


ORIGINAL CONTRIBUTION

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Diallyl trisulfide modulated autophagy in isoproterenol induced acute myocardial infarction

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Abstract

Background: Acute myocardial infarction (AMI) is the most serious manifestation of coronary artery disease. The initial ischemia in AMI causes biochemical and metabolic alterations in cardiomyocytes.

Objectives: The present study aimed to investigate the biomolecular mechanisms underlying cardioprotective effects of diallyl trisulfide (DATS) as well as captopril (CAP) in isoproterenol (ISO) induced AMI focusing on autophagy & PI3K/Akt signaling.

Methods: Seventy male Albino rats were divided into seven groups as follows: Normal control, ISO, ISO + LY294002 (PI3K inhibitor), DATS+ISO, CAP+ISO, DATS+LY294002 + ISO, and CAP+LY294002 + ISO. All treatments (40 mg/kg DATS, 50 mg/kg CAP & 0.3 mg/kg LY294002) were given daily for two weeks before ISO injection (85 mg/kg for 2 days). At the end of the experiment, serum and cardiac tissues were collected. Serum cardiac troponin I (cTnI), and creatine kinase MB (CK-MB) were measured. Cardiac glutathione peroxidase (GSH-px), malondialdehyde (MDA), hypoxia-inducible factor 1 alpha (HIF-1α), autophagy proteins (P62 & LC3IIB) and gene expression of PI3K, Akt, FOXO-1, and eNOS were assessed. Histopathological examination of heart tissue was performed.

Results: DATS and CAP significantly ($p < 0.01$) decreased serum CK-MB and cTnI, cardiac levels of MDA, HIF-1α, p62 and LC3IIB along with an increase in GSH-px activity compared with ISO group. Moreover, DATS and CAP significantly up-regulated PI3K, Akt, and eNOS gene expression but down-regulated FOXO-1 expression compared to ISO group. However, LY294002 reversed DATS and CAP cardioprotective effects.

Conclusion: DATS and CAP prior treatment proved cardioprotective effects via modulation of autophagy, PI3K/Akt signaling, eNOS and FOXO-1 downregulation in ISO induced AMI rat model.

Keywords: Autophagy, Captopril, DATS, Isoproterenol, Acute myocardial infarction, PI3K/Akt pathway

Introduction

Cardiovascular illnesses are the leading cause of death worldwide, accounting for almost 33% of all deaths, with acute myocardial infarction (AMI) being a major contributor [53]. During an AMI, the first ischemic

insult initiates a cascade of unanticipated biochemical and metabolic changes inside the heart tissue. The production of reactive oxygen species (ROS) has been identified as the primary cause of reperfusion damage. Furthermore, oxidative damage, inflammation, calcium excess, cellular autophagy and apoptosis exacerbate the condition [25, 55, 56].

Autophagy is a lysosomal breakdown pathway that is crucial for cell survival, homeostasis, and function. Cardiac hypertrophy, myocardial infarction,

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diabetic cardiomyopathy, and heart failure are all linked to autophagy dysregulation in cardiomyocytes [50].

Phosphoinositide-3 kinase (PI3K) is a key signaling protein in cell metabolism, and serine/threonine kinase (Akt) is the primary target molecule downstream of PI3K. PI3K/Akt is an intracellular signaling system involved in cell survival, death, and proliferation, among other physiological tasks [57]. Moreover, PI3K/Akt-mammalian target of rapamycin (mTOR) is one of the two detrimental signaling pathways that control autophagy. It is a classic inhibitory pathway that stimulates the activation of mTOR complex (mTORC1) via Akt pathway, thus inhibiting the formation of the Atg1 complex [61].

Cardiomyocyte apoptosis leads to blocked arterial flow to the heart, damage to cardiac muscle, AMI and eventually heart failure and sudden cardiac death [62]. The PI3K/Akt pathway is well-known to be anti-apoptotic which upon activation, it sequentially promotes recruiting several downstream targets such as; pro-apoptotic forkhead box O-1 (FOXO-1) transcription factors, Bcl-2 interacting mediator of cell death (BIM), and several anti-apoptotic effectors such as B-cell lymphoma-2 (Bcl-2) and some pro-apoptotic factors such as Caspase 3 [48].

Moreover, activation of the PI3K/Akt signaling pathway can decrease reactive oxygen species and lipid deposition, preventing plaque formation and reversing atherosclerosis progression [62]. The downstream regulator of PI3K/Akt is hypoxia-inducible factor-1 α (HIF-1 α) which is a master regulator of gene expression in hypoxia, and it has the ability to influence cellular adaptive responses to hypoxia or ischemia. Cell survival during ischemia/reperfusion is governed by HIF-1 α -dependent genes such as heme oxygenase-1, inducible nitric oxide synthase, cyclooxygenase-2, and vascular endothelial growth factor. HIF-1 α has also been linked to the regulation of cardiomyocyte apoptosis in other studies [29].

The clinically most effective treatment for AMI is still thrombolytic therapy or percutaneous coronary intervention [33]. Although the implementation of a reperfusion method and effective secondary prevention drugs has improved clinical outcomes for AMI patients, the mortality and re-hospitalization rates remain high [26]. Thus, there is an urgent need for new agents that could be used as potential cardioprotective adjuvant therapy in patients at risk or suffering from MI injury.

The renin-angiotensin system (RAS) is implicated in blood pressure regulation, inflammation, and oxidative stress. Captopril (CAP) is the prototype of angiotensin-converting enzyme inhibitor (ACEI), which prevents the formation of angiotensin II (Ang II) and inhibits the breakdown of bradykinin. Hypertension, congestive heart failure, and diabetic nephropathy are all treated with CAP [43].

The sulfhydryl (SH) group in CAP's structure protects tissue from harm caused by oxidative stress and inflammation in many experimental models [11]. Moreover, CAP can boost the activity of antioxidant enzymes like superoxide dismutase (SOD) and glutathione peroxidase (GPx) [3, 41]. CAP also lowers Ca⁺² levels by suppressing Ca⁺² transit through the cell membrane, preventing intracellular Ca⁺² overload and hence cell damage [2].

Garlic (*Allium sativum*) is a naturally occurring herb that has antioxidant, cardioprotective, anti-atherosclerotic, antihypertensive, and immunomodulatory properties. Garlic may benefit heart health by reducing cell damage, controlling cholesterol, and prevent artery plaque accumulation. Additionally, prior researchers demonstrated that the quality of white or brown epicardial adipose tissue (EAT) surrounding the coronary arteries may be altered by garlic supplementation as EAT is a physiologically active tissue that affects rates of inflammation and atherosclerosis progression [46].

Diallyl sulfide, diallyl disulfide, and diallyl trisulfide (DATS) are polysulfide chemicals found in garlic [22]. Allitridin or DATS, is the most powerful polysulfide identified in garlic. It has three sulfur atoms, which help to slow down hydrogen sulfide release (H₂S) and has antioxidant activity [24, 30].

