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Original Article

Acute myocardial infarction and myocardial ischemia-reperfusion injury: a comparison

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Abstract: Myocardial infarction (MI) denotes the death of cardiac myocytes due to extended ischemia. Myocardial reperfusion is the restoration of coronary blood flow after a period of coronary occlusion. Reperfusion has the potential to salvage ischemic myocardium but paradoxically can cause injury, a phenomenon called as 'reperfusion injury' (IR). Standard histologic, immunohistochemical and Elisa techniques were used to study the histopathologic, oxidative, apoptotic and inflammatory changes in MI and IR. The IL-6 levels in the LV of the MI group were significantly raised as compared to the IR group (*P=0.0008*). Plasma IL-6 was also significantly increased in the MI group as compared to the IR group (*P=0.031).* MI model was also associated with increase in the neutrophil polymorphs number in the infarction related myocardium as compared to the re-perfused myocardium. A significant increase in troponin I level in the MI group as compared to the IR group is also seen (*P=0.0001)*. Our IR model showed enhanced pro-apoptotic mediators like cleaved caspase-3 *(P=0.005*) and cytochrome c in the myocardium as compared to the MI model. In conclusion, myocardial damage in MI is mainly due to ischemic necrosis and inflammatory mechanisms while apoptosis is the main mechanism of cell death in IR in addition to limited ischemic necrosis.

Keywords: Heart, acute myocardial infarction, ischemia-reperfusion injury

Introduction

Coronary heart disease affects the heart due to the detrimental effects of acute myocardial infarction (MI) and Ischemia-reperfusion injury (IR). Understanding the mechanisms underlying these two processes is important as these two types of injuries are interrelated as well as different. Extending the period of acute myocardial ischemia for more than 20 minutes causes a "wave front" of cardiomyocyte death that begins in the subendocardium and extends transmurally toward the pericardium [1]. This is the reason that when a patient presents with an acute MI, the most effective therapeutic intervention is timely myocardial reperfusion using thrombolytic therapy or primary percutaneous coronary intervention to salvage the ischemic myocardium. The process of reperfusion, the very event critical for survival, can itself cause injury to the cardiomyocytes, a phenomenon called as the 'reperfusion injury' [2-4].

During acute myocardial ischemia, the lack of oxygen switches the cell metabolism to anaerobic respiration, with lactate accumulation, ATP depletion, Na^+ and Ca^{2+} overload and inhibition of myocardial contractile function [5, 6]. Reperfusion results in reactivation of electron transport chain which generates reactive oxygen species (ROS). ROS induces opening of mitochondrial permeability transition pores, contributing to intracellular Ca^{2+} overload, lipid peroxidation of cell membrane, and oxidative damage to DNA. In addition there is neutrophil accumulation in response to ROS, cytokines and complements. All these processes can independently induce cardiomyocyte death of the acutely ischemic myocardium [2-4].

Oxidative stress, apoptosis and inflammation are the most important mechanisms that are initiated during ischemia and continue over several hours into reperfusion [5]. Understanding the contribution of these processes to MI and IR is essential to look for therapeutic measures

that can help reduce the myocardial infarct size. In order to investigate these processes, we compare the histopathologic, oxidative, apoptotic and inflammatory changes in these two models of MI and IR. Our MI model has permanent left anterior descending (LAD) artery ligation for 24 hours and our IR model has LAD ligation for 30 min followed by reperfusion for 24 hours.

Material and methods

Murine model of myocardial infarction and ischemia reperfusion injury

Male C57B6/J mice (n=24) were divided into 24 hour MI group (n=8), IR group (n=8) and sham operated group (n=8). All experimental animal procedures are approved by the Animals Ethics Committee of the College of Medicine and Health Sciences, UAE University, protocol A12/10.

Male C57B6/J mice (male, age: 12-16 weeks; wt: 20-25 g) were anesthetized by an intraperitoneal injection of a combination of ketamine (100 mg/kg) and xylazine (10 mg/kg). The mice were then intubated by transesophageal illumination using a modified 22-gauge plastic cannula and fixed on the operating pad in the supine position by taping all four extremities. The mice were connected to a mouse ventilator (Harvard apparatus Minivent Hugo Sachs Electronik) which supplied room air supplemented with 100% oxygen (tidal volume 0.2 ml/min., rate 120 strokes/min). Rectal temperature was continuously monitored and maintained within 36-37°C using a heat pad. The lead II ECG (ADInstrument multi-channel recorder interfaced with a computer running Power lab 4/30 data acquisition software) was recorded from needle electrodes inserted subcutaneously. MI was induced in the mice by permanently occluding the LAD artery as described earlier [7-10].

Briefly, the chest was opened with a lateral incision at the 4th intercostal space on the left side of the sternum. Next, the chest wall was retracted for better visualization of the heart. With minimal manipulation, the pericardial sac was removed and the LAD was visualized with a stereomicroscope (Zeiss STEMI SV8). An 8-0 silk suture was passed under the LAD and ligated 1 mm distal to left atrial appendage. Occlusion was confirmed by observing immediate blanching of the left ventricle at post ligation site. An accompanying ECG recording showed characteristic ST-Elevation which further confirmed ischemia.

For our IR model, after the 8-0 silk suture was passed under the LAD, a small 1 mm polyethylene tubing (PE) was placed on top of the LAD and the suture was ligated on the top of the PE tubing without damaging the artery. Ischemia was confirmed by observing immediate blanching of the left ventricle (LV) at post ligation site. An accompanying ECG also showed corresponding ST-elevation. After 30 minutes of ischemia the ligature is removed by cutting the knot on top of this PE tube. Reperfusion was confirmed visually and by ECG changes.

