Research Article

Cryo-infarction: a method of choice for the establishment of a rat model of myocardial infarction

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Abstract

Ligation of the left anterior descending (LAD) coronary artery has been commonly employed to induce myocardial infarction (MI) in animals. However, this technique has its own drawbacks and is associated with significant mortality rates, caused by high bleeding and unpredictable areas of

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necrotic damages. Cryo-infarction is considered as a better alternative method for creation of myocardial injury with the advantage of increased survival rate of the animal models in comparison with the conventional methods. Current study was designed to to further improve this method as a convenient model for research. First, ventilated male Wistar rats underwent Open-chest surgical procedure with subsequent coronary artery ligation or cryo-induced heart infarction. To validate the MI model, we conducted TTC (2,3,5-triphenyltetrazolium chloride) and immunofluorescence staining, evaluated serum troponin levels, and analyzed Electrocardiography (ECG) data. The severity of MI was also tested by, echocardiography, hematoxylin and eosin staining, and Masson's trichrome staining. Results proved that the Cryo method of inducing myocardial infarction offers several advantages including simplicity, reproducibility, and lower mortality rate. Since true potentials of this method has not been employed very well in research and development, our results could inspire further investigations for establishment of better standard animal models for cardiac research programs in future. This would benefit both basic and translational research in the field.

Abbreviations

LAD: left anterior descending

MI: myocardial infarction

ECG: Electrocardiography

CAD: coronary artery disease

PL: permanent ligation

AS: atherosclerosis

ISO: isoproterenol

LDL: low-density lipoprotein

ROS: reactive oxygen species

PL: permanent ligation

AAR: area at risk

LV: left ventricular

TBS: Tris-Buffered Saline

FS: Fractional Shortening

EF: Ejection Fraction

LVdys: left ventricular dysfunction

Introduction

Myocardial infarction is a condition of acute ischemia leading to irreversible damage to cardiac muscle tissue. This is commonly caused by blood clots that obstruct a coronary artery [1][2]. The clot formation or thrombosis that triggers myocardial infarction can result from disrupted endothelial function, platelet activation, or coagulation factor imbalances, among other factors. The resulting ischemic injury to cardiomyocytes leads to cardiac dysfunction and potential complications such as arrhythmias, heart failure, or sudden cardiac death [3][4]. Myocardial infarction, commonly known as a heart attack, is a condition that researchers often seek to replicate in laboratory animals to study its causes and explore therapeutic interventions.To study the process of the disease in detail, animal models have played very important role in laboratory investigations for many decades [5]. Among these models, rats have gained significant attention due to several advantages, including cost-effectiveness, ease of handling, and genetic similarities to humans. These animals have helped researchers to create MI models and develop many novel therapeutic methods [6]. There are different methods to create the MI model and the success rate of the investigators depends on each method.The common procedures for the MI induction includes; permanent ligation (PL), Chemical Method (Isoproterenol Induced MI Model), and Cryoinjury Method. Also, the high-fat diet method is used to study the pathology of coronary artery disease (CAD) in animals. The high-fat diet method involves feeding experimental animals with rich cholesterol foods for a relatively long time, which induces hyperlipidemia, AS (atherosclerosis), and sclerotic plaques, resulting in stenosis in the blood vessels and myocardial ischemia. Animal models created with a high-fat diet most accurately represent real CAD patients**.** Nonetheless, this approach is labor-intensive and presents challenges in precisely controlling myocardial ischemia. Rats are popular choices to serve asanimal models in CAD investigations because they are easy to maintain, have high survival rates, and are inexpensive to keep. However, the absence of a gallbladder and and their spontaneous anti-AS formation make them less than ideal as perfect models for CAD research. The later character of rats makes it difficult to induce AS lesions by a high-fat diet alone. Therefore, most researches have preferred to use a combination of drugs and/or injury to form AS lesions. Although the morphology of the AS lesion and the rupture position of the plaque in rats are similar to those in humans, this mode of modeling is still different from the natural pathogenesis of human CAD. Drug induction is very simple and usually uses abdominal, caudal, or sublingual vein injection of pituitrin or isoproterenol, which can result in short-term MI.

The isoproterenol (ISO)-induced acute myocardial infarction (AMI) model is a widely used and well-established non-surgical approach in rats. ISO is a chemical compound that produces maximum necrosis in the ventricular subendocardial region and interventricular septum. This model offers several advantages over surgical models, such as a low mortality rate, simplicity, and no chance of post-surgical infections. Rats are commonly preferred for this model, but several other species, like mice and rabbits, are also reported to be used. The ISO-induced MI model is widely used in assessing the cardio-protective activity of natural as well as synthetic compounds [6][7]. However, this model is unsuitable for investigating autolysis recanalization in clinical settings. In the PL technique, an anesthetized rat undergoes left thoracotomy followed by LAD occlusion. This method requires surgical left thoracotomy in anesthetized rats, where the LAD is ligated. This induces total ischemia, leading to irreversible oxygen deprivation, significant AAR infarction, and permanent scar formation. This scarred region is susceptible to pathological remodeling, ultimately contributing to the progression of heart failure. Furthermore, the anatomical site of coronary occlusion significantly influences infarction extent, with proximal ligations yielding larger infarcts than distal occlusions; occlusion closer to the heart's base results in a larger and more severe injury. The PL technique typically produces extensive myocardial damage, with infarcts generally encompassing 30-40% of the total myocardial area [6][8]. A significant benefit of employing the PL method is that it results in a substantial infarction, leading to clearer distinctions in cardiac function between sham-operated and MI animals [9]. The surgical ligation model is a widely used method for inducing acute myocardial infarction by ligating different regions of the coronary artery, with the LAD coronary artery ligation being the most common. This method was first introduced in 1954 by Johns and Olson et al and has since been modified for use in both small and large animals. Mice and rats are frequently utilized in laboratory settings for surgical ligation due to their numerous benefits compared to larger animals. The main limitations of this model are high mortality rate, post-surgical infection, variation in infarct size from 4-59%, requirement of expert hands, and an artificial ventilator. Performing this technique on mice is challenging and necessitates advanced surgical proficiency with microsurgical techniques. Complications can arise due to tissue damage, high perioperative mortality rates, intraoperative bleeding, lung damage or collapse, ligation errors, or inadequate ventilation. Cryoinjury is another method of inducing permanent myocardial injury. In this model, a pre-cooled rod at 190°C is placed on the heart, multiple times, to cause injury, which leads to progressive

structural reorganization and volumetric expansion of the left ventricular chamber [9]. The cryoinjury technique models myocardial infarction through controlled focal freezing of cardiac tissue. The cryo-infarction technique has been explored as a viable method for inducing myocardial infarction in experimental models. The cryoinjury model produces well-defined myocardial damage characterized by histological features typical of infarction, including coagulation necrosis, hemorrhagic regions, inflammatory responses, and subsequent tissue repair with scar formation. This pathological progression ultimately results in heart failure, closely mimicking the outcomes observed in LAD ligation models, while providing a valuable alternative for investigating cardiac injury. Compared to surgical occlusion techniques, the cryodamage approach demonstrates superior technical simplicity and experimental reproducibility, as it eliminates the need for intricate vascular interventions [4]. We propose that cryoinjury provides an optimal balance between reproducibility and physiological relevance for modeling myocardial infarction and associated immune responses in rats. To our knowledge, no prior study has systematically compared this approach with the previously established MI induction methods.

Results

Rat model of Cryo myocardial infarction

Surgical Set-up

The animal is intubated with an endotracheal tube and placed supine on a surgical bed while anesthesia is maintained by Ketamine and Xylazine.

Macroscopic Characteristics Post Cryo-MI

A bright white and sunken area was obvious in the area of cold infarction. After the surgery with the Cryo method, heart attack occurred in the experimental animals (Figures 1, 2). The size of the infarcted area is represented in Figure 2.

Biochemical Indices of the Infarcted Rats

CD68 and caspase 3 were more accumulated in the infarcted heart tissues in comparison to the sham group (Figures 3, 4).

Survival Ratio in the Operated Rats

Qualitative troponin evaluation was performed on all rats, yielding positive results for those in the Cryo and LAD groups, while all rats in the sham group were recorded negative. Out of the 95

rats that underwent Cryosurgery, 28 died within 24 hours after the procedure, while 67 survived. In the case of the 102 rats, operated using the LAD method, 64 did not survive the first 24 hours, leaving only 38 survivors (Table 1)

Table 1: Survival ratio

ECG Analysis of MI Rats

In the current study, we tried ECG Imaging to create further data. Changes in ST-segment elevation are shown in Figure 5. The ECG waveforms of the MI group, after inducing MI, are shown in Figures 5A, 5B. The ECG waveforms of the MI group were completely different, compared to the normal ECG (Figures 5). The ST segment of the ECG, shown in Figures 5A, 5B, was noticeably elevated, suggesting that MI was successfully induced.

