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Recommended Citation

Hashmi, S., Al-Salam, S. (2013). Loss of dystrophin staining in cardiomyocytes: A novel method for detection early myocardial infarction. *International Journal of Clinical and Experimental Pathology*, 6(2), 249-257.

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Original Article

Loss of dystrophin staining in cardiomyocytes: a novel method for detection early myocardial infarction

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Received November 11, 2012; Accepted December 12, 2012; Epub January 15, 2013; Published February 1, 2013

Abstract: Myocardial infarction (MI) is the most frequent diagnosis made in majority of sudden death cases subjected to clinical and medicolegal autopsies. When sudden death occurs at a very early stage of MI, traditional macroscopic examination, or histological stains cannot easily detect the myocardial changes. For this reason we propose a new method for detecting MI at an early stage. Murine model of MI was used to induce MI through permanent ligation of left anterior descending branch of left coronary artery. Five groups of C57B6/J mice were used for inducing MI, which includes 20 minutes, 30 minutes, one hour, four hours and 24 hours post MI groups. One naïve group and sham-operated groups were used as controls. There is loss of dystrophin membranous staining in cardiac myocytes occurs as early as 20 minutes post myocardial infarction. This can be used as a novel method to diagnose early myocardial infarction in post mortem cases where diagnosis is unclear. In conclusion, evaluation of immunohistochemical expression of dystrophin represents a highly sensitive method for detecting early myocardial infarction due to the loss of staining in the infarcted areas. Dystrophin immunostaining can also be used to assess myocardial architecture.

Keywords: Heart, myocardium, ischemia, myocardial infarction, early detection, dystrophin

Introduction

Myocardial infarction (MI) is the most frequent diagnosis made in majority of sudden death cases subjected to clinical and medicolegal autopsies. The diagnosis of MI is accepted in many instances when significant narrowing of one or more major coronary vessels is found at autopsy without visualization of the infarcted myocardial tissue. This criterion is usually applied in cases where the patient did not survive for sufficient time for either macroscopic or microscopic features of MI to appear after sustaining fatal ischemic attack [1].

In these situations where sudden death occurs at a very early stage of MI, the myocardial changes are non-specific and cannot easily be detected by traditional macroscopic examination, or histological stains.

A comparative study [2] on various immunohistochemical methods for detection of early myo-

cardial infarction reviewed various markers that can help diagnose early myocardial infarction. Complement complex C5b-9 [3], desmin, Masson's trichrome stain [4], myoglobin, actin, tropomyosin [5] and fibronectin [6] have contributed to the attempts of early detection of MI; either alone or in combination with other techniques [7]. Immunohistochemical detection of Cardiac troponin I [8] and Heart fatty acid binding protein (H-FABP) [9] have claimed high sensitivity and specificity for detecting myocardial infarction in postmortem cases, but search for a novel marker that can reliably diagnose early myocardial infarction at autopsy is an area of ongoing research and controlled animal studies are an essential part in clarifying this role.

Myocardial ischemia/infarction is a multifactorial injurious event that involves all the components of the cardiac myocyte [10]. The transition of reversible to irreversible acute myocardial ischemic injury is characterized by cell swelling;

