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Original Paper

Galectin-3 Mitigates Cardiomyocytes Injury through Modulation of Left Ventricular Cathepsins B, D, L and S at 24-Hour Post Myocardial Infarction

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Key Words
Heart • Myocardial infarction • Galectin 3 • Cathepsins

Abstract
Background/Aims: Galectin 3 (GAL-3) is a beta galactoside binding lectin that has different roles in normal and pathophysiological conditions. GAL-3 was found to be up regulated in animal models of myocardial infarction (MI). Cathepsins are intracellular lysosomal proteases that degrade proteins. The objective of his study is to investigate if high GAL-3 after myocardial infarction has a protective role on the heart through its modulation of lysosomal Cathepsins in ischemic myocardium. ***Methods:*** Male C57B6/J mice and GAL-3 knockout (KO) mice were used for permanent ligation of the left anterior descending artery of the heart to create infarction in the anterior myocardium. Hearts and plasma samples were collected 24 hours after the induction of MI and were used for enzyme linked immunosorbent assay and immunofluorescent staining. ***Results:*** Our results show that the significant increase in GAL-3 levels in the left ventricle at 24-hour following MI is associated with significant lower levels of cathepsins B, D, L and S in GAL-3 wild MI group than GAL-3 KO MI group. We also report a significant lower plasma level of Troponin I in GAL-3 wild MI group than GAL-3 KO MI group. ***Conclusion:*** The increased levels of GAL-3 at 24-hour following MI regulates the process of cardiomyocytes injury through modulation of lysosomal cathepsins B, D, L and S.

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Introduction

Myocardial infarction (MI) is a grave medical event that develops following complete occlusion of coronary arteries or their branches. The acute hypoxic event affecting ischemic myocardium will induce a series of complex processes that involve many intracellular signaling pathways leading to structural and contractile dysfunction [1]. Cathepsins are lysosomal proteases that degrade proteins. There are around 15 cathepsins and each of them degrades a different protein, have different structures and work through different mechanisms [2, 3].

The expression of Cathepsins genes was reported to be increased in cardiac stress, remodeling, and dysfunction [4]. Cathepsin S was found to be increased during myocardial pressure overload in rodents and humans with hypertension-induced heart failure [4].

In addition, active Cathepsins S, and L can degrade extracellular matrix (ECM) proteins, including laminin [5], fibronectin [6], elastin [7], and collagens [8]. It is well known that matrix metalloproteinases (MMPs) can degrade all ECM proteins and activate cysteine cathepsins [9]. These outcomes support the notion that cathepsins may participate in cardiac remodeling by mediating ECM degradation in cooperation with other proteases such as MMPs and serine proteases.

Furthermore, the levels of the cathepsin B and S genes have been shown to be increased in the hypertrophic and failing myocardium [4].

In humans, the levels of cathepsins B, L and S, were increased in patients with dilated and hypertrophic cardiomyopathies compared with normal controls [10].

Cathepsin D was found to be elevated in the plasma and hearts of patients following MI [11].

Galectin-3 (GAL-3) is a beta galactoside binding lectin that plays diverse roles in normal and pathological conditions [12]. GAL-3 has been linked with heart failure in recent years [13]. GAL-3 has been shown to coordinate intracellular processes for lysosomal repair and replacement [14].

Our previous results have shown substantial increase in GAL-3 levels in the cardiomyocyes, endothelial cells and neutrophil polymorphs in the heart as well as plasma at 24-hour post MI [15]. We hypothesized that high level of GAL-3 at 24- hour post MI time has a protective role on the heart through modulating myocardial injury which is reflected by lower levels of cathepsins in the myocardium. We used male C57BL6 mice and GAL-3 KO mice with the same background strain to look for cathepsins levels in the left ventricle, in the presence and absence of GAL-3, at 24-hour post MI heart samples.

Materials and Methods

Murine model of myocardial infarction

Male C57B6/J mice (*n=16*) and GAL-3 KO mice (*n=16*) were divided into 24-hour MI group and sham operated groups. GAL-3 wild and KO mice (male, age: 12-16 weeks; wt: 20-25g) were anesthetized by an intraperitoneal injection of Phenobarbitone (70mg/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 (AD Instrument multi-channel recorder interfaced with a computer running Power lab 4/30 data acquisition software) was recorded from needle electrodes inserted subcutaneously. Myocardial infarction was induced in the mice by permanently occluding the left anterior descending coronary artery (LAD) as described earlier [16, 17].

Briefly, the chest was opened with a lateral incision at the 4th intercostal space on the left side of the sternum. Then the chest wall was retracted for better visualization of the heart. With minimal manipulation, the pericardial sac was removed and the left anterior descending artery (LAD) was visualized with a

stereomicroscope (Zeiss STEMI SV8). An 8-0 ophthalmic silk suture was passed under the LAD and ligated 1mm distal to left atrial appendage. Occlusion was confirmed by observing immediate paleness of the left ventricle post ligation. An accompanying ECG recording showed characteristic ST-Elevation which further confirmed ischemia. The chest wall was then closed by approximating the third and fourth ribs with one or two interrupted sutures. The muscles were 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 2mg/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 hours after induction of myocardial infarction. The hearts were washed in ice cold PBS, right ventricle and both atria were dissected away and left ventricle was 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.

Protein extraction from samples

Total protein was extracted from heart samples by homogenizing with lysis buffer and collecting the supernatant after centrifugation. For total tissue homogenate, the Left ventricular (LV) heart samples were thawed, weighed and put in cold lysis buffer containing 50mM Tris, 300mM NaCl, 1mM $MgCl_2$, 3mM EDTA, 20mM β-glycerophosphate, 25mM NaF, 1% Triton X-100, 10%w/v Glycerol and protease inhibitor tablet (Roche Complete protease inhibitor cocktail tablets). The hearts were homogenized on ice by a homogenizer (IKA T25 Ultra Turrax). The samples were then centrifuged at 14000 RPM for 15 minutes at 4 °C, supernatant collected, alliquoted and stored at -80 °C until further analysis. Total protein concentration was determined by BCA protein assay method (Thermo Scientific Pierce BCA Protein Assay Kit).

