Evaluation of morphological changes in experimental models of myocardial infarction: Electron and light microscopical evidence

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Electron and light microscopy are most widely used scientific techniques to assess the morphological changes that have incurred in the myocardium following acute myocardial infarction. The present chapter discusses about pathological changes in two well established experimental models of acute myocardial infarction i.e. Isoproterenol (ISO) and Ischemia-reperfusion (I-R) injury induced myocardial infarction. Pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) protein expression in these two models have also been highlighted using immunohistochemistry staining. In addition, Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was also done to evaluate the DNA fragmentation by labeling the terminal end of nucleic acids. Henceforth, this chapter provides an insight to the readers regarding evaluation of morphological changes using the above said parameters in sham, ISO control, I-R control and drug treated group.

Key words: Isoproterenol, Ischemia-reperfusion injury, Acute myocardial Infarction, TUNEL, Electron microscopy.

1. Introduction

Acute myocardial infarction (MI) being a disorder of cardiovascular disease is an immense health problem globally causing mortality and morbidity across the developing and industrialized countries. Acute MI is a state of pathological condition which is associated with ventricular remodelling and occurs as a result of increased myocardial metabolic demand and/or decreased delivery of oxygen and nutrients to the myocardium via coronary circulation. As the insult progresses, a sequel of morphological changes initiates the process of necrosis (traumatic cell death), apoptosis (programmed cell death) and autophagy which ultimately leads to progressive decline in left ventricular performance.

Since MI being a chronic response and involves an interface of multiple pathophysiological processes, it necessitates the use of those in vivo models for the study of MI which mimics the response as that of human MI. For many years, surgical rodent models acts as a tool to understand MI and attracted a lot of considerable attention in translation of various pathophysiologically findings to humans. Impaired cardiac energetics, activation of renin-angiotensin system, inflammatory response and activation of sympathetic nervous system are the few examples which mirrors to those of human response. Although, there are different in vivo models of acute MI but the most popular and well documented model for MI is the left anterior descending coronary artery ligation and subsequently reperfusion model of rat. Furthermore, in this chapter we will also study the histopathological and ultrastructural changes that occur in isoproterenol induced model of myocardial damage that causes global ischemia and necrosis. Both the models, derives their popularity because of their reproducibility, ease of use, feasibility to evaluate an array of biochemical and hemodynamic variables and most significantly, the resemblance of developed infarct to that seen in human MI. Therefore, aim of this chapter is to provide readers a brief overview of the microscopic evidence of morphological changes that occur in two models of myocardial infarction viz. isoproterenol and ischemia reperfusion injury induced myocardial infarction.

Isoproterenol, a synthetic non-selective β-adrenoceptor agonist, owing to its positive chronotropic and inotropic effects has been reported to produce ischemia-like condition; infarct-like lesions particularly marked in subendocardial regions of left ventricle and interventricular septum, myocyte damage and contractile dysfunction together which contribute to the development of acute MI.

On the other hand, induction of myocardial infarction in rats by coronary artery ligation was first introduced way back in 1960’s. Initially it was done without mechanical ventilation which consequently resulted in large mortality. But thereafter advances in techniques have not only improved survival rate but also helps the researchers to understand the disease status more precisely. Pathological changes such as complete disruption of myofilaments with hyper-contracted bands and swelling of mitochondria are hallmark of I-R injury. In particular, angiotensin-converting enzyme (ACE) inhibitors which serve as the first line drug in the treatment of MI provided significant evidence for long term survival rates in this model only, which were later replicated in humans.
2. Methods and materials

2.1 Experimental protocol

All the animals were divided into six groups with 14 animals in each group.

Group 1 rats (Sham): Received oral administration of 0.5% hydroxyethyl cellulose (3 ml/kg/day) for 14 days and on 13th and 14th day normal saline (0.3 ml, s.c.) was given at an interval of 24 h.

Group 2 rats (Vehicle + isoproterenol): Received oral administration of 0.5% hydroxyethyl cellulose (3 ml/kg/day) for 14 days along with concurrent administration of isoproterenol (85 mg/kg, s.c. at 24 h interval) on 13th and 14th day.

Group 3 rats (Ramipril 1.25mg/kg + isoproterenol): Received oral administration of ramipril suspended in 0.5% hydroxyethyl cellulose for 14 days and on 13th and 14th day isoproterenol (85 mg/kg, s.c.) was given at an interval of 24 h.