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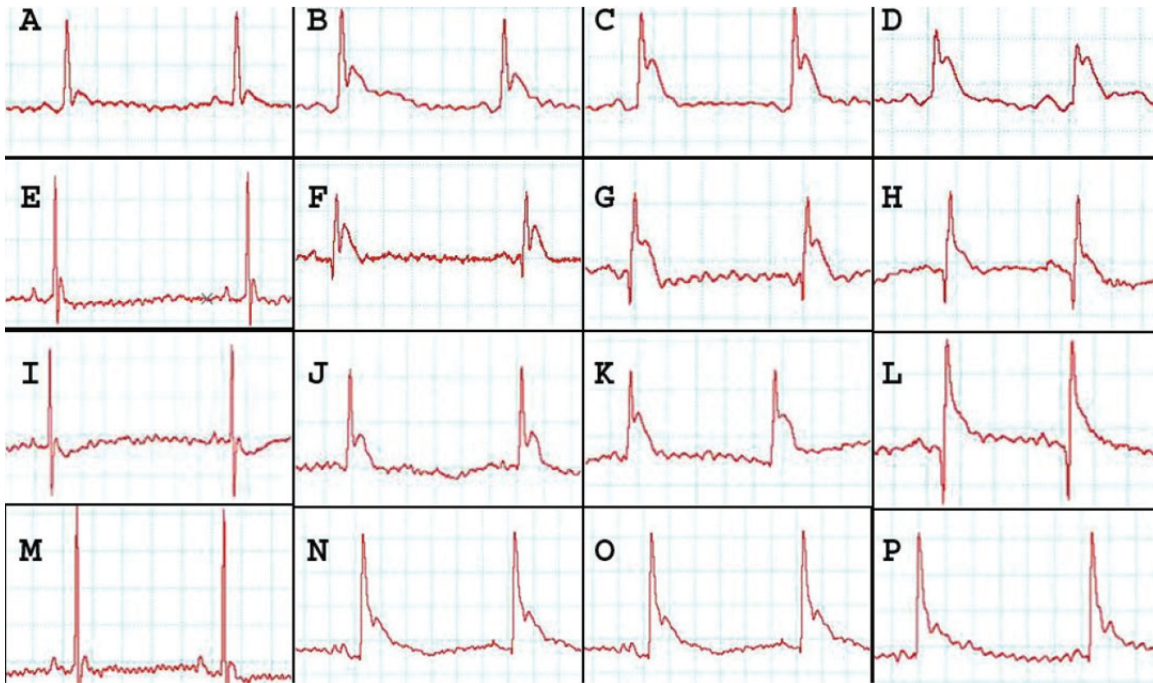


Figure 1. Representative ECG records of the 4 groups of murine model of myocardial infarction. A, B, C, D: 20 minutes post MI group; A: baseline record showing unremarkable changes, B, C & D: for 5, 10 & 20 minutes post LAD ligation respectively showing ST elevation with widening of QRS. E, F, G, H: 30 minutes post MI group; E: baseline record showing unremarkable changes; F, G & H: for 10, 20 & 30 minutes post LAD ligation respectively, showing ST elevation with widening of QRS. I, J, K, L: 60 minutes post MI group; I: baseline record showing unremarkable changes. J, K & L: for 10, 20 & 60 minutes post LAD ligation respectively, showing ST elevation with widening of QRS. M, N, O, P: 4 hours post MI group; M: baseline record showing unremarkable changes, N, O, & P: for 5, 10 and 15 minutes post LAD ligation respectively showing ST elevation with widening of QRS.

severe damage to the membrane-associated proteins and sarcolemma and altered transcription and translation processes [11, 12].

Proteins in cardiomyocytes that provide structural stability to the cell membrane and links the extracellular and intracellular environments can be divided into three groups: the dystrophin-associated protein complex; the vinculin-integrin link; and the spectrin-based submembranous cytoskeleton [13, 14].

Dystrophin, a 427-kD cytoskeletal protein, is a member of the β -spectrin/ α -actinin protein family [15]. It has an F-actin binding site at its N-terminus and a β -dystroglycan binding site at its C-terminus. Dystrophin and the dystrophin-associated proteins (dystroglycans, sarcoglycans, sarcospan, dystrobrevin, and syntrophins) form a complex [16], which is involved in contractile force transmission and stabilization of the plasma membrane [17]. Dystrophin links the transmembrane dystrophin complex and the laminin receptor to intracellular cytoskeletal actin and talin [18, 19].