Echocardiography Analysis

Echocardiographic measurements were conducted to assess heart function. Notably, significant changes in FS (Fractional Shortening) and EF (Ejection Fraction) parameters were recorded in the Cryo and LAD groups, when compared with the sham group.The FS and EF values decreased during the time intervals after surgery in both Cryo and LAD groups, while remained consistently within a certain range for the sham group (Figures 6, 7).

Sham, LAD-MI, and Cryo-MI operated heart samples

Following surgical procedures, aimed at creating the myocardial infarction model, a reduction in the diameter of the heart wall was observed. The Histological analysis demonstrated a normal architecture and morphology of cardiac myocytes, as shown in Figure 8-I. The histopathological study of heart tissue in the MI models, created by both LAD and Cryo methods, revealed that some sort of necrosis, with infiltration of mononuclear cells, happened in cardiomyocytes (Figures 8-II, 8-III.9.10). The Cryo-MI method exhibits a larger area of fibrosis, compared to the LAD-MI one. Additionally, the extent of the blue area, signifying collagen creation, is 10.99% in the samples of Cryo-MI, whereas it is 6.19% in the LAD-MI samples. These findings indicate that collagen production is more pronounced in the Cryo-MI approach (Figure 11). In the sham gropup, the blue color, indicative of collagen fibers, is absent. However, in the samples with myocardial infarction, there is a substantial and clear increase in collagen fibers within the damaged area, indicating the extent of the damage (Figure 12).

Discussion

Cryoinjury and LAD ligation representing two widely adopted techniques for generation of myocardial infarction in rat models. The cryoinjury technique utilizes controlled application of subzero temperatures to create localized tissue necrosis, resulting in well-defined myocardial infarction. This can be achieved by placing a Cryoprobe directly onto the heart tissue, resulting in the formation of ice crystals and subsequent cellular damage. This method, with a certain level of Cryoprobe and a defined duration of contact with the tissue, allows a controlled anatomical localization and magnitude of the induced tissue damage. On the other hand, LAD method involves surgical occlusion of the left anterior descending coronary artery. This is a major blood vessel that supplies oxygen-rich blood to a significant portion of the heart muscle. By temporarily blocking this artery, blood flow is restricted, leading to ischemia (lack of oxygen) and subsequent myocardial infarction. In comparison, Cryo methods offer the advantage of controlled and localized tissue damage, allowing researchers to target specific regions of the heart. In terms of reproducibility, LAD methods have been extensively utilized and standardized in the scientific community, making them a widely accepted approach. However, Cryo methods, with precise control over the inflicted damage, reduce the death rate significantly. In this study, the Cryo method was shown to significantly reduce the rate of premature mortality. This reduction is attributed to the decreased probability of heart bleeding compared to the LAD method. In the LAD method, there is a high risk of the needle tip to hit the heart and cause bleeding. Successful coronary occlusion demands: (1) stereomicroscopic visualization, (2) precision microinstruments, and (3) extensive surgical expertise. Operator skill remains the primary determinant of procedural success [10]. Inexperienced operators may produce highly variable infarct sizes (10-45% of LV area). Surgeon experience significantly reduces procedural variability, with expert operators achieving consistent infarct sizes (35±5% LV) in murine models. Infarct size directly predicts LV remodeling severity. Notably, murine models exhibit distinct pathophysiological responses compared to humans. However, mice exhibit remarkable compensatory hypertrophy that preserves ejection fraction, even with large infarcts (>30% LV), unlike clinical observations in humans. This gives better chance to researchers for long-term assessment of severe injury post-MI. It is particularly advantageous when investigating long-term post-MI remodeling and testing the efficacy of novel therapies. Nonetheless, the occurrence of permanent occlusion and the resulting extensive infarcts, as generated by this model, is less common in human cases [11][9][12]. The LAD method, on the

other hand, involves surgically occluding the left anterior descending coronary artery, one of the primary arterial conduits delivering oxygenated blood to the cardiac tissue. By temporarily blocking blood flow through this artery, researchers can simulate a heart attack and induce myocardial infarction in the rat model. This method allows for precise control over the location and severity of the resulting damage [13][14][15]. The present study aimed to ferther compare the positive and negative points for these methods in experimental rat animals. We found that 29.48 percent of the rats, under operation with the cryoinjury technique, died after intubation, whereas the mortality rate was 61.24 percent in LAD rats. The 2nd Global MI Task Force defines parameters like the level of troponin-specific biomarkers, ECG abnormalities, and echocardiography as main standards for MI assessment. MI diagnosis requires elevated cTnI levels accompanied by either characteristic electrocardiographic abnormalities and/or clinical symptoms consistent with myocardial ischemia [7]. Accordingly, troponin level and ECG were assessed in current experiments. In addition, histological examinations were performed by hematoxylin-eosin staining and Masson's trichrome staining. TTC staining was also applied to evaluate the size of the infarct. Disruption of the integrity of normal cardiac myocyte membranes may lead to the release of a wide range of biologically active intracellular proteins such as troponins, which serve as established biomarkers of myocardial damage and increase the accuracy of MI diagnosis [16][17]. Today, sensitive and specific markers of heart damage, such as cardiac troponins, which are established biomarkers of myocardial damage and increase the accuracy of MI assessment, are considered as early arrhythmia indicators after MI. Troponins are a set of three proteins (troponin C, troponin T (CTnT), and troponin I (CTnI)) that exist in cardiac and skeletal muscle and regulate the interaction of actin and myosin through calcium. Following myocardial damage, cTnI is liberated into the circulation within 4 to 6 hours after the onset of pain. Given the exceptional tissue specificity and diagnostic sensitivity of cardiac cTnI, it has emerged as the gold-standard biomarker for detecting myocardial infarction in clinical practice. The rapid troponin I test is a simple diagnostic test that uses a combination of particles coated with an anti-troponin I antibody and a marker reagent capable of detecting troponin I in whole blood or serum with a detection limit of 1 ng/ml. The test shows the presence of troponin I in the body through the formation of a colored line on the strip [18]. In current experiments, troponin levels were positive in all rats of the MI model, indicating the correct surgical method and confirming the occurrence of proper MI in the model animal, while this test proved negative for the troponin level in the sham group of rats.

Normal ranges for FS and EF falls within about 30-35% and 65-70% respectively in rats. However, the post-MI echocardiography analyses in our experiments indicated that these values faced major drop of to about 20-25% and 50% for FS and EF respectively.For a more comprehensive evaluation of the efficiency of Cryo method in MI generation, we evaluated the level of CD68 and caspase 3 markers, by immunofluorescent analisis, in the hearts of the examined rats. The level of both marker proteins increased following Cryo-MI induction in the rats compared to the control ones. CD68 marker is an indicator protein for the accumulation of immune cells in the target tissues, showingthe active presence of immune cells where inflammation is most intense. On the other hand, the caspase 3 is a key protein for the signalling of the final stages of cell death and destruction (apoptosis). In the present study, the accumulation of this marker in the heart of the infarcted rats indicates the presence of severe intracellular stress in these cells with possible consequence of cellular death. . Further tissue analysis reveals pronounced inflammatory infiltrates, characteristic of severe inflammatory responses, leading to increased rate of necrosis in the heart over time. Based on these results, it is more likely that the increased cell infiltration may occur as a side effect of acute inflammation, leading to the destruction and death of adjacent cells and further infiltration [20][21]. We conclude here that while Cryoinjury method for MI induction produces similar left ventricular dysfunction as happens with LAD ligation method, it carry some better advantages. For example, using the Cryoinjury method caused attenuated remodeling, more compact scar tissue, and higher survival rates in comparison to the LAD ligation method. Notably, cryoinjury scars persist indefinitely, making this model particularly valuable for studying scar region pathophysiology [4][9]. Cryoinjury generates reproducible, uniformly sized, and uniformly shaped infarcts with precise spatial specificity relative to LAD ligation, making them frequently employed in experimental studies in therapeutic intervention studies in rodents.

