1000 µL of distilled water. For each determination, we calibrated the reaction blank containing 15 µg protein in 1000 µL distilled water.

This kinetic method is based on the decrease in the absorbance of 2,6-DCIP determined at 600 nm (Thermo Scientific, Genesys model) at 25 °C (16,18). The enzymatic activities of complexes I and II were calculated using a molar extinction coefficient of 2,6-DCIP of 19.1 $mM^{-1.}\ cm^{-1}$ in samples assayed in triplicate (19,20). The enzymatic activity of the complexes is expressed in $nmol{\cdot}min^{-1.}\ mg^{-1}$ protein.

STATISTICAL ANALYSES

All results are expressed as mean and standard error of the mean (mean ± SEM, n=8). Statistical analyses were performed using GraphPad Prism 8, differences were compared using the unpaired t-test, and p-values < 0.05 as significant.

RESULTS

INDUCTION OF MYOCARDIAL INFARCTION EFFICACY

To assess the efficacy of this technique in inducing ischemia, cardiac tissue samples from both the control and I/R groups were subjected to histological analysis. The results obtained with TTC staining showed that the percentage of ischemic area was approximately 8.5 times higher in the I/R group than in the control group (51.07±5.21 and 6.20±1.43% of infarction, respectively) (Figure 2A, 2B).

In control hearts stained with Hematoxylin & Eosin, the cardiomyocytes were well defined, with a central nucleus with lax chromatin and cross striations. Ischemic tissue changes were observed, including intracellular edema, intracellular edema, and loss of tissular organization (Figure 2C, 2D).

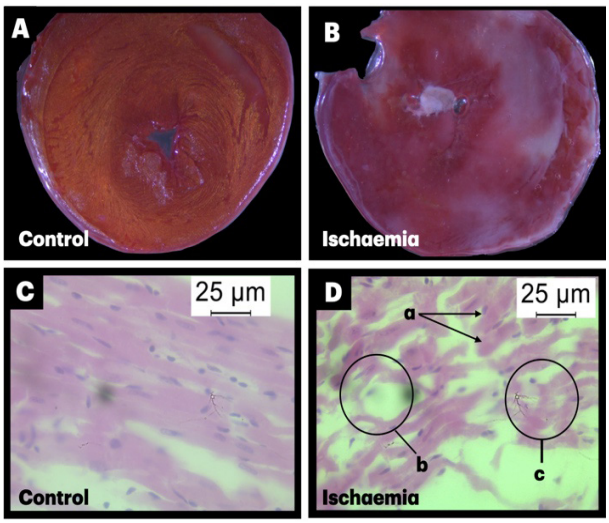

Figure 2. TTC Staining detected the area of myocardial infarction (A and B). H&E staining was used to detect myocardial injury as follows: a) intracellular edema, b) intracellular edema and c) loss of tissular organization (C and D).

OXIDATIVE STRESS

SOD AND CAT ACTIVITY IN PERFUSED RAT HEART TISSUE

A non-significant increase in SOD activity was observed in heart tissue homogenates subjected to the I/R process (12.94±1.06 U/mL) compared with SOD for the control group (10.81±0.79 U/mL) (Figure 3A).

Determination of CAT activity in hearts subjected to I/R injury is intended to demonstrate the efficacy of pharmacological intervention or to determine the impact of a pathological condition(13,21). We observed a significant decrease in CAT values in hearts subjected to ischemic conditions (37.56±4.67 U/mg protein) when compared with data obtained in the control group (55.37±4.13 U/mg protein) (Figure 3B).

LIPID PEROXIDATION IN CARDIAC TISSUE SUBJECTED TO I/R

In samples obtained from hearts subjected to I/R we observed a TBARS value equivalent to 4.21±0.49 nmol/mg protein, that was significantly higher than control group (2.24±0.33 nmol/mg protein) (Figure 3C).