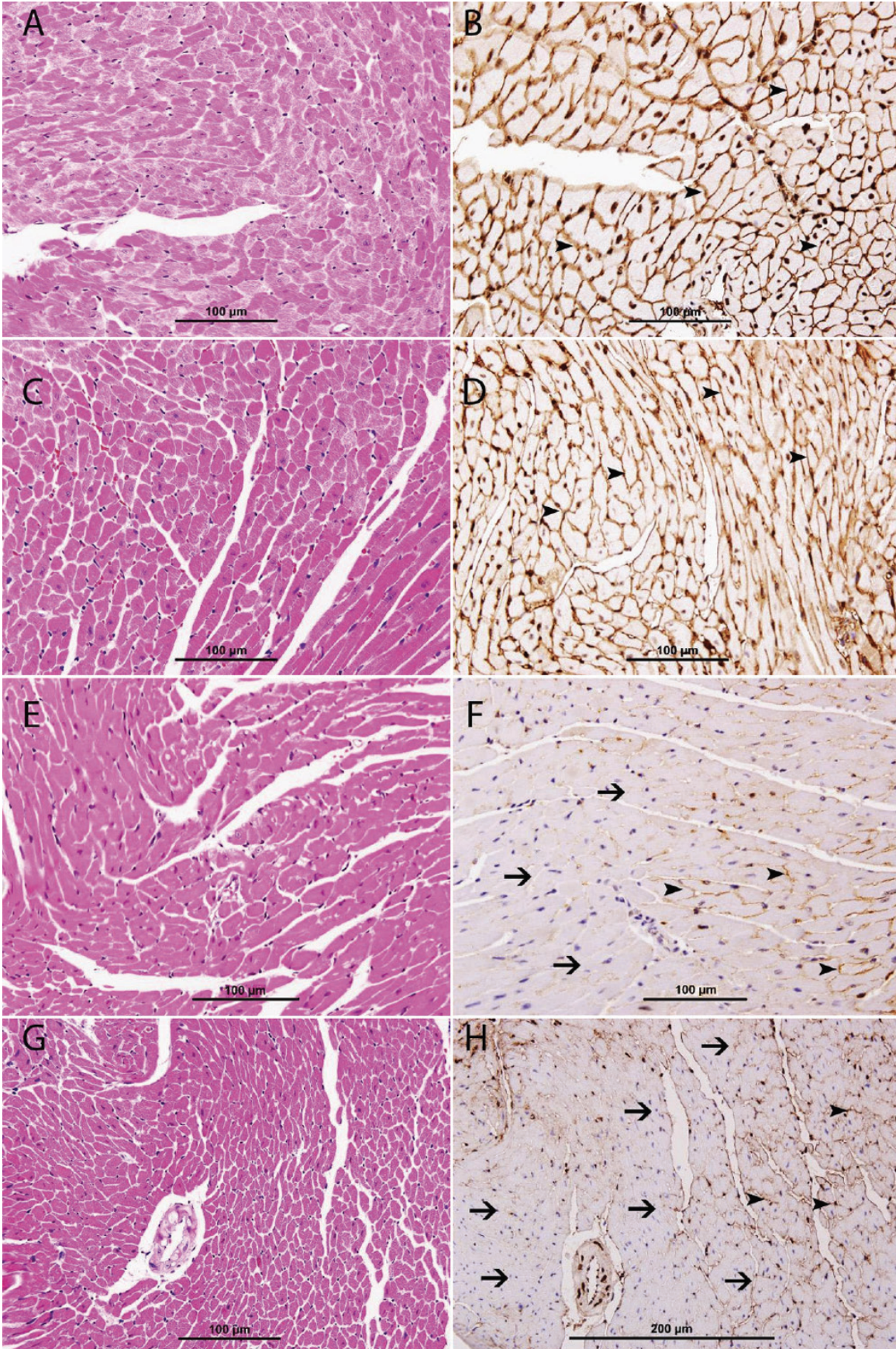
In myocardial ischemia, sarcolemmal separation causing sub-sarcolemmal bleb formation is facilitated by dissociation of dystrophin from the membrane [20]. Here we show that loss of dystrophin membranous staining in cardiac myocytes occurs as early as 20 minutes post myocardial infarction in a murine model of myocardial infarction. This can be used as a novel method to diagnose early myocardial infarction in post mortem cases where diagnosis is unclear.