Group 4 rats (I+R Sham group): Normal saline was administered for 7 days and then only thread was passed beneath coronary artery but not ligated.

Group 5 rats (I-R control group): Normal saline was administered for 7 days prior to 45 min of ischemia followed by 1hr of reperfusion.

Group 6 rats (I-R drug treated group): Ramipril 1.25 mg/kg was given for 7 days prior to 45 min of ischemia followed by 1hr of reperfusion.

2.2 Induction of experimental myocardial infarction

2.2.1 Isoproterenol induced myocardial damage

Isoproterenol (85 mg/kg) was injected subcutaneously (s.c.) to rats daily for two consecutive days i.e. on 13th and 14th day respectively with 24 h interval to induce experimental myocardial infarction.

2.2.2 Ischemia- reperfusion injury induced myocardial infarction

Rats of all the experimental groups were anesthetized intraperitoneally with pentobarbitone sodium (60 mg/ kg). Atropine was co-administered with the anesthetic to keep the heart rate elevated especially during the surgery protocol and reduce broncho-tracheal secretions. The body temperature was monitored and maintained at 37°C throughout the experimental protocol. The neck was opened with a ventral midline incision, and a tracheostomy was performed and the rats were ventilated with room air from a positive pressure ventilator (Inco, India) using compressed air at a rate of 70 strokes/min and a tidal volume of 10 ml/kg. The right carotid artery was cannulated and the cannula filled with heparinised saline was connected to the cardiac output monitor CARDIOSYS CO-101 (Experimetria, Hungary) via a pressure transducer for measurement of MAP and HR. The left jugular vein was cannulated with polyethylene tube for continuous infusion of normal saline solution.

A left thoractomy was performed at the fifth intercostal space and the pericardium was opened to expose the heart. The left anterior descending coronary artery (LAD) was ligated 4–5 mm from its origin by a 5-0 silk suture with a traumatic needle and ends of this ligature were passed through a small vinyl tube to form a snare. After the completion of the surgical procedure, the heart was returned to its normal position in the thorax. Myocardial ischemia was induced by one stage occlusion of the LAD for 45 min by pressing the polyethylene tubing against the ventricular wall and then fixing it in place by clamping the vinyl tube with a hemostat. A wide bore (1.5 mm) sterile metal cannula was inserted into the cavity of the left ventricle from the posterior apical region of the heart. The cannula was connected to a pressure transducer (Gould Statham P231D) for measuring the hemodynamic parameters.

The animals then underwent 45 min of ischemia, confirmed visually in situ by the appearance of regional epicardial cyanosis and ST-segment elevation. The myocardium was reperfused by releasing the snare gently for a period of 60 min. Successful reperfusion was confirmed by visualization of arterial blood flow through the artery, appearance of hyperemia over the surface of the previously ischemia cyncotic segment. At the end of reperfusion period, animals were sacrificed for immunohistochemical, histological and electron microscopy studies by an overdose of anaesthesia.

2.3 Histopathological studies

At the end of the experiment, myocardial tissue was immediately fixed in 10% buffered formalin solution. The sections obtained were stained with hematoxylin and eosin (H&E) and visualized under light microscope to study the light microscopic architecture of the myocardium. The pathologist performing the histological evaluation was blinded to treatment allocation.
2.4 Ultrastructural studies by Transmission Electron Microscope (TEM)

The Karnovsky's fixed tissues were washed in phosphate buffer (0.1 M, pH 7.4, 6 °C) and post fixed for 2 h in 1% osmium tetroxide in the same buffer at 4°C. The specimens were then washed with phosphate buffer, dehydrated with graded acetone and then embedded in araldite CY212 to make tissue blocks. Semithin (1 µm) as well as ultrathin sections (70–80 nm) was cut by ultra microtome (Ultracut E, Reichert, Austria). The sections were stained with uranyl acetate and lead acetate and examined under a Transmission Electron Microscope (Morgagni 268D, Fei Co., The Netherlands) operated at 60 kV by a morphologist blinded to the groups studied. At least three hearts from each group were examined for ultrastructural changes.

