Biochemistry and Biophysics Reports 5 (2016) 77–88

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

Diallyl disulfide ameliorates isoproterenol induced cardiac hypertrophy activating mitochondrial biogenesis via eNOS-Nrf2-Tfam pathway in rats

Tarak Nath Khatua [a,b], Amit K. Dinda [c], Uday K. Putcha [d], Sanjay K. Banerjee [a,b,*]

[a] Division of Medicinal Chemistry and Pharmacology, Indian Institute of Chemical Technology, Hyderabad 500607, India
[b] Drug Discovery Research Center, Translational Health Science and Technology Institute, Faridabad 121001, India
[c] Department of Pathology, All India Institute of Medical Sciences, New Delhi 110029, India
[d] Division of Pathology, National Institute of Nutrition, Hyderabad 500607, India

ARTICLE INFO

Article history:
Received 21 April 2015
Received in revised form
27 October 2015
Accepted 9 November 2015
Available online 24 November 2015

Keywords:
DADS
Garlic
Hypertrophy
Isoproterenol
Mitochondria

ABSTRACT

The beneficial effect of garlic on cardiovascular disease is well known. However, the use of raw garlic against cardiac hypertrophy is not established. In the present study we explored whether raw garlic and its compound, diallyl disulfide (DADS) could inhibit hypertrophy through H_2S and/or mitochondrial biogenesis. Cardiac hypertrophy was induced in rat by giving isoproterenol at the dose of 5 mg/kg/day subcutaneously for 14 days through alzet minipump. Aqueous garlic homogenate, DADS and NaHS (liberate H_2S) were fed to third, forth and fifth group of rats for 14 days at a dose of 250 mg/kg/day, 50 mg/kg/day, 14 μM/kg/day respectively. Garlic and DADS reduced cardiac hypertrophy markers and normalized mitochondrial ETC-complex activities, mitochondrial enzyme activites and mitochondrial biogenetic and apoptotic genes expression. Garlic and DADS enhanced eNOS and p-AKT level in rat heart along with increased NRF2 protein level and Tfam gene expression. However, normalization was not observed after administration of NaHS which generates H_2S in-vivo. In conclusion, garlic and DADS induces mitochondrial biogenesis and ameliorates cardiac hypertrophy via activation of eNOS-Nrf2-Tfam pathway in rats.

1. Introduction

Cardiac hypertrophy is a health problem of global concern. It is characterized by an increase in cardiac muscle mass due to increase in cardiomyocyte size and accumulation of myocardial scarring (collagen) without increasing its pumping ability. Cardiac hypertrophy is the response to stress or disease such as hypertension, valvular heart disease, heart muscle injury, myocardial infarction, heart failure or neurohormones. Its risk increases with age, smoking, hypercholesterolemia, diabetes, and hypertension, and is more common in men than in women [1].

Many scientific literatures have indicated the involvement of reactive oxygen species in cardiac hypertrophy. In addition to their ability to directly damage cellular macromolecules, ROS play a significant role in activating stress-sensitive signaling pathways that regulate gene expression leading to cellular damage. The source of excess ROS production in hypertrophy heart is mitochondria [2].

Mitochondria play critical roles in both the life and death of cells. It contains the enzymes of the citric acid cycle, the electron transport chain, and oxidative phosphorylation. Mitochondrial dysfuction covers large number of clinical problems related to tissues having high energy requirements such as heart, brain, muscle, kidney and endocrine systems [3]. Primary function of cardiac myocytes is to provide ATP through oxidative phosphorylation to meet the high energy demands of the beating heart. Mitochondrial dysfunction plays an important role in cellular senescence, oxidative stress and cardiovascular inflammation [4].

Cardiac hypertrophy is a progressive and chronic disease and can be modified by nutritional and other life style modification. Our focus is to find the role of widely used nutritional agent for the prevention of cardiovascular diseases through mitochondrial biogenesis. In recent literatures it is reported that garlic and its preparations are used to prevent cardiovascular diseases. Role of garlic in reducing hyperlipidemia, hypertension and platelet aggregation has been well established. Some recent studies are shown that isolated compound from garlic or garlic derived sulphur compounds can reduce cardiac hypertrophy [5]. However, the role of garlic to reduce cardiac hypertrophy through modulation of mitochondria was not observed yet. The present study explored if garlic and DADS (diallyl disulfide), garlic derived active

* Corresponding author at: Drug Discovery Research Center (DDRC), Translational Health Science and Technology Institute (THSTI), 3rd Milestone, Faridabad-Gurgaon Expressway, Faridabad 121001, Haryana, India.
E-mail address: skbanerjee@thsti.res.in (S.K. Banerjee).
URL: http://www.thsti.res.in/ (S.K. Banerjee).

http://dx.doi.org/10.1016/j.bbrep.2015.11.008

compound can reduce cardiac hypertrophy in rat through activation of mitochondrial biogenesis pathways.

2. Material and methods

2.1. Garlic homogenate

Garlic (*Allium sativum*) bulbs were purchased from a local market. Cloves were peeled, sliced, ground into a paste and then suspended in distilled water. Rats were fed with freshly prepared aqueous garlic homogenate every day. Diallyl disulfide and sodium hydrogen sulphide were purchased from Sigma Aldrich.

2.2. Animals

All animal experiments were undertaken with the approval of Institutional Animal Ethics Committee of Indian Institute of Chemical Technology, Hyderabad. Forty male Sprague-Dawley rats (250 g) were obtained from National Center for Laboratory Animal Sciences (NICLAS), Hyderabad, India. The animals were housed in BIOSAFE, an animal quarantine facility of Indian Institute of Chemical Technology (IICT), Hyderabad. The animal house is maintained at temperature 22 ± 2 °C with relative humidity $50 \pm 15\%$ and 12 h dark/light cycle throughout the study. Rats had free access to food (pellet diet supplied from NICLAS, Hyderabad) and water adlibitum.

2.3. Experimental protocol

Weight matched (250 g) male Sprague-Dawley rats were randomly divided into five groups with eight rats each. Control rats were fed normal saline daily for 14 days. Aqueous garlic homogenate, diallyl disulfide and sodium hydrogen sulphide were fed to third, forth and fifth groups rats by oral gavage everyday at a fixed time (10:00 AM) for 14 days at a dose of 250 mg/kg/day [6,7], 50 mg/kg/day [8], 14 μM/kg/day [9] respectively. Four test groups (2nd, 3rd, 4th, 5th) were given isoproterenol by placing alzet pumps subcutaneously for 14 days at a dose of 5 mg/kg/day. At the end of 14 days animals are sacrificed and hearts were stored at −80 °C for estimation of all tissue parameters.

Experimental groups

SL No.	Groups	Description
1	Con	Normal saline for 14 days released from alzet pump placed subcutaneously.
2	Iso	Isoproterenol 5 mg kg^{-1} day^{-1} for 14 days released from alzet pump placed subcutaneously.
3	Iso+Gar	Oral administration of garlic homogenate 250 mg kg^{-1} day^{-1} for 14 days along with isoproterenol as administered in Iso group.
4	Iso+DADS	Oral administration of diallyl disulfide 50 mg kg^{-1} day^{-1} for 14 days along with isoproterenol as administered in Iso group.
5	Iso+NaHS	Oral administration of sodium hydrogen sulfide 14 μM kg^{-1} day^{-1} for 14 days along with isoproterenol as administered in Iso group.

2.4. Cardiac hypertrophy parameter

Isoproterenol through minipump at a dose of 5 mg/kg body weight for 14 days creates cardiac hypertrophy in rat [10]. In each group the heart weight/body weight ratio and the heart weight/Tail length ratio were measured on the day of sacrifice. Heart weight was measured after keeping the heart in ice cold saline and squeezing out the blood. Heart weight/body weight ratio and heart weight/tail length ratio are primary indicators of cardiac hypertrophy. BNP and β MHC gene expression in heart were also measured as markers of cardiac hypertrophy.

2.5. Myocardial endogenous antioxidant and lipid peroxidation status

Myocardial glutathione (GSH) content in heart homogenate was measured by biochemical assay using dithionitrobenzoicacid (DTNB) method as described by Ellman [11]. Data were expressed as mg per gm heart weight.

Myocardial catalase activity was determined by measuring the decomposition of hydrogen peroxide at 240 nm, according to the method described by Aebi [12]. Data were expressed as milliunits per microgram protein.

Total antioxidant activity was measured by DPPH assay as described earlier [13]. DPPH solution was prepared at the concentration of 0.195 mg/ml in methanol. Fifty μl heart homogenate was mixed with 100 μl DPPH solution and 100 μl tris buffer. After incubating for 30 min, absorbance was read at 517 nm by UV spectrophotometer. Methanol was used as the blank. Optical densities were used to calculate the free radical scavenging activity. Heart homogenate with lower optical density indicate higher antioxidant status.

The extent of lipid peroxidation in heart was determined by measuring malondialdehyde (MDA) content according to modified method of Okhawa et. al. [14] based on the reaction with thiobarbituric acid. Data were expressed as nanomoles per gm heart weight using extinction co-efficient of 1.56×10^5 M^{-1} cm^{-1}.