Tissue Processing

Hearts were excised, washed with ice-cold phosphate buffer saline (PBS), blotted with filter paper and weighed. 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.

Immunofluorescent double labeling

Five-um sections were deparaffinized with xylene and rehydrated with graded alcohol. Sections were placed in EnVisionTM FLEX Target Retrieval Solution with a high PH (PH 9) (DAKO Agilent, USA) in a water bath at 95 °C for 30 minutes hour. Sections were washed with distilled water for 5 minutes followed by PBS for 5 minutes. Sections were then incubated with anti-cathepsin D antibody (Rabbit Polyclonal, 1:100, Thermo Fisher Scientific, USA), anti-cathepsin B (Rabbit monoclonal, 1:100, Cell Signaling Technology, USA), anti-cathepsin L (Rabbit monoclonal, 1:100, Abcam, USA), anti-cathepsin S (Rabbit monoclonal, 1:100, Abcam, USA), anti-galectin-3 (Rabbit polyclonal, 1:100, Santa Cruz Biotechnology) overnight at room temperature. Sections were subsequently washed with PBS and incubated with donkey anti-rabbit Rhodamine labeled secondary antibody (Jackson Immune Research Laboratories, USA, 1:100) for one hour at room temperature. Sections were then washed with PBS and incubated with the second primary antibody anti-desmin (Mouse monoclonal, 1:100, DAKO Agilent, USA), overnight at room temperature followed by washing with PBS and incubation with donkey anti-mouse Alexa Fluor 488, (Jackson Immune Research Laboratories, USA, 1:100) antibody for one hour at room temperature. Sections were then washed and mounted in Dapi-water-soluble mounting media (Abcam, USA) and viewed with Olympus Fluorescent microscope. Appropriate positive control sections 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. Positive and negative controls were used in every batch of stained slides (not shown in figures).

Morphometric analysis

Morphometric analysis of GAL-3, cathepsin B, cathepsin D, cathepsin L, and cathepsin S expression in left ventricular cells was done at 24-hour following MI using ImageJ software (http://rsbweb.nih.gov/ij/).

Cathepsin B, cathepsin D, cathepsin L, and cathepsin S labeling was determined by counting the number of positively stained lysosomes within cells in randomly-selected high power fields (HPF) in the left

ventricle. The mean numbers of positive lysosomes were converted from per HPF to per mm2 (Each mm2= 4HPF). GAL-3 labeling was determined by counting the number of positively stained cells in randomly-selected high power fields (HPF) in the left ventricle. The mean numbers of positive cells were converted from per HPF to per mm2 (Each mm2= 4HPF).

For, cathepsin B, cathepsin D, cathepsin L, and cathepsin S labeling, cells were considered positive when there was a dotted cytoplasmic staining pattern of lysosomes. For GAL-3 labeling, cells were considered positive when there was a cytoplasmic or nuclear staining pattern or both.

Enzyme linked immunosorbent assay

Left ventricular myocardial concentration of GAL-3, Cathepsin B, Cathepsin D, Cathepsin L, Cathepsin S and Troponin-I were determined using enzyme linked immunosorbent assay (ELISA) development kits: [galectin-3 (DY1197) R&D Systems, Minneapolis, MN, USA], Cathepsin B, (DY2176) R&D Systems, Minneapolis, MN, USA], Cathepsin D, (ab239420) Abcam, Boston, MA, USA], Cathepsin L, (DY952) R&D Systems, Minneapolis, MN, USA], Cathepsin S, (DY1183) R&D Systems, Minneapolis, MN, USA], and cardiac Troponin-I Elisa kit (2010-1-HSP, Life Diagnostics, Inc.), 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-Immune Plate MaxiSorp Surface (NUNC Brand Products, A/S, Roskilde, Denmark), were coated with antibody specific to 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.

Statistical analysis

All statistical analyses were done using GraphPad Prism Software version 5. Multiple comparisons between the various groups were achieved by one-way analysis of variance (ANOVA), followed by Newman-Keuls post hoc multiple range tests. Data are presented in mean ± standard error (S.E). P values < 0.05 are considered significant.

Results

Plasma Troponin I at 24- hour post MI

Plasma Troponin I was measured by ELISA at 24-hour post MI in GAL-3 wild and GAL-3 KO MI groups and corresponding sham groups. There were significantly higher levels of plasma Troponin I in both GAL-3 wild (*P<0.001*) and GAL-3 KO (*P<0.001*) MI groups as compared to their sham control groups. This confirms acute myocardial infarction in mice in both groups. Moreover, there was a significantly higher levels of plasma Troponin I in GAL-3 KO MI group (12.83 ± 0.7312 ng/ml) than GAL-3 wild MI group (5.992 ± 0.312 ng/ml), (*P<0.001*), (Fig. 1). This indicates absence of GAL-3 is associated with more damage to the cardiac myocytes and suggests a protective role of GAL-3 at 24-hour post MI.

Galectin-3 is increased in the LV at 24-hour post MI

Galectin-3 was measured by ELISA in tissue homogenate of LV at 24-hour post MI hearts in the GAL-3 wild type mice. Our results show GAL-3 level is significantly higher in GAL-3 wild MI group than GAL-3 wild sham group (14950 ± 1662 vs 4628 ± 345.1 pg/mg, *P<0.0001*), (Fig. 2).

Immunofluorescent double labeling has shown higher expression of GAL-3 in cardiomyocytes, endothelial cell and infiltrating neutrophil polymorphs in areas around the infarction (Fig. 3A-C) when compared to the expression of GAL-3 in areas away from the infarction (Fig. 3D and E).

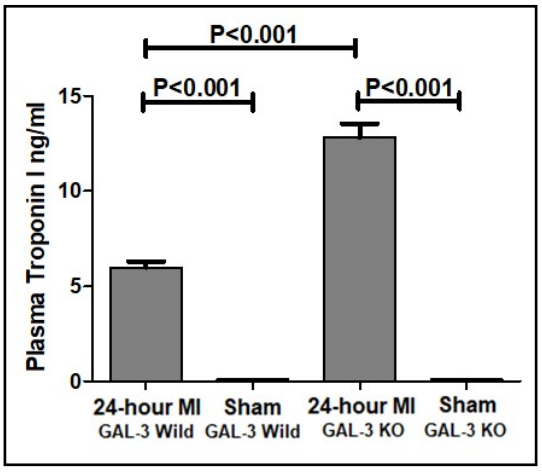


Fig. 1. The graph represents plasma concentrations of troponin I at 24-hour following MI in GAL-3 wild and GAL-3 KO MI mice compared to their control sham. P value<0.05 is statistically significant.