2.5 Apoptotic studies

2.5.1 Immunostaining for the localization of Bax and Bcl-2 proteins

A monoclonal mouse anti-human Bcl-2 and Bax proteins as the primary antibody were used for Bcl-2/Bax immunohistochemical staining. The ImmunoCruz Staining Systems utilizes a horseradish peroxidase (HRP)-streptavidin complex for staining of formalin-fixed paraffin-embedded myocardial sections. Briefly, 4–6µ thick fixed paraffin tissue sections were subjected to the immunohistochemical procedure for the localization of Bax and Bcl-2 proteins using specific mouse monoclonal primary antibodies. Sections are first blocked and then incubated in primary antibody. Biotinylated secondary antibody is added followed by the addition of HRP-streptavidin complex. The target protein (Bax/Bcl-2) was visualized by incubation in peroxidase substrate (H2O2) using DAB (3,3′ dianinobenzidine) as the chromogen. Observation of 50 myocytes for each specimen and six specimens were included, i.e. total 300 myocytes (approximately) counted for each group. The change in Bcl-2 and Bax percentage was defined as: (%) = Bcl-2 and Bax cells/total cells X 100.

2.5.2 Terminal Deoxyribonucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL Assay)

TUNEL technique was used to quantify the myocardial apoptosis which was evaluated by detection of DNA fragmentation. The protocol for obtaining cryosections was same as under the histopathology section. DNA fragmentation in the tissue sections was determined using an in situ cell death detection kit, POD (Roche, Germany). Briefly, the sections were fixed in 4% paraformaldehyde in phosphate-buffered saline for 15 min at room temperature and then washed with PBS for 15 min. The sections were incubated with proteinase K (1 µg/ml) for 30 min to permeabilize the cell membrane, again washed with PBS and treated with 3% H2O2 to block the endogenous peroxidase. The slides were incubated with TUNEL mixture (containing terminal deoxynucleotidyl transferase (TdT) and fluorescein-dUTP), the enzyme TdT catalyses the attachment of fluorescein-dUTP to free 3′OH ends in the DNA. The incorporated fluorescein was then detected by using anti-fluorescein antibody conjugated with POD (peroxidase substrate) that generates a brown-colored product with diaminobenzidine (DAB). The sections were counterstained with hematoxylin to visualize normal nuclei. The slides were then dehydrated in graded alcohols, mounted with DPX and visualized under light microscope. Total cell counts and TUNEL-positive cells in the specimens were determined by means of a light microscope. The cells with clear nuclear labeling were defined as TUNEL positive cells. The apoptotic cells i.e. TUNEL positive cells were expressed as percentage of normal nuclei.

Observation of 50 myocytes for each specimen and six specimens were included, i.e. total 300 myocytes (approximately) counted for each group. The apoptotic index was defined as follows: apoptotic index (%) = apoptotic cells/total cells X 100.

3. Key findings

Morphological evaluation as confirmed by histopathology and electron microscopic studies at both cellular and subcellular levels in the rat myocardium is as follows:

3.1 Light microscopic features of sham-operated rat hearts revealed normal structural architecture of myofibrils (NMF) without any fraying or infarction (Fig. 1-A). Contrastingly, in Fig. 1-B histopathological section showed severe necrotic patches (NP) of myocardial tissue along with interstitial edema (IE) presented the dilapidated state of myocardium in ISO control group. In addition focal confluent necrosis of muscle fibers with inflammatory cell infiltration (ICI), vacuolar degeneration and myophagocytosis along with extravasation of red blood cells (RBC) was also observed. In drug treated group (Fig. 1-C), there were only focal necrosis with mild interstitial edema and lesser inflammatory cell infiltration as compared to ISO treated group.
3.2. Electron micrographs of ISO treated hearts showed moderate to severe ultrastructural alterations. The myocytes revealed sarcolemmal rupture (SR) and irregular or even disrupted myofilaments (DMF) pattern. Furthermore, swelling of mitochondria (SM), disoriented and dissolved cristae (DC) and increase in number of granules (G) was also a hallmark of ISO mediated injury (Fig 6-B). The sham and drug treated group resulted in almost similar ultrastructural alteration with normal structure of myofilaments (F), well preserved mitochondria and lesser number of granules (Fig 6-A, 6-C).
3.3 In sham-operated group, TUNEL negative reaction was observed (3-A, 4-A). Similarly, in drug treated group, we did not observe any TUNEL positive cells (TP) (3-C, 4-C). Paradoxically, ISO and I-R treated rats showed TUNEL positive cardiomyocyte which indicates DNA extract contained necrotic and few apoptotic cells (3-B, 4-B). In addition, changes in TUNEL positivity in different experimental groups are depicted in Table 1.
3.4. Histopathology of sham-operated hearts revealed normal and well preserved normal integrity of cardiomyocytes without any pathological changes (Fig. 2-A). In ischemic-reperfused hearts marked coagulative necrosis (MCN), hyper-contracted myofibrils (contraction band, CB), inter myocardial fiber hemorrhage, extravasation of both polymorphonuclear and mononuclear cells (ICI), vacuolization and edema (E) was observed (Fig. 2-B). Drug pretreatment significantly reduces the coagulative necrosis, hyper-contracted myofibrils and edema (Fig. 2-C).
Fig. 2. Light photomicrographs of representative rat heart sections (H&E, 200X) from different experimental groups: 1-A: Sham group, 1-B: I+R control group, 1-C: Drug treated group.