The chest wall was closed by approximating the third and fourth ribs with one or two interrupted sutures. The muscles returned back to their original position and the skin closed with 4-0 prolene suture. The animal was gently disconnected from the ventilator and spontaneous breathing was seen immediately. Post-operative analgesic (Butorphanol 2 mg/kg, s/c, 6 hourly) was given at the end of the procedure. Sham operated mice underwent exactly the same procedure described above, except that the suture passed under the LAD is left open and untied. According to the experimental protocol, mice were sacrificed 24 hour after induction of myocardial infarction. The hearts were washed in ice cold PBS, right ventricle and both atria dissected away and left ventricle immediately frozen in liquid nitrogen and later stored in -80°C freezer. Blood was also collected in EDTA vacutainers and centrifuged at 3000 RPM for 15 minutes. The plasma was collected, alliquoted and stored at -80°C until further analysis. Heart samples from the same time point following LAD ligation were fixed in 10% buffered formal-saline for 24 hours.

Histopathology

Hearts were excised, washed with ice-cold PBS and weighed. Each heart was sectioned into 4 equal transverse (coronal) sections, cassetted and fixed directly in 10% buffered formalin. Sections were dehydrated in increasing concentrations of ethanol, cleared with xylene and embedded in paraffin. Three-um sections were prepared from paraffin blocks and stained with haematoxylin and eosin (H&E).

H&E stain was performed by de-waxing sections with xylene, rehydration with graded alcohol and washing in running tap water for 5 minutes. Tissue sections were then incubated in haematoxylin for 5 minutes followed by washing in tap water. The slides were checked for bluing of the nuclei. If the intensity of blue color is high, the slides were given a quick dip in 1% acid alcohol solution. Sections were then rinsed in running tap water until satisfactory blue color of nuclei is achieved. The slides were then stained with Eosin for 1 minute, washed in running tap water, dehydrated, cleared and mounted with DPX.

Immunohistochemistry

Three-micrometer sections were prepared and mounted on aminopropyltriethoxysilane (APES) coated slides. After dewaxing with xylene and rehydration with graded alcohol, slides were placed in a 0.01 M citrate buffer solution (pH=6.0) and pre-treatment procedures to unmask the antigens was performed in a water bath at 90°C for 60 minutes. Sections were then treated with peroxidase block for 30 minutes followed by protein block for 30 minutes. Sections were later incubated overnight with anti-cleaved caspase-3 (Rabbit polyclonal, ASP 175, Cell Signaling Technology, USA), anti-Bcl2 (Mouse monoclonal, SP66, Cell Marque, USA), anti-Myeloperoxidase (MPO) (Rabbit Polyclonal, 1:2000, Santa Cruz biotechnology, USA), and anti-Cytochrome c (Rabbit Polyclonal, 1:400, Santa Cruz biotechnology, USA) at 4°C. After conjugation with primary antibodies, sections were incubated with biotin-labeled secondary antibody (Thermo Scientific, USA) for 20 minutes at room temperature. Finally, sections were incubated with streptavidin-peroxidase complex for 20 minutes at room temperature (Thermo Scientific, USA), DAB chromogen (Thermo Scientific, USA) was then added and counter staining done with haematoxylin. Appropriate positive controls were used. For negative control, the primary antibody was not added to sections and the rest of the procedure was carried as above. Positive and negative controls were used in every batch of slides that were stained.

Morphometric analysis

Morphometric analysis of the number of neutrophil polymorphs at 24-hour following MI and IR was done using ImageJ software (http://rsbweb.nih.gov/ij/).

The number of neutrophil polymorphs was determined by counting the number of neutrophil polymorphs in 10 randomly selected fields in the left ventricle in areas supplied by LAD in all specimens followed by measuring the mean neutrophil polymorphs number in each specimen.

The apoptotic index was determined by counting the number of cells expressing cleaved caspase-3 in 10 randomly selected fields in the left ventricle in areas supplied by LAD in all specimens following by measuring the mean apoptotic cells in each specimen.

For cleaved-caspase labeling, cells were considered positive when there was a nuclear and cytoplasmic staining pattern.

Enzyme linked immunosorbent assay

Left ventricular myocardial concentration of IL-6, IL-1β, cleaved caspase-3, Wnt-3, and total Akt-1 and plasma levels of IL-6 were determined using DuoSet enzyme linked immunosorbent assay (ELISA) Development kit (Mouse IL-6 (DY406), Mouse IL-1β/IL-1F2 (DY401), Mouse Akt-1 (DYC1775-5), Mouse Wnt-3 (DY1324), Mouse cleaved caspase-3 (Asp175) (DYC835-5), R&D Systems, Minneapolis, MN, USA) for sandwich ELISA, using standard procedure according to the manufacturer's instructions. The levels were normalized to total protein concentrations. Briefly, 96-well plates (Nunc-Immuno Plate MaxiSorp Surface (NUNC Brand Products, A/S, Roskilde, Denmark), were coated with antibody specific for our proteins of interest. Biotinylated detection antibody and streptavidin conjugated horseradish peroxidase were used for detection of captured antigens. The plates between steps were aspirated and washed 3 times using ELISA plate washer (BioTek ELx50). Captured proteins were visualized using tetramethylbenzidine (TMB)/hydrogen peroxide. Absorbance readings were made at 450 nm, using a 96-well plate spectrophotometer (BioTek ELx800). Concentrations in the samples were determined by interpolation from a standard curve. Standards and samples were assayed in duplicate.