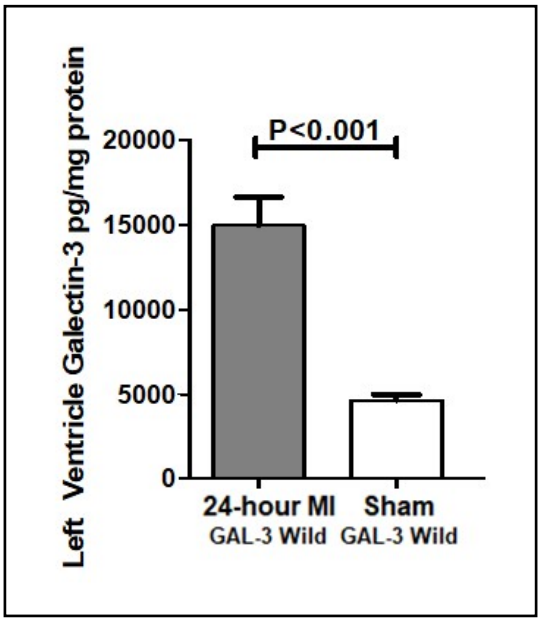


Fig. 2. The graph represents left ventricular concentrations of GAL-3 at 24-hour following MI in GAL-3 wild MI mice compared to their control sham. P value <0.05 is statistically significant.

LV Cathepsins at 24-hour post MI

Cathepsin B Activity. Cathepsin B activity was measured by ELISA in the tissue homogenate of LV of 24- hour post MI hearts in GAL-3 wild and GAL-3 KO MI groups and corresponding sham groups.

There were significantly higher levels of cathepsin B protein in both GAL-3 wild (*P<0.001*) and GAL-3 KO (*P<0.001*) MI groups as compared to their sham control groups. Moreover, there was a significantly higher levels of cathepsin B in GAL-3 KO MI group (256.6 ± 4.192 pg/mg) than GAL-3 wild MI group (181.9 ± 2.392 pg/mg), (*P<0.001*), (Fig. 4).

Immunofluorescent double labeling has shown increase in the expression of cathepsin B in cardiomyocytes and endothelial cells at 24-hour following MI both in GAL-3 wild and KO MI groups (Fig. 5A and C and Fig. 6A). The pattern of staining was dotted lysosomal staining. There was a significantly higher expression of cathepsin B in areas around infarction than areas away from the infarction in both GAL-3 wild and KO MI groups (Fig. 5A-D and Fig. 6A). We also show a significantly higher expression of cathepsin B in areas around infarction in GAL-3 KO than GAL-3 wild MI groups (Fig. 5A-D and Fig. 6A).

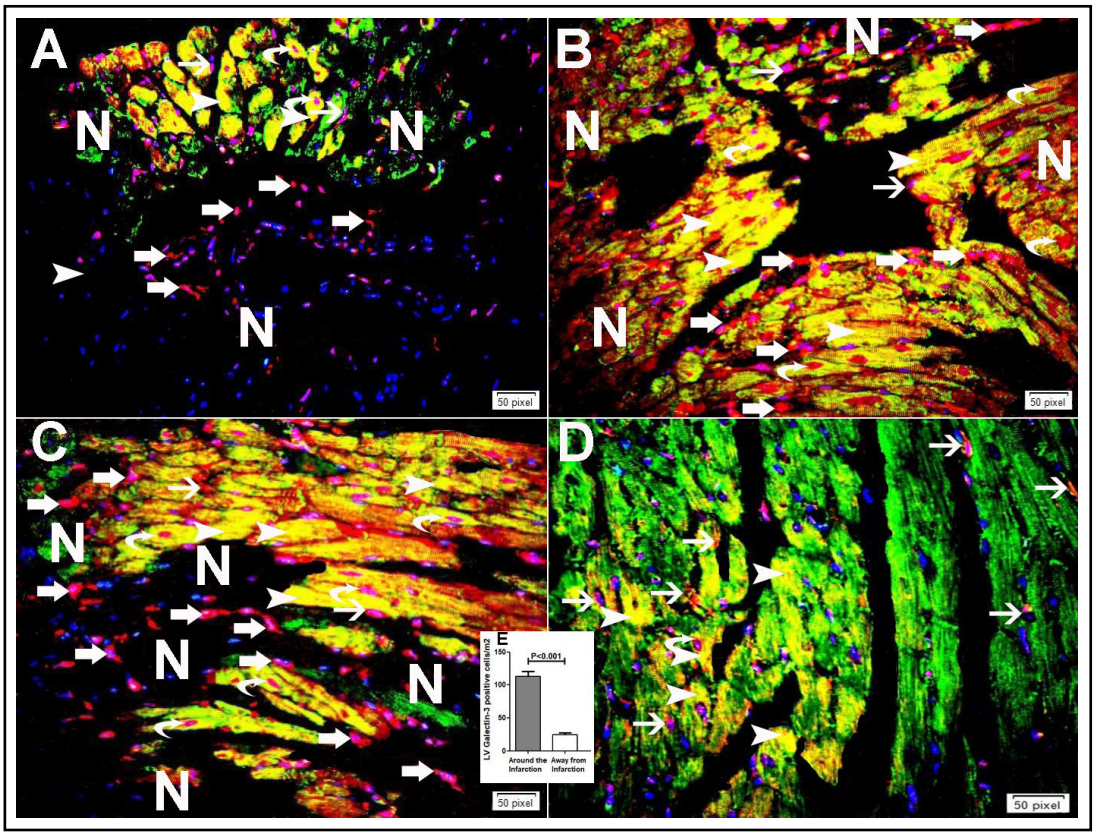
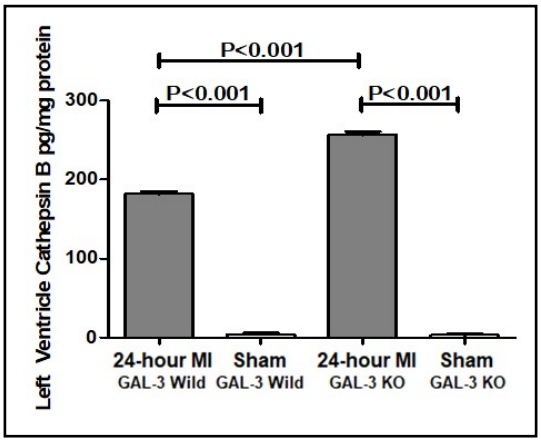