DATS can inhibit apoptosis in cardiomyocytes triggered by high glucose levels via reducing ROS production. Furthermore, DATS was found to protect rats' brains from doxorubicin-induced inflammation and oxidative damage [28]. DATS also inhibits thrombin-induced cell signaling and cell surface activation, suggesting that it could be used as a dietary component for antithrombotic therapy or arteriosclerosis prevention [21]. However, the mechanisms underlying DATS cardioprotection remain unexplained. Consequently, this current study aimed to investigate the role of autophagy and apoptosis as well as PI3K/Akt signaling pathway in the pathogenesis of isoproterenol (ISO)-induced AMI. Additionally, we looked at the underlying mechanisms and the potential cardioprotective benefits of DATS or CAP alone or in combination in the ISO-induced AMI rat model.

Materials and methods

Drugs and chemicals

Captopril (purity $\geq 98\%$) was obtained as a gift from Cairo Company for Pharmaceutical and Chemical Industries (Cairo, Egypt). It was prepared by dissolving in normal saline 0.9% to obtain 15 mg/mL [11]. LY294002 (PI3K inhibitor, purity $\geq 99\%$) was purchased from AdooQ Bioscience company (Irvine, USA), and prepared by dissolving in 1% DMSO to obtain 0.09 mg/mL. DATS (purity $\geq 98\%$) was purchased from Henan Sunlake Enterprise

Company, Ltd., (China), and was diluted with corn oil to obtain 12 mg/mL Yu et al (2017) [56]. Corn oil was chosen as a vehicle for DATS because it has wide safety margin for rats (LD₅₀ > 100 mL/kg). Also, corn has no significant impact either on immune response or intestinal bacterial community structure in rats [16]. Isoproterenol hydrochloride (ISO) (purity ≥ 98%) was purchased from Sigma-Aldrich (St. Louis, USA), and prepared by dissolving in normal saline 0.9% to obtain 25 mg/mL [53].

Animals & experimental design

The study was performed in accordance with the Guidelines for the Care and Use of Laboratory Animals approved by the Research Ethical Committee, Faculty of Pharmacy, Tanta University, Egypt (FPTU-REC, 115/2018/620). Seventy male Albino rats aged 6–8 weeks old and weighing approximately 150–200 g were purchased from the National Research Center (NRC, Cairo, Egypt). Rats were fed standard pellet chow (El-Nasr Chemical Company, Cairo, Egypt) and allowed free access to food and water. Animals were housed in standard temperature (25 °C ± 2) and normal dark/light cycle. After one week of acclimatization, rats were randomly and equally divided for treatments.

Induction of AMI

A pilot study was performed for induction of AMI using different doses of ISO (85 mg/kg [8, 9, 19], 100 mg/kg [1, 4], 150 mg/kg [12, 47]) that were injected subcutaneously (s.c) in 5 rats for two days. Then, animals were anesthetized and scarified. Heart tissue was examined histopathologically with H&E. The results of the pilot study showed death of rats at doses of 150 mg/kg ISO and after the second dose of 100 mg/kg ISO. However, 85 mg/kg ISO for two days was adequate to induce AMI in rat model as confirmed by histopathological examination without death.

Experimental rat groups

- Normal control group: Rats were given the vehicle (0.5 mL corn oil orally + 0.5 mL 1% DMSO intraperitoneally (i.p.) daily for 14 days.
- ISO group: AMI was induced in rats by isoproterenol (ISO) injection, which was freshly prepared in normal saline and injected at a dose of 85 mg/kg, subcutaneously for two days at 24 h interval (on the day 13th and 14th) [53].
- LY294002 + ISO group: Rats were given 0.3 mg/kg LY294002, intraperitoneally and daily for two weeks before AMI induction [57].
- DATS + ISO group: Rats were given 40 mg/kg DATS, orally and daily for two weeks before AMI induction [24].
- CAP + ISO group: Rats were given 50 mg/kg CAP, orally and daily for two weeks before AMI induction [11].
- DATS + LY294002 + ISO group: Rats were given DATS & LY294002 simultaneously with the same doses for two weeks before AMI induction.
- CAP + LY294002 + ISO group: Rats were given CAP & LY294002 simultaneously with the same doses for two weeks before AMI induction.

On the day 15th, rats were anesthetized and scarified. Blood was collected from the heart and serum was separated by centrifugation at × 800 g for 20 min at 4 °C (Sigma centrifuge 3K15 SIGMA Laborzentrifugen GmbH- Germany). Serum was used for estimation of cardiac troponin I (cTnI), and creatine kinase MB (CK-MB). The heart was removed, washed with normal saline, and cut into small portions. One portion was fixed in 10% formalin (Iso Chem Co. Egypt) for histopathological examinations. The other portions were kept at – 80 °C for the analysis of glutathione peroxidase (GSH-px), malondialdehyde (MDA), p62, microtubule-associated proteins 1A/1B light chain 3B (LC3IIB), hypoxia inducible factor 1 alpha (HIF-1α), phosphatidylinositide 3-kinases (PI3K), serine and threonine kinase (Akt), forkhead box protein O (FOXO-1), and endothelial nitric oxide synthase (eNOS).

Preparation of heart tissue homogenate

100 mg of cardiac tissue was homogenized in one mL PBS (Iso Chem Co. Egypt) (pH 7.4) using polytron homogenizer (PT 3100, Switzerland) at × 800 g for 20 min at 4 °C and the supernatant was separated immediately and stored at – 80 °C for analysis.

Determination of serum cardiac biomarkers

Serum cTnI [8, 9] was determined using rat cardiac troponin I ELISA kit (Sunred Biological Technology Co., China), according to the manufacturer's instructions. Serum cTnI level was expressed in ng/L. CK-MB activity was determined spectrophotometrically by immune-inhibition UV method [37] using CK-MB kit (BioSystems®, Spain). CK-MB activity was expressed in U/L. The detection limit for cTnI was 4–1000 ng/L and 2–2000 U/L for CK-MB.

Determination of cardiac autophagy biomarkers

Rat p62 and LC3IIB were assayed in heart tissue homogenate [35] using rat p62 and LC3IIB (ELISA) kits (Shanghai Sunred Biological Technology Co., China) according to

the manufacturer's instructions. p62 level was expressed in pg/ml and LC3IIB in ng/ml. The detection limit was 15-2000 pg/mL for p62 and 0.3-90 ng/mL for LC3IIB.

Determination of cardiac oxidative stress biomarkers

MDA, the lipid peroxidation biomarker, and GSH-px activity were measured in cardiac tissue homogenate using colorimetric kits (Biodiagnostics Co., Giza, Egypt) according to the manufacturer's protocols. MDA content was expressed in nmol/g tissue. GSH-px activity was expressed in U/g tissue. Rat HIF-1 α was detected in heart tissue homogenate [54] using rat HIF-1 α ELISA kit (Shanghai Sunred Biological Technology Co., China) according to the manufacturer's instructions. HIF-1 α level was expressed in pg/mL. The detection limit for MDA was up to 100 nmol/mL and 15-4200 pg/mL for HIF-1 α .