Material and methods

Animal groups

C57B6/J mice are divided into 5 groups. Group I (n = 5): 20 min post MI, Group II (n=5): 30 min post MI, Group III (n=5): 1 hour post MI, Group IV (n=5): 4 hour post MI and Group V (n=5): 24 hour post MI. Samples from non-operated animals (Normal mice; n = 5), and from sham operated animals (n = 2) for each mentioned time points are also studied and used as controls. The Animals Research Ethics Committee of the

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Figure 2. A, C: Naïve and sham heart sections showing preservation of morphology of cardiac myocytes and cardiac tissue architecture, H & E. B, D: Naïve and sham heart sections showing thick sarcolemmal staining of dystrophin completely encircling cardiac myocytes (arrow head) forming a fishnet pattern of normal architecture. Immunoperoxidase streptavidin-biotin method. E, G: Representative sections from hearts of 20 minutes and 30 minutes post LAD ligation respectively, showing preservation of cardiac myocytes morphology and architecture, H&E. F, H: There is focal complete loss of sarcolemmal dystrophin staining (thin arrows) and focal loss of the fishnet pattern. There is partial loss or interrupted sarcolemmal staining of dystrophin in cardiac myocytes surrounding the area of complete loss of staining (arrow head), Immunoperoxidase streptavidin-biotin method.

Faculty of Medicine and Health Sciences, UAE University approves all experimental animal procedures.

Murine model of myocardial infarction

C57B6/J mice (male, age: 12-16 weeks; wt: 20-25g) are anesthetized by intraperitoneal injection of a combination of Ketamine (100mg/kg) and Xylazine (10mg/kg). The mice are 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 are connected to a mouse ventilator (Harvard apparatus Minivent Hugo Sachs Elektronik) which supplied room air supplemented with 100 % oxygen (tidal volume 0.2 ml/min., rate 120 strokes/min). Rectal temperature is continuously monitored and maintained within 37-38°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) is recorded from needle electrodes inserted subcutaneously. Myocardial infarction is induced in the mice by permanently occluding the left anterior descending coronary artery (LAD) as described earlier [21, 22].

Briefly, the chest is opened with a lateral incision at the 4th intercostal space on the left side of the sternum. Next the chest wall is retracted for better visualization of the heart. With minimal manipulation, the pericardial sac is removed and the left anterior descending artery (LAD) is visualized with a stereomicroscope (Zeiss STEMI SV8). An 8-0 silk suture is passed under the LAD and ligated 1mm distal to left atrial appendage. Occlusion is confirmed by observing immediate blanching of the left ventricle post ligation. An accompanying ECG recording will show characteristic ST-elevation, which will further confirm ischemia. The chest wall is closed by approximating the third and fourth ribs with one or two interrupted sutures. The muscles are returned back to their original

position and the skin closed with 4-0 prolene sutures. The animal is gently disconnected from the ventilator and spontaneous breathing is seen immediately. Postoperative analgesic (Butorphanol 2mg/kg, s/c, 6 hourly) is given at the end of the procedure. According to the experimental protocol, mice are sacrificed after induction of myocardial infarction with desired time of ischemia. Sham operated mice underwent exactly the same procedure described above, except that the suture passed under the LAD is left open and untied.

Tissue processing: Hearts were excised, washed with ice-cold phosphate buffer saline (PBS), and blotted with filter paper. Each heart was sectioned into coronal slices of 2mm thickness then cassetted and fixed directly in 10% neutral formalin for 24 hours, which was followed by dehydration in increasing concentrations of ethanol, clearing with xylene and embedding with paraffin. Three- μ m sections were prepared from paraffin blocks and stained with Hematoxylin and eosin (H&E). The stained sections were evaluated by the histopathologist that participates in this project using Olympus light microscopy.