Fig. 3. A, B, C: Representative sections from LV from areas around infarction stained with double immunofluorescent labels for desmin and GAL-3 showing high expression of GAL-3 in cardiomyocytes cytoplasm (arrow head) and nuclei (curved arrow). GAL-3 is also highly expressed by endothelial cells (thin arrow) and neutrophil polymorphs (thick arrow). N indicates areas of necrosis. D: Representative sections from LV from areas away from infarction stained with double immunofluorescent labels for desmin and GAL-3 showing focal staining of cardiomyocytes with GAL-3 (arrowhead) and endothelial cells (thin arrow). E: The graph represents the number of left ventricular GAL-3 positive cells/m^2 at 24-hour following MI in GAL-3 wild MI mice compared to their control sham. P value<0.05 is statistically significant.

Fig. 4. The graph represents left ventricular concentrations of cathepsin B at 24-hour following MI in GAL-3 wild and GAL-3 KO MI mice compared to their control sham. P value<0.05 is statistically significant.



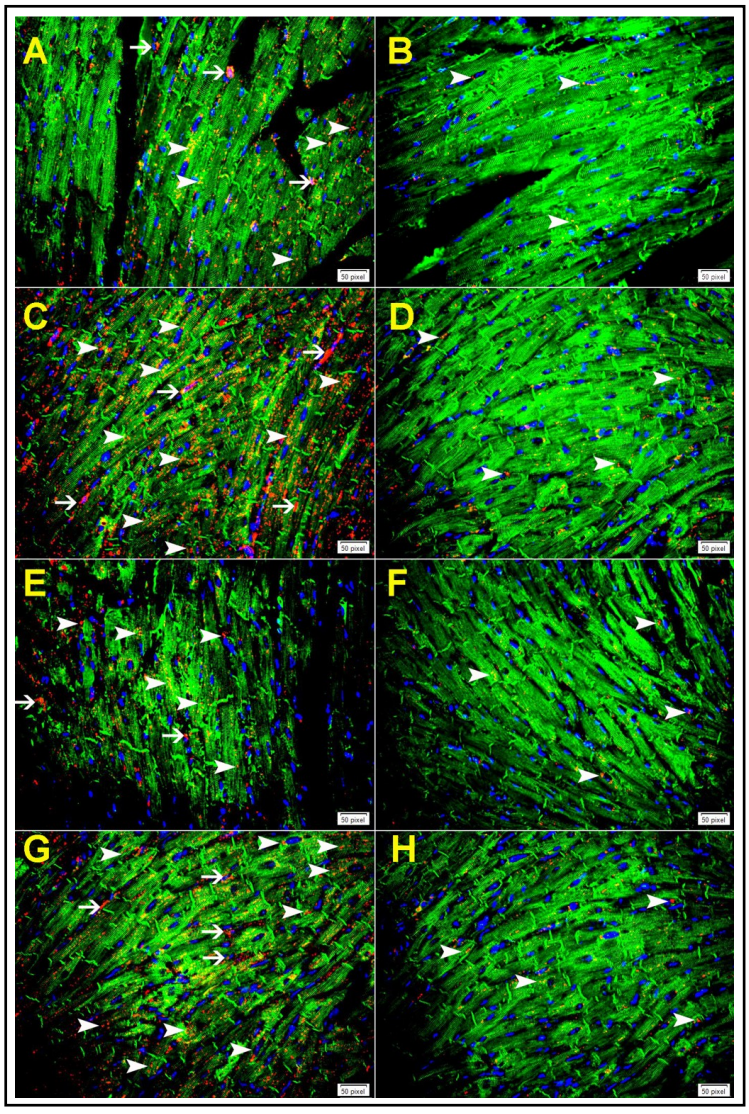


Fig. 5. A. Representative sections from LV from areas around infarction stained with double immunofluorescent labels for desmin and cathepsin B showing high expression of cathepsin B in lysosomes within cardiomyocytes (arrow head) and endothelial cells (thin arrow) in GAL-3 wild MI mice at 24-hour following MI. B. Representative sections from LV from areas away from infarction stained with double immunofluorescent labels for desmin and cathepsin B showing expression of cathepsin B in lysosomes within cardiomyocytes (arrow head) in GAL-3 wild MI mice at 24-hour following MI. C. Representative sections from LV from areas around infarction stained with double immunofluorescent labels for desmin and cathepsin B showing high expression of cathepsin B in lysosomes within cardiomyocytes (arrow head) and endothelial cells (thin arrow) in GAL-3 KO MI mice at 24-hour following MI. D. Representative sections from LV from areas away from infarction stained with double immunofluorescent labels for desmin and cathepsin B showing expression of cathepsin B in lysosomes within cardiomyocytes (arrow head) in GAL-3 KO MI mice at 24-hour following MI. E. Representative sections from LV from areas around infarction stained with double immunofluorescent labels for desmin and cathepsin D showing high expression of cathepsin D in lysosomes within cardiomyocytes (arrow head) and endothelial cells (thin arrow) in GAL-3 wild MI mice at 24-hour following MI. F. Representative sections from LV from areas away from infarction stained with double immunofluorescent labels for desmin and cathepsin D showing expression of cathepsin D in lysosomes within cardiomyocytes (arrow head) in GAL-3 wild MI mice at 24-hour following MI. G. Representative sections from LV from areas around infarction stained with double immunofluorescent labels for desmin and cathepsin D showing high expression of cathepsin D in lysosomes within cardiomyocytes (arrow head) and endothelial cells (thin arrow) in GAL-3 KO MI mice at 24-hour following MI. H. Representative sections from LV from areas away from infarction stained with double immunofluorescent labels for desmin and cathepsin D showing expression of cathepsin D in lysosomes within cardiomyocytes (arrow head) in GAL-3 KO MI mice at 24-hour following MI.

