Remodeling in early myocardial infarction: alteration of extracellular matrix; Collagen-1, Collagen-3, α-SMA, and α-Actinin in Porcine heart model

Muhammad Arza Putrā1,2*, Idrus Alwi3, Tri Wisesa Soetisna4, Gunanti5, Normalina Sandora6, Pribadi Wiranda Busro7, Supomo8, Nur Amalina Fitria9, Tyas Rahmah Kusuma6

ABSTRACT

Background: Myocardial infarction (MI) leads to remodeling due to altered cardiac structure and function. The study of MI histopathology in humans or large animals mimicking the human heart is limited. Using the porcine heart model, our study investigated the composition of myocardial extracellular matrix (ECM) in collagen-1, collagen-3, α-smooth muscle actin (α-SMA), and α-actinin before and after myocardial infarction.

Methods: This study used two groups of domestic pigs: the Infarct group (n=4) and the Sham group (n=4). MI was induced by permanent ligation of the proximal branch of the posterior left ventricular artery. Cardiac enzymes, electrocardiography, and echocardiography data were collected before and after ligation. Cardiac tissue was harvested from the infarcted area after 60 minutes of ligation and stained with hematoxylin-eosin and Movat's Pentachrome. Collagen-1, collagen-3, and α-SMA were identified with immunohistochmical labeling, and the labeled area was measured using ImageJ. Meanwhile, α-actinin was visualized using immunofluorescence.

Results: Expression of collagen-1, collagen-3, and α-SMA in the infarct group were significantly decreased after 60 minutes of infarction compared with those in the sham group (p<0.01). The α-actinin was fragmented and diminished in the infarct group.

Conclusion: Myocardial remodeling was detected 60 minutes after infarction with mild alteration in myocardial histoarchitecture and significant deterioration of ECM composition of collagen-1, collagen-3, α-SMA, and fragmented α-actinin fibers in the porcine heart model.

Keywords: Collagen, ECM, Myocardial Infarction, α-SMA, α-actinin.


INTRODUCTION

Cardiovascular diseases have been a global health issue constantly escalating, with 17.6 million cases per year, and it is predicted to hit 23 million in 2030.1,2 Similarly, according to the Sample Registration System data, ischemic heart disease accounts for 13.3% of total mortality in Indonesia.3

The major cause of MI is atherosclerosis of the coronary artery, which, once it is blocked, causes asphyxia and cardiomyocyte death.1,4 MI prognosis depends on the infarct size, collaterals presence, and interval of the occluded artery revascularization.1 Cardiac remodeling refers to various cardiac structural and functional changes following MI.6 Remodelling causes protein accumulation in the ECM and alters the physiological structure and function.7

Cardiac remodeling correlates to collagen accumulation and causes fibrosis scarring that disrupts electrical conduction, eventually causing arrhythmia. Fibrosis is correlated to sudden death and arrhythmia.6 Development of pathological remodeling after infarction is correlated to collagen amount, particularly type 1 and 3. Collagen 1 and collagen 3 are the major ECM components of the myocardium.8,9

The content of mature scars is mainly collagen-1, which forms dense fibers with a slow turnover rate, while collagen-3 is a thinner fiber with rapid turnover. During infarct healing, reserve cardiac fibroblasts will transform into α-smooth muscle actin/ α-SMA+ myofibroblasts recruited into the injury site, proliferate, and produce ECM to develop collagen-rich scar tissue that prevents ventricular rupture.10 Nonetheless, the massive elevation of collagen-1 forms

1Doctoral Program in Medical Sciences, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia;
2Division of Thoracic, Cardiac and Vascular Surgery, Department of Surgery, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia;
3Division of Cardiology, Department of Internal Medicine, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia;
4Division of Surgery and Radiology, School of Veterinary Medicine and Biomedical Sciences, Universitas Institut Pertanian Bogor, Bogor, Indonesia;
5Indonesian Medical Education and Research Institute, Universitas Indonesia, Jakarta, Indonesia;
6Division of Adult Cardiac Surgery, Harapan Kita National Cardiovascular Center, Jakarta, Indonesia;
7Division of Surgery and Radiology, School of Veterinary Medicine and Biomedical Sciences, Universitas Institut Pertanian Bogor, Bogor, Indonesia;
9*Corresponding author: Muhammad Arza Putra; Doctoral Program in Medical Sciences, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia; putramuhammadarza@gmail.com