Immunohistochemistry: Three- μ m sections were prepared and mounted on aminopropyltriethoxysilane-coated slides. After dewaxing with xylene and rehydrating 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 microwave oven for 10 minutes. Then, sections were treated with peroxidase block for 60 minutes followed by protein block for 60 minutes. Sections were over night incubated with Dystrophin (rabbit polyclonal antibody 1:300, Thermo Scientific, USA) at 4°C. Then sections were washed with PBS for 15 minutes in three changes then incubated with biotin-labeled secondary antibody (Thermo Scientific, USA) for 20 minutes at room temperature, which was followed by washing in PBS for 15 minutes in 3 changes. After that, sections were incubated

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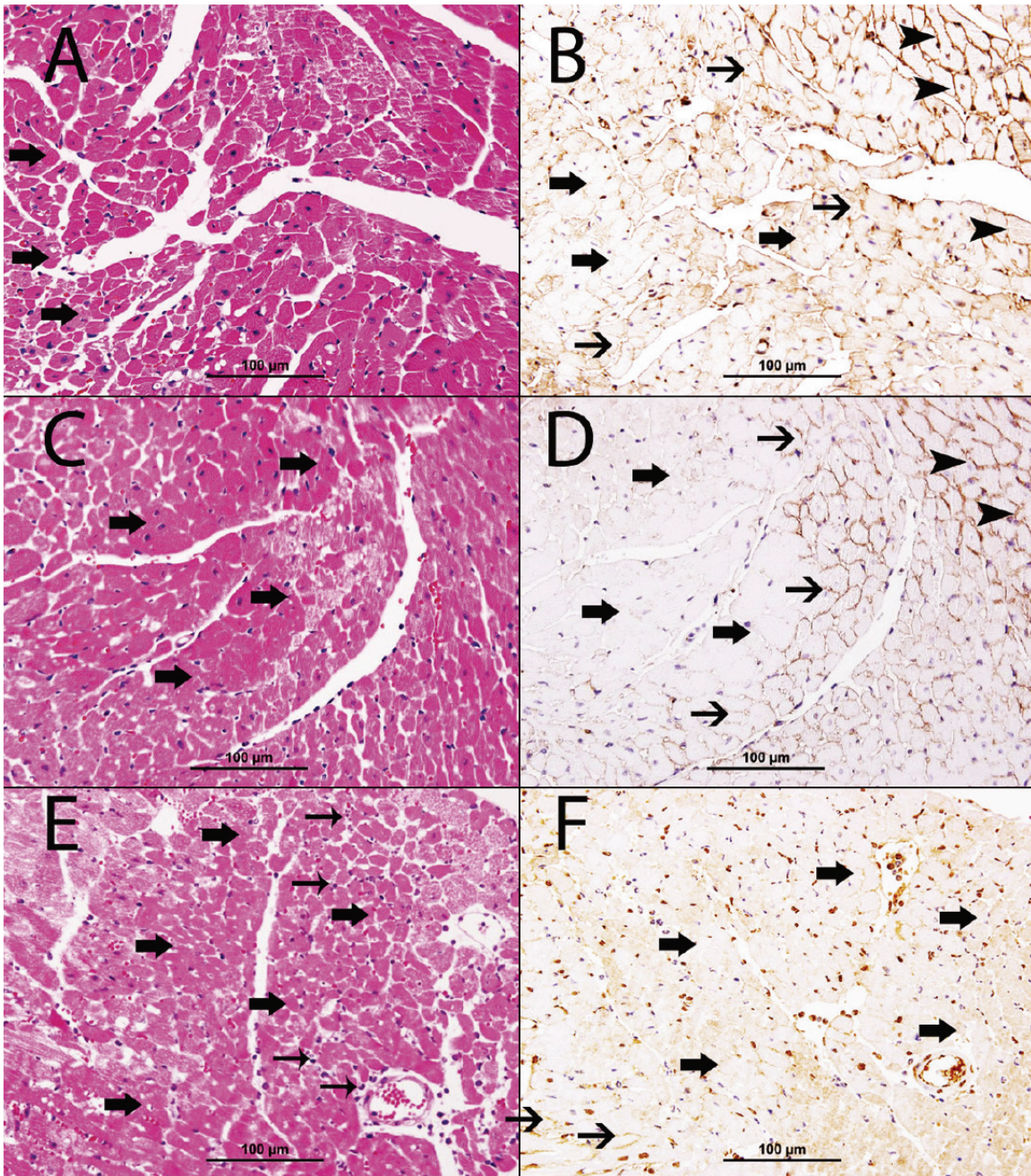