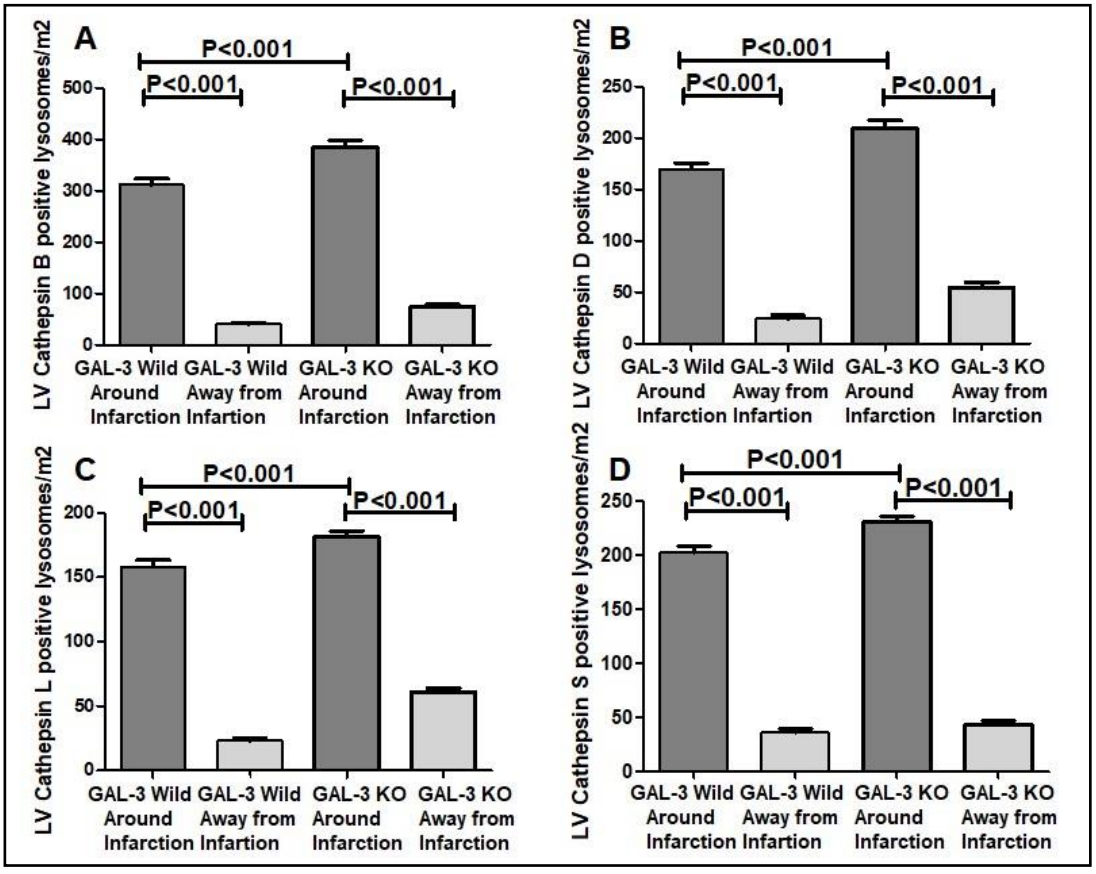


Fig. 6. A. The graph represents the number of left ventricular cathepsin B positive lysosomes/m^2 at 24-hour following MI in GAL-3 wild MI and GAL-3 KO MI mice compared to their control sham. P value<0.05 is statistically significant. B. The graph represents the number of left ventricular cathepsin D positive lysosomes/m^2 at 24-hour following MI in GAL-3 wild MI and GAL-3 KO MI mice compared to their control sham. P value<0.05 is statistically significant. C. The graph represents the number of left ventricular cathepsin L positive lysosomes at 24-hour following MI in GAL-3 wild MI and GAL-3 KO MI mice compared to their control sham. P value<0.05 is statistically significant. D. The graph represents the number of left ventricular cathepsin S positive lysosomes at 24-hour following MI in GAL-3 wild MI and GAL-3 KO MI mice compared to their control sham. P value<0.05 is statistically significant.

Cathepsin D Activity. Cathepsin D activity was measured by ELISA in the tissue homogenate of LV of 24- hour post MI hearts in GAL-3 wild and GAL-3 KO MI groups and corresponding sham groups.

There were significantly higher levels of cathepsin D protein in both GAL-3 wild (*P<0.01*) and GAL-3 KO (*P<0.001*) MI groups as compared to their sham control groups. Moreover, there was a significantly higher levels of cathepsin D in GAL-3 KO MI group (64.36 ± 1.801 ng/mg) than GAL-3 wild MI group (44.11 ± 2.424 ng/mg), (*P<0.001*), (Fig. 7).

Immunofluorescent double labeling has shown increase in the expression of cathepsin D in cardiomyocytes and endothelial cells at 24-hour following MI both in GAL-3 wild and KO MI groups (Fig. 5E and G and Fig. 6B). The pattern of staining was dotted lysosomal staining. There was a significantly higher expression of cathepsin D in areas around infarction than areas away from the infarction in both GAL-3 wild and KO MI groups (Fig. 5E-H and Fig. 6B). We also show a significantly higher expression of cathepsin D in areas around infarction in GAL-3 KO than GAL-3 wild MI groups (Fig. 5E-H and Fig. 6B).

Cathepsin L Activity. Cathepsin L activity was measured by ELISA in the tissue homogenate of LV of 24- hour post MI hearts in GAL-3 wild and GAL-3 KO MI groups and corresponding sham groups.

There were significantly higher levels of cathepsin L protein in both GAL-3 wild (*P<0.001*) and GAL-3 KO (*P<0.001*) MI groups as compared to their sham control groups. Moreover, there was a significantly higher levels of cathepsin L in GAL-3 KO MI group (804.3 ± 14.18 pg/mg) than GAL-3 wild MI group (740.1 ± 9.980 pg/mg), (*P<0.001*), (Fig. 8).

