Construction of an Isolated Myocardial Ischemia-Reperfusion Model in Experimental Tree Shrews (Postprint)

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Abstract

Objective: To establish an isolated model of myocardial ischemia-reperfusion in experimental tree shrews. Methods: The Langendorff isolated heart perfusion system was employed to establish a myocardial ischemia-reperfusion model in experimental tree shrews. Experiments were divided into 5 groups based on different ischemia and reperfusion durations. Enzyme-linked assays were used to measure alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH). The immunoinhibition method was used to determine creatine kinase MB isoenzyme (CK-MB). 2,3,5-triphenyltetrazolium chloride staining (TTC) was used to measure the infarct area of sections. Results: Detection of myocardial enzymatic indicators and infarct area revealed that the 30-min ischemia/30-min reperfusion group and the 30-min ischemia/60-min reperfusion group exhibited significantly higher levels of perfusate CK-MB, perfusate LDH, tissue ALT, tissue CK-MB, and tissue LDH compared to the other 3 groups ($P<0.05$), but there was no statistically significant difference between these two groups ($P>0.05$). Electrocardiographic analysis revealed that the heart rate in the 30-min ischemia/60-min reperfusion group was significantly lower than that in the continuous perfusion group, the 15-min ischemia/30-min reperfusion group, and the 30-min ischemia/30-min reperfusion group ($P<0.05$), while there was no statistically significant difference in heart rate between the 30-min ischemia/30-min reperfusion group and the continuous perfusion group or the 15-min ischemia/30-min reperfusion group ($P>0.05$). The average heart rate of isolated hearts in the 30-min ischemia/30-min reperfusion group was closer to the physiological indices of experimental tree shrews. Conclusion: The isolated Langendorff model of myocardial ischemia-reperfusion in experimental tree shrews was successfully established, with the 30-min ischemia/30-min reperfusion model demonstrating the optimal results.

Full Text

Preamble

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Abstract

Objective To establish an ex vivo model of myocardial ischemia-reperfusion injury in tree shrews. **Methods** Using the Langendorff isolated heart perfusion system, we established a myocardial ischemia-reperfusion model in tree shrews with five different ischemia and reperfusion time protocols. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) levels were measured by enzymatic assay, creatine kinase MB isoenzyme (CK-MB) was detected by immunoinhibition method, and infarct size was determined by 2,3,5-triphenyltetrazolium chloride (TTC) staining. **Results** Analysis of myocardial enzyme markers and infarct area revealed that both the 30-min ischemia with 30-min reperfusion group and the 30-min ischemia with 60-min reperfusion group showed significantly higher levels of CK-MB and LDH in perfusate, as well as ALT, CK-MB, and LDH in myocardial tissue, compared with the other three groups ($P<0.05$), though no significant differences were observed between these two groups ($P>0.05$). Electrocardiographic analysis demonstrated that the heart rate in the 30-min ischemia with 60-min reperfusion group was significantly lower than in the continuous perfusion, 15-min ischemia with 30-min reperfusion, and 30-min ischemia with 30-min reperfusion groups ($P<0.05$), while heart rates among the latter three groups were comparable ($P>0.05$). Notably, the average heart rate of isolated hearts in the 30-min ischemia with 30-min reperfusion group most closely approximated the physiological heart rate of tree shrews. **Conclusion** We successfully established an ex vivo Langendorff model of myocardial ischemia-reperfusion in tree shrews, with the 30-min ischemia followed by 30-min reperfusion protocol proving optimal.

Keywords: myocardial ischemia-reperfusion; ex vivo model; tree shrew

Introduction

Cardiovascular disease poses a severe threat to human health worldwide, with its incidence and mortality now exceeding those of malignant tumors to become the leading cause of death, while the age of onset continues to trend younger [1]. Accumulating animal experiments and clinical observations have demonstrated that myocardial injury worsens following reperfusion of ischemic myocardium, a phenomenon known as myocardial ischemia-reperfusion injury (MIRI), which represents a major factor affecting prognosis in acute myocardial infarction [2]. Consequently, developing effective measures to prevent or mitigate reperfusion

injury has become a research priority, necessitating the establishment of stable and reliable experimental animal models for comprehensive investigation.

Currently, most myocardial ischemia/reperfusion injury models utilize rats, with some reports also describing models in macaques, Kunming mice, Japanese white rabbits, and beagle dogs [3-6]. The tree shrew represents a novel experimental animal model characterized by small size, rapid reproduction, ease of capture and husbandry, low breeding costs, and high evolutionary status, with gross anatomy, metabolism, and molecular/cellular characteristics showing high similarity to humans [7-8]. Tree shrews have been widely used in biomedical research and drug safety evaluation, yet a tree shrew model of cardiac ischemia-reperfusion remains lacking. In this study, we employed the Langendorff isolated heart perfusion system to establish a myocardial ischemia-reperfusion model in tree shrews, combining this novel experimental animal with a well-established experimental apparatus. We assessed model establishment by measuring myocardial enzyme changes in perfusate and tissue following reperfusion and calculating infarct area from tissue sections, aiming to identify optimal experimental conditions and establish a reliable ex vivo model of myocardial ischemia-reperfusion for future mechanistic studies.