Troponin-I assay

Mouse cardiac troponin I levels in plasma were measured by using a high sensitivity mouse cardiac troponin-I Elisa kit (2010-1-HSP, Life Diagnostics, Inc.) according to the manufacturer's instructions.

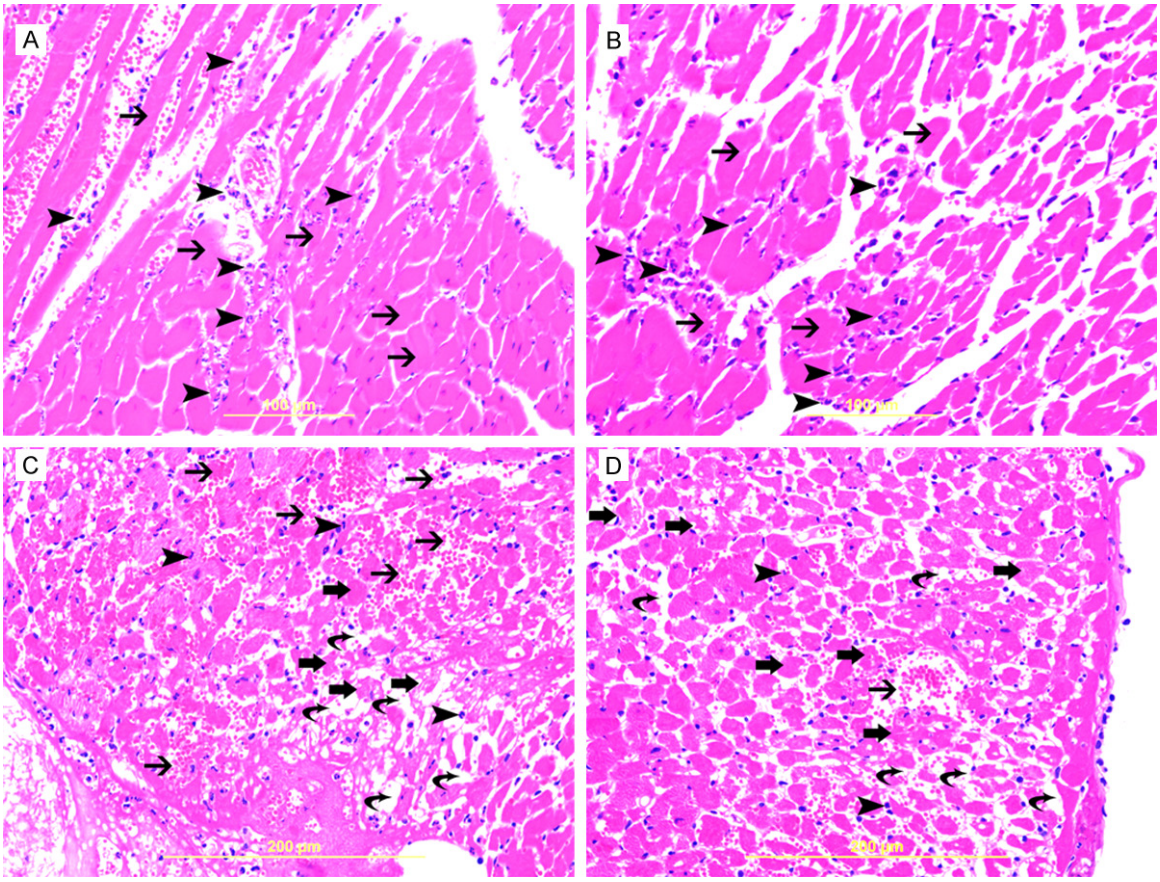

Figure 1. A & B. Represent high power views of MI heart sections showing ischemic cardiomyocytes appearing deeply eosinophilic with loss of cross striations (thin arrow) and neutrophil polymorphs(arrow heads) flooding the infarcted area. C & D. Represent high power views of IR heart sections showing injured cardiomyocytes (thick arrows) with prominent interstitial edema (curved arrows), RBCs in the interstitial spaces (thin arrows) and neutrophil polymorphs infiltration (arrow heads).

Glutathione and superoxide dismutase (SOD) activity assay

Total glutathione level in the heart protein extract was measured by a Glutathione Assay kit (CS0260 Sigma-Aldrich). Glutathione standard solutions were used to generate a standard curve and GSH levels calculated using Magellan 6 software. SOD activity was measured using the SOD determination kit (19160 Sigma-Aldrich). Appropriate blanks were set up and SOD activity was calculated according to the manufacturer's instructions.

Statistical analysis

Statistical analysis is done using IBM SPSS Statistics version 20. Data are presented in mean ± standard error (S.E). Statistically significant differences ($P<0.05$) were calculated between groups by Student t test.

Results

Histological changes in MI and IR models

The main histologic change in acute MI at 24-hour time point is coagulative necrosis of cardiomyocytes with heavy neutrophil polymorphs infiltration (**Figure 1A**, **1B**). The ischemic cardiomyocytes appear deeply eosinophilic with loss the cross striation and disappearance of the nuclei. LV sections studied at 24-hour following IR injury show focal necrosis of cardiomyocytes with prominent interstitial edema and accumulation of RBCs in the interstitial spaces with scattered neutrophil polymorphs infiltration of the injured myocardium (**Figure 1C**, **1D**).