Immunofluorescent double labeling has shown increase in the expression of cathepsin L in cardiomyocytes at 24-hour following MI both in GAL-3 wild and KO MI groups (Fig. 9A and C and Fig. 6C). The pattern of staining was dotted lysosomal staining. There was a significantly higher expression of cathepsin L in areas around infarction than areas away from the infarction in both GAL-3 wild and KO MI groups (Fig. 9A-D and Fig. 6C). We also show a significantly higher expression of cathepsin L in areas around infarction in GAL-3 KO than GAL-3 wild MI groups (Fig. 9A-D and Fig. 6C).

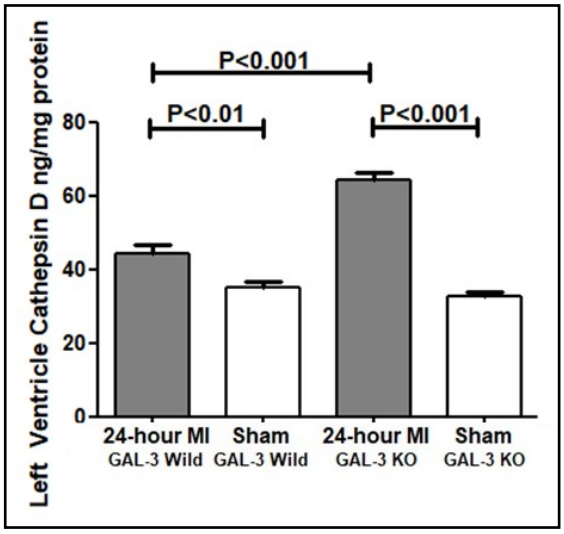


Fig. 7. The graph represents left ventricular concentrations of cathepsin D at 24-hour following MI in GAL-3 wild and GAL-3 KO MI mice compared to their control sham. P value<0.05 is statistically significant.

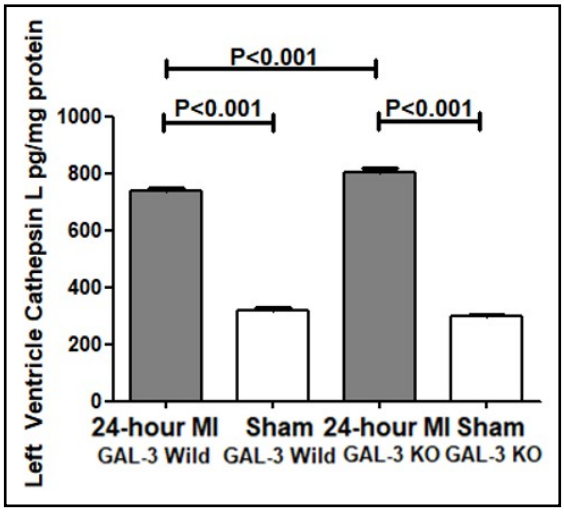


Fig. 8. The graph represents left ventricular concentrations of cathepsin L at 24-hour following MI in GAL-3 wild and GAL-3 KO MI mice compared to their control sham. P value<0.05 is statistically significant.