Received: 2023-06-24
Accepted: 2023-08-20
Published: 2023-09-12
thick cardiac scarring and causes rigid myocardial walls, eventually reducing cardiac output.\textsuperscript{8}

The α-SMA, a specific isoform of actin, is predominantly found in vascular smooth muscle cells and plays a crucial role in differentiating cardiac fibroblasts into myofibroblasts contributing to the increased contractile force generated by myofibroblasts.\textsuperscript{11} Myofibroblasts are activated fibrogenic partitions characterized by elevated extracellular matrix levels and the de novo expression of α-SMA. These cells control contractile properties and activate latent transforming growth factor-β1 (TGF-β1), a fibrogenic growth factor in the development of fibrosis.\textsuperscript{12,13} α-SMA also transmits external forces to the extracellular matrix through interactions with integrins, focal adhesion molecules, and actin-binding proteins such as α-actinin.\textsuperscript{14} α-actinin is a highly conserved protein that can cross-link actin filaments, playing a role in skeletal and cardiac muscle and non-muscle cells. Its preferential binding pattern involves bipolar cross-linking, enabling structural reinforcement in various orientations.\textsuperscript{15}

Although the study of the remodeling process in early MI is still limited, understanding the impact duration of MI on cardiac remodeling is essential for further studies in early treatment and procedures to treat MI. A postmortem autopsy of the human heart indicated that gross and microscopic changes showed granulation tissue with extensive collagen deposition approximately 10–14 days after MI.\textsuperscript{16} Another study of infarct size in rat models of chronic infarction showed remodeling occurred after six weeks of coronary artery ligation.\textsuperscript{17} PorceMI models mimicking the pathology of the human heart are required to evaluate the early remodeling process after MI. Our study aimed to investigate whether 60 minutes of MI affected the composition of ECM, focusing on collagen-1, collagen-3, α-SMA, and α-actinin, using the porcine heart as the model.

**METHODS**

**Animal subjects**

The methods used in this study were reviewed and approved by the Animal Ethics Committee, Faculty of Veterinary Medicine, IPB University (No. 158/KEH/SKE/XII/2019). This study was an experimental animal study using porcine since porcine has more significant similarity to humans in this regard and has only a few collateral anastomoses so that in coronary occlusion, it will form a transmural infarction of the appropriate size. The study was conducted from July 2021 to December 2022 in the Veterinary Teaching Hospital, Faculty of Veterinary Medicine, IPB University and at the Indonesia Medical Education and Research Institute (IMERI), Faculty of Medicine, Universitas Indonesia.

Sampling using non-probability consecutive sampling. A total of eight large white domestic pigs (Sus scrofa domesticus) were grouped into two; the sham (n=4) and the Infarct group (n=4), obtained from a local farm in Kuningan, West Java, Indonesia, weighed around 50–65 kg, 2–4 months of age, and showed no bacterial or parasitic infections. Upon arrival at the Veterinary Teaching Hospital, all pigs were acclimated for seven days to eliminate infections and allow them to adapt; feed and water were accessed ad libitum. Four pigs were induced for MI by ligating the proximal branch of the left ventricle/PLV artery; the first branch of the left circumflex artery/LC was terminated after 60 minutes. All pigs were at 21.25 ± 2.36 weeks old, weighing around 59.50 ± 5.26 kg, while the Sham group was at 19.50 ± 3.11 weeks and 53.75 ± 11.03 kg. No significant difference was found in the study subjects’ age or weight of porcine.