Real-time quantitative reverse transcriptase polymerase chain reaction (RT-qPCR)

Total RNA was extracted from heart tissue using RNeasy[®] total RNA extraction kit (Qiagen Co, Hilden, Germany) according to the manufacturer's instructions. Concentration and purity of mRNA at A₂₆₀/A₂₈₀ nm were determined using Nanodrop spectrophotometer (Analytik Jena, Italy). The extracted mRNA was converted to complementary DNA (cDNA) using RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, Qiagen Co., Germany). The cDNA was amplified using QuantiTect SYBR Green I PCR (Qiagen Co., Germany). Primers were purchased from Biosearch Technologies co. (USA). Sequences of primers [18, 31, 44] are shown in Table (1). The RT-qPCR program was 94°C for 15 sec (denaturation), then 60°C for 30 sec (annealing), and 72°C for 30 sec (extension). Each sample was analyzed and normalized to the level of the housekeeping gene (β -actin), and expressed as fold change. Threshold cycle (Ct) values of samples were calculated and the relative content of the gene amplification product was calculated using the $2^{-\Delta\Delta Ct}$ method [6].

Histopathological examination of cardiac tissue

Sections from the left ventricle of the heart were fixed in 10% formalin (Iso Chem Co. Egypt) for 24h then transferred to ascending grades of alcohol (Iso Chem Co, Egypt), cleared in xylene (Iso Chem Co. Egypt), and then embedded in paraffin (ALEXANDRIA Paraffin Wax Co., Egypt) to form blocks. Four μ m sections were prepared using a microtome (Leica RM2135, Germany), deparaffinized, stained by hematoxylin and eosin (H&E) stain (Newcomer supply, Middleton, USA) and examined using a light microscope (Leica DM 500, Switzerland) at $\times 200$ magnification by an expert histopathologist, who

Table 1 Primers' Sequences

Gene of interest	Sequences
PI3K	Forward: 5'-AGCATTGGGACCTCACATTACACA-3' Reverse: 5'-ACTGGAAACACAGTCC ATGCACATA-3'
Akt	Forward: 5'-GCTGGACGATAGCTTGA-3' Reverse: 5'-GATGACAGATAGCTGGTG-3'
FOXO-1	Forward: 5'-AATTTGCTAA GAGCCGAGGA-3' Reverse: 5'-AAGTCATCATTGCTGTGGGA-3'
eNOS	Forward: 5'-TGCACCCTTCCGGGATTCT-3' Reverse: 5'-GGATC CCTGGAAAAGCGGT-3'
β-actin	Forward: 5'-ACTGGCATTGTGATGGACTC-3' Reverse: 5'-CAGCACTGTGGCATAGA-3'

PI3K Phosphatidylinositol 3-Kinases, *AKT* Serine and Threonine Kinase, *FOXO-1* Forkhead Box Protein O, *eNOS* Endothelial Nitric Oxide Synthase

was blind to the treatment. The histopathological alterations including necrosis, apoptosis, and inflammatory cells infiltration were assessed and scored [8, 9]. Apoptotic index was determined in stained cardiac sections by dividing the total number of apoptotic bodies by the total number of intact cardiac cells and multiplying by 100 [14, 15]. The apoptotic cells showed cellular shrinkage, condensation, and deep eosinophilia of the cytoplasm and pyknotic, round to crescentic or irregular nucleus [10].

Statistical analysis

Analysis of data was performed with statistical package for social science (SPSS) software version 22 [11]. Data are presented as mean \pm SD and as a percent of change. Statistical comparison among groups was performed by one-way analysis of variance (ANOVA) using Fisher's least significant differences (LSD) method for comparison between two groups. Statistical significance was set at $p < 0.05$.

Results

Effect of treatment on cardiac injury biomarkers

CK-MB, and cTnI are identified as essential diagnostic markers of ischemic injury of the myocardial tissues that leak out from damaged myocardium to blood [7]. As shown in Table (2), significant increase in the levels of CK-MB and cTnI were detected in ISO group compared with the normal control group ($p < 0.01$). However, DATS and CAP pretreatment significantly decreased serum CK-MB and cTnI ($p < 0.01$) in DATS+ISO and CAP+ISO groups compared with ISO group. When LY294002 was applied to treated groups, the levels of CK-MB were elevated in DATS+LY294002+ISO and CAP+LY294002+ISO compared with the DATS+ISO and CAP+ISO pretreated groups ($p < 0.01$). On the other hand, there was non-significant change in the levels of cTnI in LY294002 co-treated groups relative to

Table 2 Effect of treatment on serum cardiac biomarkers of studied rat groups

Group	Normal Control	ISO	LY294002 + ISO	DATS + ISO	CAP + ISO	DATS + ISO + LY294002	CAP + ISO + LY294002
Serum cTnl level	198.24 ± 7.1	264.09 ± 11.76 ^a	253.81 ± 8.97 ^a	208.93 ± 37.13 ^{b,c}	215.04 ± 16.38 ^{b,c}	201.95 ± 32.8 ^{b,c}	201.79 ± 26.44 ^{b,c}
Serum CK-MB	6.6 ± 1.81	25.86 ± 3.24 ^a	14.86 ± 1.81 ^{a,b}	7.7 ± 0.85 ^{b,c}	7.98 ± 2.43 ^{b,c}	12.66 ± 1.35 ^{a,b,c}	11.83 ± 1.93 ^{a,b,c}

Data are presented as mean ± SD, significance was set at $P < 0.05$, $n = 6$. ISO: Isoproterenol induced AMI group (85 mg/kg/d), LY294002 + ISO: PI3K inhibitor group (0.3 mg/kg/d), DATS+ISO: Diallyl trisulfide pretreated group (40 mg/kg d), CAP+ISO: Captopril pretreated group (50 mg/kg/d), DATS+LY294002 + ISO: Diallyl trisulfide and PI3K inhibitor pretreated group, CAP+LY294002 + ISO: Captopril and PI3K inhibitor pretreated group. a: Significant versus normal control group. b: Significant versus ISO group. c: Significant versus LY294002 + ISO group

DATS+ISO and CAP+ISO pretreated groups. Also, there was significant decrease in CK-MB and cTnl levels in DATS+LY294002 + ISO, and CAP+LY294002 + ISO groups as compared with LY294002 + ISO pretreated group ($p < 0.01$).

Effect of treatment on myocardial autophagy

As shown in Fig. (1), myocardial tissue levels of both p62 and LC3IIB were significantly increased in ISO group compared with normal control group ($p < 0.01$). As compared with ISO group, DATS+ISO and CAP+ISO pretreatment significantly decreased both p62 and LC3IIB levels ($p < 0.01$). When LY294002 was applied, there was a significant decrease in p62 level in both DATS+LY294002 + ISO, and CAP+LY294002 + ISO groups compared with LY294002 + ISO pretreated group ($p < 0.01$), whereas DATS+LY294002 + ISO, and CAP+LY294002 + ISO groups showed non-significant

change in LC3IIB level compared with LY294002 + ISO pretreated group ($p < 0.01$).