Figure 3. A, C: Representative sections from hearts of 60 minutes and 4 hours post LAD ligation respectively, showing focal area of interstitial edema and increased eosinophilia of cardiac myocytes (thick arrows), H&E. B, D: There is focal complete loss of sarcolemmal dystrophin staining (thick arrows) and focal loss of the fishnet pattern. There is partial loss or interrupted sarcolemmal staining of dystrophin in cardiac myocytes surrounding the area of complete loss of staining (thin arrows) and maintaining membranous dystrophin pattern at the periphery (arrow heads). Immunoperoxidase streptavidin-biotin method. E: Representative section from hearts of 24 hours post LAD ligation, showing coagulative necrosis of cardiac myocytes with loss of cross striation, deep eosinophilia (thick arrows), interstitial edema and many neutrophil polymorphs (thin arrows), H & E. F: There is complete loss of sarcolemmal dystrophin staining (thick arrows) and loss of the fishnet pattern. Only few foci of residual faint sarcolemmal staining (thin arrows) are seen, Immunoperoxidase streptavidin-biotin method.

with streptavidin -peroxidase complex (Thermo Scientific, USA) for 20 minutes at room temper-

ature followed by washing in PBS for 15 minutes in 3 changes. DAB chromogen (Thermo

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Scientific, USA) was added to all sections for 5 minutes, which was followed by washing in PBS and counter staining with haematoxylin. Sections were dehydrated, cleared and mounted in DPX. Appropriate positive controls were used. For negative control, the primary antibody was not added to sections and the whole procedure carried out in the same manner as mentioned above.

Results

Naïve and sham operated heart samples

The H&E- stained sections revealed normal architecture and morphology of cardiac myocytes (**Figure 2A** and **C**). The immunohistochemical stain with anti-dystrophin antibody showed thick membranous (sarcolemmal) brown staining which completely encircled the cardiac myocytes forming a fishnet pattern (**Figure 2A** and **C**). The intensity of staining is uniformly strong and diffusely distributed across all cardiomyocytes (**Figure 2B** and **D**). The ECG record was unremarkable (Data is not shown).

Heart samples 20 and 30 minutes post myocardial infarction

The H&E- stained sections of both 20 and 30 minutes post MI heart samples revealed conservation of normal architecture and morphology of cardiac myocytes. There was no neutrophil polymorph infiltration of the left ventricle (**Figure 2E** and **G**). There was no demarcated area of coagulative necrosis or any loss of cardiac myocytes within the left ventricle. Wavy cardiac myocytes were seen focally in the apex of the heart (Data is not shown).

The immunohistochemical stain with anti-dystrophin antibody showed well demarcated foci of complete loss of membranous (sarcolemmal) staining of dystrophin in cardiac myocytes in the left ventricle and within the region of LAD supply (**Figure 2F** and **H**). Areas of partial loss of membranous staining of dystrophin were seen in vicinity to those with absent dystrophin staining (**Figure 2F** and **H**). These areas delineate foci of coagulative necrosis as a result of ligation of LAD, which were not identified by the routine H&E staining (**Figure 2E** and **G**). In addition, areas with interstitial edema identified with H&E stain was observed to be associated with partial absence of dystrophin staining

(**Figure 2F** and **H**). Areas of loss of dystrophin were larger in 30 minutes than that seen in 20 minutes post MI sections. The ECG showed persistent ST elevation at 5, 10, 15, 20 and 30 minutes post ligation of the LAD (**Figure 1B-H**) confirming acute MI.