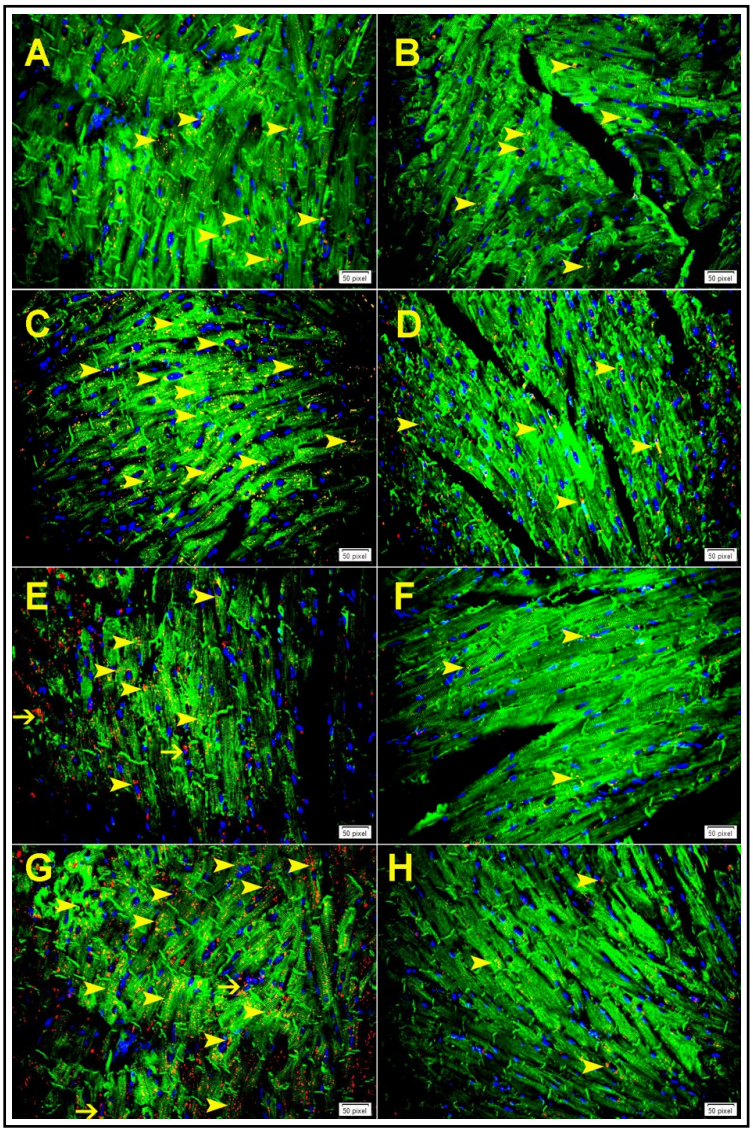


Fig. 9. A. Representative sections from LV from areas around infarction stained with double immunofluorescent labels for desmin and cathepsin L showing high expression of cathepsin L in lysosomes within cardiomyocytes (arrow head) in GAL-3 wild MI mice at 24-hour following MI. B. Representative sections from LV from areas away from infarction stained with double immunofluorescent labels for desmin and cathepsin L showing expression of cathepsin L in lysosomes within cardiomyocytes (arrow head) in GAL-3 wild MI mice at 24-hour following MI. C. Representative sections from LV from areas around infarction stained with double immunofluorescent labels for desmin and cathepsin L showing high expression of cathepsin L in lysosomes within cardiomyocytes (arrow head) in GAL-3 KO MI mice at 24-hour following MI. D. Representative sections from LV from areas away from infarction stained with double immunofluorescent labels for desmin and cathepsin L showing expression of cathepsin L in lysosomes within cardiomyocytes (arrow head) in GAL-3 KO MI mice at 24-hour following MI. E. Representative sections from LV from areas around infarction stained with double immunofluorescent labels for desmin and cathepsin S showing high expression of cathepsin S in lysosomes within cardiomyocytes (arrow head) and endothelial cells (thin arrow) in GAL-3 wild MI mice at 24-hour following MI. F. Representative sections from LV from areas away from infarction stained with double immunofluorescent labels for desmin and cathepsin S showing expression of cathepsin S in lysosomes within cardiomyocytes (arrow head) in GAL-3 wild MI mice at 24-hour following MI. G. Representative sections from LV from areas around infarction stained with double immunofluorescent labels for desmin and cathepsin S showing high expression of cathepsin S in lysosomes within cardiomyocytes (arrow head) and endothelial cells (thin arrow) in GAL-3 KO MI mice at 24-hour following MI. H. Representative sections from LV from areas away from infarction stained with double immunofluorescent labels for desmin and cathepsin S showing expression of cathepsin S in lysosomes within cardiomyocytes (arrow head) in GAL-3 KO MI mice at 24-hour following MI.

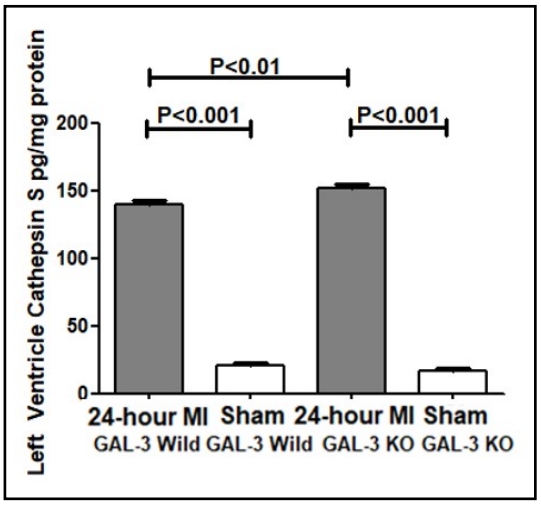


Fig. 10. The graph represents left ventricular concentrations of cathepsin S at 24-hour following MI in GAL-3 wild and GAL-3 KO MI mice compared to their control sham. P value<0.05 is statistically significant.

Cathepsin-S Activity. Cathepsin S activity was measured by ELISA in the tissue homogenate of LV of 24- hour post MI hearts in GAL-3 wild and GAL-3 KO MI groups and corresponding sham groups.

There were significantly higher levels of cathepsin S protein in both GAL-3 wild (*P<0.001*) and GAL-3 KO (*P<0.001*) MI groups as compared to their sham control groups. Moreover, there was a significantly higher levels of cathepsin S in GAL-3 KO MI group (151.5 ± 2.818 pg/mg) than GAL-3 wild MI group (139.6 ± 2.920 pg/mg), (*P<0.01*), (Fig. 10).

Immunofluorescent double labeling has shown increase in the expression of cathepsin S in cardiomyocytes and endothelial cells at 24-hour following MI both in GAL-3 wild and KO MI groups (Fig. 9E and G and Fig. 6D). The pattern of staining was dotted lysosomal staining. There was a significantly higher expression of cathepsin S in areas around infarction than areas away from the infarction in both GAL-3 wild and KO MI groups (Fig. 9E-H and Fig. 6D). We also show a significantly higher expression of cathepsin S in areas around infarction in GAL-3 KO than GAL-3 wild MI groups (Fig. 9E-H and Fig. 6D).

Discussion

Cathepsins are intracellular proteases that are active in an acidic environment of the lysosomes. Serine proteases; cathepsins A and G, aspartic proteases; cathepsins D and E and cysteine proteases; cathepsins B, C, F, H, K, L O, S, V, X and W were traditionally considered as intracellular enzymes required for non-specific, bulk proteolysis in the lysosomal compartment where they degrade intracellular and extracellular proteins [18, 19]. This view is, however, changing. Newer studies have shown a diverse role of these proteases. Localization of cathepsins in the nucleus, cytoplasm and plasma membrane have been found; for example, cathepsin L is also localized in the nucleus and is shown to take part in the regulation of cell-cycle progression [20], proteolytic processing of histone H3 tail [21] and interaction with other specific histones and protease inhibitor [22]. Cathepsins are also known to be responsible for the remodeling of the extracellular matrix with strong elastolytic and collagenolytic activities [23].

Numerous studies have been done so far linking cathepsins to cardiovascular diseases. Patients with coronary artery stenosis have been shown to have elevated serum cathepsin L levels compared to those without lesions detectable by quantitative angiography [24]. Cathepsin D is shown by proteomic analysis to be increased both in plasma and monocytes of patients with acute coronary syndrome [25]. Elevated levels of cathepsin B is observed in patients in the acute phase of myocardial infarction who have had no past history of ischemic heart disease nor were they on any medication for the same [26]. Studies on the development

of atherosclerosis in apolipoprotein E-deficient (ApoE−/−) mice have shown expression of cathepsins B, D and L in macrophages- derived foam cells in the necrotic area of the plaques and fibrous cap of the atherosclerotic lesions [27].

Our results have shown that cathepsins B, D, L, and S are increased in the LV after 24 hours of MI as compared to the sham operated LVs. Immunofluorescent double labeling has also shown that there is significantly higher expression of cathepsins B, D, L, and S in areas around infarction than in areas away from the infarction. Moreover, we have shown that cathepsins B, D, L, and S are expressed in cardiomyocytes and endothelial cells by immunofluorescent double labeling.