For estimating conjugated dienes, heart tissue homogenates were treated with chloroform: methanol (2:1) followed by vigorous vortexing and centrifugation at 2000 rpm for 10 min. The upper layer obtained was discarded along with proteins, while the lower chloroform layer was dried under a stream of nitrogen at 45 °C. The residue obtained was dissolved in cyclohexane and the absorbance was taken at 233 nm against a cyclohexane (standard 1 O.D.=37.5 nmoles) [15].

Reactive oxygen species (ROS) was measured fluorometrically in heart tissue homogenates using 2, 7-dichlorofluorescein diacetate (DCF-DA) according to Maity et al. [16]. Briefly 100 mM of DCF-DA and tissue homogenate was incubated for 30 min at room temperature in dark. After incubation, the volume of the reaction was adjusted with phosphate buffer saline (PBS, 0.1 M, pH-7.4) and fluorescence was measured at 488 nm excitation and 525 nm emission wavelength. The data obtained was expressed as percentage of control.

2.6. Histopathology

Masson's Trichrome is a commonly used for staining collagen and detecting cardiac fibrosis. Heart tissue was fixed in 10% formalin, routinely processed and embedded in paraffin. Paraffin sections (3 μm) were cut on glass slides, stained with Masson's Trichrome and examined under a light microscope to find myocardial fibrosis. Two sections of heart from each group were analysed for histopathology in blinded manner.

2.7. Mitochondrial enzyme in heart

Mitochondrial enzymes e.g. citrate synthase, isocitrate dehydrogenase, α ketoglutarate dehydrogenase and β Hydroxylacyl CoA dehydrogenase were estimated to measure the extent of

Table 1
Primers sequences.

Name	Direction	Sequences	Productsize (bp)	Temperature (°C)
RPL32	Forward	AGATTCAAGGGCCAGATCCT	175	57
	Reverse	CGATGGCTTTTCGGTTCTTA		
BNP	Forward	GCAGAAGATAGACCGGATCG	101	53
	Reverse	CAGAGTCAGAAGCCGGAGT		
βMHC	Forward	TGGAGCTGATGCACCTGTAG	163	55
	Reverse	ACTTCGTCTCATTGGGGATG		
CO1	Forward	GGAGCAGTATTCGCCATCAT	450	59
	Reverse	CGGCCGTAAGTGAGATGAAT		
PGC1α	Forward	ATGTGTCGCCTTCTTGCTCT	179	60
	Reverse	ATCTACTGCCTGGGGACCTT		
Tfam	Forward	AGCTGATGGGCTTAGAGAAGG	170	62.5
	Reverse	ATTTCCCCTGAGCTGACTCAT		
Cyb	Forward	ACGCTCCATTCCCAACAAAC	189	53
	Reverse	GTTGGCCTCCGATTCATGTT		
	Reverse	GGTTGAGCTTGTCCTTCCAG		
Bcl2	Forward	CCATGACTGAGGGACCAACT	223	61
	Reverse	CTCTTCTTCCTGCCCTTCCT		
Caspase3	Forward	AGGCCGACTTCCTGTATGCT	155	67
	Reverse	TCCGGTTAACACGAGTGAGG		
Caspase9	Forward	AAGACCATGGCTTTGAGGTG	210	60
	Reverse	CAGGAACCGCTCTTCTTGTC		
BAD	Forward	CGTGAGCTCCGAAGAATGAG	149	51
	Reverse	TCCTTTCCCCAAATTTCGAT		
α Skeletal actin	Forward	CACGGCATTATCACCAACTG	200	57–62
	Reverse	CCGGAGGCATAGAGAGACAG		
MMP2	Forward	GGGTGGTGGTCACAGCTATT	155	54.5
	Reverse	CGGTGTGCAGTGAAGATTGT		
β-2 microglobulin (B2m)	Forward	TCAGAAAACTCCCCAAATTC	185	57
	Reverse	AGAAAGACCAGTCCTTGCTG		

mitochondrial dysfunction.

Citrate synthase activity was measured at 37 °C in 0.1 M Tris–HCl (pH 8.3) assay buffer containing 0.12 mM 5,5′-dithio-bis 2-nitrobenzoic acid and 0.6 mM oxaloacetate as described previously [17]. The reaction was initiated by adding 3.0 mM acetyl-CoA, and the change in absorbance was measured every 10 s for 7 min at 412 nm.

Mitochondrial isocitrate dehydrogenase activity was measured according to the method of Duncan et al. [18] by measuring the reduction of NAD to NADH at 340 nm with the help of a UV–vis spectrophotometer. One milliliter assay volume contained 50 mM phosphate buffer pH 7.4, 0.5 mM isocitrate, 0.1 mM $MnSO_4$, 0.1 mM NAD and enzyme. The enzyme activity was expressed as units/mg protein.

Mitochondrial alpha-ketoglutarate dehydrogenase activity was measured spectrophotometrically according to the method of Duncan et al. [18] by measuring the reduction of 0.35 mM NAD to NADH at 340 nm using 50 mM phosphate buffer, pH 7.4 as assay buffer, and 0.1 mM α-ketoglutarate as substrate. The enzyme activity was expressed as units/mg protein.

Beta-hydroxyacyl CoA dehydrogenase activity was measured at 37 °C in assay buffer containing 0.1 M triethanolamine-HCl, 5 mM EDTA, and 0.45 mM NADH (pH 7.0) as described previously [17]. The reaction was initiated by adding 0.1 mM acetoacetyl-CoA, and the change in absorbance was measured every 10 s for 5 min at 340 nm.

2.8. Mitochondrial complexes in heart tissue

Activity of NADH dehydrogenase (Complex I) was estimated as described by King and Howard [19]. Forty microlitre of mitochondrial preparation was added to the reaction mixture containing 6 mM NADH, 0.2 M glycyl-glycine (pH 8.5), 0.02 M $NaHCO_3$ and 1 mM oxidized cytochrome C. NADH dehydrogenase catalyzed reduction of cytochrome C and the decrease in absorbance was followed spectrophotometrically at 550 nm for 1 min. Result was expressed as nmol NADH oxidized/ min/mg protein.

Cytochrome C oxidase (Complex IV) activity was measured at 25 °C in 0.03 M potassium phosphate buffer containing reduced cytochrome C (2 mg/ml) and 4 mM sodium hydrosulfite as described previously [17]. The reaction was initiated by adding sample and the change in absorbance was measured every 10 s for 5 min at 550 nm.

Mitochondrial F1F0 synthase (Complex V) activity was measured as described by Griffiths and Houghton [20]. Reaction was started by adding appropriate amount of mitochondrial suspension in ATPase buffer (50 mM Tris and 5 mM MgCl2, pH 7.5) at 37 °C with 5 mM ATP for 10 min. The reaction was stopped by adding 10% (*w*/*v*) trichloroacetic acid. The contents were centrifuged at 3000 g for 20 min, and an appropriate volume of supernatant was mixed with water. Phosphate produced was measured by kit from fluca. Results were expressed as nmol of ATP hydrolyzed/min/mg protein.

The reduction of MTT (total dehydrogenase) to blue formazan by dehydrogenases present in the mitochondrial suspension was also monitored to assess mitochondrial functions [21]. To appropriate (3 μl) homogenate, MTT (0.1 mg/ml) was added, mixed and incubated at 37 °C for 30 min and then centrifuged to obtain formazan pellet. The pellet was dissolved in ethanol and the mixture was again centrifuged at 2000 g for 10 min. The absorbance of the supernatant was measured at 595 nm.

2.9. Mitochondrial DNA content

20 mg of heart tissue was crushed into small pieces and put these in 500 μl extraction buffer. It was heated at 55 °C for 24 h after adding 50 μl proteinase-K. After removing from oven, 800 μl phenol chloroform isoamyl alcohol solution was added and vortex 5 min. After centrifugation aqueous layer was taken and mixed with 50 μl 3MNaAC. Then mixed with 100% ethanol and kept in 15 min −20 °C freezer. DNA pallet was precipitated. From this DNA, we did qPCR to measure mitochondrial CO1 and Cyb DNA

expressions. β2-microglobulin (β2 M) was taken as reference standard [22].

2.10. Real-time quantitative PCR (QPCR)

Total RNA was isolated from rat heart with TRIzol (sigma). Reverse transcriptase reactions were performed for cDNA synthesis according to the method described by Banerjee et al. [23]. Primers (Table 1) for real-time quantitative PCR (QPCR) analysis were designed using published sequence information, avoiding regions of homology with other genes. For each gene, 10 ng of cDNA were analyzed on an ABI PRISM 7700 using Absolute SYBR Green ROX PCR Master Mix (Thermo Scientific). Fold-change analysis was based on normalizing to RPL32 transcript levels in each sample [24].