**Myocardial infarction induction in pigs**

All pigs prepared for myocardial infarction were anesthetized using 2 mg.kg\textsuperscript{-1} Xylazine (Randlab, Australia), 20 mg.kg\textsuperscript{-1} Ketamine (Agrovet Market, Canada), 5 mg.kg\textsuperscript{-1} Propofol (Finusolprima Farma International, Indonesia), and 0.02–0.05 mg.kg\textsuperscript{-1} Fentanyl (Mahakam Beta Farma, Indonesia). Ventilation was maintained using an RWD Vet Anesthesia R640-S1 inhalation machine (RWD Life Science Co., Ltd, Shenzhen, China), bagging manually 60 times per minute through an endotracheal tube (no. 6 and no. 6.5). Electrocardiography/ ECG data was collected using Fukuda ME C120 (Fukuda Denshi Co., Ltd, Tokyo, Japan), and the echocardiography data using Chison EBit60 VET Ultrasound (Chison Medical Imaging Co., Ltd, Wuxi Jiangsu, China). The blood serum was acquired before and after infarct induction to confirm MI. An expert cardiology veterinarian performed transthoracic echocardiography through the right lateral recumbent position. The pig heart was exposed through the fifth to sixth intercostal space thoracotomy, and the PLV artery was suture ligated once identified (Figure 1A), using polyprene suture (Premilene no. 6-0, B.Braun, Germany), and marked using PTFE pledget polymer (Covidien, USA). Infarction was visible immediately after the ligation, with a patch of a darkened area beyond the ligation (Figure 1B).

**Specimen retrieval**

The heart was harvested after 60 minutes of infarction, verified by the ECG and echocardiography. Specimens for histology were obtained from the center of the infarcted area, 5x5x5 mm\textsuperscript{3}, fixed at room temperature in the neutral buffer formalin 10% (v/v) (Leica, Germany). Tissue samples were automatically dehydrated and infused with wax in the tissue processor for 23 hours. Each specimen was prepared as a paraffin wax block, with sections at 5 µm for histological staining and labeling.

**Measurement of cardiac troponin T and CK-MB levels**

For ELISA, porcine blood (10 mL) was obtained from the peripheral auricular vein and collected in serum separator tubes (BD Bioscience, CA, USA). Serum was separated from the erythrocytes by centrifugation at 3000 rpm for 10 minutes and stored at -80°C until all samples had been collected. ELISA kit to measure the cardiac troponin (cTnT) and creatinine kinase – myocardial band (CK-MB) (CUSABIO, Wuhan, China) was performed according to the manufacturer’s instructions. The color measured the concentration of each sample yielded using Varioskan™ LUX multimode microplate reader (Thermo Fisher, USA), analyzed at 450 nm and 540 nm wavelengths in optical densities (OD). The final value was obtained from the subtraction of OD value
visualized under a brightfield microscope and captured using Zeiss Axio Imager (Zeiss, Germany). Immunohistochemistry (IHC) labeling toward collagen-1, collagen-3, and α-SMA followed the biotin-avidin labeling protocol against the extracellular matrix protein; collagen-1 (1:100 dilution, Invitrogen, USA), collagen-3 (1:50 dilution, Abcam, USA) also the α-SMA (1:400 dilution, Sigma, USA). The antigen for α-SMA was retrieved using heat-induced in the microwave at 95–100°C, 10 min, in 10 mM citric acid, pH 6, while the collagen-1 and collagen-3 using enzymatic reaction Proteinase K (Dako, Denmark) at 25°C for 10 min. Antibody labeling towards α-SMA was incubated at 25°C for 1 hour, except the collagen-1 and collagen-3 were overnight at 4°C. The labeled sections were visualized using the microscope Brightfield Zeiss (AxioVert.A1, Zeiss, Germany). Cardiac actin-myosin was visible by identification of α-actinin, using immunofluorescence (IF) with Alexa Fluor 555nm as the fluorophore at 1:500 ratio (Invitrogen, USA), visualized using confocal microscopy (Leica LSM 090, Germany) filtered at 600 nm, excitation at 555 nm. The sections were also counterstained with 4′,6-diamidino-2-phenylindole (DAPI) to identify the cell nucleus of the cardiac actin-myosin, visualized at the light excitation at 359 nm, emission at 457 nm.