Materials and Methods

1.1 Experimental Animals

Sixty healthy adult male tree shrews (Tupaia belangeri chinensis) aged 4-6 months and weighing 120-150 g were obtained from the Tree Shrew Germplasm Resource Center at the Institute of Medical Biology, Chinese Academy of Medical Sciences (license number: Dian Fa Xun Fan [92-29]). Animals were maintained according to our laboratory' s established husbandry protocols and fasted overnight prior to surgery with free access to water.

1.2 Experimental Procedures

1.2.1 Equipment The Langendorff isolated heart perfusion system (PowerLab, ADInstruments) was utilized, which enables direct observation of myocardial ischemia-reperfusion injury under conditions that exclude neurohumoral interference.

1.2.2 Experimental Groups The study was divided into five groups (n=5 each): (1) continuous perfusion control group, (2) 15-min ischemia followed by 30-min reperfusion group, (3) 15-min ischemia followed by 60-min reperfusion group, (4) 30-min ischemia followed by 30-min reperfusion group, and (5) 30-min ischemia followed by 60-min reperfusion group.

1.2.3 Model Establishment Animals were anesthetized via intraperitoneal injection of chloral hydrate (1 mL/animal) and anticoagulated with intraperitoneal heparin (1,000 U/animal). The chest was rapidly opened to isolate the

heart, and the aorta was transected 4-5 mm from its origin along with other vessels. The heart was immediately placed in 0-4°C solution to evacuate residual blood from the cardiac chambers and promptly transferred to the Langendorff perfusion apparatus. The aorta was cannulated retrogradely while a balloon was positioned in the left ventricle. Hearts were perfused with Krebs-Henseleit (K-H) buffer (pH 7.35-7.45) at constant temperature (37°C) and pressure (60 mmHg) with coronary flow maintained at 6-12 mL/min. The pulmonary artery root was incised to ensure unobstructed coronary venous drainage, and effluent was collected in a graduated cylinder timed to represent coronary flow.

K-H buffer composition: NaCl 6.92 g/L, KCl 0.35 g/L, KH PO 0.35 g/L, NaHCO 2.1 g/L, MgSO 0.296 g/L, glucose 2 g/L, CaCl 0.28 g/L, and EDTA 0.187 g/L. The pH was adjusted with HCl. The K-H buffer was continuously equilibrated with 95% O and 5% CO throughout the perfusion period. Recording electrodes were placed in the right atrium and cardiac apex and connected to a multi-channel electrophysiology recorder for continuous ECG monitoring. Following stabilization of cardiac perfusion, hearts were removed immediately after perfusion completion, rinsed in ice-cold saline to remove blood, blotted dry with filter paper, and cleared of vascular and atrial tissue. After freezing for 2 hours, six myocardial slices were cut at 2-mm intervals along the coronary plane. Ventricular tissue was then divided into 0.2-g blocks, placed in cryovials, snap-frozen in liquid nitrogen, and transferred to a -80°C freezer for subsequent experiments.

1.2.4 Evaluation Criteria for Successful Model Establishment Successful model establishment was evaluated based on three criteria: (1) Electrocardiographic and perfusion stability: the isolated heart maintained stable ECG with heart rate >200 beats/min without significant bradycardia, sustained ventricular arrhythmias, or cardiac arrest, while perfusion remained stable at constant temperature (37°C) and pressure (60 mmHg) with coronary flow of 6-12 mL/min. (2) Myocardial enzyme analysis: levels of ALT/GPT, AST/GOT, CK-MB, and LDH were measured in perfusate and myocardial tissue samples at different time points to compare changes before and after ischemia-reperfusion within groups and assess the impact of different ischemia and reperfusion durations, with myocardial damage severity evaluated based on enzyme content. (3) Myocardial infarct area comparison: myocardial tissue sections were stained with TTC, photographed digitally, and infarct area was calculated for inter-group comparison.