Materials and Methods

Animal subjects

Adult male Wistar rats (weighing 250-300g) were kept under standard conditions. The rats were bred in the animal house of Ferdowsi University of Mashhad. They were kept in stable physical conditions with free access to standard food and drinking water at a temperature of 23 °C±2 and a 12-hour light/dark cycle. We conducted all procedures in compliance with the applicable

guidelines and regulations, including the ARRIVE 2.0 guidelines. Fifteen male rats, aged between 60 and 90 days, were randomly allocated to three groups: sham (group 1, n=5), LAD group (group 2, n=5), and Cryo group (group 3, n=5). During the experiments, the animals were randomly divided into three groups using Excel software. This work was supported by the Faculty of Science Research Council at Ferdowsi University of Mashhad [Grant No. 59386] Additionally, This study was supported by the National Institute for Medical Research Development (NIMAD, Grant No 957797) of Iran. The funding body had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.The study was conducted in accordance with ethical standards and received approval from the university's Research Ethics Committee [Approval No: IR.UM.REC.1401.269].

Pre- and post-surgical procedures

Prior to anesthesia, the body weight and temperature of the rats were recorded. Anesthesia was induced with a combination of ketamine (100 mg/kg; Alfasan 10%) and xylazine (1 mg/kg; Interchemie 2%) in a 2:1 ratio. Additionally, meloxicam (1 mg/kg; RazakPharma 2%) was administered intramuscularly as a preemptive analgesic. The depth of anesthesia was assessed by the absence of the toe-pinch reflex before the procedure and monitored periodically throughout.Endotracheal intubation was performed by placing an angiocath (Vaccess I.V.Cannula green) in the trachea and viewing the trachea with a flashlight in a dark room. Mechanical ventilation was performed by connecting the endotracheal tube to the modified Babylog 8000 Drager ventilator (O2-Vol=60, insp. Flow V=2(l/min), Pinsp=30(mbar), (TI=0.2s). The aseptic condition was maintained during the survival surgery. Before surgery, eye ointment was applied to the eyes of the rats to prevent dry eyes. After the surgery, the animal is monitored for body temperature, heart rate, and signs of pain until it is on its feet. The rat is then removed from the ventilator and placed in a cage with a heating pad at one end to keep it warm. Water is provided to the rat during this time. If the rat shows signs of dehydration after surgery, 0.5 ml of sterile saline is injected subcutaneously. The antibiotic enrofloxacin is prescribed immediately after the surgery and during the postoperative recovery at a dose of 10 mg/kg [4][22]. For the rats of all groups, before the surgery, meloxicam was injected at a dose of 1 mg/kg. Also, after surgery and

successful recovery, antibiotic injections were given every 12 hours and painkillers every 24 hours for 3 days [22][23].

Induction of myocardial infarction

Animals were left to reach steady breathing and their chests were opened at the fourth left intercostal space using surgical scissors. The space was opened without cutting the tissue to reduce the risk of bleeding. Then a chest opener was placed in the fourth intercostal space. The pericardium was then opened but remained in place whenever possible [22][24]. The LAD was identified and tied using a 6-0 Prolene (polypropylene blue monofilament 209127) suture [25]. In the Cryo-MI rat, a Cryoprobe, consisting of a 3 mm diameter copper rod, was immersed in liquid nitrogen for two minutes. Following the identification of the heart and removal of the pericardium, Cryoinfarction was induced by applying the Cryoprobe tip to the anterior free wall of the left ventricle for 10 seconds [4]. In sham control rats, the procedure was the same, except that the LAD was not occluded via either method. Then the chest opener was removed and the ribs were sutured using 20 mm 0-4 Prolene (20mm polyglycolate coated violet) suture. After closing the ribs, the ventilator output was cut off for a short time (1-2 seconds) to ensure proper breathing. The skin was closed using a 20 mm 0-4 Prolene suture with a continuous suture pattern [25].

Infarct Size Measurement

Myocardial infarction size was evaluated by TTC staining. Hearts were rapidly excised, flash-frozen at -80°C, and sectioned transversely into 2 mm-thick slices. The sections were incubated in 2% TTC solution (37°C, 30 minutes), followed by 24-hour fixation in 10% neutral buffered formalin. After saline rinsing, the unstained infarcted regions were clearly demarcated from viable brick-red stained myocardium, enabling precise topographic documentation of ischemic damage [26][27].

Immuno-histochemistry Analysis

Excised tissue specimens were immediately fixed in 10% neutral buffered formalin and left for 24-72 hours. A graded ethanol series was then employed to dehydrate the aqueous tissue components, prior to xylene clearing and wax infiltration (70% alcohol for 50 min, 80% alcohol for 50 min, 90% alcohol for 50 min, and 100% alcohol for 50 min). Following xylene treatment, paraffin was applied to solidify and prepare the tissue for shaping and subsequent cutting. The samples were cut with a microtome device (Leica RM2135) to a thickness of 5 μm and placed on a silanized slide. The tissue sections were subjected to antigen retrieval by microwaving in 1X

Tris-Buffered Saline (TBS; Sigma, T5912) until reaching boiling boil, followed by a 20-minute incubation in the heated solution. After three washes (5 minutes each) with phosphate-buffered saline (PBS; Sigma, P4417), samples were permeabilized with 0.3% Triton X-100 (Sigma, T8787) for 30 minutes. Following additional PBS washes, non-specific binding sites were blocked with 10% goat serum (Sigma, G9023) for 45 minutes. Primary antibodies against CD68 (orb388936) and caspase-3 (orb10237), diluted 1:100 in PBS, were applied to sections and incubated for 24 hours at 2-8°C in a humidified chamber to prevent dehydration. After incubation, sections underwent four 5-minute washes with PBS before proceeding to secondary antibody application (CD68:orb388936, caspase3:orb688925). Then, the secondary antibody was added with a dilution of 1:150 and incubated in a 37°C incubator (AriaTeb model) for 1.5 h in the dark. The sample was transferred from the incubator to a dark room and washed 3 times. DAPI (SIGMA-D9542) was added to the sample for 20 min, followed by a washing step (PBS 1X). Glycerol and PBS solution were poured on to the sample, and the slide was subjected to fluorescent photography (Olympus).

Biochemical Analysis of troponin in serum

The blood required for the troponin test was collected 24 hours after surgery from the retroorbital sinus of rats. After collection, it was centrifuged at 3000 × g for 5 minutes at 4°C. The resulting plasma was immediately used to evaluate troponin levels. To check for the presence of troponin in the rat serum, we employed the Cardiac Troponin I Cassette Tape (Vitrotec CTN00-08). This cassette is a rapid and qualitative test based on immunoassay chromatography, specifically designed for detecting troponin I in both whole blood and serum samples. A colored line in the control area confirms the presence of a sufficient sample amount and ensures the correct test performance. For serum samples, 2 drops (equivalent to 50 microliters) were added into the sample compartment, followed by a single drop (40 microliters) of cTnl buffer.

Electrocardiography Analysis

On day 7, electrocardiography was performed on the rats. For this, rats were anesthetized with ketamine and xylazine (ketamine 75 mg/kg and xylazine 10 mg/kg).The experiment was conducted using the PowerLab Electrophysiological Instrument (ML11) from Australia. To record the ECG, two electrodes were connected to the right and left hands of the rat and values for FS and EF parameters were recorded. Echocardiography-based FS is widely used to evaluate left ventricular

dysfunction (LVdys). It is a measure of the percentage of blood ejected each time the heart is compressed (systole). EF is a measure of the heart's pumping efficiency and is expressed as a percentage. It quantifies the fraction of blood ejected from the left ventricle with each heartbeat. EF is calculated by dividing the stroke volume (the amount of blood pumped out of the heart) by the end-diastolic volume (the total amount of blood in the heart's left ventricle at the end of diastole) and multiplying it by 100. A normal ejection fraction in Rats typically ranges between 65% and 75%, indicating a healthy heart that effectively pumps blood. A heart that fails to supply sufficient blood to satisfy the body's demands (i.e., heart failure) has a reduced EF. FS and EF parameters provide vital information about cardiac function and are often used in clinical practice to assess heart health, diagnose heart failure, monitor treatment efficacy, and guide therapeutic interventions. Rats were anesthetized with ketamine and xylazine (ketamine 75 mg/kg and xylazine 10 mg/kg), and their chest hair was removed. Echocardiography was performed one day before surgery and on days 1, 28, and 55 after surgery (MyLab 30Gold VET, with an 8-10 MHz transducer).

Histology

Hematoxylin-Eosin staining Staining

Hematoxylin-Eosin staining was used to observe the morphological changes of the heart tissue and to confirm the myocardial infarction. Tissue samples were collected 7 and 55 days post-surgery and placed in 10% formalin. 5 µm sections were prepared and stained with hematoxylin-Eosin to examine the areas of necrosis, and image analysis was done with a microscope (Olympus BX-51 camera).