Heart samples 60 minutes and 4 hours post myocardial infarction

The H&E- stained sections from heart samples 1 hour and 4 hours post MI revealed foci of interstitial edema with increased eosinophilia of cardiac myocytes (**Figure 3A** and **C**). There was no neutrophil polymorph infiltration of the left ventricle (**Figure 3A** and **C**) similar to the 20 and 30 min post MI samples. Wavy cardiac myocytes were seen in the apex of the heart as was seen in the previous time points with additional wavy fibers seen at anterior wall of left ventricle in 4 hour post MI samples (Data not shown).

The immunohistochemical stain with anti-dystrophin antibody showed well demarcated areas of complete loss of membranous (sarcolemmal) staining of dystrophin in cardiac myocytes (**Figure 3B** and **D**). The identified areas of complete loss of dystrophin staining were larger than that seen in 30 minutes post MI sections. Incomplete loss of dystrophin was also seen around areas of complete loss of dystrophin and in cardiac myocytes surrounded by interstitial edema (**Figure 3B** and **D**). Foci of deep eosinophilia in cardiac myocytes at 4h post MI corresponded well with areas of complete loss of dystrophin (**Figure 3C** and **D**). The ECG showed persistent ST elevation at 5, 10, 15, 20, 30 and 60 minutes (**Figure 1J-L, N-P**) confirming acute MI.

Heart samples 24 hours post myocardial infarction

The H&E- stained sections of 24 hour post MI heart samples showed loss of normal architecture and morphology of cardiac myocytes in the left ventricle within the areas supplied by LAD (**Figure 3E**). There was well demarcated area of coagulative necrosis with loss of nuclei and cross striation (**Figure 3E**). There was heavy neutrophil polymorphs infiltration of the infarcted area associated with prominent interstitial edema (**Figure 3E**). Areas around coagulative necrosis showed interstitial edema with com-

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plete detachment of some cardiac myocytes which attained rounded morphology (**Figure 3E**).

The immunohistochemical stain with anti-dystrophin antibody showed well demarcated areas of complete loss of membranous staining of dystrophin in cardiac myocytes in the left ventricle and within LAD supply region (**Figure 3F**). A characteristic difference that we observed was that the areas showing loss of dystrophin staining are larger than the infarcted area seen by the H&E staining. In addition, areas with loss of dystrophin staining are larger than that seen in 4-hour post MI. Rounded cardiac myocytes that are seen in areas of interstitial edema show complete loss of dystrophin staining (**Figure 3E and F**).

Discussion

Myocardial ischemia for 20-24 minutes causes irreversible cellular injury which is marked by leaky cell membranes and release of proteolytic enzymes and other molecules [23]. Ischemic cell death involves morphological changes in the sarcolemmal membrane which include formation of subsarcolemmal blebs, detachment of sarcolemmal membranes from the basement membrane, focal attenuation of the glycocalyx, loss of an amorphous electron dense layer from the internal surface of the sarcolemma and appearance of focal discontinuities in the lipid bilayer [20].

Detecting an acute MI in autopsy prior to neutrophilic infiltration is often very difficult on H&E staining. Examination of H&E stained sections will not identify ischemic lesions until more than 6 hours post-infarction [1]. Wavy fibers seen one to three hours after irreversible ischemic injury are thought to be due to intercellular edema separating the dead myocytes as the surrounding normal contracting myocardium pulls on them [1]. In our study, wavy fibers are seen as early as 20 minutes post MI and are seen mainly in the apex. Contraction bands with loss of cross striations may be seen at the same time as wavy fibers, and are present at the edge of the infarct [1]. Area of wavy fibers, contraction bands and loss of cross striations are non-specific and may be easily missed especially if they are small. Neutrophilic infiltration begins after 6 hours which peaks at two to

three days with true coagulation necrosis in the myocardium seen after 18 hours [1, 5, 24].