Effect of treatment on oxidative stress biomarkers

Figure 2 shows the levels of MDA, HIF-1 α , and GSH-px activity in cardiac tissue. In ISO group, there was a significant increase in MDA, HIF-1 α levels, along with a significant decrease in GSH-px activity compared to normal control group ($p < 0.01$). On the other hand, DATS and CAP pretreatment significantly decreased both MDA and HIF-1 α levels with a marked elevation of GSH-px activity compared with ISO group ($p < 0.01$). Moreover, there were significant decrease of MDA and HIF-1 α levels in both DATS+LY294002 + ISO, and CAP+LY294002 + ISO groups compared with LY294002 + ISO pretreated group ($p < 0.01$). Also, DATS+LY294002 + ISO, and CAP+LY294002 + ISO groups showed significant increase in GSH-px activity

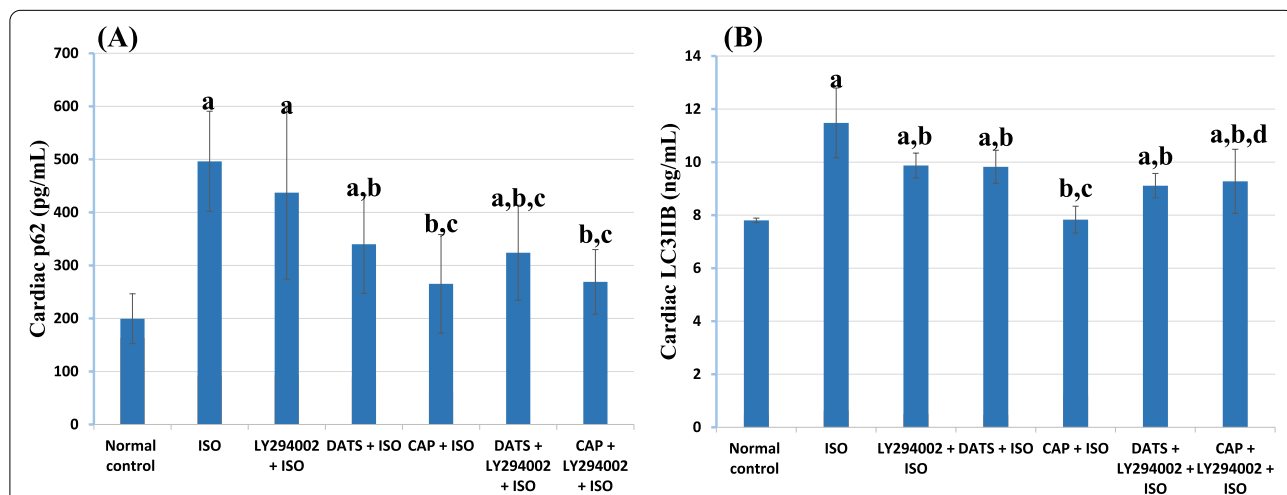
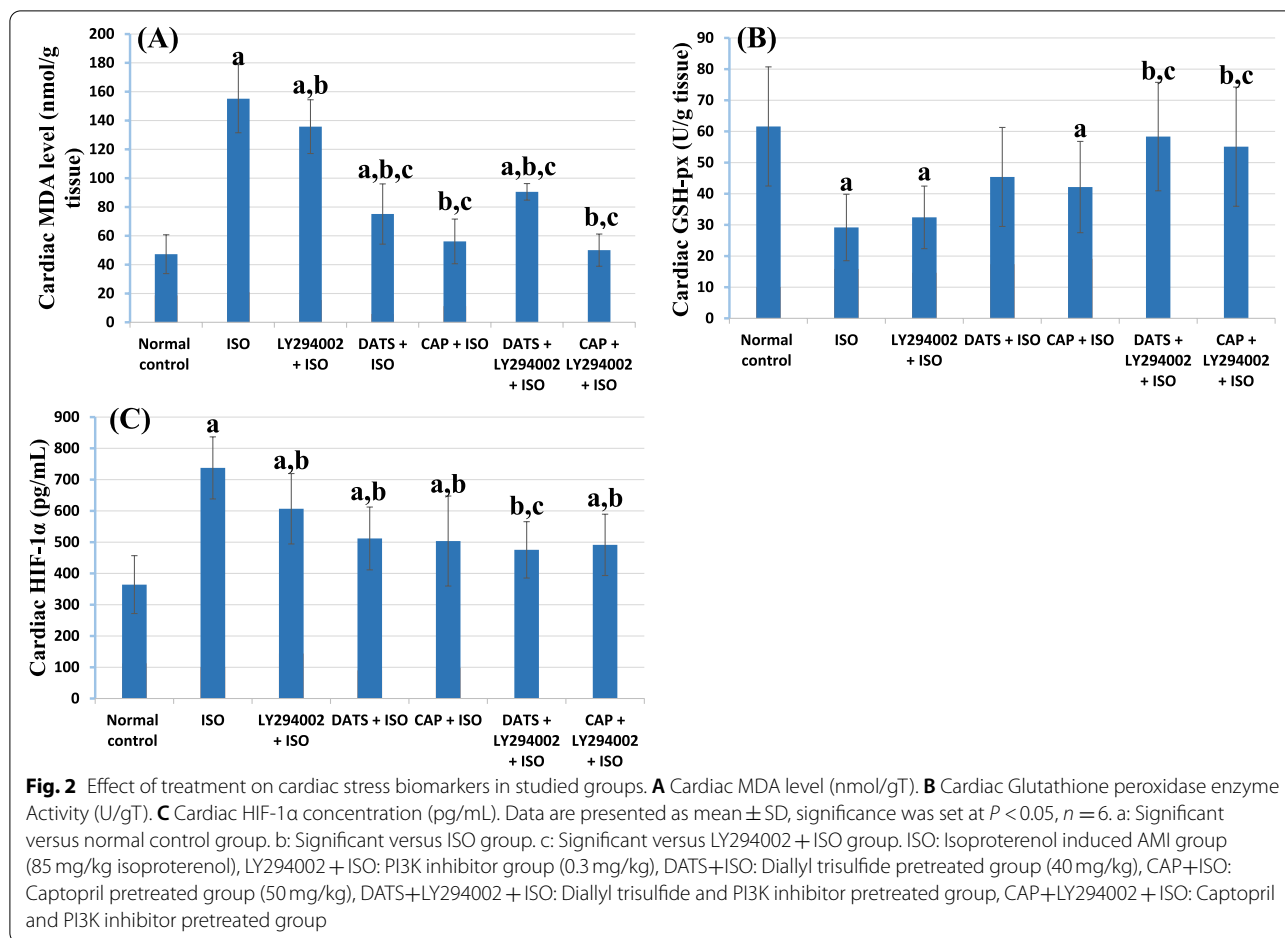