Masson’s trichrome

This method is used to detect collagen fibers in heart tissue. Collagen fibers are colored blue, nuclei are colored black, and the background is colored red. For this staining, tissues were collected 55 days post-surgery, fixed in 10% neutral buffered formalin for 24 hours, and embedded in paraffin. Serial 5-µm sections were then obtained from regions of interest using a microtome [28][29]. Images of heart tissue sections were taken with an OLYMPUS loupe (SZH-ILLK, JAPAN).

Statistical analysis

Cell image expression percentages were measured using ImageJ software, and statistical analyses for the Caspase3 and CD68 factors were performed using PRISM software. These analyses were conducted for both the sham and Cryo-MI groups.

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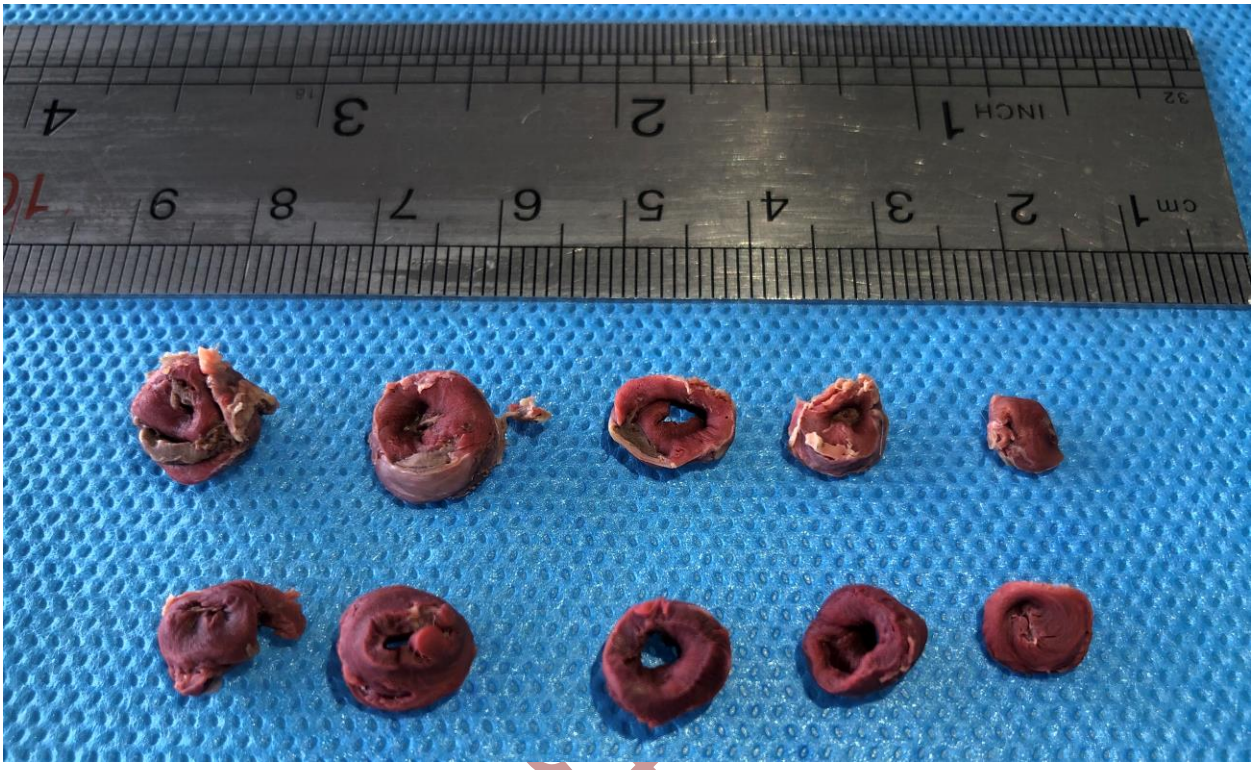

Figure 1: TTC staining of MI in rat heart tissue induced by the Cryo method(up) compared to the sham group (down) at 24 hours post-surgery.

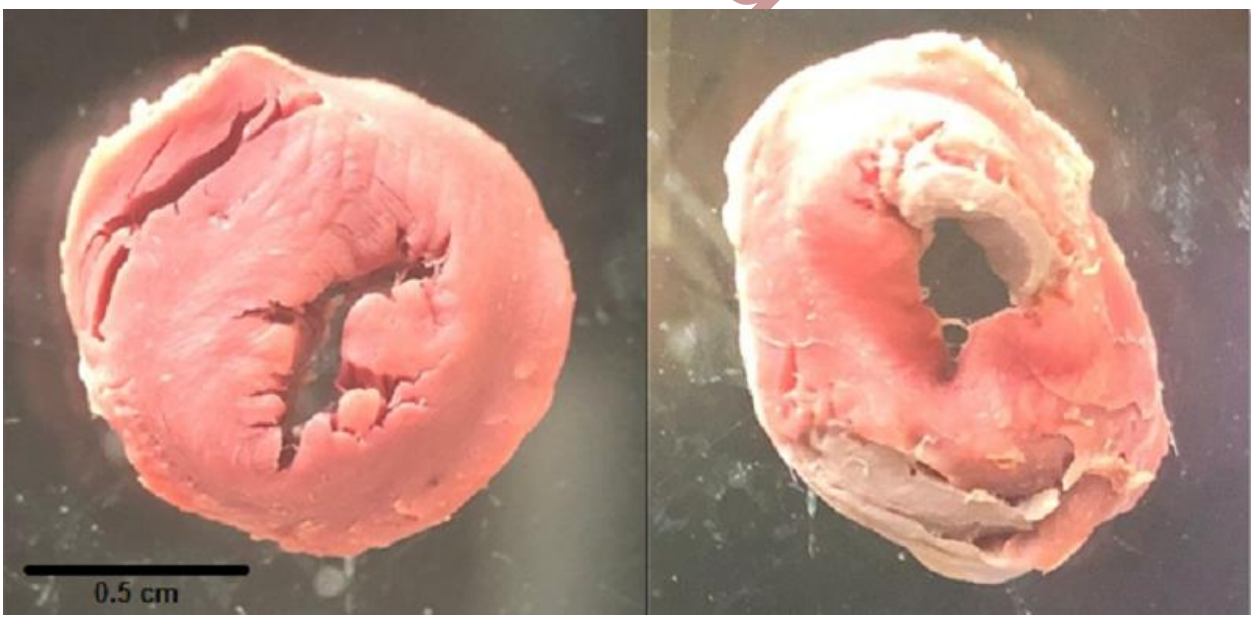

Figure 2: Representative TTC-stained heart sections from sham (left) and Cryo-MI (right) groups at 24 hours post-surgery. The infarcted area (white) in the Cryo-MI sample was quantified as 12.52% using ImageJ software.