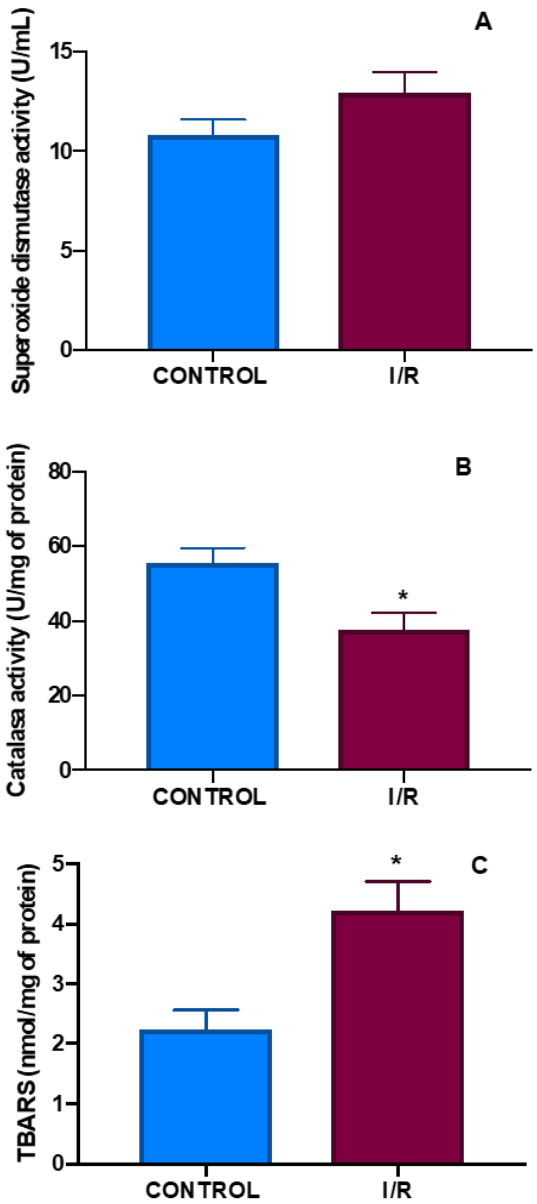

Figure 3. Activity of superoxide dismutase (A), catalase (B) and TBARS (C) in control group and IR group of perfused rat hearts. Values expressed as mean ± SEM (N=5). *p<0.05 vs control

KINETICS OF COMPLEX I AND II ACTIVITY IN CARDIAC TISSUE SUBJECTED TO I/R

The mitochondrial complex I activity was 1822±352.4 nmol·min^{-1}· mg^{-1} protein in hearts perfused under physiological conditions. The activity of this complex was not significantly reduced in the I/R group (1584±313.4 nmol·min^{-1}· mg^{-1} protein) (Figure 4A). In contrast, the activity of mitochondrial complex II was significantly decreased in the I/R group compared with that in the control group (1512±245.8 and 2326±156.6 nmol·min^{-1}· mg^{-1} protein; respectively) (Figure 4B).

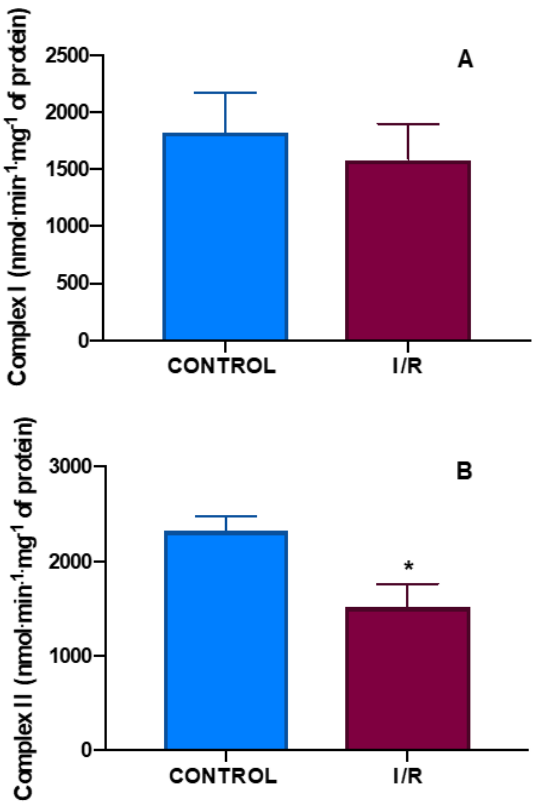

Figure 4. Activity of mitochondrial complexes I (A) and II (B) in control and IR groups of perfused rat hearts. Values are expressed as the mean ± SEM (N=5). *p<0.05 vs control.

DISCUSSION

The development of a perfused rat heart model using the Langendorff system is the first step in initiating research on ischemic heart disease (IHD).

I/R injury is a widely used experimental model to study the understanding and treatment of IHD (22).

In the standardization of the I/R technique, we observed that when perfusion suppression was used as a mechanism to induce ischemia, cardiac activity did not recover during reperfusion and the heart showed massive ischemia (data not shown). These results led us to reconsider the methodological aspects, considering the establishment of a variation in the protocol for inducing ischemia. Therefore, we used an oxygen-deprived nutrient solution to induce I/R injury (23). We evaluated the efficacy of this design to induce ischemia and established its relationship with variations in biochemical parameters that have been described as markers of oxidative stress in the I/R injury model (23).