Data analysis
Cardiac enzymes were presented in the histogram, and the difference between before and after ligation was analyzed using the paired-t test. The area that expressed collagen-1, collagen-3, and α-SMA after IHC labeling was measured using ImageJ (NIH, USA). Four examiners analyzed and performed five fields with three repetitions in each section. The data collected from the observations were verified and compiled using SPSS software ver.20, presented as a descriptive table. Data normality and statistical hypothesis tests for each dependent variable were also done with the same software. An independent T-test was used for normally distributed data. For data that are non-normally distributed, the Mann-Whitney test was used for analysis. Data analysis was done using GraphPad Prism version 7.
Some areas showed broken myocardial fibers, even though most sections retained parallel structures of myocardial fibers. The cytoplasm surrounding the nuclei was enlarged in several regions; meanwhile, matrix denaturation was not recognized (Figure 3D).

The sarcomeric pattern of myocardial fibers indicated the actin-myosin was also visible in 400x mag (Figure 3C), while after 60 minutes of infarction, most sarcomeric patterns had disappeared. Scars were also visible as collagen-stained yellow between broken fibers, with red blood cell extravasation spilled all over the fibers (Figure 4D). Scars stained yellow were visible after 60 minutes of infarction, indicating damage to myocardial fibers, with a diminished sarcomere pattern.

Immunohistochemical labeling towards collagen-1, collagen-3, and α-SMA indicated decreased expressions in the infarct sections compared to the sham group. Collagen-1 disappeared in several areas, with RBC extravasation seen (highlighted in a black circle) with irregular muscle fibers (Figure 3F) compared to the sham (Figure 3E). All controls verified these results (Figure 3G, H). Sections of collagen-1 positive labeling of the sham and infarct groups were calculated into µm² area using the ImageJ application. They indicated that after 60 minutes of myocardial infarction, collagen-1 significantly diminished (1,767.87 ± 249.12 µm²) compared to the sham group (4,409.59 ± 628.01 µm²) (p<0.01) (Figure 4). The expression of collagen-3 was also decreased after 60 minutes of infarction compared to the sham group. Their controls verified these. The sham group had an area positive to collagen-3 up to 4,424.49 ± 400.57 µm², significantly higher compared to the infarct group, 1,792.53 ± 99.53 µm² (p<0.01).

Myocardial actin-myosin fibers, epicardial and myocardial cells, smooth muscle cells, pericytes, and cardiac fibroblasts express α-SMA, labeled homogeneous smears across the section. In our study, α-SMA was found to decrease after 60 minutes of infarction (1,430.79 ± 167.61 µm²) compared to the sham group (4,991.47 ± 459.14 µm²) (p<0.01) (Figure 4).

In our study, α-actinin was identified using IF with Alexa fluor 555 nm, showed

**RESULTS**

**Results of infarct modeling in pigs**

All pigs induced for MI after 60 minutes of PLV artery ligation had recorded ST elevation from ECG [Figure 2B] compared to the sham group [Figure 2A]. At the same time, the echocardiography demonstrated anterior wall motion abnormality, increasing serum cTnT to 479.28 ± 82.94 pg.mL⁻¹ (CI 95% 429.16–529.40, p = 0.002) or 55.01-fold from pre-ligation and the CK–MB up to 69.26 ± 12.92 mU.mL⁻¹ (CI 95% 61.45–77.72, p = 0.001) or 12.41-fold [Figure 2B, C]. Hence, the MI was achieved in all subjects.

**Results of histopathology staining, IHC, and IF**

This study highlighted the changes in ECM after 60 minutes of PLV ligation. Sections of the infarct area stained with H&E indicated acute bleeding with extravasation of red blood cells scattered all over the matrices near the torn capillary, compared to the sham sections (Figure 3B).
the infarct group showed fragmented fibers expressing α-actinin, with fewer nuclei stained positive to DAPI, compared to the sham group.