Inflammatory mediators in MI and IR models

IL-6 levels in the LV of the MI group were significantly raised as compared to the IR group

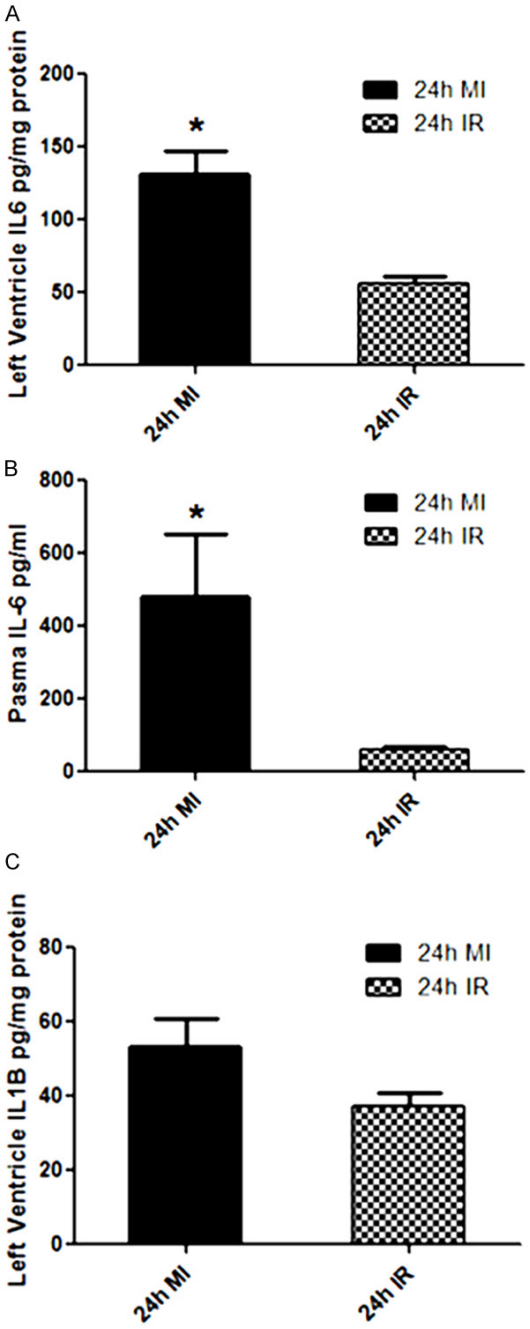

Figure 2. The graphs represent (A) left ventricular IL-6 concentrations (B) Plasma IL-6 levels and (C) left ventricular IL-1β concentrations in 24 hour MI and IR groups. *Shows P<0.05.

(130.9 ± 16.79 vs. 56.17 ± 4.63 pg/mg, *$P=0.0008$) (**Figure 2A**). Plasma IL-6 was also significantly increased in the MI group as compared to the IR group (479.3 ± 174.9 vs. 59.68 ± 9.61 pg/ml, *$P=0.031$) (**Figure 2B**). Heart LV IL-1β concentrations were not significantly different between the groups (**Figure 2C**).

Immunohistochemical staining of the heart section with MPO showed significant differences in the number as well as the intensity of staining of neutrophil polymorphs between the MI and IR groups (**Figure 3**). Morphometric analysis of the number of neutrophils was significantly higher in the MI group (109.6 ± 13.73) as compared to the IR group (61.10 ± 3.990), *$P=0.0032$ (**Figure 4**).

Apoptotic markers in MI and IR models

Heart LV cleaved caspase-3 levels were significantly increased in the IR group as compared to the MI group (2027 ± 93.47 vs. 1600.49 ± 89.44 pg/mg protein. *$P=0.0053$) (**Figure 5A**). Morphometric analysis of apoptotic cells is significantly higher in IR group than in MI group (18.08 ± 2.930 vs. 9.364 ± 1.274 *$P=0.0152$) (**Figure 5B**). Immunohistochemical staining of the heart sections with cleaved caspase-3 also showed more apoptotic cells in the IR group (**Figure 6K**, **6L**) as compared to the MI group (**Figure 6I**, **6J**). Cytochrome c was also seen to be higher in the heart sections from the IR group (**Figure 6B**, **6D**) as compared to the MI group (**Figure 6A**, **6C**) by immunohistochemistry. The expression is cytoplasmic seen predominantly in cardiomyocytes, but endothelial cells and neutrophil polymorphs also stained positive for it. The anti- apoptotic protein Bcl-2 was also seen to be expressed by cardiomyocytes and endothelial cells. The expression was higher in the MI group (**Figure 6E**, **6G**) as compared to the IR groups (**Figure 6F**, **6H**).

Total AKT-1 protein in MI and IR models

Heart LV AKT-1 levels were significantly higher in the MI group as compared to the IR group (755.8 ± 70.58 vs. 417.4 ± 48.47 pg/mg protein. *$P=0.0014$) (**Figure 5C**).

Total Wnt-3 protein in MI and IR models

Heart LV Wnt-3 levels were significantly higher in the MI group as compared to the IR group (32130 ± 1979 vs. 24420 ± 2704 pg/mg protein. *$P=0.0402$) (**Figure 5D**).