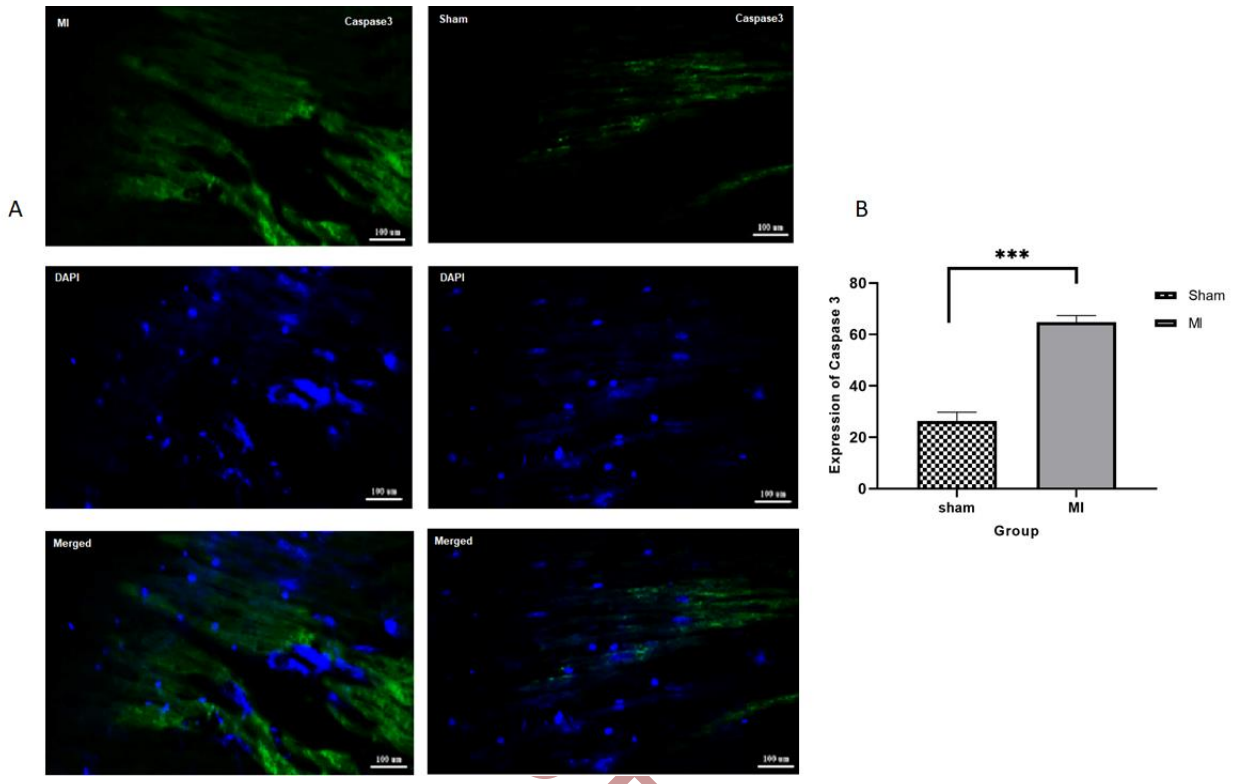

Figure 3: A: Immunofluorescence analysis of caspase-3^{+} cells in sham and Cryo-MI groups. Caspase-3^{+} cells (green) were quantified and expressed as a percentage of total nuclei (DAPI, blue). B: Caspase-3 expression is significantly increased in Cryo-MI compared to sham group.

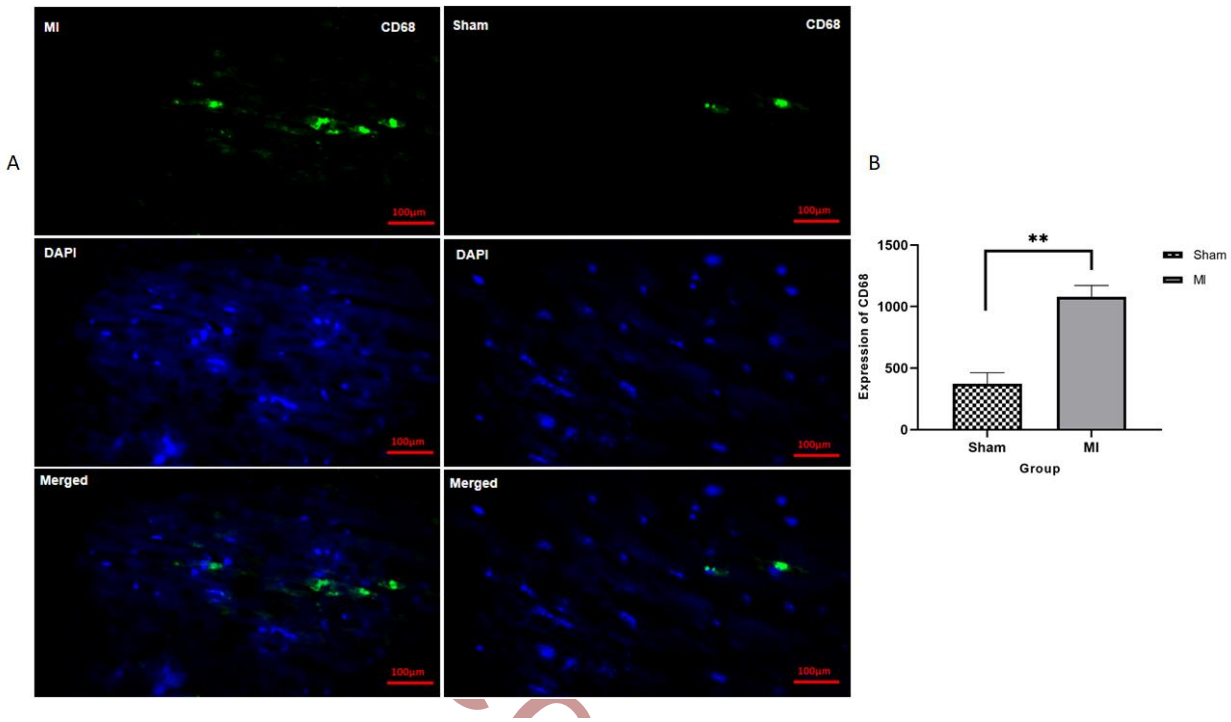

Figure 4: A: Immunofluorescence staining and quantification of CD68⁺ macrophages in sham and Cryo-MI groups. CD68⁺ cells (green) were quantified as a percentage of total nuclei (DAPI, blue). B: CD68+ macrophage infiltration is markedly elevated in Cryo-MI versus sham controls.

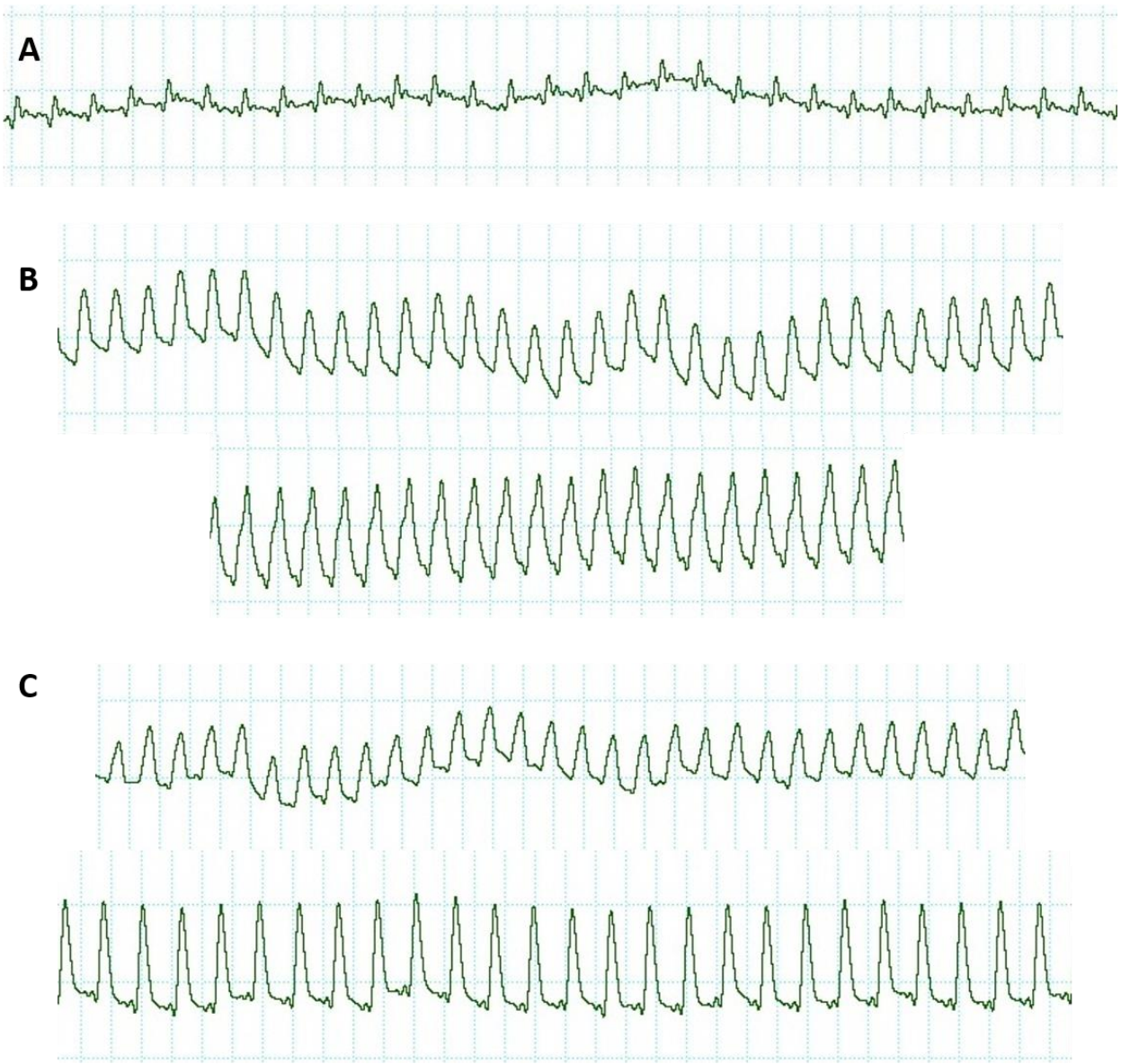

Figure 5: A: Representative ECG waveforms from sham-operated control group. B: Representative ECG waveforms following LAD-induced myocardial infarction. C: Representative ECG waveforms following Cryo-induced myocardial infarction.