1.2.5 Common Experimental Issues Potential complications included anesthetic accidents resulting in animal death, inadequate heparinization leading to blood coagulation and thrombosis in coronary vessels or ventricular chambers, failure to maintain constant temperature (37°C) and pressure (60 mmHg) during perfusion, incorrect buffer composition or pH adjustment, equipment malfunction such as tubing blockage or leakage, poor-quality homemade latex balloons affecting left ventricular pressure measurement,

coronary artery damage or size mismatch between cannula and aorta during heart suspension, improper aortic cannula depth causing perfusate leakage from aortic branches or obstruction of coronary ostia with potential aortic valve injury, and prolonged experimental duration (>6 hours) due to unskilled manipulation.

1.2.6 Measurement of ALT, AST, CK-MB, and LDH Samples were collected from perfusate and myocardial tissue at different time points. Measurements were performed using a microplate reader, biochemical analyzer, micropipettes, and vortex mixer. Reagents included ALT/GPT assay kit (Nanjing Jiancheng, catalog C009-2, microplate method), AST/GOT assay kit (Nanjing Jiancheng, catalog C0010-2, microplate method), CK-MB assay kit (Nanjing Jiancheng), and LDH assay kit (Nanjing Jiancheng, catalog C020-2, microplate method).

1.2.7 TTC Staining for Infarct Area Measurement Myocardial tissue samples from different time points were used. Equipment included culture dishes, transfer pipettes, 37°C incubator, glass slides, and TTC stain (Nanjing Jiancheng). The principle underlying TTC staining involves reduction by normal tissue respiratory chain enzymes to produce a red color, whereas ischemic tissue with decreased enzyme activity remains unstained and appears pale, enabling clear distinction between infarcted and normal/ischemic myocardium. This method is widely used in isolated heart ischemia/reperfusion injury research due to its high sensitivity and clear visualization [9]. Staining results showed non-infarcted regions in red, infarcted areas in gray-white, and ischemic zones at the border between red and gray regions.

1.3 Statistical Analysis

Normally distributed continuous data are expressed as mean ± standard deviation. Independent samples t-test was used for intra-group comparisons, and one-way ANOVA was employed for inter-group comparisons. $P<0.05$ was considered statistically significant.

Results

Laboratory measurements of CK-MB, LDH, ALT, and AST in coronary effluent and myocardial homogenates at different time points, along with TTC staining for myocardial infarction assessment, revealed highly significant differences among the five groups in perfusate and tissue enzyme levels and infarct area ($P<0.01$, Table 2 , Table 3). Pairwise comparisons showed that, with few exceptions, myocardial enzyme levels in group 2 were significantly higher than in group 1; group 3 was significantly higher than groups 1 and 2; group 4 was significantly higher than groups 1, 2, and 3; and group 5 was significantly higher than groups 1, 2, and 3 ($P<0.05$). No significant differences were observed between groups 4 and 5 ($P>0.05$). Infarct area analysis demonstrated

that groups 2 and 3 were significantly higher than group 1, while groups 4 and 5 were significantly higher than the preceding three groups ($P<0.05$), with no significant difference between groups 4 and 5 ($P>0.05$, Table 2, Table 3).

Heart rate analysis revealed that the average heart rate in group 3 was slower than in groups 1 and 2, while group 4 was faster than group 3, and group 5 was slower than groups 1, 2, and 4, with all differences being statistically significant ($P<0.05$). Post-reperfusion heart rates measured in different groups were: group 1 (305.12±20.77) BPM, group 2 (307.98±14.88) BPM, group 3 (266.89±14.10) BPM, group 4 (301.37±12.38) BPM, and group 5 (271.59±19.72) BPM, with significant differences among the five groups ($P<0.05$).

Intra-group Comparisons Before and After Reperfusion

CK-MB levels in perfusate increased significantly after reperfusion in groups 2 and 4 ($P<0.05$), decreased significantly in group 1 after continuous perfusion ($P<0.05$), and showed no significant changes in groups 3 and 5 compared with pre-reperfusion values ($P>0.05$, Figure 1 [Figure 1: see original paper]). No significant differences were observed in tissue CK-MB levels before and after reperfusion in any group ($P>0.05$, Figure 2 [Figure 2: see original paper]).

LDH levels in perfusate increased significantly after reperfusion in groups 2, 3, and 5 ($P<0.05$), with no significant changes in groups 1 and 4 compared with pre-reperfusion values ($P>0.05$, Figure 3 [Figure 3: see original paper]). Tissue LDH levels showed no significant differences before and after reperfusion in any group ($P>0.05$, Figure 4 [Figure 4: see original paper]).

ALT levels in perfusate increased significantly after reperfusion in groups 2, 3, 4, and 5 ($P<0.05$), with no significant change in group 1 ($P>0.05$, Figure 5 [Figure 5: see original paper]). Tissue ALT levels increased significantly after reperfusion in groups 3, 4, and 5 ($P<0.05$), with no significant differences in groups 1 and 2 compared with pre-reperfusion values ($P>0.05$, Figure 6 [Figure 6: see original paper]).