2.11. Immunoblot analysis

Total protein extraction and immunoblotting were performed as described previously [25]. Protein concentration was determined by Bradford method. An equal amount (40 μg) of protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). After electrophoresis, protein was transferred to PVDF membranes (Millipore, USA). The membranes were then blocked in Tris-buffered saline Tween-20 (TBS-T; 10 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween-20) and 5% non-fat dry milk for 1 h, and subsequently washed and incubated with primary antibodies in TBST with 3% non-fat dry milk at 4 °C for overnight. The following polyclonal antibody and titre was used: Nrf2 and cytochrome C (1:1000, Cell Signaling Technology). After washing with TBS-T, membrane was incubated with Goat Anti-Rabbit IgG–HRP (1:4000 dilutions, Cell Signaling Technology, # SC 2004) horseradish peroxidase conjugated secondary antibody with 2.5% non-fat dry milk at room temperature for 2 h. After washing with TBS-T, immunoreactions were visualized with a chemiluminescence detection kit (Super signal® west Pico chemiluminescent substrate, Thermo Scientific). Then the blots were exposed to chemiluminecence instrument and developed. Gel stained with coomassie blue served as an equal loading control. Quantification of band intensity was performed using the Image J Software (NIH, Bethesda, MD, USA).

2.12. Immunoprecipitation analysis

2.12.1. Binding of Antibody (Ab) with beads

Dynabeads were resuspended by pipetting and transfered 50 μl dynabeads to a tube, placed on magnet and removed supernatant. Then tube was removed from magnet and resuspend the dynabeads in 200 μl Ab binding and washing buffer containing either Ab of eNOS or Phospho AKT and Incubated 10 min with rotation at room temperature. Then tube was placed on magnet and removed supernatant. After removing tube from magnet, dynabeads-Ab complex was washed by resuspending in 200 μl Ab binding and washing buffer.

2.12.2. Immunoprecipitation of Antigen (Ag)

By keeping tube on magnet, supernatant was removed and then tissue protein sample was added to the dynabeads-Ab complex and gently resuspend by pipetting and incubated 10 min at room temperature with rotation. After placing tube on magnet and transfering supernatant to a clean tube, the dynabeads-Ab-Ag complex was washed 3 times, using 200 μl washing buffer for each wash. Dynabeads-Ab-Ag complex was resuspended in 100 μl washing buffer and the suspension was transfered to a clean tube.

2.12.3. Elution of Ab/Ag complex

By placing tube on magnet and removing supernatant, the dynabeads-Ab-Ag complex was resuspend in 20 μl elution buffer and incubated two minutes at room-temperature. Then putting tube on magnet, supernatant/sample was transferred to a clean tube. Finaly sample was prepared for western blot to measure eNOS and phosphor AKT level in heart tissue.

2.13. Cell culture

H9C2 cells were purchased from Lonza, USA and cultured using DMEM media containing10% fetal bovine serum (FBS). Cells from passages 2–7 were used for all the experiments. H9C2 cells were in different treatment protocol as shown below.

SL no.	Groups	Descriptions
1	Con	Only vehicle
2	Iso	Cells were treated with isoproterenol in 25 μM for 48 h
3	Iso + Gar	Cells were treated with isoproterenol in 25 μM for 48 h along with fresh aqueous garlic extract in 0.25 mg/ml concentration.
4	Iso + Gar + LNAME	Cells were treated with isoproterenol in 25 μM for 48 h along with fresh aqueous garlic extract in 0.25 mg/ml and LNAME in 100 μg/ml concentration.
5	Iso + DADS	Cells were treated with isoproterenol in 25 μM for 48 h along with DADS in 50 μM concentration
6	Iso + DADS + LNAME	Cells were treated with isoproterenol in 25 μM for 48 h along with DADS in 50 μM concentration and LNAME 100 μg/ml concentration
7	Iso + NaHS	Cells were treated with isoproterenol in 25 μM for 48 h along with NaHS in 100 μM concentration
8	Iso + NaHS + LNAME	Cells were treated with isoproterenol in 25 μM for 48 h along with NaHS in 100 μM and LNAME in 100 μg/ml concentrations.

2.14. Nitric oxide

Nitric oxide was measured according to Griess Reaction. 30 μl of each experimental sample and 30 μl of 1% Sulfanilamide solution were added to wells in duplicate and incubated for 10 min. Then 30 μl of 0.1% N-1-napthylethylenediamine dihydrochloride solution was added to all wells. After incubation for 30 min, absorbance was measured 540 nm. Nitric oxide was calculated in nMol/μg protein.

2.15. Statistical analysis

All values were expressed as mean ± SEM. Data from more than two groups were statistically analyzed using one way ANOVA for multiple group comparison followed by Bonferroni post hoc test. Differences between two groups were compared by Student's *t* test. Significance was set at $p < 0.05$.

3. Result

3.1. DADS and garlic treatment attenuated cardiac hypertrophy in rat heart

Heart weight/body weight ratio and heart weight/tail length ratio were evaluated as a measurement of cardiac hypertrophy. A significant ($p < 0.05$) increase in heart/body weight ratio and heart weight/tail length ratio was observed in Iso group compared to control group. However, there was significant ($p < 0.05$) decrease in these ratios in Iso+garlic, Iso+DADS and Iso+NaHS group compared to Iso group (Fig. 1A and B).

BNP and β-MHC gene expression were measured as markers of cardiac hypertrophy. BNP gene expression was significantly ($p < 0.05$) increased in Iso group compared to control group and significantly ($p < 0.05$) decreased in Iso+garlic and Iso+DADS but not in Iso+NaHS group compared to Iso group (Fig. 1C).

Similarly, β-MHC gene expression was significantly ($p < 0.05$) increased in Iso group compared to control group and significantly ($p < 0.05$) decreased in Iso+garlic and Iso+DADS but not in Iso+NaHS group compared to Iso group (Fig. 1D).

3.2. DADS and Garlic treatment ameliorated gene expression of collagen, α skeletal actin and MMP2 which are increased in disease condition in hypertrophy heart

Collagen, α skeletal actin and MMP2 expression were measured as markers of myocardial remodeling. A significant ($p < 0.05$) increase in expression of collagen was observed in Iso group compared to control group. However, significant ($p < 0.05$) decrease in expression of collagen was observed in Iso+garlic, Iso+DADS and Iso+NaHS group compared to Iso group (Fig. 2A).

A significant ($p < 0.05$) increase in expressions of α skeletal actin and MMP2 were observed in Iso group compared to control group. However, significant ($p < 0.05$) decrease in expression of these genes were observed in Iso+garlic and Iso+DADS group but not in Iso+NaHS group compared to Iso group (Fig. 2B and C).

3.3. DADS and garlic treatment attenuated oxidative stress in hypertrophy rat heart

A significant ($p < 0.05$) decrease in myocardial GSH level was observed in Iso group compared to control group. However, there was significant ($p < 0.05$) increase in GSH level observed in Iso-+garlic and Iso+DADS group but not in Iso+NaHS group compared to Iso group (Fig. 3A).

A significant ($p < 0.05$) decrease in myocardial catalase activity was observed in Iso group compared to control group. However, a significant ($p < 0.05$) increase in myocardial catalase activity was observed in Iso+garlic and Iso+DADS group but not in Iso+NaHS group compared to Iso group (Fig. 3B).

The total antioxidant capacity of heart homogenate was measured by DPPH assay. There was no change in total antioxidant capacity between Iso and control group. However significant ($p < 0.05$) increase in total antioxidant capacity was observed in Iso+garlic, Iso+DADS and Iso+NaHS group in comparison to Iso group (Fig. 3C).

A significant ($p < 0.05$) increase in myocardial TBARS level was observed in Iso group compared to control group. However, there was significant ($p < 0.05$) decrease in myocardial TBARS level in Iso+garlic, Iso+DADS and Iso+NaHS group compared to Iso group (Fig. 3D).

A significant ($p < 0.05$) increase in myocardial conjugatated diene level was observed in Iso group compared to control group. However, there was significant ($p < 0.05$) decrease in myocardial conjugatated diene level in Iso+garlic and Iso+DADS group but not in Iso+NaHS group compared to Iso group (Fig. 3E).