3.5. Electron microscopy of I+R hearts showed myofibrillar derangement (MD) and degeneration, swollen mitochondria (SM) with thickened, disoriented and dissolved cristae, flocculent density deposition, breaks in the outer mitochondrial membrane, disassembly of nuclear lamina, markedly clumped chromatin (CC) randomly dispersed in the nuclei (N), vacuoles (V), lipid droplets and infiltrative cells (Fig. 5-B). All these changes are hallmark of necrosis. Apoptotic features such as condensation and margination of chromatin and nuclear membrane breakage (NMB), mitochondria with disarrayed cristae were also observed simultaneously (Fig. 5-B). Drug treatment group shows region of normal architecture with well preserved integrity (Fig. 5-C). In sham treated groups normal architecture of myocardial filament (F) with well preserved mitochondrial integrity was observed (Fig. 5-A).
3.6. Bcl-2 and Bax are the members of family of proteins that regulate apoptosis. Light micrograph pictures of changes in Bax and Bcl-2 protein expression using immunohistochemical staining are enlisted in Figure 7 and 8. In addition, changes in Bax and Bcl-2 protein expression in different experimental groups are depicted in Table 1.
Fig. 7. Representative photomicrographs of ventricular tissue stained for Bax protein (H&E, 200X) from different experimental groups: 7-A: Sham group, 7-B: ISO control group, 7-C: Drug treated group, 7-D: I-R control group, 7-E: Drug treated group
Fig. 8. Representative photomicrographs of ventricular tissue stained for Bcl-2 protein (H&E, 200X) from different experimental groups: 8-A: Sham group, 8-B: ISO control group, 8-C: Drug treated group, 8-D: I+R control group, 8-E: Drug treated group

Table 1. Changes in Bax, Bcl-2 and TUNEL positivity in different experimental groups.

<table>
<thead>
<tr>
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<th>Bax (%)</th>
<th>Bcl-2 (%)</th>
<th>TUNEL (%)</th>
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<tbody>
<tr>
<td>Sham</td>
<td>2.4±0.2</td>
<td>0.81±0.11</td>
<td>0.17±0.02</td>
</tr>
<tr>
<td>ISO</td>
<td>7.3±0.32*</td>
<td>0.59±0.09</td>
<td>3.4±0.32</td>
</tr>
<tr>
<td>Drug</td>
<td>3.6±0.62#</td>
<td>0.48±0.02</td>
<td>1.6±0.09</td>
</tr>
<tr>
<td>I-R</td>
<td>11.4±0.87*</td>
<td>1.2±0.08</td>
<td>5.1±0.19</td>
</tr>
<tr>
<td>Drug</td>
<td>3.5±0.39‡</td>
<td>0.9±0.05</td>
<td>2.7±0.21</td>
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Each value is expressed as Mean ± S.D. of six experiments. P*<0.001 vs. Sham group; P#<0.01 vs. ISO control group. P‡<0.01 vs. I-R control group. Total cell counts and Bax, Bcl-2 and TUNEL positive cells in the specimens were determined by means of a light microscope. The Bax, Bcl-2 and TUNEL positive cells were expressed as percentage of normal nuclei.