**DISCUSSIONS**

Our study aimed to investigate the ECM of the myocardium after 60 minutes of infarction. Sixty minutes is the golden period for reperfusion therapy in patients with ST-elevation myocardial infarction/STEMI, according to the ACC/AHA guidelines. Investigating the alteration in the histoarchitecture of early MI contributes to ongoing research in stem cell therapy for MI to reverse myocardial remodeling by specific-targeted therapy. Our initial study successfully generated MI in the porcine heart model by suture ligating the PLV artery. All subjects in this study were confirmed for MI by cardiac troponin I elevation to 18–75%.

Our study indicated that after 60 minutes of infarction, the infarcted area and might kill the experimental animals immediately. Cardiac myofibroblast that was positive to vimentin and α-SMA but negative to calponin and desmin increased significantly after 3 to 7 days of one-hour infarction followed by reperfusion.

Myofibroblast absence also emphasized the matrix destruction phase in our study, while collagen-1 and collagen-3 were reduced. It is believed that during the myocardial healing process after infarction, the reserve cardiac fibroblast will transform into myofibroblast, express high α-SMA, and activate focal adhesion kinase (FAK) to generate large amounts of ECM proteins.

A study of forty-six human autopsies from sudden death due to an MI within the first two hours showed wavy fibers from early infarction as first order to third order. The late-phase infarction showed necrotic coagulation and contraction bands were found after 12 to 36 hours of infarction with signs of vascular occlusion in the area of infarction. In our study, 60 minutes of infarction had no signs, such as wavy fibers and necrotic coagulation. A study of MI in rabbit models showed a decrease in neutrophils after 6 hours. In contrast, necrotic coagulation occurred 7 hours post-infarction, the peak of fibrosis formation appeared after 36 hours, and the mature wound formed after 56 hours of infarction.

Another study using the Sprague-Dawley rat heart as an MI model after ligation of the left anterior descending coronary artery and after 1-, 2-, 3-, and 4-weeks ligation showed an increase of collagen-1 by 1.4-, 1.5-, 2.9-, and 3.9-fold compared to the sham, collagen-3 increased by 1.2-, 1.7-, 2.8- and 3.9-fold, and elastic fiber gradually diminished from 2, 77, 86, or 97% reduction, respectively to the timeline stated above. MI has compromised cardiac function following the formation of scars, decreased elastin fibers in the infarcted zone, increased stiffness, and reduced heart flexibility.

Our study indicated that after 60 minutes of infarction, the infarcted area had expressed less collagen-1, 3, and α-SMA in the infarct group, significantly compared to the sham group. The expression of α-actinin identified by IF was also reduced in the infarcted area of the infarct group compared to the sham group. Myocardial ischemia leads to inflammatory cascades...
involving reactive oxygen species/ROS. This series of cascades occurs with the activation of collagenase, which activates the matrix metalloproteinase that digests ECM components. ECM damages and cardiomyocyte death after ischemia were due to hypoxic injury to the cardiomyocytes, resulting in a decrease in NAD+, leading to mitochondria dysfunction and energy depletion, and eventually, increasing ROS, which causes Ca++ imbalance and activates protease. Protease is a catalytic enzyme that digests collagen matrix, causes fibers fragmentation, and induces fibrogenic reaction. Reports showed that cytoskeleton disintegration and matrix fragmentation happen initially before cardiomyocyte damage in MI is massive, with up to ten billion cell deaths. Massive cell death releases pro-inflammatory cytokines and damage-associated molecular patterns (DAMPs) into the infarct area until hours after the infarction, leading to severe local and systemic inflammation. Pro-inflammatory cytokines attract mononuclear cells to become macrophages and digest dead cells, especially cardiomyocytes. Non-cardiomyocyte cells such as fibroblasts, endothelium, and macrophages are much more susceptible to hypoxic injury. As a result, a severe infarct area eventually will become hypocellular, having homogeneous and dense scars in histological analysis.