Fig. 1 Effect of treatment on cardiac autophagy biomarkers in studied groups. **A** Cardiac P62 concentration (pg/mL). **B** Cardiac LC3IIB concentration (ng/mL). Data are presented as mean ± SD, significance was set at $P < 0.05$, $n = 6$. a: Significant versus normal control group. b: Significant versus ISO group. c: Significant versus LY294002 + ISO group. d: Significant versus CAP + ISO. ISO: Isoproterenol induced AMI group (85 mg/kg isoproterenol), LY294002 + ISO: PI3K inhibitor group (0.3 mg/kg), DATS+ISO: Diallyl trisulfide pretreated group (40 mg/kg), CAP+ISO: Captopril pretreated group (50 mg/kg), DATS+LY294002 + ISO: Diallyl trisulfide and PI3K inhibitor pretreated group, CAP+LY294002 + ISO: Captopril and PI3K inhibitor pretreated group



compared with LY294002 + ISO pretreated group ($p < 0.01$) (Fig. 2).

Effect of treatment on gene expression of PI3K/AKT downstream mediators and apoptotic genes

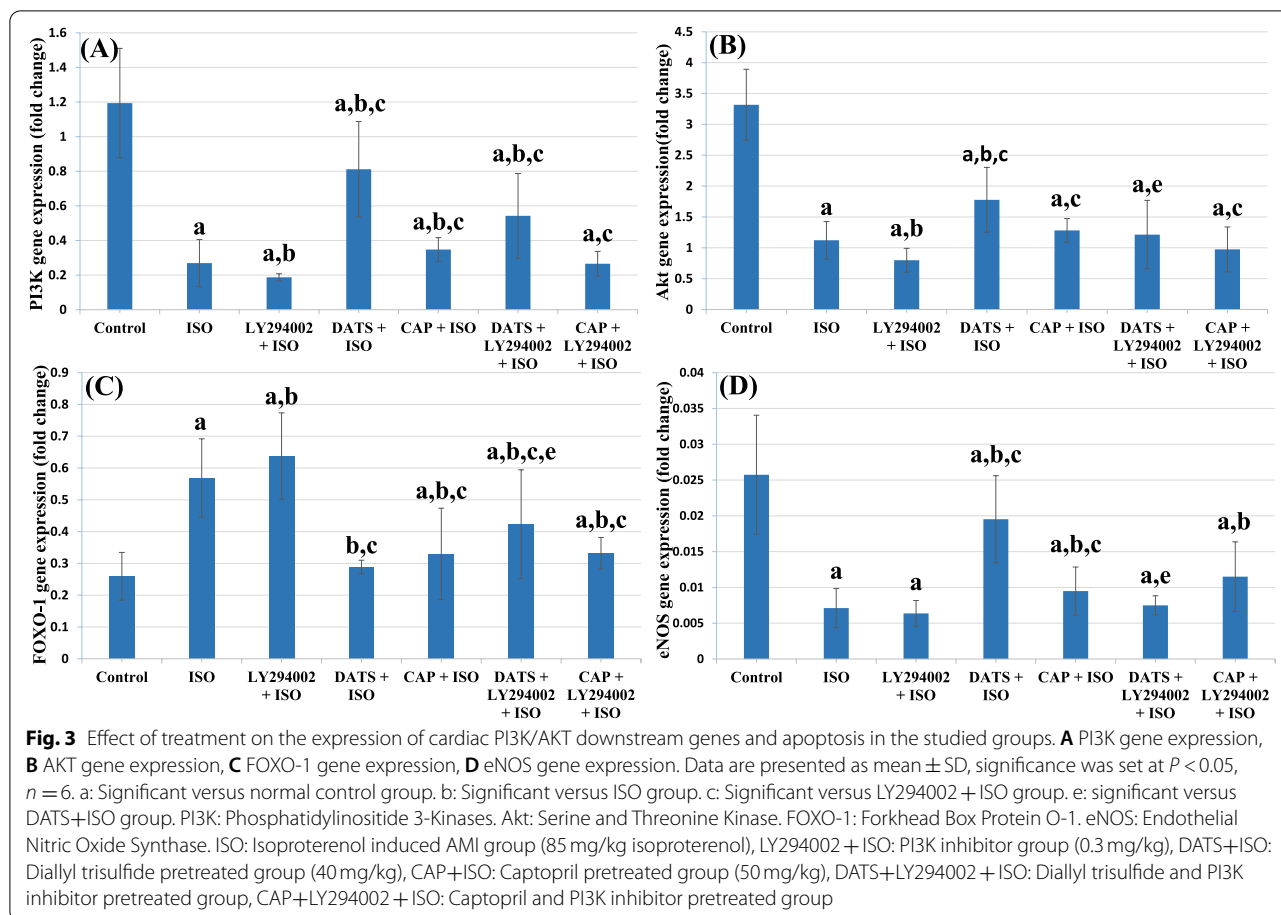
To clarify whether the PI3K/Akt pathway was involved in the cardioprotective effect of DATS and CAP in AMI induced rat model, gene expression of PI3K, Akt, FOXO-1 and eNOS in the ischemic myocardial tissue was detected by RT-qPCR. As shown in Fig. 3 (A, B & D), there was a significant decrease in PI3K, Akt and eNOS gene expressions in ISO group compared with normal control group ($p < 0.01$). However, DATS and CAP pretreatment significantly upregulated gene expression of PI3K, Akt and eNOS ($p < 0.01$) when compared with ISO group. The present results showed also that administration of LY294002 down-regulated PI3K, AKT and eNOS activation induced by both DATS and CAP pretreatment (Fig. 3A, B, D).

Herein, we detected whether the administration of DATS and CAP could affect the expressions of apoptotic-related genes of FOXO-1 in myocardial tissue. A

significant increase in FOXO-1 gene expression was observed in the ISO group compared with normal control group ($p < 0.01$). However, DATS and CAP pretreatment significantly downregulated FOXO-1 expression in comparison to the ISO group ($p < 0.01$) but LY294002 antagonized the effects of DATS and CAP on FOXO-1 gene expression ($p < 0.01$) Fig. 3(C).

Effect of treatment on cardiac histology

As shown in Fig. (4), cardiomyocytes structure of normal control group showed typical normal pattern of cardiac muscle fibers and normal vascular structures (Figs. 4A & a). However, ISO group demonstrated marked congestion of coronary and intermuscular blood vessels together with focal atrophic and apoptotic changes in a variable number of cardiomyocytes (about 15-20%), degenerative and necrotic changes in some muscle fibers (about 25-30%), characteristic inflammatory reactions, fatty degeneration and cardiomyolysis as compared with normal control group (Figs. 4B& b). The myocardial damage caused by isoproterenol was ameliorated in the DATS+ISO and CAP+ISO pretreated



groups. Figure 4(D, d) & (E, e) show few mild cardiomyocytic degenerative, atrophic, and apoptotic changes, beside mild focal interstitial edematous inflammatory reaction. However, rats co-treated with LY294002 in DATS+LY294002 + ISO, and CAP+LY294002 + ISO groups (Fig. 4E, f & G, g) showed protective effects on the myocardial structure, and mild lyse of muscle fibers with moderate infiltration of lymphocytes and macrophages when compared to LY294002 + ISO pretreated group (Fig. 4C & c).