Total troponin I in MI and IR models

Plasma troponin I levels were significantly increased in the MI group as compared to the

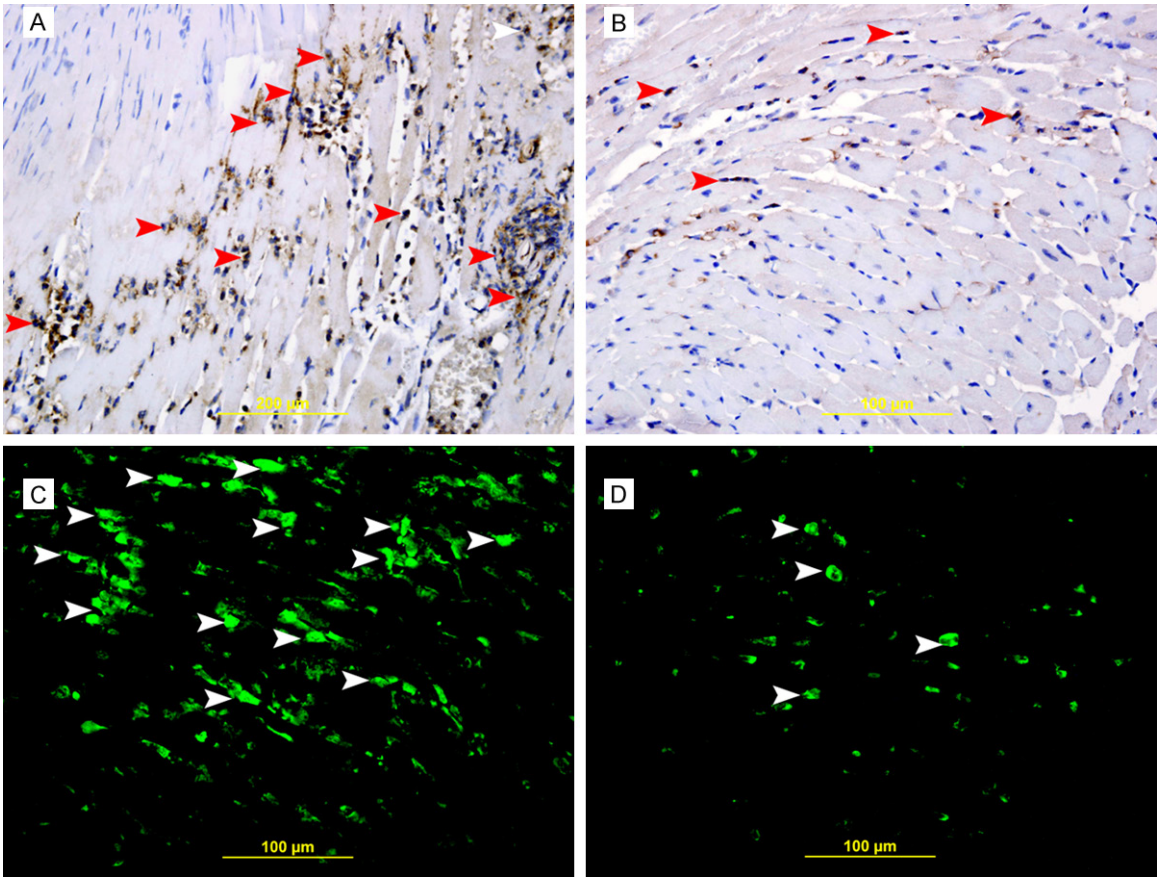

Figure 3. A & C. Representative section from LV in an area supplied by LAD at 24-hour following ligation of LAD showing many neutrophil polymorphs infiltrating the myocardium and expressing myeloperoxidase (arrow heads). B & D. Representative section from LV in an area supplied by LAD at 24-hour following ligation of LAD and reperfusion showing fewer number of neutrophil polymorphs infiltrating the myocardium and expressing myeloperoxidase (arrow heads). A & B. Immunoperoxidase streptavidin-biotin method. C & D. Alexa Fluor 488 immunoflyorescent technique.

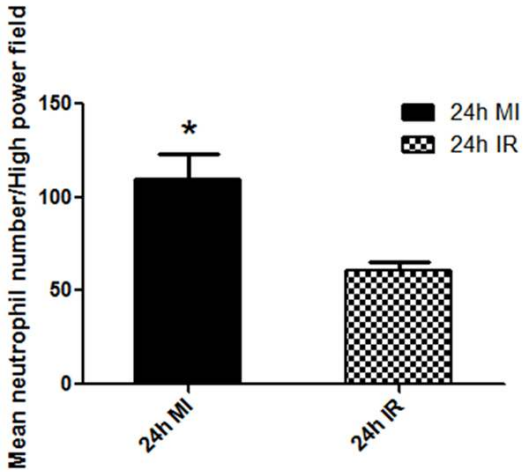

Figure 4. Comparison of Mean number of neutrophil polymorphs/high power field in the left ventricle in areas supplied by LAD between MI and IR.

IR group (12.58 ± 0.8158 vs. 3.727 ± 1.168 pg/mg protein. *P=0.0001) (**Figure 7**).

Antioxidant enzyme levels in MI and IR models

The levels of antioxidant enzymes are not significantly different between the MI group and the IR group. Heart LV Total Glutathione levels was 4.55 ± 0.31 nmol/mg in the MI group as compared to the IR group, which showed 5.76 ± 0.51 nmole/mg, P=0.06. Heart LV SOD inhibition activity was also not different among the MI and IR group (23.71 ± 0.76% vs. 23.49 ± 0.49%, P=0.81) (**Figure 8**).