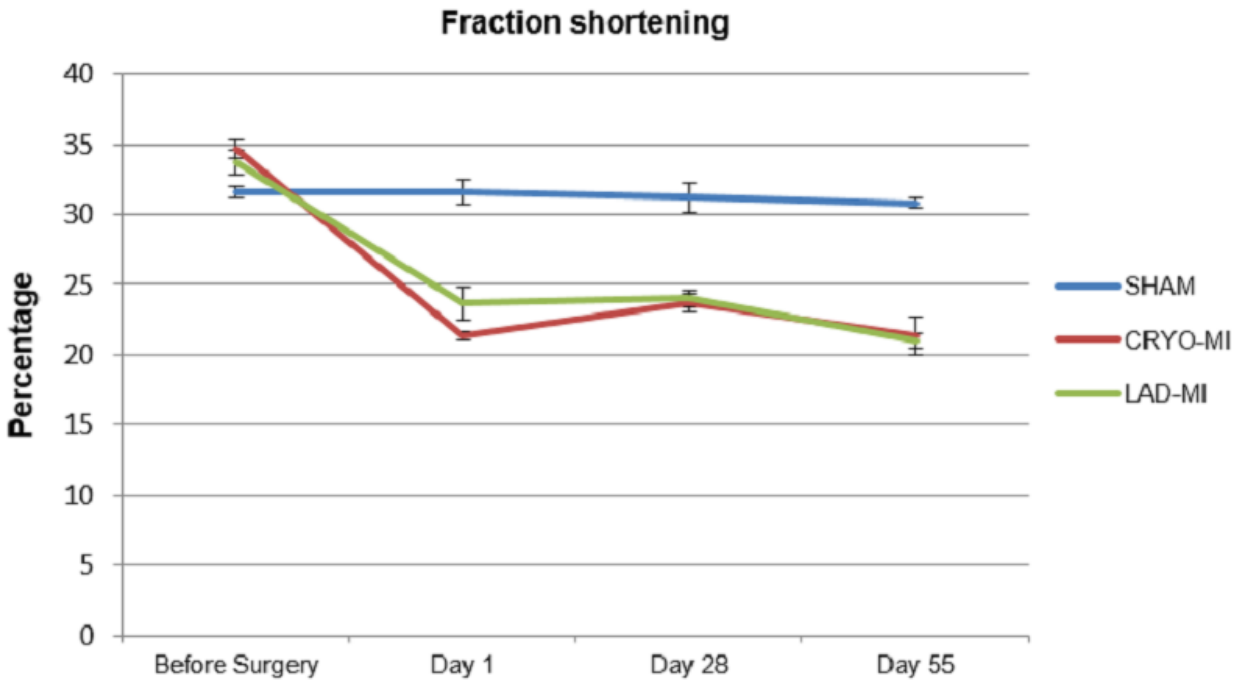

Figure 6: Longitudinal changes in FS following myocardial infarction. Data show FS measurements at pre-surgery baseline, day 1, day 28, and day 55 post-procedure for sham (n=5 for FS), Cryo-MI (n=3), and LAD-MI (n=3) groups.

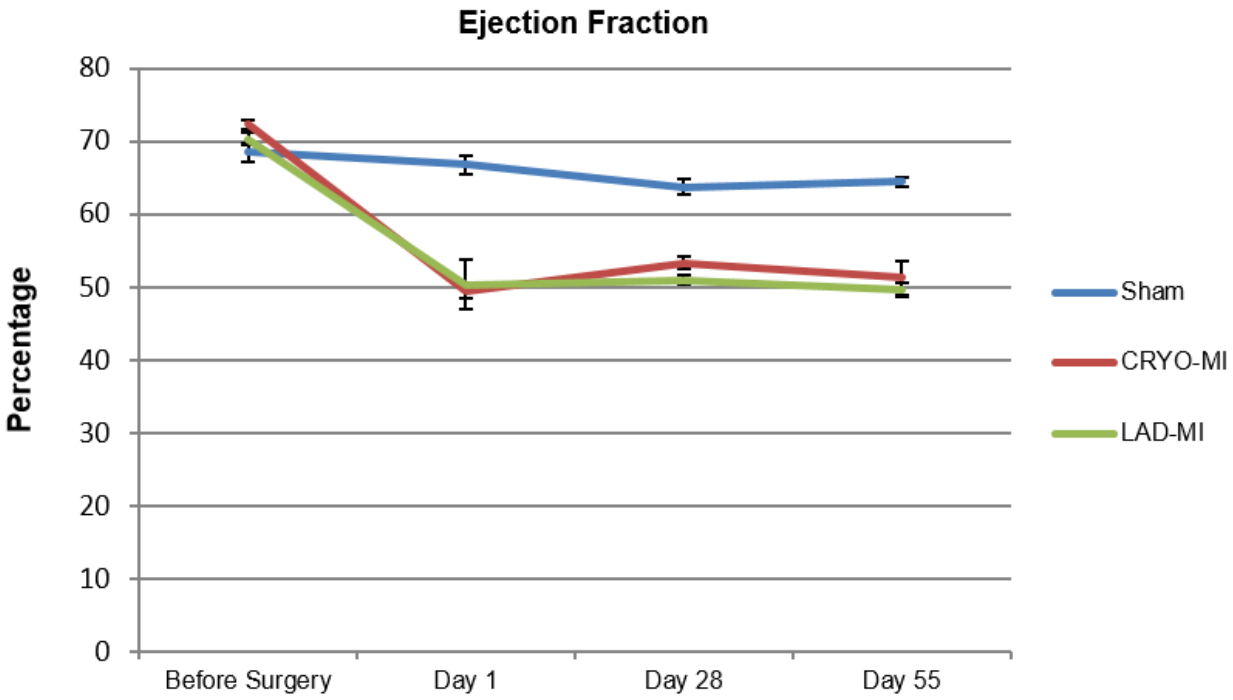

Figure 7: Longitudinal changes in EF following myocardial infarction. Data show EF measurements at pre-surgery baseline, day 1, day 28, and day 55 post-procedure for sham (n=4 for EF), Cryo-MI (n=3), and LAD-MI (n=3) groups.