A significant ($p < 0.05$) increase in myocardial reactive oxygen

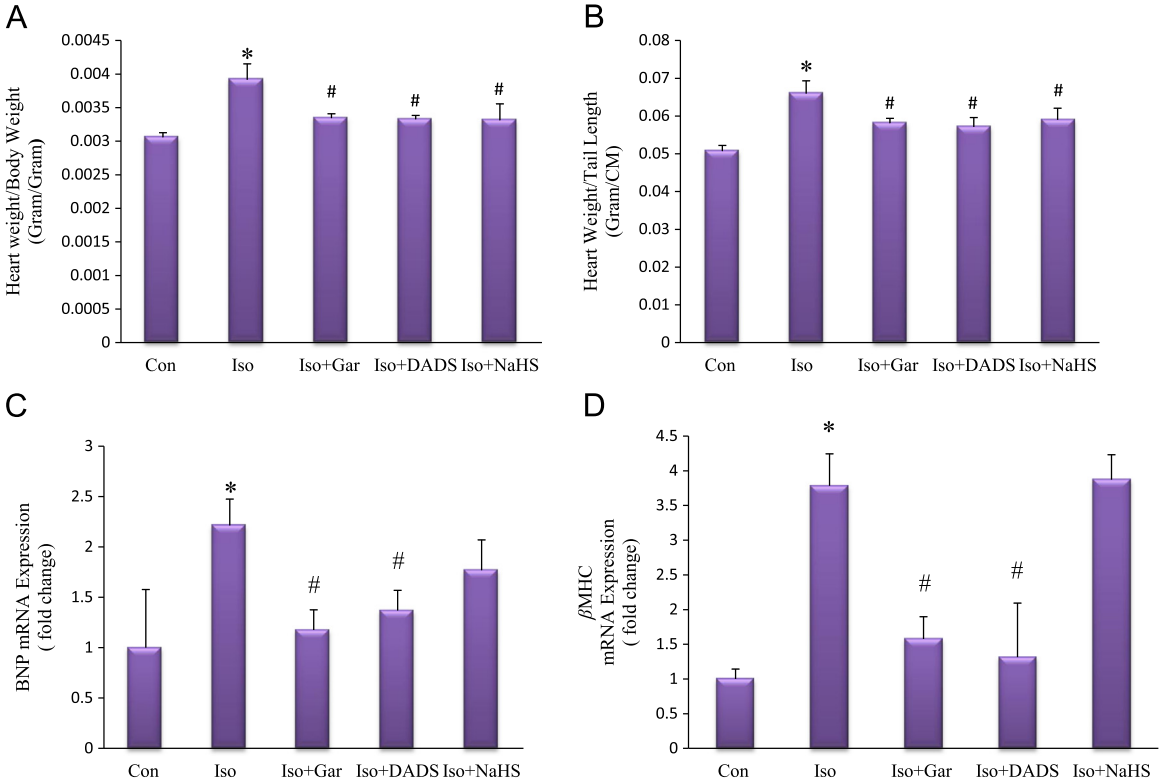

Fig. 1. Effect of garlic, DADS and NaHS on isoproterenol-induced cardiac hypertrophy. (A) Representation of cardiac hypertrophy by heart weight and body weight ratio. (B) Representation of cardiac hypertrophy by heart weight and tail length ratio. (C) Expression of BNP mRNA as a marker of cardiac hypertrophy. (D) Expression of β-MHC mRNA as a marker of cardiac hypertrophy. Data were shown as mean ± SEM ($N=8$), *$p < 0.05$ versus Con, #$p < 0.05$ versus Iso.

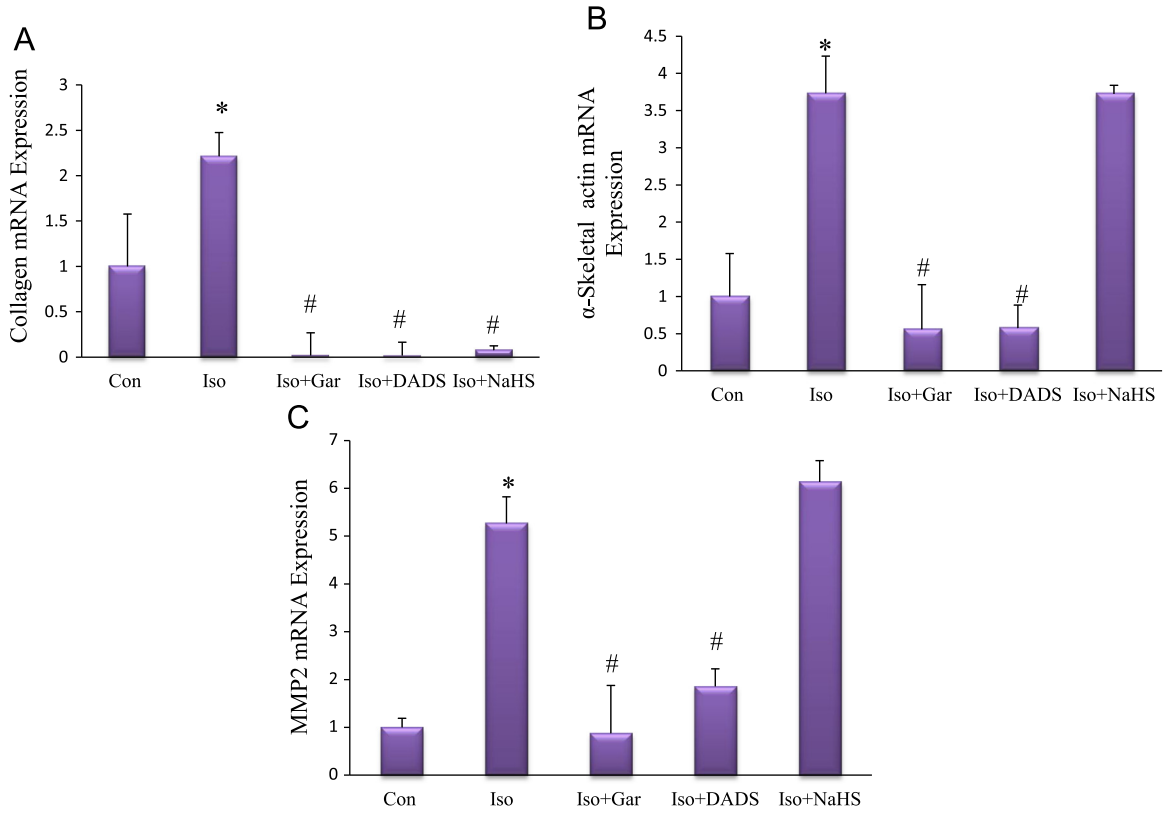

Fig. 2. Effect of garlic, DADS and NaHS against isoproterenol induced change in gene expression. (A) Collagen mRNA expression. (B) α-skeleton actin mRNA expression. (C) MMP2 mRNA expression. Data were shown as mean ± SEM ($N=3$), $^{*}p<0.05$ versus Con, $\#p<0.05$ versus Iso.

species level was observed in Iso group compared to control group. However, there was significant ($p<0.05$) decrease in myocardial reactive oxygen species level in Iso+garlic, Iso+DADS and Iso+NaHS group compared to Iso group (Fig. 3F).

3.4. DADS and garlic treatment normalized histopathologycal changes in hypertrophy heart

We observed myocardial fibrosis in Iso group after staining with Masson's trichrome. While light micrograph of control heart showed normal architecture and no fibrosis, hypertrophic heart (Iso) showed fibrosis in cardiac muscle along with infiltration of acute and chronic inflammatory cells. However, our finding showed that fibrosis was reduced in Iso+garlic and Iso+DADS but not in Iso+NaHS group compared to Iso group (Fig. 4).

3.5. DADS and garlic treatment enhanced mitochondrial enzymes activities in hypertrophy heart

Citrate synthase, isocitrate dehydrogenase, α keto glutarate dehydrogenase and β hydroxyl acyl CoA dehydrogenase activities were measured as markers of mitochondrial function. A significant ($p<0.05$) decrease in these enzyme activities was observed in Iso group compared to control group. However, significant ($p<0.05$) increase in the activity of all those enzymes was observed in Iso+garlic and Iso+DADS group but not in Iso+NaHS group except β hydroxyl acyl CoA dehydrogenase compared to Iso group (Fig. 5).

3.6. DADS and garlic treatment enhanced activity of mitochondrial complexes in hypertrophy heart

Enzymes from mitochondrial complexes i.e., NADH dehydrogenase, cytochrome C oxidase, F1F0 synthase activities, MTT reduction were measured as markers of mitochondrial function. A significant ($p<0.05$) decrease in three enzyme (NADH dehydrogenase, total dehydrogenase and cytochrome C oxidase) activities was observed in Iso group compared to control group. However, significant ($p<0.05$) increase in the activity of all these enzymes was observed in Iso+garlic and Iso+DADS group but not in Iso+NaHS group compared to Iso group (Fig. 6).

However, we have observed significant ($p<0.05$) increased of F1F0 synthase activity in Iso group compared to control group. Significant ($p<0.05$) decrease of this enzyme activity was observed in Iso+garlic and Iso+NaHS but not in Iso+DADS group compared to Iso group (Fig. 6).

3.7. DADS and garlic treatment normalized the apoptotic gene expression in hypertrophy heart

Bcl2 gene expression was measured as markers of cell survival. A significant ($p<0.05$) increase in expression of Bcl2 gene was observed in Iso group compared to control group. However, significant ($p<0.05$) decrease expression of this gene was observed in Iso+garlic, Iso+DADS and Iso+NaHS group compared to Iso group (Fig. 7A).

Caspase3, caspase9 and BAD expression were measured as markers of apoptosis. A significant ($p<0.05$) decrease expression of these genes were observed in Iso group compared to control group. However, significant ($p<0.05$) increase expression of these genes were observed in Iso+garlic and Iso+DADS group but not in Iso+NaHS group compared to Iso group (Fig. 7B–D).