Discussion

For many years, it was thought that myocardial reperfusion is only beneficial and that there was no cell death related to it [2, 11, 12]. Later when cardiomyocytes death was seen in the reperfused myocardium it was postulated that they are the already irreversibly damaged cardiomyocytes that were fated to die during ischemia [13]. The concept of 'reperfusion

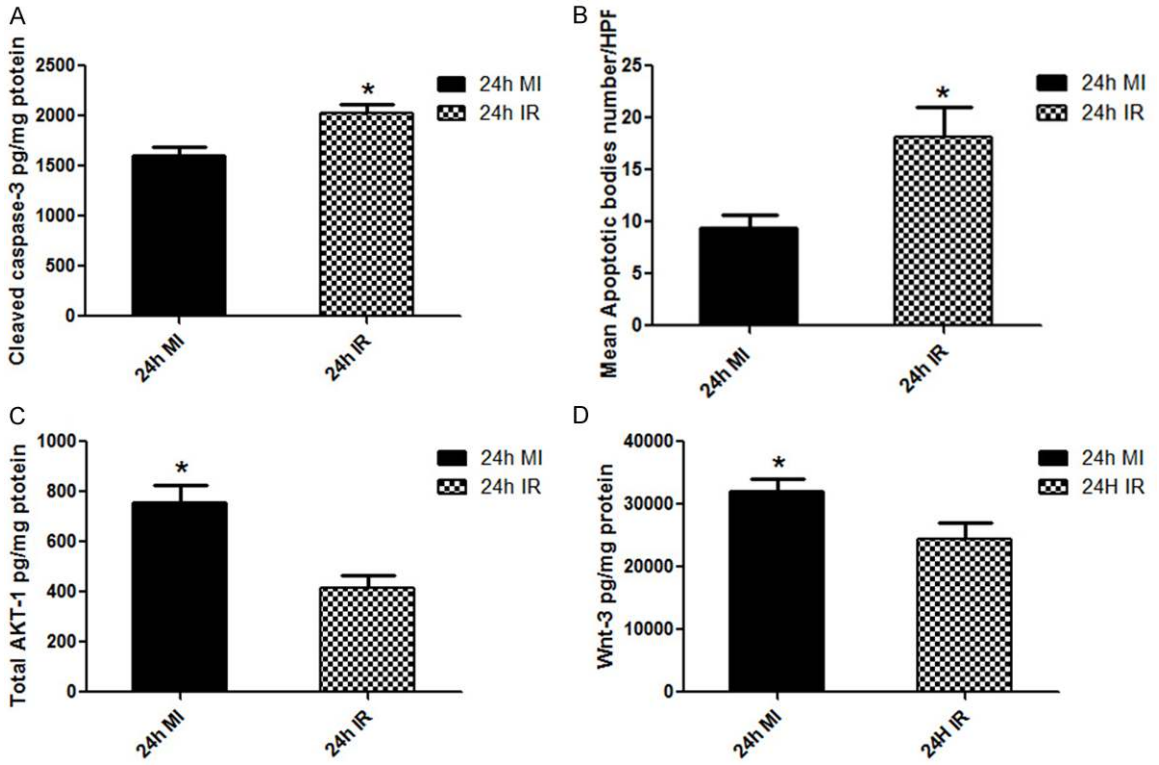

Figure 5. The graphs represent (A) left ventricular cleaved caspase-3 concentrations (B) Left ventricular mean Apoptotic bodies number/high power field (HPF), (C) Left ventricular total AKT-1 protein (D) Left ventricular Wnt-3 protein in 24 hours MI and IR groups. *Shows $P<0.05$.

injury' was presented when it was shown that reperfusion induced death in cardiomyocytes that were viable during ischemia [14]. Comparisons between these two types of injuries is still continuing till today because of two main reasons: First, it is almost impossible to estimate the own effects of reperfusion [15] and second, despite advances in antithrombotic, anti-platelet and PCI technologies, there is still no effective way to prevent the myocardial reperfusion injury [5].

Our study attempts to show substantial differences in the local microenvironment of the myocardium between these two modes of injury and analyze these changes.

The MI group shows a significantly higher inflammatory activity associated with induction of IL-6 than IR group both in the LV and plasma. The MI group is also associated with a significantly higher number of infiltrating neutrophil polymorphs in the infarcted myocardium as compared to the re-perfused myocardium. Neutrophil polymorphs that infiltrate the infarcted myocardium at 24 hour following MI will secret their enzymes to digest necrotic tissue as well as will have undesired damaging effects on adjacent survived cardiomyocytes which may potentiate the inflammatory response. We also show a significant higher level of troponin I in the MI group when compared with the IR group. Troponin I level is an indirect estimate of the infarct size [16-18]; hence, a higher level of troponin I in the MI group when compared with IR group indicates more necrosis in the myocardium in MI than in IR. The results comparing the MI and IR groups show that in MI there is increased myocardial damage via ischemic necrosis and inflammatory mechanisms. The IR group also shows inflammation but it is unclear whether the inflammatory response that accompanies an acute MI, during the first 30 minutes following ligation of LAD, contributes to the pathogenesis of myocardial reperfusion injury or whether it is a microinvironmental reaction to the acute myocardial injury [19].