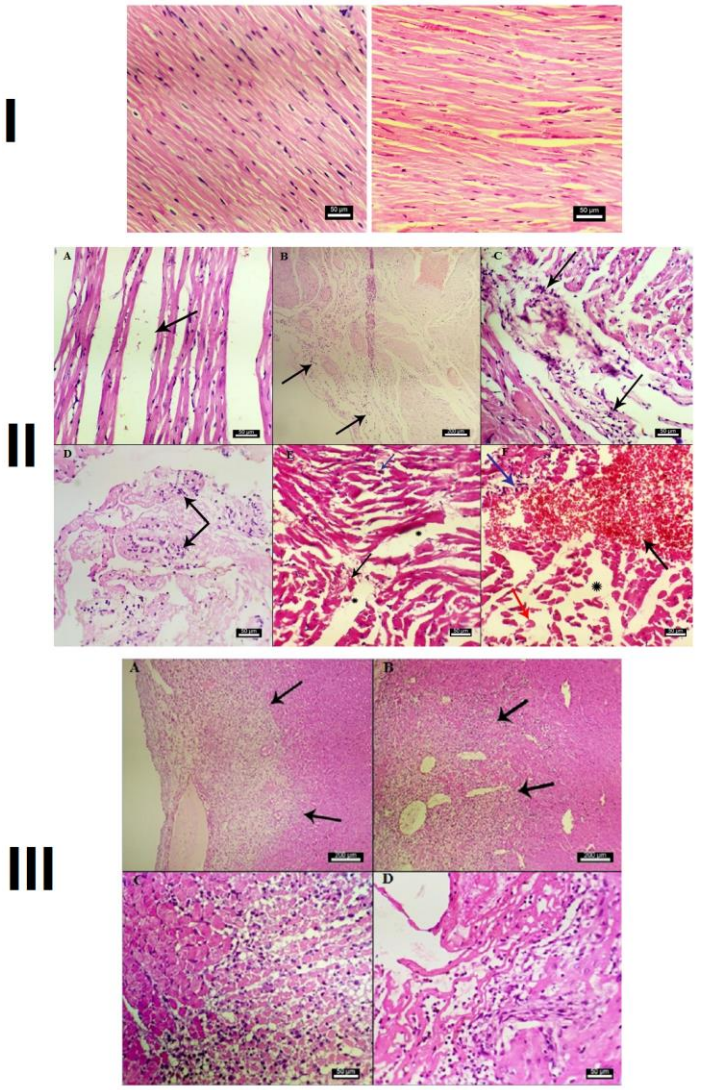

Figure 8: I. Sham group heart tissue showing normal histological architecture. (H&E, 400× magnification). Representative micrograph demonstrates preserved cardiomyocyte morphology and intact tissue organization with no pathological alterations. II. Histopathological features of myocardial tissue 7 days post-LAD ligation. (A) Interstitial edema with widened intramuscular spaces (arrow; H&E, 400×). (B) Pericardial mixed inflammatory infiltrate (arrows; H&E, 100×). (C-D) Cardiomyocyte degeneration and necrosis with mononuclear cell infiltration (arrows; H&E, 400×). (E) Co-localization of myocyte necrosis (black arrow), inflammatory infiltrate (blue arrow), and interstitial edema (stars; H&E, 400×). (F) Hemorrhagic necrosis showing degenerated cardiomyocytes (red arrow), hemorrhage (black arrow), inflammation (blue arrow), and edema (star; H&E, 400×). All images represent characteristic findings from the infarct border zone. III. Histopathological features of myocardial tissue 7 days post-Cryo injury. (A,B) Necrotic cardiomyocytes with mixed inflammatory infiltrate (mononuclear and polymorphonuclear leukocytes, arrows; H&E, 100×). (C) High-magnification view of necrotic myocardium with leukocyte infiltration (H&E, 400×). (D) Pericardial mixed inflammatory cell infiltration (H&E, 400×). All sections show characteristic interstitial edema and eosinophilic changes in the cryo-injured region.

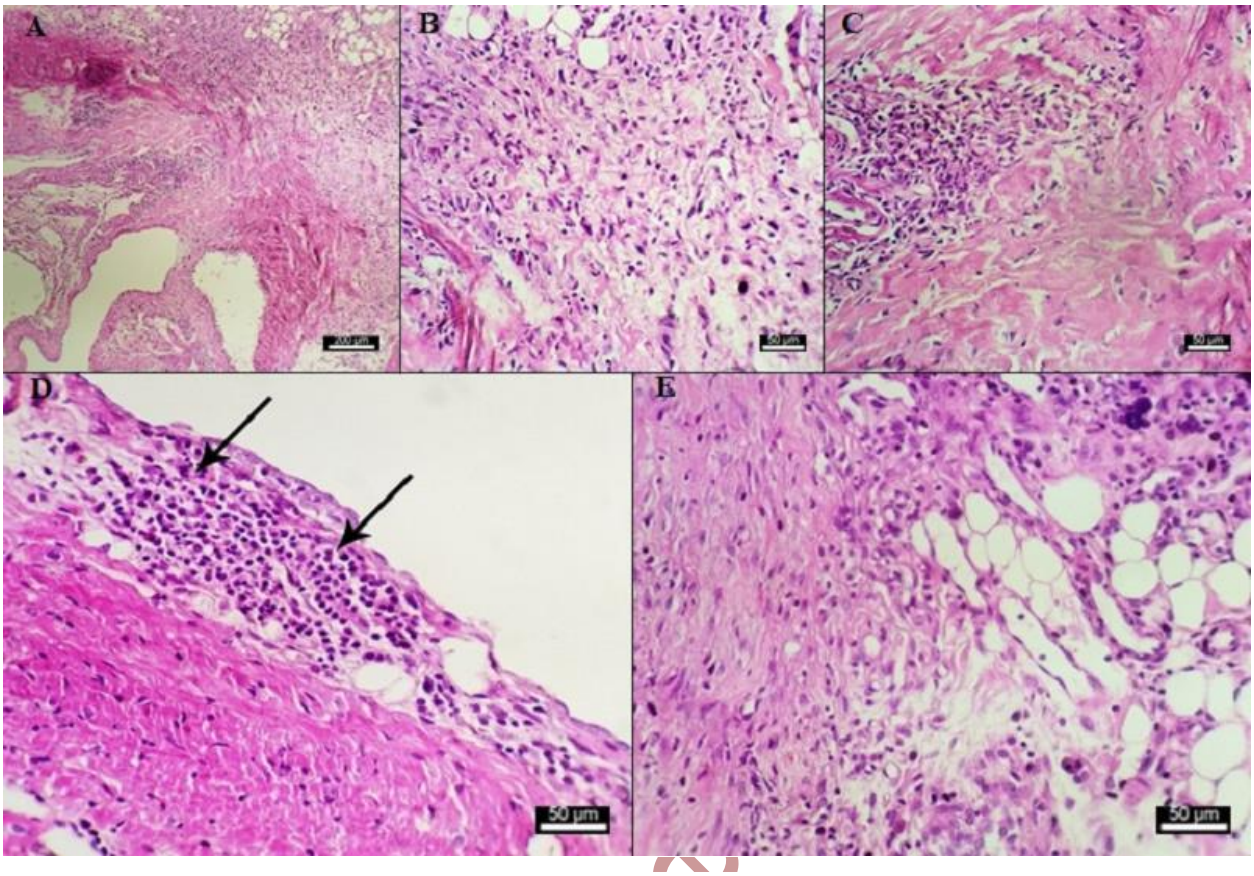

Figure 9: The representative heart sections obtained 55 days after LAD ligation. A, B, C: Cardiomyocyte necrosis associated with granulocytes and mononuclear cells infiltration and also, granulation tissue formation including fibroblast proliferation and early collagen (H&E, ×100). D: Mixed inflammatory cell infiltration in the pericardium (arrows) (H&E, ×400). E: Mixed inflammatory cell infiltration into the thickened and fibrotic pericardium (H&E, ×400).