Table (3) also shows marked significant increase in necrosis and inflammation of cardiac tissue of untreated ISO group compared with normal control group. However, DATS and CAP pretreatment significantly decreased necrosis and inflammatory cells infiltration compared with ISO group. Moreover, adding LY294002 to DATS+ISO group, and CAP+ISO group caused significant increase in necrosis and inflammation when compared with untreated DATS+ISO and CAP+ISO groups. The histopathological alterations induced in hearts of LY294002 + ISO group was significantly ameliorated by DATS and CAP pretreatment in ISO group.

Figure (5) shows apoptotic index in cardiomyocytes of treated rat groups. Myocardial apoptosis was significantly increased in ISO group compared with the normal control group ($p < 0.01$). DATS+ISO and CAP+ISO pretreated groups showed significant decrease in apoptotic index as compared with ISO group ($p < 0.01$). addition of LY294002 caused non-significant changes in apoptosis in both DATS+LY294002 + ISO, and CAP+LY294002 + ISO groups compared with LY294002 + ISO pretreated group ($p < 0.01$).

Discussion

Acute myocardial infarction and heart failure are the major causes of death all over the world [60]. When blood supply is abruptly blocked-in coronary artery, a large number of free radicals are created, which react with cardiomyocytes membrane lipids, proteins, and DNA, causing an oxidative stress state. In addition, mitochondria have their own circular DNA, which is very vulnerable to damage from excessive ROS emission. Moreover, oxidative stress damage to mitochondrial DNA (mtDNA) leads to a continual loop

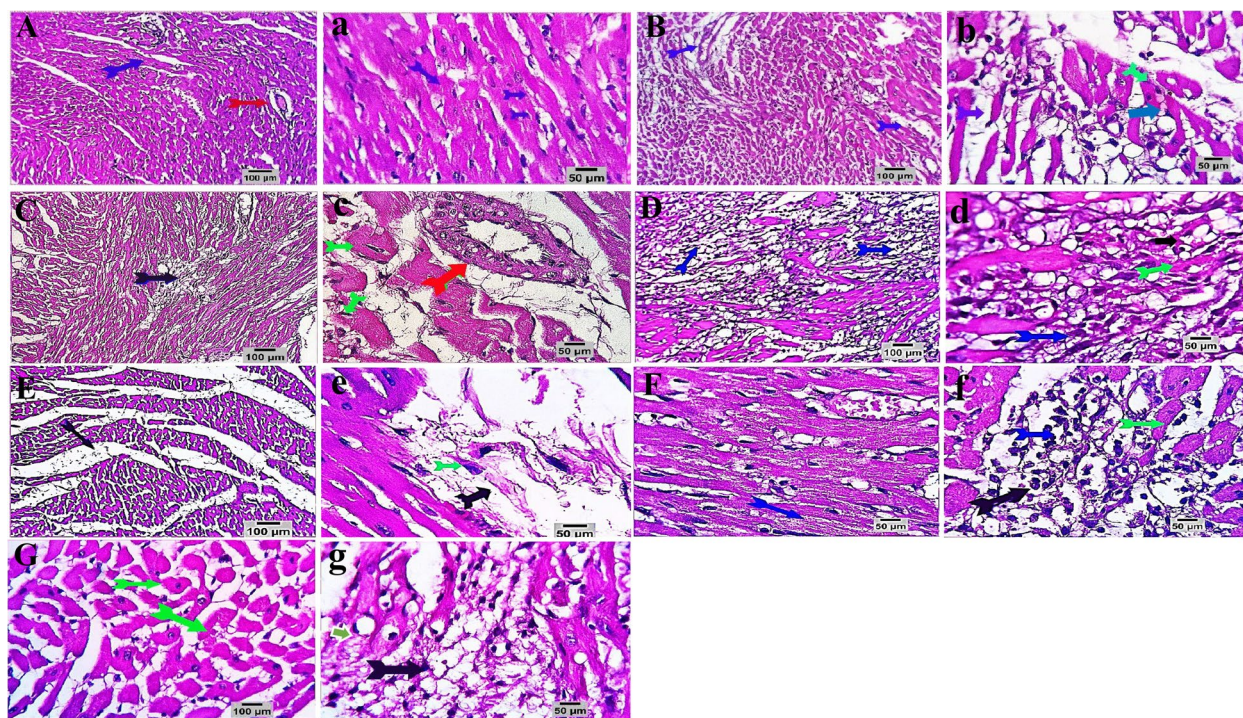


Fig. 4 Representative photomicrographs of cardiac tissue sections of rat groups stained with H&E. ($\times 200$). **(A,a)**: Normal control group showing typical normal pattern of cardiac muscle fibers (arrow) and normal vascular structures (bi-forked thick arrow). **(B,b)**: ISO group showing focal atrophic, apoptotic, degenerative and necrotic changes in some muscle fibers (light head arrow). Interstitial and perivascular edematous reactions, fatty degeneration and cardiomyolysis are also seen (arrow & curved thick arrow). **(C,c)**: LY294002 + ISO group showing focal atrophic, degenerative and apoptotic changes in some cardiomyocytes (light-head arrow), beside focal interstitial and perivascular edematous reaction (thick arrow) with degenerative changes in some vascular walls (bi-forked thick arrow). Normal heart muscles, intercalated discs and sarcolemmal cells are also seen (bent arrow). **(D,d)**: DATS+ISO group showing most of the cardiomyocytes and the sarcolemmal cells were apparently normal (arrow), along with mild focal cardiomyocytic atrophic and degenerative changes (light-head arrow), and a few cardiomyocytes shows fatty degenerative change (curved arrow). **(E,e)**: CAP+ISO group showing normal cardiomyocytes, sarcolemmal cells with interstitial tissue (bent arrow). Mild cardiomyocytic degenerative, apoptotic changes and focal intestinal edematous inflammatory process (light-head arrow & thick arrow respectively). **(F,f)**: DATS+LY294002 + ISO group showing marked histopathologic changes represented by cardiomyocytic atrophic, degenerative and apoptotic changes (light-head arrow), with a moderate interstitial edematous inflammatory reaction (thick arrow). Other cardiac muscles and structures were apparently normal (arrow). **(G,g)**: CAP+LY294002 + ISO group showing cardiomyocytes suffered atrophic, degenerative and apoptotic changes (thick arrow) and a few cells with fatty degeneration (curved arrow). Other cardiac muscles and structures are apparently normal (light-head arrow). A,B,C,D,E,F,G; scale bars 100um. a,b,c,d,e,f,g; scale bars 50um