Our IR group shows higher cleaved caspase-3 activities in the LV than in MI group. We also

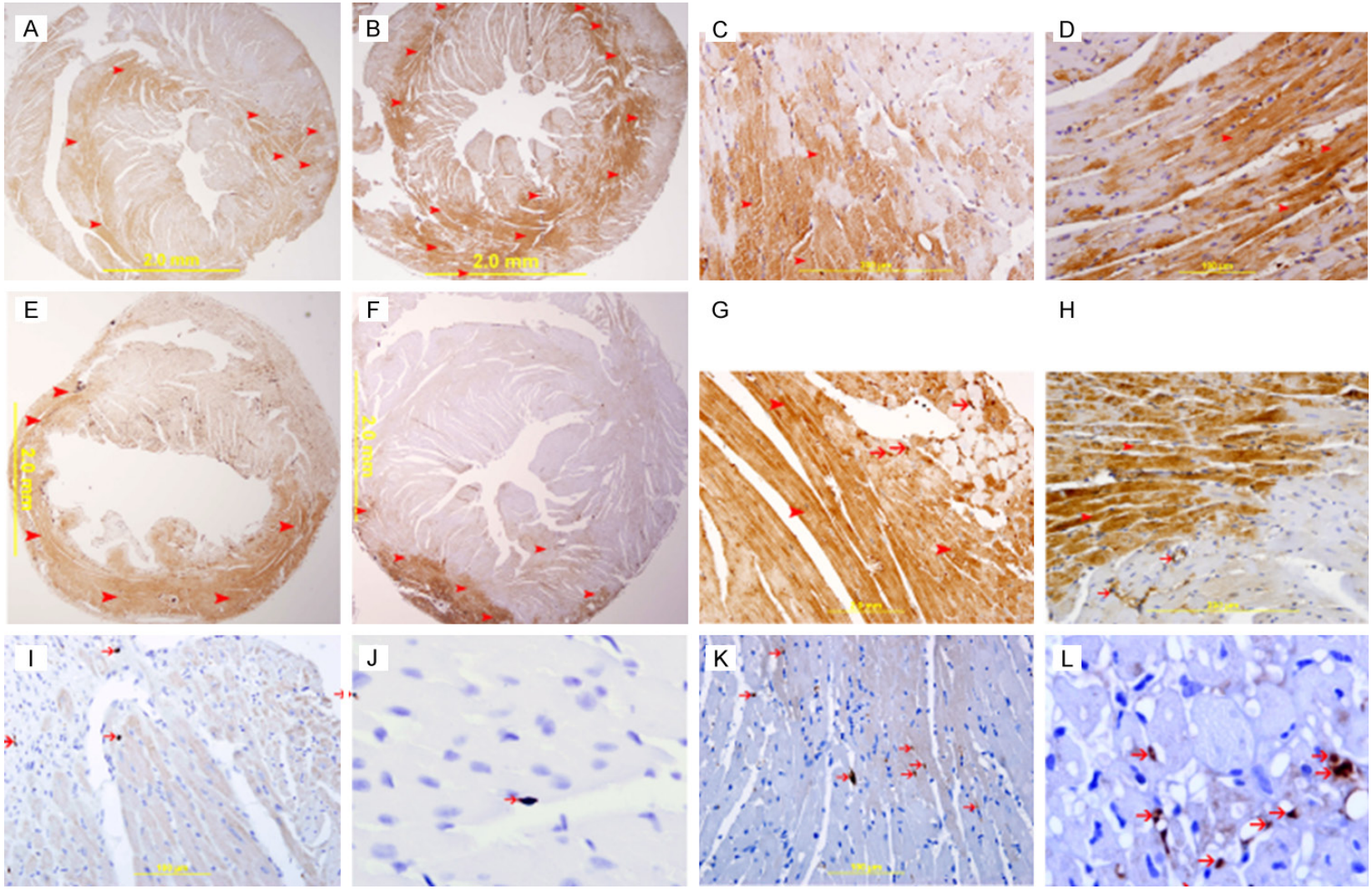

Figure 6. (A & B) Represents low power view of heart sections showing cytochrome c expression in MI (A) and IR (B) groups. (C & D) Show high power view of MI (C) and IR heart section expressing cytochrome c in the cytoplasm of cardiomyocytes. The intensity and number of positive staining in IR group is higher than the MI. Streptavidin-biotin immunoperoxidase method. (E & F) Represents low power view of heart sections expressing bcl2 in MI (E) and IR (F) groups. (G & H) Are the high power views of these sections showing increase in bcl2 immunostaining in cardiac myocytes (arrow heads) and endothelial cells (thin arrows) in the MI group (G) compared to IR (H) group, Streptavidin-biotin immunoperoxidase method. (I & J) Represent high power views of heart sections from the MI group showing cleaved caspase-3 expression in apoptotic cells (thin arrows) in the left ventricle from areas supplied by LAD. (K & L) Represent high power views of heart section from the IR group showing number of apoptotic cells expressing cleaved caspase-3 (thin arrows) in the left ventricle from areas supplied by LAD, Streptavidin-biotin immunoperoxidase method.

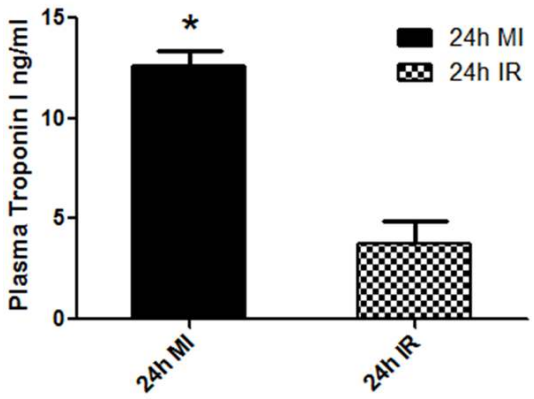

Figure 7. The graph represent plasma Troponin I concentrations in MI and IR models.