3.8. DADS and garlic treatment ameliorated mitochondrial gene expression in hypertrophy heart

PGC1α and Tfam expression were measured as markers of mitochondrial biogenesis. A significant ($p<0.05$) decrease expression

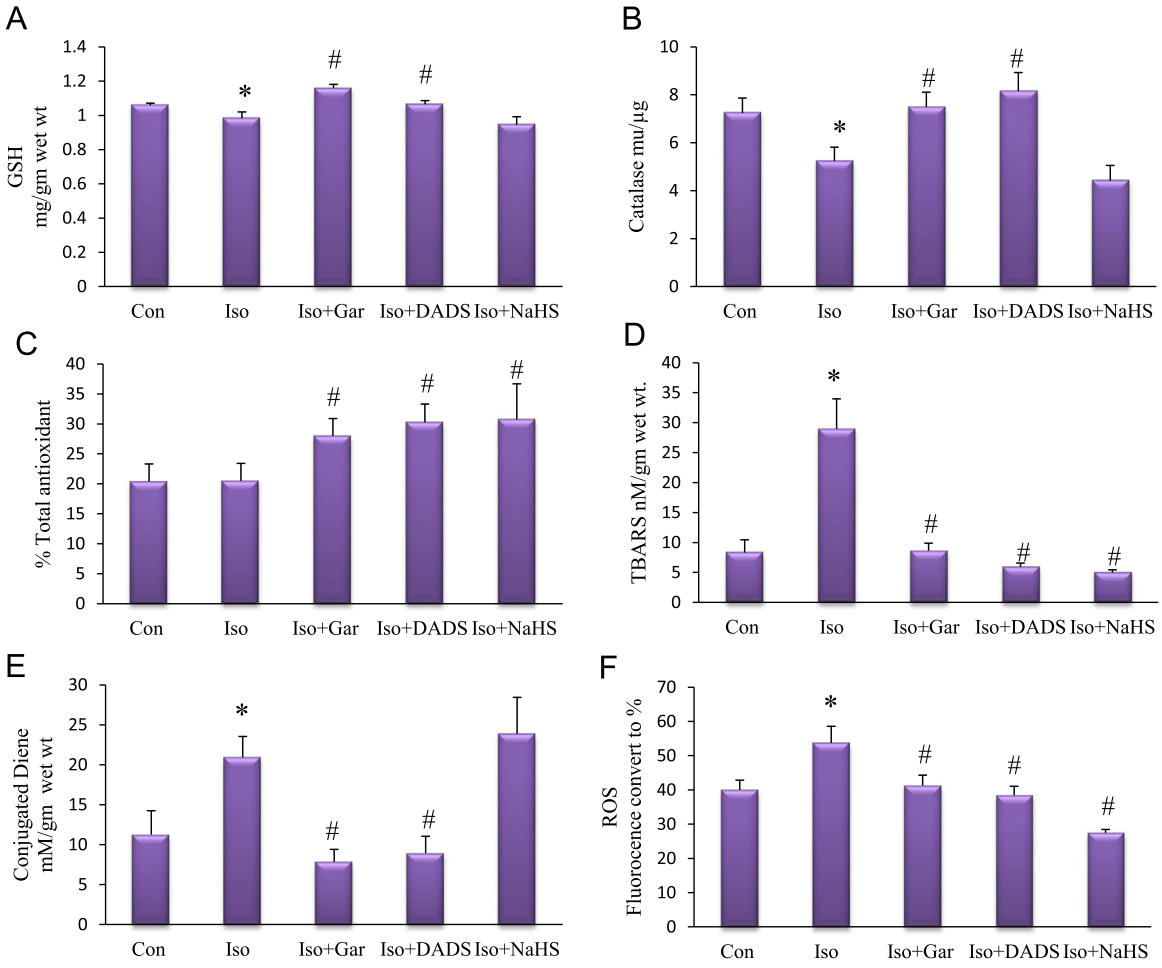

Fig. 3. Effect of garlic, DADS and NaHS on endogenous antioxidants, conjugated dienes and total antioxidants. (A) Myocardial GSH level. (B) Myocardial catalase. (C) Myocardial total antioxidant capacity as measured by DPPH assay. (D) Myocardial TBARS. (E) Myocardial conjugated diene. (F) Myocardial ROS generation. Data were shown as mean ± SEM ($N=8$), $^{*}p<0.05$ versus Con, $\#p<0.05$ versus Iso.

of these genes was observed in Iso group compared to control group. However, significant ($p<0.05$) increase in expression of these genes were observed in Iso+garlic and Iso+DADS group but not in Iso+NaHS group compared to Iso group (Fig. 8A and B).

Mitochondrial CO1 and Cyb DNA expressions were measured as markers of mitochondrial biogenesis. A significant ($p<0.05$)

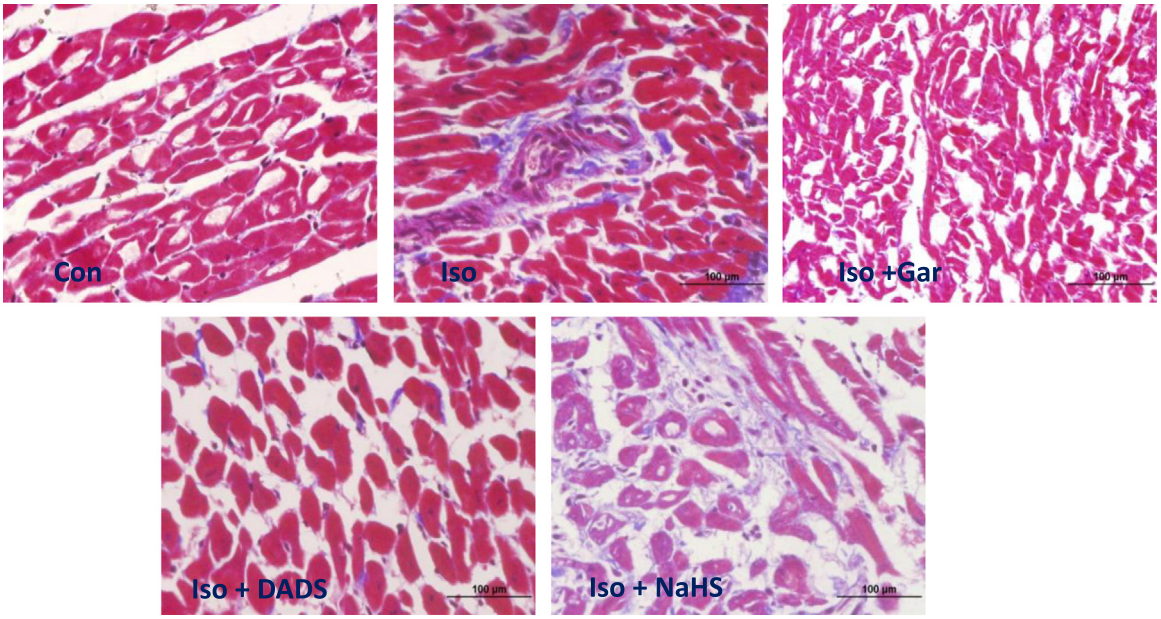

Fig. 4. Effect of garlic, DADS and NaHS against isoproterenol induced histopathological changes in different groups.

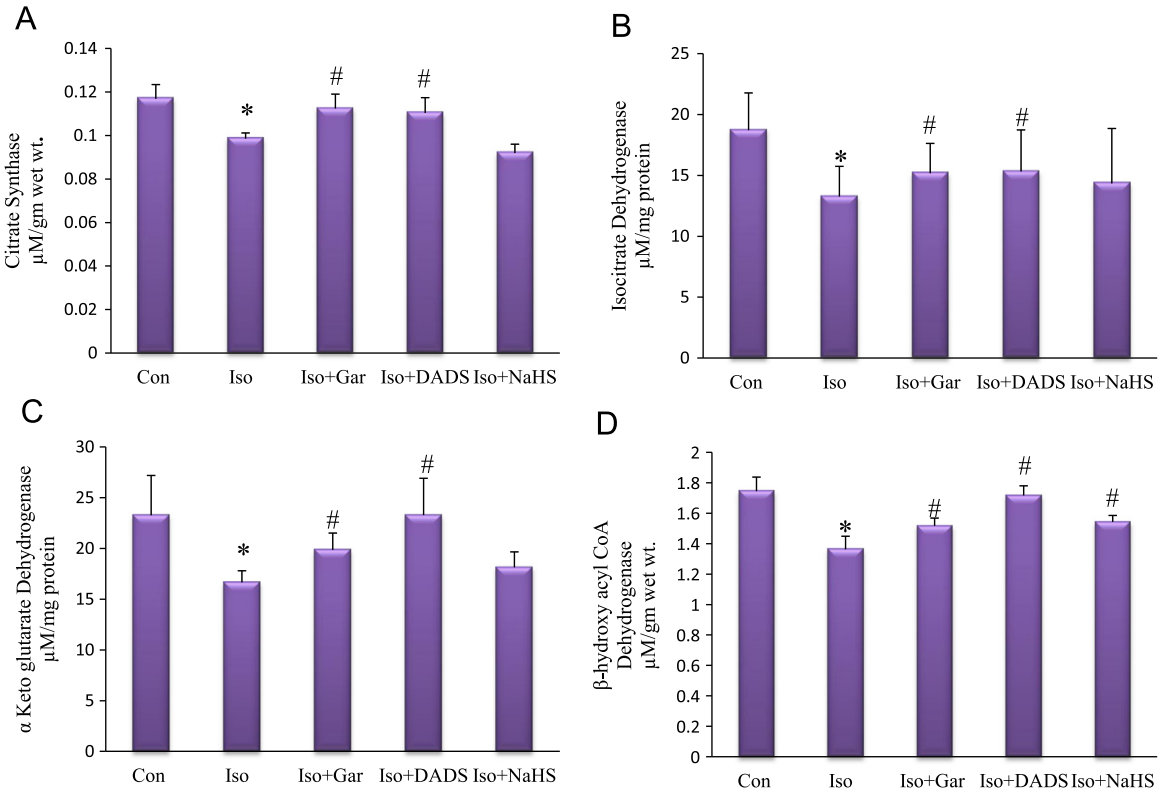

Fig. 5. Effect of garlic, DADS and NaHS against isoproterenol induced decreased in the activites of mitochondrial enzymes. (A) Citrate synthase (B) Isocitrate dehydrogenase (C) α-Ketoglutarate dehydrogenase (D) β-Hydroxy acyl CoA dehydrogenase. Data were shown as mean ± SEM ($N=8$), $^{*}p<0.05$ versus con, $\#p<0.05$ versus Iso.