Myocardial fibers consist of actin-myosin that is cross-linking during contraction/depolarisation. A healthy myocardial expresses α-actinin in the form of sarcomeric bands with I and A band arranged in a lattice position to form a Z-line structure with a 2.8 μm interval. Massive death of cardiomyocytes released massive ROS and released protease that digests collagen fibers and is seen as broken rod-shaped fibers between vacuoles and seen as spotted positive to α-actinin. Our study was parallel to other reports that showed a decrease in the ratio of α-actinin in skeletal muscle to α-actinin in heart muscle decreased in two weeks after MI. In a chronic MI, α-SMA is highly expressed in cells identified as myofibroblast. A study of MI using a murine model showed that cells positive to α-SMA were primarily found at the infarct border and associated with scar contraction. Others report abundant expression of α-SMA in the infarct area with contractile properties identified as myofibroblasts. It is believed that α-SMA is essential to contract and remodel post-infarction cardiac fibrosis healing. Their study concluded that severe inflammation reduced myofibroblast recruitment and delayed necrosis coagulation. Areas with α-SMA cell-positive will develop into fibrotic scars that alter the anatomy and physiology of the heart and, eventually, heart failure. Cardiofibroblast is believed to be the cardioprotectant against hypertrophy due to overload by expressing the KLF5 gene and releasing IGF-1 to grow the heart muscle. The infarct area will remodel once the inflammation has settled and hibernated, while chronic hibernation results in a permanent mature scar. Infarct healing aims to refrain myocardial rupture; nonetheless, mature scars resulted from the end of this process are very stiff and increase overload stress to the heart. Again, the force increases collagen production and cross-linking, resulting in mature scars that reduce cardiac compliance and decrease cardiac output. Therefore, an infarct area in the ventricle might grow as the force of the stiff heart wall causes an adjacent area with cardiomyocyte hypertrophy, fibrosis interstitials, and left ventricle hypertrophy. Nonetheless, remodeling can be reversed to hinder permanent hear wall stiffness.

On the other hand, another study in swine conducted for infarction with mid-LAD coronary artery occlusion was conducted in 20 pigs, involving one group within 90 minutes of ligation without reperfusion and two groups with reperusions and termination after 1 and 4 weeks (n=5). Elastic fibers were found in the infarcted area and scarred myocardium compared to the sham, with disorganized cardiomyocytes. The study showed no changes in collagen-1 and collagen-3 compositions, measured using Fast Fourier transform analysis. Laminin and fibronectin were found in the basement and around the fascicles. The expression of laminin and fibronectin was also higher in the infarct group compared to the healthy heart. This study confirmed a remodeling by 90-minute infarction.

CONCLUSION
Myocardial remodeling had been detected as early as 60 minutes after infarction with mild alteration in myocardial histoarchitecture and significant deterioration of ECM composition of collagen-1, collagen-3, α-SMA, and fragmented α-actinin fibers in the porcine heart model.

CONFLICT OF INTEREST
The authors declare that there is no competing interest regarding the manuscript.

ETHICAL CONSIDERATIONS
The methods used in this study were reviewed and approved by the Animal Ethics Committee, Faculty of Veterinary Medicine, IPB University, with Number 158/KEH/SKE/XII/2019.

FUNDING
None.

AUTHOR CONTRIBUTION
MAP and G were responsible for infarct modeling and harvesting specimens;
NS, NAF, and TRK were responsible for specimen verifications. MAP, G, NS, TRK, IA, TWS, PWB, and S contributed to the drafting and editing.

ACKNOWLEDGMENT
Authors want to thank the Badan Riset dan Inovasi Nasional-DIKTI (National Research and Innovation Agency-Ministry of Education, Culture, Research, and Technology) for their grants support Number: 008/E4.1/AK.04.PRN/2021. The authors also expressed gratitude to Universitas Indonesia, the national research priority flagship program for college institutions in 2021, who funded this research (No. PKS-190/UN2.INV/HKP05/2021).

REFERENCES
2. Benjamin EJ, Blaha MJ, Chiuve SE, Cushman HKP. College Institutions in 2021, who funded research priority flagship program for Universitas Indonesia, the national research priority flagship program for college institutions in 2021, who funded this research (No. PKS-190/UN2.INV/HKP05/2021).