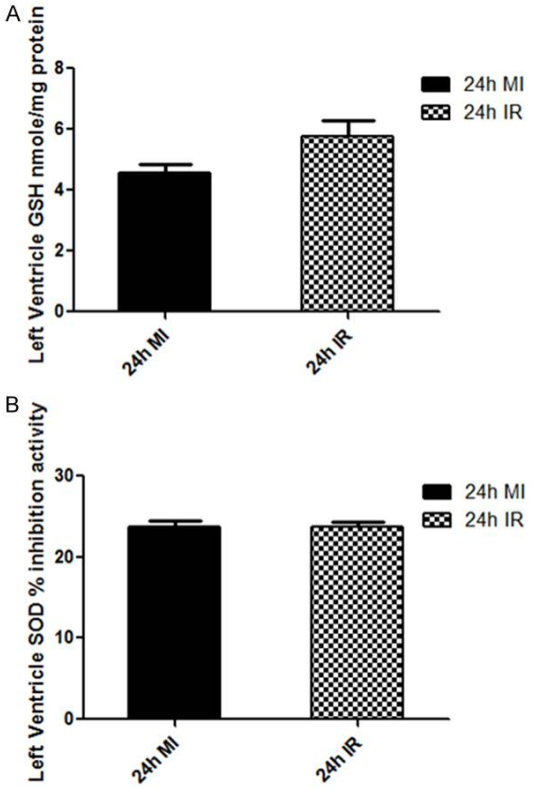

Figure 8. The graphs represent (A) left ventricular Total glutathione concentration and (B) left ventricular SOD% inhibition activity in 24 hour MI and IR groups.

show a significantly higher AKT-1 in the LV in the MI group than IR group. AKT-1 activation has anti-apoptotic activities on cardiac myocytes in MI and IR [20]. Many downstream targets of AKT-1 have been shown to contribute to its pro-survival effects such as phosphorylation of BCL-2 family members [21]. Activation of AKT-1 has been shown to modulate pro-apoptotic proteins through the phosphorylation of BCL-2 family members BAX and BAD leading to blockage of pro-apoptotic protein function and initiation of protective signaling cascades resulting in anti-apoptotic effects [22, 23]. This will contribute to the higher cleaved caspase-3 activity in IR group than MI group.

We also show significantly higher Wnt-3 levels in MI group than IR group. Activation of Wnt-3 has shown to be associated with an anti-apoptotic effect [24, 25]. This will also contribute to the higher cleaved caspase-3 activity in IR group than MI group.

The higher apoptotic activity in IR group than MI group suggests that the main mode of cardiomyocyte death in IR is apoptosis in addition to ischemic necrosis. It was shown by Lieberthal*et al* that the severity and duration of ATP depletion determines the mechanism of death: cells with an intracellular ATP concentration below a certain threshold become necrotic, whereas an ATP value above that threshold induces apoptosis [26, 27]. As MI model is associated with more ATP depletion as compared to IR model, where reperfusion may replenish the ATP stores, the main mechanism of cell death is caspase activated apoptosis in IR model.

Perhaps the most interesting observation made in our study is the level of antioxidant enzymes measured in the MI and IR models. We did not find significant differences between the groups signifying that the level of antioxidant enzymes is approximately the same whether the myocardium is subjected to 24 hours of permanent ischemia or whether it is subjected to 30 min of ischemia followed by 24 hours of reperfusion. We think that this observation should be looked at from a different angle. There is ample evidence that the level of oxidative stress generated in reperfusion injuries is considerably more than mere ischemic injury. In the first few minutes of myocardial reperfusion, a burst of oxidative stress [14, 28] is produced by a variety of sources. Organelles may begin to produce reactive oxygen species. Myocytes produce both hydrogen peroxide and superoxide radicals [29]. The electron transport chain of mitochondria is also a potential source of free radicals in both the endothelial cell and myocyte [30]. This detrimental oxidative stress mediates myocardial injury and cardiomyocyte death. The same oxidative

stress can lead to increase in the antioxidant capacity and so the level of antioxidant enzymes capacity may reflect the oxidative stress [31]. The level of oxidative stress and subsequent anti oxidant protection in IR is dependent on the time of ischemia before reperfusion is initiated and the reperfusion time [4, 32, 33]. However controversies do exist in this respect [34]. Our MI and IR model showing the same level of antioxidant capacity at 24-hour following LAD ligation may be related to the particular ischemia and reperfusion time in our models. Our results are significant in terms of IR injuries as the main goal in treatment of acute infarction is early revascularization with reperfusion. Reperfusion affects a larger portion of the left ventricle than infarction alone [35] so reperfusion injury may act as an independent determinate of cardiac remodeling in addition to infarct size.

In conclusion, the myocardial damage in MI is mainly due to ischemic necrosis with accompanying inflammation while apoptosis is the main mechanism of cell death in IR in addition to limited ischemic necrosis.

The differences observed in these two models as seen in our experiments show that these two processes of cardiomyocyte injury are indeed very distinct and the local microenvironment of the myocardium determines the particular roles of molecules and enzymes that are part of their pathogenesis.

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Disclosure of conflict of interest

None.

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