decrease in expressions of CO1 and Cyb were observed in Iso group compared to control group. However, significant ($p<0.05$) increase expressions of CO1 and Cyb DNA were observed in Iso+garlic and Iso+DADS but not in Iso+NaHS group compared to Iso group (Fig. 8C and D).

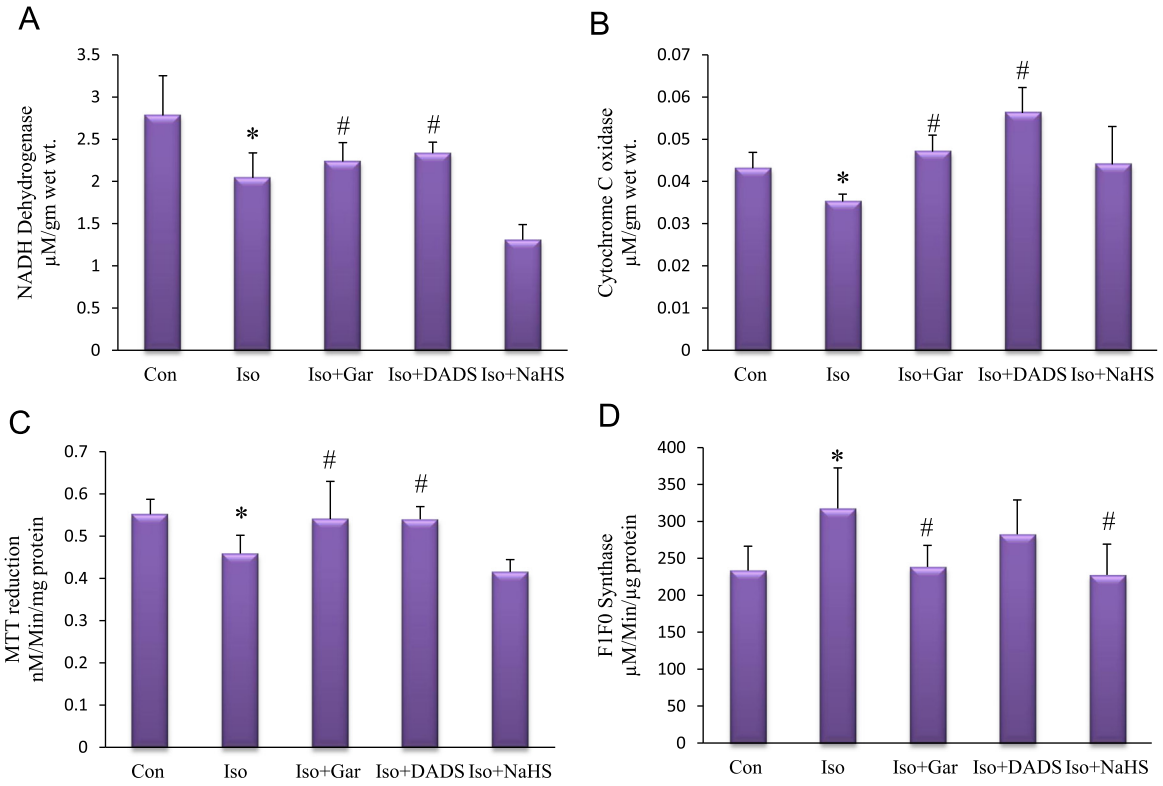

Fig. 6. Effect of garlic, DADS and NaHS against isoproterenol induced decreased of mitochondrial complexes activites. (A) NADH Dehydrogenase. (B) Cytochrome C oxidase. (C) MTT reduction (D) F1F0 Synthase. Data were shown as mean ± SEM ($N=8$), $^{*}p<0.05$ versus Con, $\#p<0.05$ versus Iso.

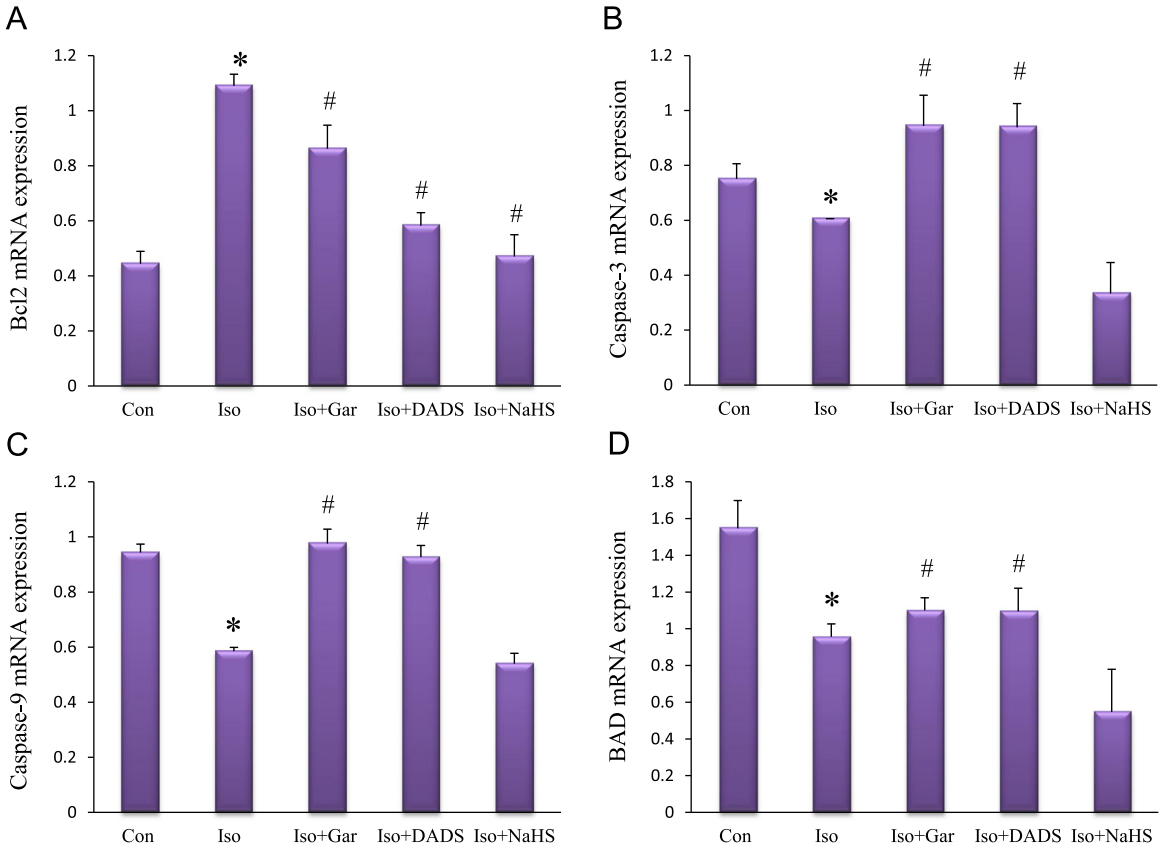

Fig. 7. Effect of garlic, DADS and NaHS against isoproterenol induced change in apoptotic gene expression. (A) Bcl2 mRNA expression. (B) Caspase-3 mRNA expression. (C) Caspase-9 mRNA expression. (D) BAD mRNA expression. Data were shown as mean ± SEM ($N=3$), $^{*}p < 0.05$ versus Con, $\#p < 0.05$ versus Iso.