AST levels in perfusate increased significantly after reperfusion in groups 3 and 5 ($P<0.05$), with no significant changes in groups 1, 2, and 4 ($P>0.05$, Figure 7 [Figure 7: see original paper]). Tissue AST levels increased significantly after reperfusion in groups 2, 3, and 5 ($P<0.05$), with no significant differences in groups 1 and 4 compared with pre-reperfusion values ($P>0.05$, Figure 8 [Figure 8: see original paper]).

TTC staining of tissue sections revealed pale infarcted regions with clear demarcation lines and hemorrhage within infarct areas. Infarct area increased significantly after reperfusion in groups 2 and 3 ($P<0.05$), with no significant differences in groups 1, 4, and 5 compared with pre-reperfusion values ($P>0.05$, Figure 9 [Figure 9: see original paper]).

Discussion

Emerging research on tree shrews has revealed their close phylogenetic relationship with primates. Previous studies have reported tree shrew electrocardiographic characteristics, hematological and biochemical parameters [10], major organ weights and coefficients [11], brain development [12], gastrointestinal transit time [13], and have established models of infectious diseases [14-16], psychiatric [17] and neurological disorders [18-19], metabolic diseases [20], and cancer [21]. However, a cardiovascular system model in tree shrews remains lacking.

The Langendorff isolated heart perfusion model offers high reproducibility, excellent stability, and relatively modest technical requirements while eliminating interference from other organs and neurohumoral systems, making it one of the most widely used experimental models in cardiovascular research [22]. This technique has enabled major breakthroughs in numerous areas including myocardial ischemia/reperfusion injury, myocardial cell therapy, and cardiac transplantation preservation. However, we recognized during experimentation that multiple factors influence model preparation, and achieving a stable and effective isolated heart model requires not only proficient technique but also careful attention to factors such as animal anesthesia, K-H buffer preparation, perfusate temperature, aortic cannula position and depth, perfusion flow or pressure, balloon size, and air bubbles in the tubing, as any error may lead to experimental failure or compromised results.

Myocardial ischemia-reperfusion injury involves a highly complex pathological process [19]. Reperfusion generates massive oxygen free radicals [5,23-25] that severely damage cell membrane structure, increase membrane permeability, and cause massive release of intracellular enzymes into the bloodstream. The degree of enzyme leakage indirectly reflects the severity of myocardial reperfusion injury and provides important value for determining infarction timing, area, recurrent myocardial infarction, and reperfusion status [27-28]. Ischemia and reperfusion duration represent the two most critical parameters in animal model construction. Studies in rat models have shown that reperfusion injury occurs with as little as 10 min of ischemia, while 40 min produces severe injury [27-28]. Considering model success rate, post-injury recovery, and potential for pharmacological intervention, an ischemia duration of 15-30 min is considered optimal for rat isolated heart models.

Furthermore, Chen et al. [30] reported that CK and LDH levels began to increase after 30 min of regional ischemia in rat Langendorff models, peaking at 15 min of reperfusion, with no significant differences between 15 min and 30 min or between 30 min and 75 min of reperfusion. TTC staining revealed that the white infarct area expanded with prolonged reperfusion, though no significant differences were observed among 30, 60, and 120 min of reperfusion. Other studies have indicated that 60 min of reperfusion is sufficient to evaluate changes in cardiac hemodynamics and infarct area in isolated heart models [30]. Based on these rat studies, we designed our model optimization using two ischemia

durations (15 and 30 min) and two reperfusion durations (30 and 60 min).

Comparisons of AST, ALT, CK, and LDH levels in perfusate and myocardial tissue before and after reperfusion, along with myocardial infarct area analysis, demonstrated varying degrees of increase in myocardial enzyme markers and infarct area following reperfusion, confirming successful model establishment. Inter-group comparisons revealed highly significant differences among the five protocols. The 30-min ischemia with 30-min reperfusion and 30-min ischemia with 60-min reperfusion groups showed significantly higher post-reperfusion enzyme levels and infarct areas than the other three groups, with the severity of reperfusion injury correlating positively with enzyme values and infarct size, indicating the most severe myocardial damage in these two groups—consistent with previous findings [27-30]. However, electrocardiographic analysis during these protocols showed that the average heart rate in the 30-min ischemia with 30-min reperfusion group more closely approximated the physiological heart rate of tree shrews, representing a clear advantage. Therefore, this protocol provides a more rational and reliable experimental model. We conclude that the ex vivo Langendorff model of myocardial ischemia-reperfusion in tree shrews was successfully established, with the 30-min ischemia followed by 30-min reperfusion protocol demonstrating optimal performance.

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Note: Figure translations are in progress. See original paper for figures.