Table 3 Effect of treatment on cardiac histopathological scoring of necrosis and inflammation in the studied groups

Group	Normal Control	ISO	LY294002 + ISO	DATS + ISO	CAP + ISO	DATS + ISO + LY294002	CAP + ISO + LY294002
Necrosis	0 ± 0	11.28 ± 1.13 ^a	10.4 ± 0.47 ^a	5.8 ± 0.78 ^{a,b,c}	6.56 ± 0.86 ^{a,b,c}	9.76 ± 0.99 ^{ab}	10.02 ± 1.14 ^{ab}
Inflammation	0 ± 0	18.56 ± 2.11 ^{ac}	14.72 ± 1.32 ^{ab}	8.18 ± 1.04 ^{a,b,c}	8.44 ± 1.2 ^{a,b,c}	11.56 ± 1.09 ^{a,b,c}	12.82 ± 2.1 ^{a,b,c}

Data are presented as mean ± SD, significance was set at $P < 0.05$, $n = 6$. ISO: Isoproterenol induced AMI group (85 mg/kg/d), LY294002 + ISO: PI3K inhibitor group (0.3 mg/kg/d), DATS+ISO: Diallyl trisulfide pretreated group (40 mg/kg d), CAP+ISO: Captopril pretreated group (50 mg/kg/d), DATS+LY294002 + ISO: Diallyl trisulfide and PI3K inhibitor pretreated group, CAP+LY294002 + ISO: Captopril and PI3K inhibitor pretreated group. a: Significant versus normal control group. b: Significant versus ISO group. c: Significant versus LY294002 + ISO group

of increased ROS production, exacerbating oxidative stress damage and further mitochondrial dysfunction. Furthermore, fragments of mitochondrial DNA are released into the bloodstream, where they serve as

pro-inflammatory chemicals, exacerbating the inflammatory injury [39, 60].

When this oxidative stress exceeds the antioxidant capacity of the internal defensive enzymatic (superoxide

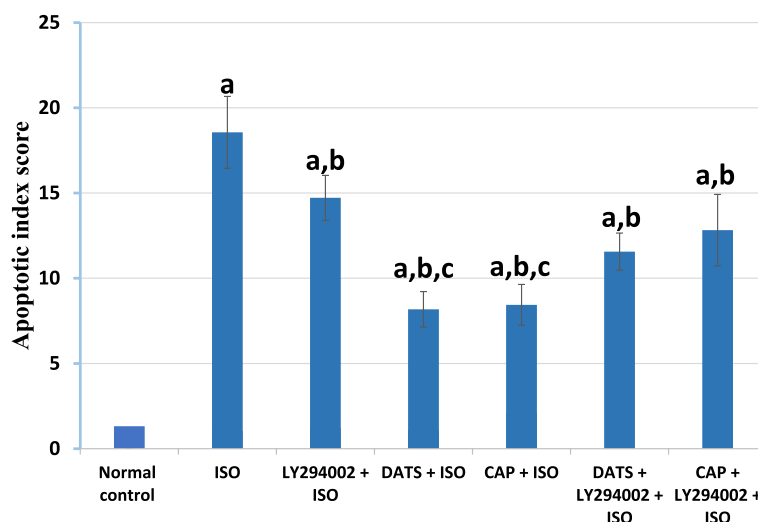


Fig. 5 Effect of treatment on cardiac histopathological scoring of apoptosis in the studied groups. Data are presented as mean \pm SD, significance was set at $p < 0.05$, $n = 6$. ISO: Isoproterenol induced AMI group (85 mg/kg/d), LY294002 + ISO: PI3K inhibitor group (0.3 mg/kg/d), DATS+ISO: Diallyl trisulfide pretreated group (40 mg/kg d), CAP+ISO: Captopril pretreated group (50 mg/kg/d), DATS+LY294002 + ISO: Diallyl trisulfide and PI3K inhibitor pretreated group, CAP+LY294002 + ISO: Captopril and PI3K inhibitor pretreated group. a: Significant versus normal control group. b: Significant versus ISO group. c: Significant versus LY294002+ ISO group

dismutase SOD, catalase CAT, and glutathione peroxidase GSH-px) and non-enzymatic (reduced glutathione GSH) antioxidants, cardiomyocyte injury and AMI occurs [34].

The present study aimed to elucidate the possible protective role of diallyl trisulfide and captopril in isoproterenol induced AMI via various pathways such as autophagy, apoptosis and PI3K/Akt pathway.

LY294002, the PI3K inhibitor was used to explore the role of PI3K/Akt pathway. LY294002 attenuates, blunts and reverses cardioprotective effects of many promising agents such as hydroxytyrosol [37], resveratrol [59] and total flavonoids of *Jinhe yangxin* prescription [7] via inhibiting PI3K/Akt pathway. Herein, LY294002 combined with each of DATS or CAP significantly reversed their action on PI3K/Akt pathway mediators as it antagonized DATS & CAP action on PI3K, Akt, eNOS and FOXO-1 gene expression.

In the present experimental study, AMI was induced in rats by subcutaneous administration of 85 mg/kg ISO for two successive days with 24 h interval in-between. The histopathological examination of ISO group showed focal atrophic, apoptotic, degenerative and necrotic changes in some muscle fibers and vascular walls. Interstitial and perivascular edematous and inflammatory reactions, fatty degeneration and cardiomyolysis are also seen. These effects were consistent with previous reports [22, 24].

Isoproterenol AMI model is a widely and commonly used experimental model of AMI [24, 39]. Isoproterenol

is a synthetic catecholamine and β -adrenergic agonist that can cause necrosis, hypoxia, hyperplasia of cardiac tissue, and AMI at supramaximal doses, [7, 35]. It also causes cardiomyocytes' energy reserves to be depleted, causing in significant biochemical and structural alterations. Furthermore, ISO induces production of ROS and disrupts tissue antioxidative defences, such as chemical scavengers and antioxidant molecules, resulting in infarction-like lesions [58].

Interestingly, the existence of myocardial necrosis is the histopathological hallmark of AMI. Herein, the present study showed significant increase in the levels of cTnI and CK-MB in ISO group. Membrane breakdown caused by lipid peroxide causes these biomarkers to seep into the bloodstream. Elevated cTnI levels, on the other hand, can predict the likelihood of cardiac mortality and subsequent infarction [7, 25].

The present results indicated that the pretreatment of rats with DATS and CAP in ISO treated rats reduced myocardial necrosis after histopathological examination as well as significant decrease in serum cTnI and CK-MB. These results indicated that DATS and CAP effectively maintains cell functional integrity and restricts the leakage of the enzyme into circulation. These findings were in line with those of Gomaa et al., [17] and Asdaq et al., [5], who reported a synergistic cardioprotective effect of captopril and garlic in rats suffering from ISO-induced cardiac injury.