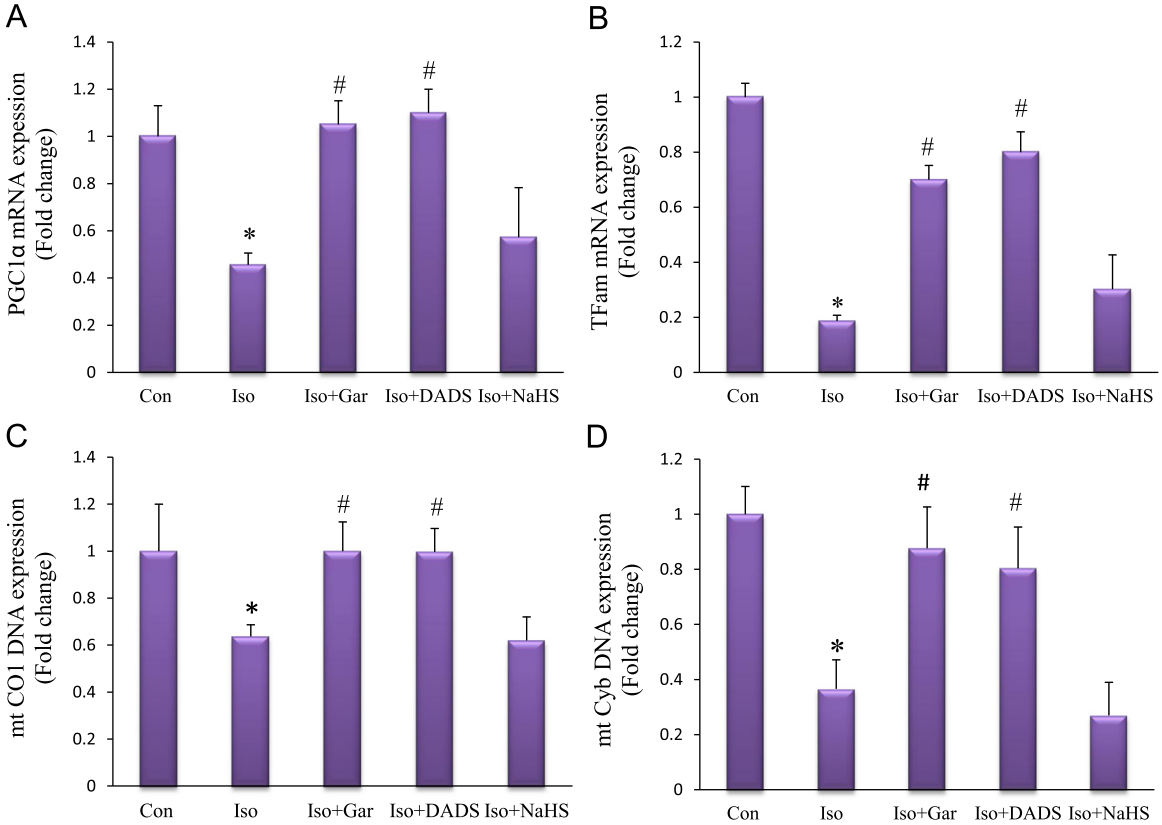

Fig. 8. Effect of garlic, DADS and NaHS against isoproterenol induced change in mitochondrial gene expression. (A) PGC 1α mRNA expression. (B) Tfam mRNA expression. (C) Mito CO1 DNA expression. (D) Mito Cyb DNA expression. Data were shown as mean ± SEM ($N=3$), $^{*}p < 0.05$ versus Con, $\#p < 0.05$ versus Iso.

3.9. DADS and garlic treatment enhanced mitochondrial protein expressions in hypertrophy heart

As eNOS was very low and not detectable by western blot in rat heart, we did immunoprecipitation followed by western blot. Our data showed that there was very low level of eNOS in both control and Iso heart. There was no difference of myocardial eNOS protein expression in Iso group compared to control group. However, few fold increase in eNOS protein level was observed in Iso+garlic and Iso+DADS group but not in Iso+NaHS group compared to Iso group (Fig. 9A). GAPDH and coomassie blue of heart homogenate before immunoprecipitation was used as a loading control.

Similarly, phospho-AKT level was measured by western blot after immunoprecipitation. Decrease in myocardial phospho-AKT protein expression was observed in Iso group compared to control group. However, a significant increase in myocardial phospho-AKT level was observed in Iso+garlic, Iso+DADS and Iso+NaHS group compared to Iso group (Fig. 9A). GAPDH and coomassie blue of heart homogenate before immunoprecipitation was used as a loading control.

We measured Nrf2 and Cytochrome C protein level in heart homogenate by western blot. No change of myocardial Nrf2 and cytochrome C protein expressions were observed in Iso group compared to control group. However, increase in Nrf2 and cytochrome C protein levels were observed in Iso+garlic, Iso+DADS and Iso+NaHS group compared to Iso group (Fig. 9B).

3.10. Garlic and DADS enhanced nitric oxide level and mitochondrial biogenesis in H9C2 cells

Our in-vitro work with H9C2 cell lines showed that nitric oxide level was reduced significantly in Iso group when compared to control group. Significant increased nitric oxide level was observed in Iso+Gar and Iso+DADS when compared to iso group. However, there was no change in cellular nitric oxide levels in Iso+Gar+LNAME, Iso+DADS+LNAME, Iso+NaHS and Iso+NaHS+LNAME groups compared to Iso group (Fig. 10).

CO1 and Cyb mRNA expression was reduced significantly in Iso group when compared to control group. Significant increase in CO1 and Cyb mRNA expressions were observed in Iso+Gar and Iso+DADS when compared to iso group. However, both gene expressions were not increased in Iso+Gar+LNAME, Iso+DADS+LNAME, Iso+NaHS, Iso+NaHS+LNAME groups when compared to Iso group (Fig. 10B and C).

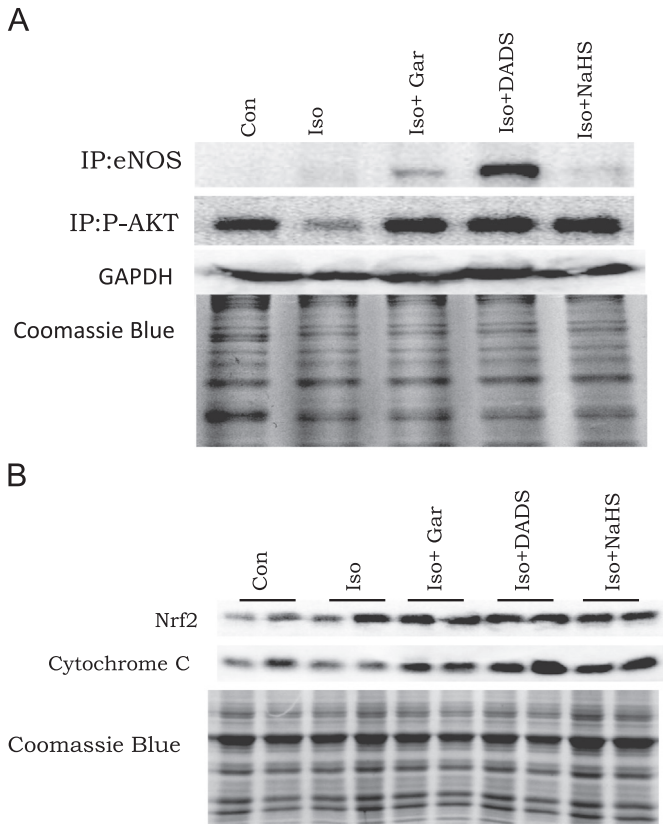

Fig. 9. Effect of garlic, DADS and NaHS against isoproterenol induced change in protein expression on rat heart. (A) Western blot of eNOS and phospho AKT after immunoprecipitation- GAPDH and coomassie are used to represent same amount of protein. (B) Western blot of Nrf2 and cytochrome C-coomassie are used to represent same amount of protein.

4. Discussion

In the present study, we evaluated the potential therapeutic and pharmacological effect of garlic and its one compound diallyl disulfide on isoproterenol induced cardiac hypertrophy and explored the possible mechanisms involved. As previously reported that H_2S generates from garlic or DADS after interacting with cellular GSH, responsible for beneficial effect of garlic, we choose NaHS to administer in rats to generate H_2S in-vivo. We demonstrated that both garlic and DADS prevented and partially reversed cardiac pathological hypertrophy by increasing mitochondrial efficiency. Garlic and DADS decreased cardiac hypertrophy as observed by reduction of heart weight/body weight rato, BNP and βMHC mRNA expression. Although heart weight/body weight rato was reduced after H_2S administration too, no change was observed in the expression of BNP and βMHC.

Different genes involved in remodeling of heart such as α-skeletal actin, MMPs and collagen level were over-expressed in hypertrophy heart [26]. Isoproterenol-induced increase in all these genes in rat heart were normalized by garlic and DADS treatment but not by hydrogen sulfide. Increased myocardial fibrosis and collagen deposition is a hallmark of cardiac hypertrophy [27]. It is mostly due to secondary response to the pathophysiological remodeling of long-standing disease. Garlic and DADS reduced fibrosis in hypertrophy heart induced by isoproterenol.