The evaluation of biochemical parameters related to oxidative stress is commonly required in this type of study. Interest in the determination of ROS and antioxidant enzymes is because cardiomyocytes switch to anaerobic metabolism during acute myocardial ischemia, which produces a lower level of adenosine triphosphate (ATP). Because of this reduced energy supply, ATP-dependent ion exchange becomes inefficient, which induces the retention of sodium inside the cells because of the reduction in the activity of the Na^+/K^+ ATPase pump. All of the changes described affect the activity of the Na^+-H^+ pump and generate a reduction in intracellular pH (4,24). The resulting cellular acidosis leads to deterioration of intracellular enzyme activity, including antioxidant enzymes such as SOD, CAT, and GPx, with overproduction of ROS. ROS can damage virtually all cellular biomolecules and promote opening of the mitochondrial permeability transition pore (mPTP) (24,25) (Figure 1).

During reperfusion, oxygen influx fuels reactive oxygen species (ROS) production, which is known as the "oxygen paradox." This phenomenon suggests that during the reoxygenation (reperfusion) of ischemic tissue, an increased amount of ROS is produced, which increases the damage induced by the initial ischemia (26). Under conditions of prolonged hypoxia and reperfusion, antioxidant mechanisms become inefficient, either because the large production of free radicals exceeds the antioxidant capacity of the organism or because the inflammatory mechanisms underlying oxidative stress lead to increased degradation of the enzymes that neutralize the reactive components (27) (Figure 1). Consequently, the final damage to a cardiac infarction is the result of both ischemia and reperfusion (28).

Oxidative stress is considered the main mechanism of I/R injury, and the results obtained in relation to the inhibition of CAT activity demonstrate that this component is involved in the model described, and that it also correlates with the histological data. Thus, with our model, we not only observed significant ischemia, but also a reduction in CAT activity, an activity that has been described by other authors who also used a total perfusion time of 120 min (29–31).

However, the results of SOD activity are contradictory, since other studies have shown that the activity of this enzyme is not modified in the heart as a consequence of the damage induced by I/R (32), while other studies have described the inhibition of the activity of this antioxidant enzyme as a representative effect of the injury (21,33). Other authors have reported an increase in its enzyme activity, explained as an effort to neutralize the overproduction of ROS induced by I/R, which has been associated with the secretion of TNFα by cardiomyocytes and macrophages resident in the cardiac tissue (34). In turn, this cytokine mediates the activation of the transcription factor NF-κB, which is capable of significantly increasing the expression of genes encoding SOD and its corresponding enzymatic activity (35,36). Thus, the latter approach may explain the increased activity reported in our study. This variability in SOD activity may be due to the conditions of the model used, as there are differences in the experimental conditions among the authors, including ischemia and reperfusion times.

In the same oxidative stress pathway, I/R injury is characterized by other cellular events in which the burst of oxidation associated with reperfusion is accompanied by lipid peroxidation (37). Thus, the results we have obtained are consistent with reports by other authors who also describe an increase in lipid peroxidation as a result of the ischemic process to which cardiac tissue is subjected in the I/R model (31,38,39). It should be emphasized that this accumulation of toxic lipid peroxidation results from both hyperactivity of the oxidation mechanism and weakening of the antioxidant mechanism (7). On the other hand, it has been described that

lipid peroxidation in I/R is one of the main mechanisms of cell damage since it can alter the properties of the mitochondrial membrane, increasing calcium permeability and cytochrome C release, that could induce cell death (4).

Finally, mitochondria play an important role in the development of I/R injury and are mostly associated with prolonged opening of the mPTP (25). It should be noted that, under physiological conditions, when mPTPs are opened in a controlled and transient manner, they play an important role in regulating signaling. However, when mPTP opening is prolonged and unregulated, results in changes of the mitochondrial membrane potential, cessation of ATP synthesis, massive release of cytochrome C and mitochondrial Ca^{2+}, leading to mitochondrial swelling and death (17). During I/R injury, mechanisms involving excessive ROS levels can modify the opening periods of mPTP and, thus, mitochondrial dynamics (10). Thus, in I/R injury, mitochondrial dysfunction occurs due to a decrease in ATP production and an increase in ROS. In studies performed with Clark-type electrodes (40) as well as spectrophotometric studies (39), complexes I and II have been identified as proteins of the electron transport chain affected by prolonged ROS-induced mPTP opening in I/R (4,9). In addition, persistent alterations in mitochondrial complexes have been associated with acidosis and tissue damage induced by lipoperoxidation (4,41). In our study, we also observed alterations in the mitochondrial complexes, which were significant for complex II in homogenates obtained from hearts subjected to I/R, highlighting the findings of other authors.

CONCLUSIONS

The model of I/R by perfusion with oxygen-deprived nutrient solution generates alterations in redox mechanisms with an increase in lipid peroxidation and antioxidant deficit, which is accompanied by inhibition of mitochondrial complexes and histological evidence of myocardial infarction.

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