Hypoxia inducible factor-1 alpha (HIF-1 α) is a transcription factor that responds to ischemia, as well as

being able to reflect the course of acute myocardial infarction to some extent [54]. The present results showed that the concentration of HIF-1 α in ISO treated group was significantly higher than that of normal control group, indicating ischemic condition induced in hearts. These observations were in agreement with Yu et al., [54]. The response to stressful hypoxic condition in ISO group increases HIF-1 α level to activate angiogenesis and thus attenuate ischemia. Moreover, Sun et al., [45], reported that HIF-1 α overexpressed exosomes promoted new vessel development in the ischemia border zone, which had a profound cardioprotective effect on the MI heart.

On the other hand, pretreatment with DATS and CAP significantly reduced HIF-1 α levels compared to the ISO treated group. This effect supports that DATS and CAP are anti-hypoxic agents during myocardial damage [54]. Interestingly, CAP inhibits the breakdown of bradykinin and lowering plasma angiotensin II, and thus has a potent vasodilation impact [32].

Consistently with many studies, oxidative damage accompanied by free radicals hastens the progression of heart injury [23]. Our findings showed increased level of MDA, the marker of lipid peroxidation, and decreased the antioxidant activity of GSH-Px in ISO treated group that indicated severe oxidative stress [27]. These results were enforced by the histopathological findings that showed characteristic inflammatory reactions, fatty degeneration and cardiomyolysis in ISO group.

The present study showed that DATS and CAP pretreatment decreased MDA level and increased GSH-Px activity. These results showed the antioxidant activity of both DATS and CAP, which was in agreement with previous studies [37, 53]. CAP's antioxidant action is due to its SH structural component, which directly scavenges ROS via hydrogen donation or electron transfer [11]. DATS, on the other hand, has three sulphur atoms, which operate as a source of H₂S [22].

Activation of the PI3K/Akt pathway has been shown to protect against myocardial injury in vivo and in vitro by reducing oxidative stress, restricting the inflammatory cascade, and inhibiting apoptosis [53]. In addition, The Class III PI3K complex is an important subunit during autophagosome formation, that leads to the membrane nucleation process and formation of the cup-shaped, lipid bilayer membrane-structured phagophore [49]. To further verify the molecular mechanisms of DATS and CAP as cardioprotective agents, we studied PI3K/Akt signaling pathway by applying LY294002 as PI3K inhibitor.

In the present study, the expression levels of PI3K, and Akt were significantly downregulated in the ISO model

rats, but upregulated in DATS and CAP pretreated ISO groups. These findings were consistent with those of Yu false [55, 56], who showed that DATS therapy reduced myocardial infarct size and prevented myocardial apoptosis via AMPK-mediated AKT/GSK-3/HIF-1 signaling. On the contrary, a significant upregulation was observed in FOXO-1 gene expression, the downstream of PI3K/Akt signaling, in ISO group and downregulated by DATS and CAP pretreatment.

The anti-apoptotic activity of both DATS and CAP was confirmed after downregulation of FOXO-1 expression. This anti-apoptotic activity is also confirmed by histopathological examination that revealed marked atrophic and apoptotic changes in cardiac tissue of ISO group. However, pretreatment with DATS and CAP significantly ameliorated these apoptotic changes.

The fundamental component of endothelial cell activity is eNOS which is involved in vasomotor function and possesses antithrombotic properties via generation of nitric oxide (NO) [57]. During AMI attack, eNOS produces superoxide anion rather than NO, a phenomenon known as "eNOS uncoupling," which may lead to excessive free radical production and a lack of cofactors required for normal NO biosynthesis [20].

In the current study, ISO induced AMI rat model showed significant decrease in eNOS gene expressions. However, DATS and CAP pretreatment significantly increased gene expression of eNOS. These observations were in consistent with Predmore et al., [38], who reported that DATS treatment in vivo activates eNOS and increases NO bioavailability. NO is a powerful cardio-protectant that may help to maintain the link between electron transport and ATP production.

Moreover, Yang et al., [52] reported that DATS treatment improved angiogenic potential, tissue viability, and ischemic tissue blood perfusion and mitigated oxidative stress in a diabetic mouse model with peripheral artery disease. Herein, CAP pretreatment, suppressed RAS and Ang II so that increased eNOS gene expression [13].

The role of autophagy in myocardial infarction remains controversial. Autophagy may be increased, which had beneficial benefits on heart fibrosis and function. On the other hand, excessive autophagy has been linked to cardiomyocyte death, due to the destruction of a significant number of organelles. Furthermore, autophagy inhibition may diminish the size of infarcted area [40, 42].

Autophagy is regulated by two detrimental signaling pathways. PI3K/Akt-mammalian target of rapamycin (mTOR) pathway is a well-known inhibitory route that stimulates the activation of the mTOR complex (mTORC1) via the Akt pathway, thus preventing the development of the Atg1 complex. The other one is AMP-activated protein kinase (AMPK). AMPK is a stress

and nutrient input sensor. To promote autophagy, AMPK activates ULK1 by inactivating mTORC1 or phosphorylating ULK1 at various residue serine kinase complexes [61].

The autophagy initiation marker is LC3IIB (microtubule-associated proteins 1A/1B light chain 3B), while the p62 protein represents autophagic flux activity. In the current study, myocardial tissue levels of both p62 and LC3IIB were significantly increased in ISO treated group compared with normal control group. These findings suggested that AMI caused autophagic flux impairment by promoting autophagosome formation, but reducing autophagosome clearance.

These findings were consistent with those of Wu et al, [50], who found that reduced autophagic flux is one of the main causes of ischemic/reperfusion damage. Furthermore, in cardiac ischemia disorders, restoring autophagic flux is critical for cardiomyocyte survival.

However, DATS and CAP pretreatment significantly decreased LC3II and p62 levels when compared with ISO group indicating that DATS as well as CAP inhibited excessive autophagy and improved autophagosome clearance. Our findings suggest that DATS and CAP suppress AMI-induced excessive autophagy and restore autophagic flux via the PI3K/Akt/mTOR pathway, which has been shown to diminish autophagic activity and improve autophagic flux [51].

Conclusion

The present study showed the role of DATS and CAP as cardioprotective agents. They suppressed oxidative stress and inflammation and thereby attenuate necrosis, apoptosis, and promote autophagy after acute myocardial infarction. The cardioprotective effect was associated by their ability to modulate PI3K/Akt signaling pathway. Taking all our data together, it may be suggested that DATS and CAP could be used as potential cardioprotective adjuvant therapy in the patients at risk or suffering from MI injury.

Authors' contributions

El-Ashmawy NE: Designed the study, analyzed the data and approved the manuscript in final version for publication. Khedr NF: Conceived the study idea, guide the experiments, analyzed the data, reviewed the manuscript and approved it for publication. Al-Ashmawy GM: Planned the experiment, analyzed the data, reviewed the manuscript and approved it for publication. Naguib MO: Conducted the research, analyzed the data and drafted the manuscript in the final form. The author(s) read and approved the final manuscript.

Declaration

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Received: 17 April 2022 Accepted: 5 November 2022
Published online: 05 December 2022

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