It is known that at post mortem examination, the gross and microscopic findings of myocardial infarction are identifiable at least 6-24 hours after MI. Hence, if the patient dies within 6 hours after sustaining MI, using the non-specific gross and microscopic criteria for diagnosing MI, is not only unreliable but it is misleading and will result in over diagnosis of MI as a cause of death.

For these reasons we propose a new method for detecting myocardial infarction at an early stage. The immunohistochemical staining in our study indicates that the depletion of dystrophin could be detected as early as 20 min after myocardial infarction in murine hearts, which strongly suggests that loss of dystrophin staining in cardiomyocytes can be used as a valuable and reliable marker for the confirmation of very early myocardial damage in autopsy material. Although, membranous loss of dystrophin was reported for the first time in a rat heart model of isoproterenol toxicity, it was not reported to be used to detect early myocardial infarction with the time points that are used in this study [25].

The murine model of myocardial infarction is a well established model to study protein and gene expressions after LAD ligation. The significance of this model in this study relies to the fact that the time points are well controlled and show the sequence of events after myocardial infarction correlating well with loss of dystrophin. In addition, tissue fixation and processing is optimum and controlled to overcome any changes due to inappropriate fixation and tissue processing that might affect the H&E or the immunohistochemical staining. Furthermore, dystrophin immunostaining can be done on fixed and paraffin-embedded tissue sections, which makes it valuable in retrospective studies. Moreover, immunohistochemical procedures on paraffin-embedded tissue are done routinely in histopathology laboratories which make the application of dystrophin staining on postmortem tissue feasible.

Besides, we show the characteristic fishnet pattern of dystrophin staining in normal myocardium. Hence dystrophin staining can also be used to study the basic architecture of cardiac

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myocyte and to detect any abnormality in myocardial architecture.

Our results show a time-dependent depletion of dystrophin after acute MI. There is increase in the area of dystrophin loss with the increase in post MI time.

Reduction or absence of dystrophin implies rupture of the physical linkage that anchors the actin-based subsarcolemmal cytoskeleton and the sarcomeres to the sarcolemma, which may be related to impairment of contractile force transmission [26]. This breakdown of the link with the cytoskeleton impairs the sarcolemmal structural support, destabilizing and rendering it more vulnerable to subsequent damage [20, 27].

The damage of the sarcolemma leads to an increase in the permeability of the membrane. The correlation between loss of dystrophin and increased sarcolemmal permeability in ischemic myocardium has been demonstrated by Armstrong et al. in tissue and isolated cells [20, 27].

The absence of immunohistochemical staining of dystrophin could occur either due to the loss of specific protein, alteration of antigenic binding sites or redistribution of the protein. Armstrong et al [20] provides evidence that the redistribution of dystrophin accounts for sarcolemmal membrane dystrophin loss during ischemia; however, a decrease in transcriptional activity of ischemic cells might also be a cause of loss of expression of dystrophin [10].

Our results also show that with the occlusion time for LAD prolonged, the depletion of dystrophin was more evident and affected area bigger. In the 24 hours post MI samples, the area with dystrophin loss was considerably larger than the area seen when examining H&E stained sections, implying that the actual area of myocardium that sustained damage was much bigger than that observed by routine H&E stains even at 24 hours MI time point.

We have no data regarding the effect of autolysis on dystrophin expression, but we can assume that if autolysis has affected the heart in post mortem cases, the dystrophin loss will be diffuse in all areas of the heart and not confined to the ventricles, as is the case in fatal myocardial infarction.

In conclusion, evaluation of immunohistochemical expression of dystrophin represents a highly sensitive method for detecting early myocardial infarction due to the loss of staining in the infarcted areas. Dystrophin immunostaining can also be used to assess myocardial architecture. Studies on human autopsy specimens are needed to fully elucidate its potential as a marker for early myocardial infarction in cases where the cause of death is unclear.

Acknowledgement

The authors would like to acknowledge Ms Manjusha Sudhadevi from Department of Pathology, Faculty of Medicine & Health Sciences, United Arab Emirates University for her technical efforts in doing the immunohistochemical staining.

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