Myocardial infarction results in ATP depletion in affected cells causing inhibition of cellular pumps, consequently leading to intracellular accumulation of Na^+ and H^+ ions. Activation of sodium calcium exchanger leads to increased intracellular Ca^{2+}, increased mitochondrial Ca^{2+}, mitochondrial permeability transition pore opening and necrosis. Mitochondrial swelling and rupture causes increased H^+ in the cytoplasm and with the inactivation of H^+ pumps leads to decrease in lysosomal pH and subsequent over activation of cathepsin proteases [28]. Lysosomal swelling and membrane rupture causes cathepsins to be released into the cytoplasm and the process of digesting of various substrates commences. Finally, the massive inflow of water into the cell by the osmotic imbalance ultimately leads to cell swelling and rupture of the plasma membrane [29].

Cathepsins have also demonstrated their role in apoptosis and autophagy [30]. Cathepsins play an active part in caspase dependent as well as independent cell death pathways. They are also shown to be important regulators of inflammatory cells like neutrophils, T and B lymphocytes [29]. Cathepsins are released from the lysosomes into the cytosol for its function either by lysosomal destabilization or lysosomal membrane permeabilization [30-32], however, the exact molecular mechanism of its release from cardiomyocyes after MI remains unknown.

Experimental evidence reveals that cathepsin D upregulation after seven days post MI contributes to enhanced myocardial autophagy [33]. The mechanism of this effect is linked to the removal of the autophagosomes in the heart [33]. Studies have overwhelmingly shown that this enhanced myocardial macroautophagy confers a protective role to cathepsin D by promoting protein and organelle quality control [34], which in turn protects against post-MI ventricular remodeling and HF progression.

GAL-3 is a member of a family of lectins with affinity for β-galactoside glycoconjugates. It is ubiquitously present in various organisms [35], as well as expressed by many organs of the body. Galectins are known for their extracellular functions; cell adhesion, migration, signaling, inflammation, fibrosis, infection, cardiovascular diseases, and cancer [36, 37].

Previously, our group has shown that GAL-3 at 24- hour post MI is protective against tissue injury by virtue of its antiapoptotic mechanisms in the myocardium [17]. We also identified that the anti-apoptotic mechanisms are likely mediated through interaction of intracellular GAL-3 with cathepsin D proteins among others. Our results in this study also show that Cathepsins D is higher in GAL-3 KO LVs after 24- hour MI when compared to wild type; in addition, we have tested three other Cathepsins, L, S and B and found the same pattern of expression. In the LVs of Sham group of both wild type and KO, the values of cathepsin, B, D, L and S in the LV are comparable, both have significantly lower values as compared to MI groups and also no significant differences between sham groups was observed.

Our results suggest that there is a possible direct or indirect link between GAL-3 signaling pathway and cathepsins since the absence of LV intracellular GAL-3 in GAL-3 KO MI mice leads to a significantly higher levels of cathepsins D, B, L and S than GAL-3 wild MI mice. We have shown a significantly higher levels of GAL-3 in the LV of GAL-3 wild MI group than sham control group. We have previously alluded to the fact the cathepsin D upregulation post MI time frame is related to its role in myocardial autophagy [33]. GAL-3 is also shown to act as a tag for autophagy receptors like tripartite motif containing 16 (TRIM16) [38, 39]; recruiting the autophagy machinery to damaged organelles after they are marked for degradation. Galectins are shown to form puncta on damaged lysosomes [40], and so serve

as markers for endolysosomal damage. Newer studies are however, showing that GAL- 3 is doing more than being just a marker for lysosomal damage.

Endosomal sorting complexes required for transport (ESCRT) is shown to play a role in repair of damaged lysosomes and promotes cell survival [41, 42]; this is especially important in the context of maintaining endolysosomal system homeostasis. GAL-3 and ESCRT Component ALIX interact and protect lysosomes from damage, evidenced by experimental approaches to measure lysosomal membrane repair [41, 42] at various time points.

GAL-3 thus switches from interplay with ESCRT, involved in membrane repair [25, 26], to Tripartite Motif Containing 16 (TRIM16) interactions, involved in autophagic removal of damaged lysosomes [38, 43].

Our results show increase in cathepsin B, D, L and S levels in GAL-3 knockout LVs after 24 hours of MI can be explained on the evidence that expressed GAL-3 during early post MI time point recruits, controls, and integrates ESCRT and autophagy pathways [27], resulting in building up a cellular response to endomembrane damage which may include membrane repair of lysosomal membrane and prevents cathepsins from being released; this explanation supports our finding of significantly lower concentrations of LV cathepsins B, D, L and S in GAL-3 wild MI mice than GAL-3 KO MI mice. Hence GAL-3 serves to activate survival mechanisms to prevent further cardiomyocyes damage this is supported by significantly lower levels of plasma troponin I in GAL-3 wild MI group than GAL-3 KO MI group suggesting that absence of intracellular GAL-3 is associated with more damage to the cardiomyocyes. Studies have shown a close correlation between the levels of cardiac troponins and infarct size in acute MI and that the level of plasma troponin is a measure of the magnitude of infarct size [44, 45]. Troponin I is a very specific cardiomyocyte protein and can only be seen in the peripheral blood when there is injury to cardiac myocytes that allows passage of troponin I to the circulation. However, as we do not have the infarct size measurement per se, we think that it is a limitation of this study and will be considered in our future studies.

So, following MI, GAL-3 is likely to orchestrate a response to either cope and repair damage or escalate its response due to accumulation of unsalvageable organelles.

Conclusion

The increased levels of GAL-3 at 24-hour following MI regulates the process of cardiomyocytes injury through modulation of lysosomal cathepsins B, D, L and S.

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Author Contributions

All authors reviewed and approved the submitted version of the manuscript. S.A. introduced the concept, designed the study, analyzed and interpreted the data, performed immunofluorescent double labeling, designed the figures, and wrote the manuscript. S.H. performed the experimental model of MI, analyzed and interpret the data, and wrote the manuscript. G.J. performed protein extraction, ELISA technique and submitted resulted data.

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Statement of Ethics

All animal experimental procedures are approved by the Animal Research Ethics Committee of the UAE University, Protocol ERA_2020_6175.

Disclosure Statement

The authors declare that no conflicts of interest exist.

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