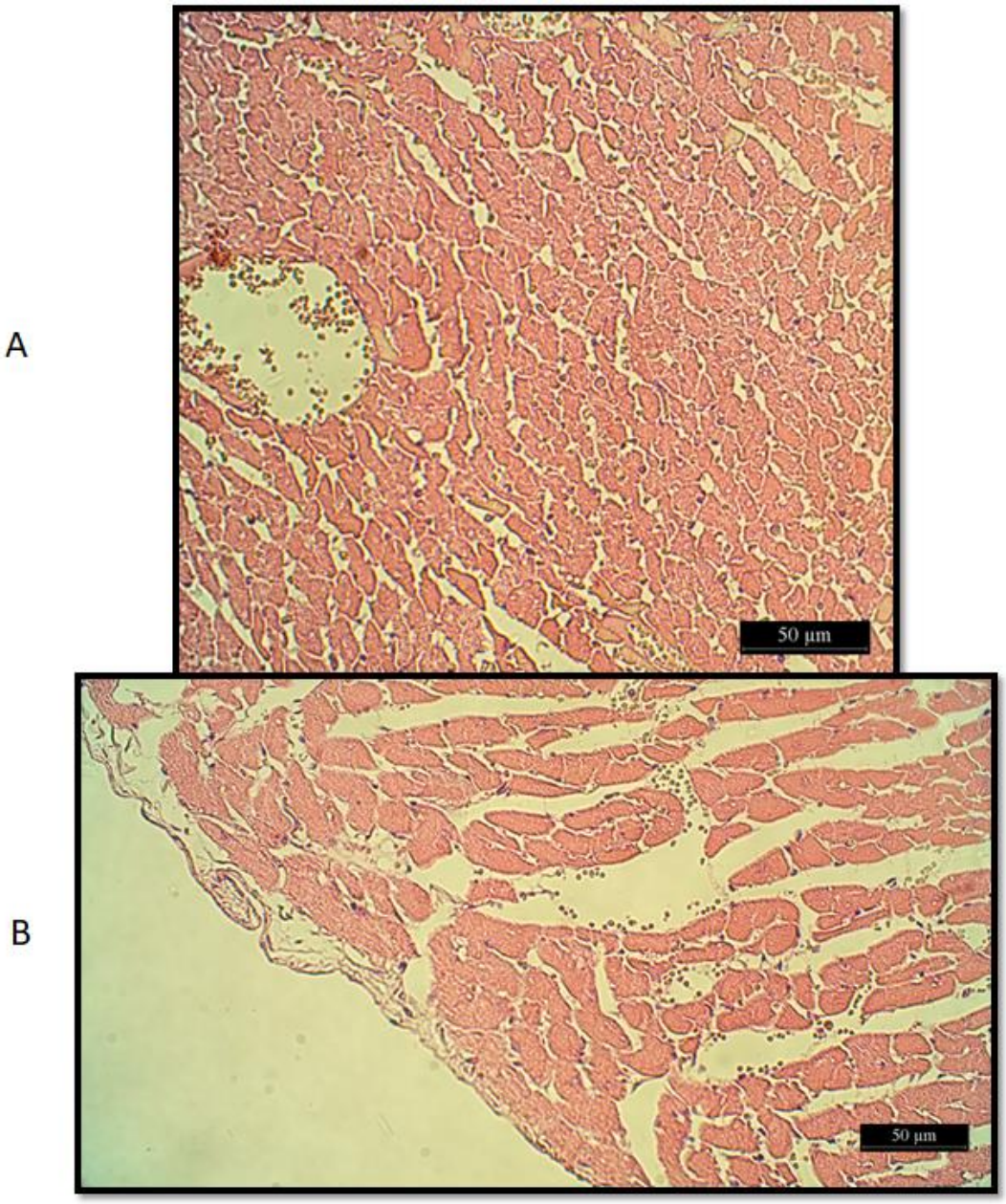

Figure 10: The representative heart sections obtained 55 days after Cryo injury. The image confirms successful model induction, illustrating the end-result of the wound healing cascade following a large myocardial infarction. The replacement of functional myocardium with a non-contractile fibrotic scar and the accompanying hypertrophic remodeling in the border zone are key pathological substrates for the development of chronic heart failure. A: The most prominent feature is a large, acellular, and densely collagenous scar tissue (indicated by the pale, homogeneous area). B: Adjacent to the fibrotic scar, the surviving myocardium shows evidence of compensatory remodeling. The cardiomyocytes appear hypertrophied (enlarged), a common adaptive response to the increased hemodynamic load on the remaining viable tissue (H&E, ×400).

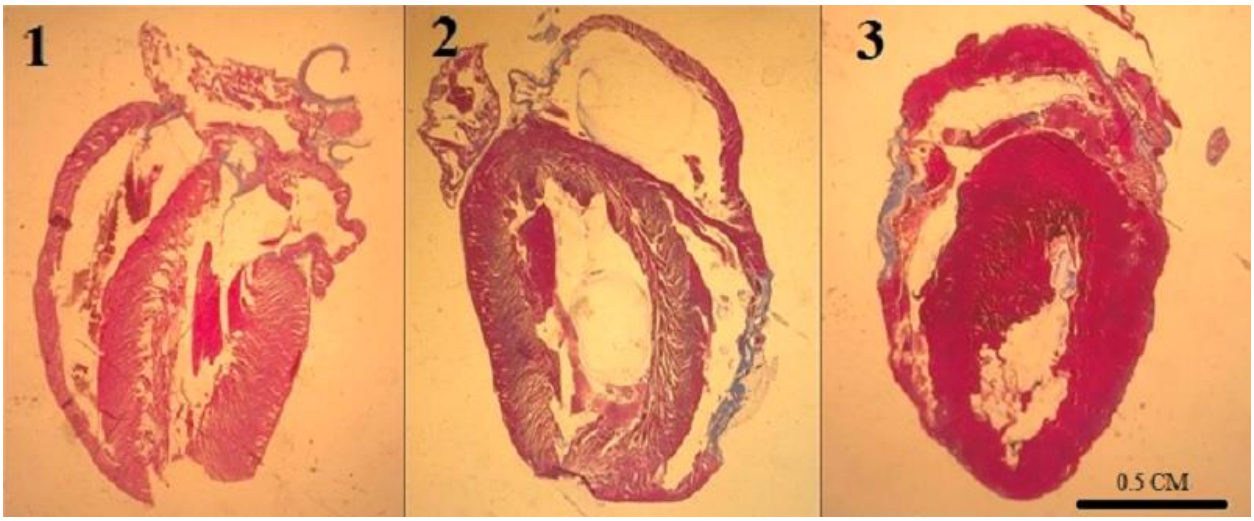

Figure 11: Fibrotic remodeling 56 days post-myocardial infarction (Masson's trichrome stain). (1) Sham group shows no detectable collagen deposition (blue). (2) Cryo-MI group demonstrates extensive fibrotic replacement (blue) with significant wall thinning. (3) LAD-MI group exhibits collagen deposition (blue) in the infarct zone.

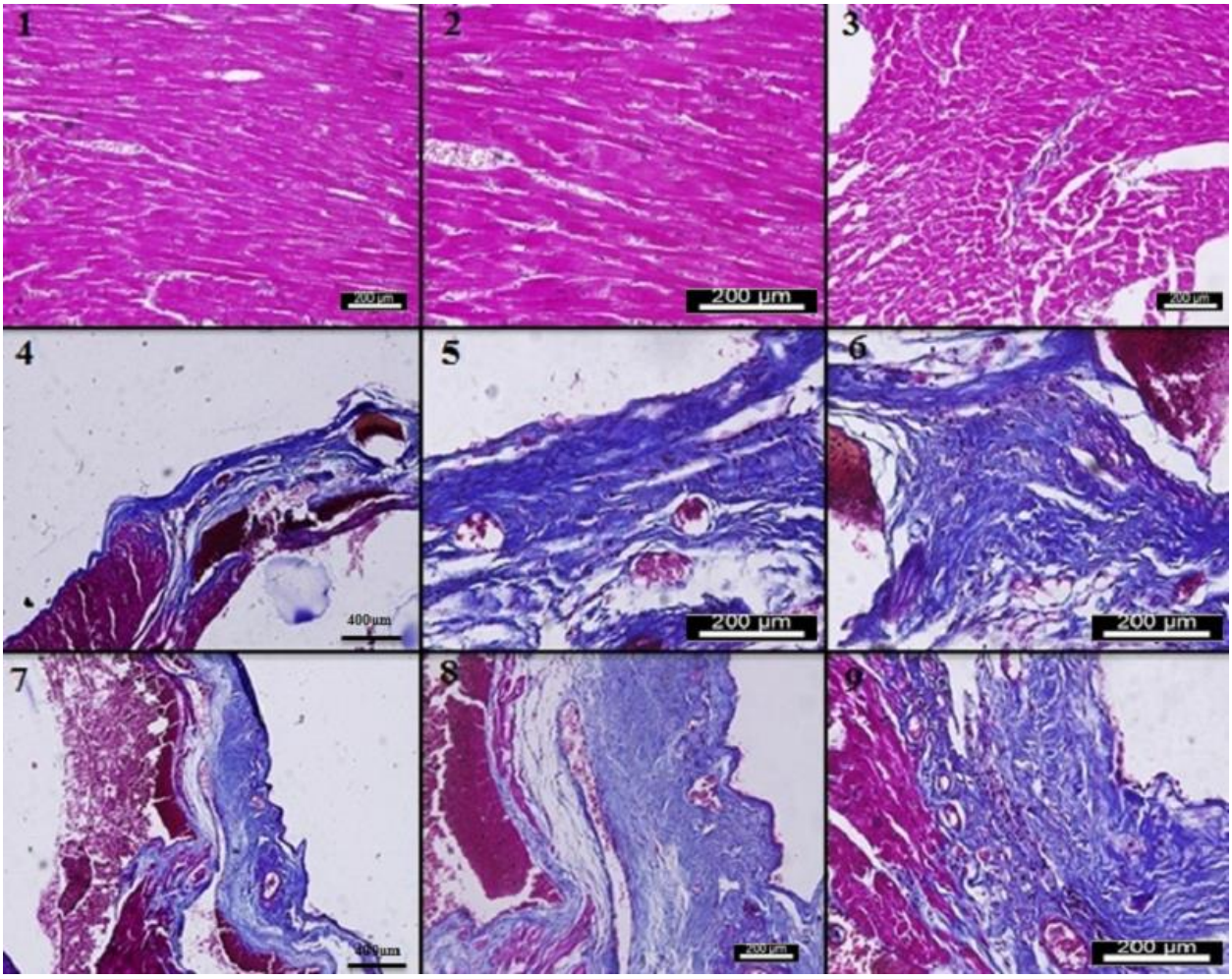

Figure 12**:** Cardiac fibrosis assessment 56 days post-surgery (Masson's trichrome). (1-3) Sham group shows normal myocardial architecture without collagen deposition. (4-6) Cryo-MI group exhibits extensive fibrotic replacement (blue) with significant wall thinning. (7-9) LAD-MI group displays collagen deposition (blue) in the infarct zone.