Chronic activation of hypertropic pathways and ROS generation in heart may affect cardiac remodeling. ROS affects myocardial growth, cellular function and matrix remodeling. ROS triggers a large variety of hypertrophy signaling transcriptional factors and reduces endogenious antioxidants [28]. In the present study, isoproterenol decreased endogenious antioxidants such as GSH and catalase, increased lipid peroxidation such as TBARS and conjugated diene, and reduced total antioxidant in heart. Garlic and DADS normalized endogenious antioxidant system and reactive oxygen species in isoproterenol-treated heart. However, H2S was not able to normalize all oxidative stress parameters.

Our data indicated that cardioprotective effects of garlic and DADS were mediated by the activation of mitochondrial biogenesis, which led to the inhibition of cardiac hypertrophy. Because mitochondria are the principal target of oxidative stress, herein, we have investigated the effect of isoproterenol on the mitochondrial enzymes and related pathways for energy metabolism. The current studies have investigated the status and activity of some of the mitochondrial Krebs cycle enzymes, particularly, citrate synthase, isocitrate dehydrogenase and alpha-ketoglutarate dehydrogenase, and some mitochondrial β oxidation enzyme mainly β-Hydroxy acyl CoA dehydrogenase following treatment of rats with isoproterenol. In each case, the activities of the enzymes were significantly inhibited in isoproterenol treated rats. Inhibition of mitochondrial Krebs cycle enzymes may enhance free radical formation as described previously [29]. Treatment with garlic

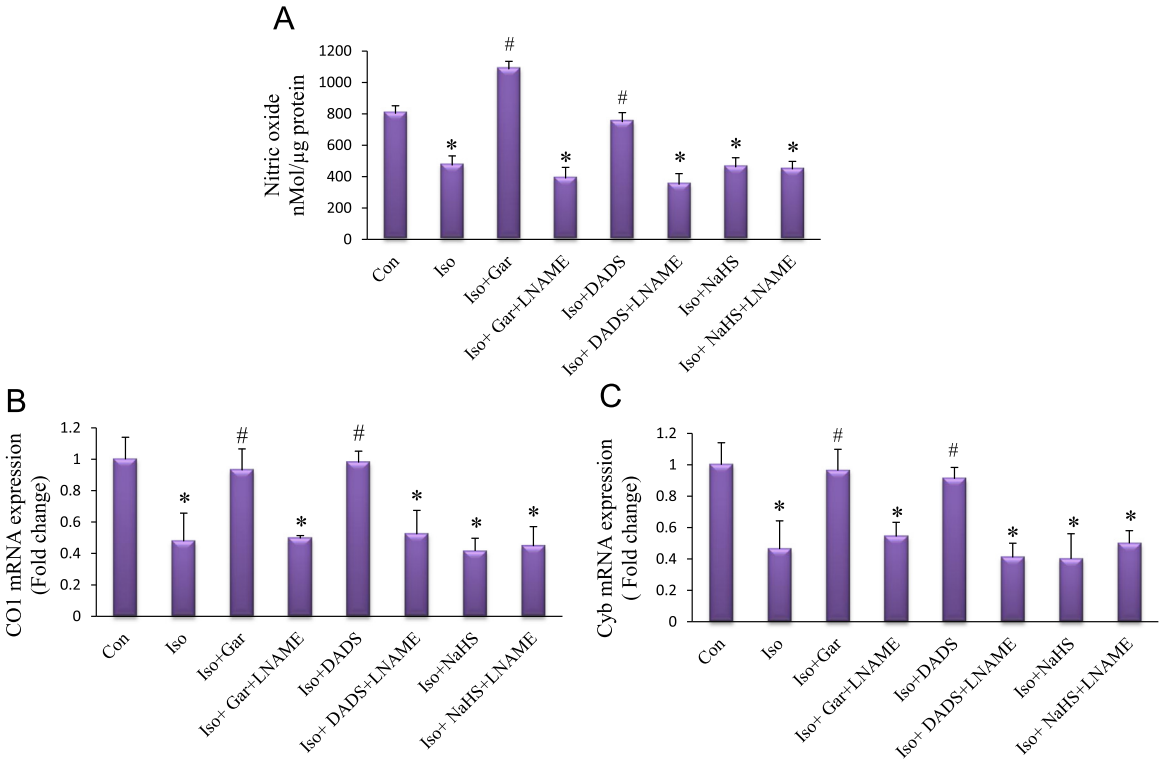

Fig. 10. Effect of garlic, DADS and NaHS on cellular nitric oxide level and mitochondrial biogenesis against isoproterenol treated H9C2 cells and its inhibition by L-NAME. (A) Nitric oxide level. (B) CO1 mRNA expression. (C) Cyb mRNA expression. Data were shown as mean ± SEM ($N=3$), $^{*}p<0.05$ versus Con, $\#p<0.05$ versus Iso.

and DADS significantly elevated the activities of these crucial enzymes. These data indicate that garlic and DADS have the ability to protect these enzymes either through scavenging the toxic reactants produced within the mitochondria in isoproterenol treated rats or protecting the substrate-binding sites of these enzymes.

A reduction in the activity of NADH dehydrogenase (Complex I), MTT reduction (all dehydrogenases) and cytochrome C oxidase (Complex IV) of the respiratory chain following isoproterenol treatment in rats is clearly indicative of reduction of cardiac mitochondrial complex activities. Treatment with garlic and DADS completely restored the activity of these enzymes indicating that garlic and DADS are capable of mitigating reduced mitochondrial complex activities after Iso treatment. Normalization of most of the mitochondrial enzymes and complexes activities were not observed after H_2S administration.

Decreased mitochondrial enzyme activity or mitochondrial efficiency is associated with increased ROS production and programmed cell death. Programmed cell death (apoptosis) is the key event in end-stage human heart failure [30]. Apoptosis is extremely rare in the normal myocardium. However, the rate of apoptosis in cardiac myocytes was increased in dilated and ischemic cardiomyopathies [31]. In initial stage, hypertrophy is considered as an adaptive mechanism and thus anti-apoptosis pathway is upregulated. Proapoptotic genes like caspage-3, caspase-9 and BAD are down regulated and antiapoptotic gene such as Bcl2 is upregulated in isoproterenol treated hypertopic heart. However, garlic and DADS but not H_2S reversed the expression of all these genes related to apoptosis.

The PPAR-gamma coactivator (PGC-1α) is a powerful regulator of mitochondrial functions and biogenesis in the heart, by regulating gene expression from both mitochondrial and nuclear genomes. Similarly Tfam, a transcription factor for mitochondria, expression is associated with increased mitochondrial number. Increased expression of PGC-1α and Tfam are considered as mitochondrial biogenesis [32]. The expression of PGC-1α and Tfam are decreased in numerous heart failure models and considered important contributor to the maladaptive energetic profile of failing hearts [33]. In the present study, mRNA expression of PGC-1α and Tfam, and mitochondrial CO1 and Cyb DNA content were downregulated in rat heart after isoproterenol treatment. Garlic and DADS increased PGC-1α and Tfam mRNA expression and increased mitochondrial CO1 and Cyb DNA copy number. We have also observed the expression of proteins linked to mitochondrial biogenesis signaling pathway after garlic and DADS treatment and thus responsible for increased PGC-1α and Tfam expression in rat heart.

Interestingly, we found higher eNOS level was observed in rat heart treated with DADS and garlic. Phospho AKT, the down stream of eNOS, was also decreased in Iso treated heart and improved after garlic and DADS treatment. Increased NO in cardiomyocites is associated with PGC-1α and Tfam expression [34]. Although, H_2S treatment did little-bit increase in eNOS level hypertrophy heart but unable to increase in PGC1 and Tfam expression. Similar to previous studies [35–37], we have also observed some protection i.e reduction of cardioac hypertrophy and oxidative stress but this protection was not due to activation of mitochondrial biogenesis. In the present study, along with the mitochondrial biogenesis pathway, two important mitochondrial proteins were measured to indicate the increase of mitochondrial number. Our data showed Nrf2 and cytochrome c, both are upregulated after garlic and DADS treatment.

To confirm our in-vivo data, we have treated H9C2 cells with garlic and DADS. Our data confirmed that nitric oxide level was decreased in isoproterenol-treated H9C2 cells but increased after garlic and DADS treatment. This increased nitric oxide level after garlic and DADS treatment was blocked when pre-treated with LNAME, nitric oxide synthase inhibitor. However, NaHS did not able to increase nitric oxide level in H9C2 cells. Increased nitric oxide level in H9C2 cells after garlic and DADS was associated with increased CO1 and Cyb mRNA expressions. This in-vitro

experiment confirmed our hypothesis that activation of nitric oxide pathway after garlic and DADS treatment is responsible for mitochondrial biogenesis and thereby protection against cardiac hypertrophy.

5. Conclusions

Garlic and diallyl disulfide induces mitochondrial biogenesis and ameliorates isoproterenol induced cardiac remodeling through eNOS-Nrf2-Tfam pathway in rats. These findings proved that garlic and DADS may be the promising candidate for therapies against cardiac hypertrophy and subsequent progression to heart failure.

Conflict of interest

The authors declare that they have no competing interest.

Acknowledgments

Financial support was provided by Ramalingaswami Fellowship fund (SKB) from Department of Biotechnology (DBT) (BT/HRD/35/02/2006), Senior Research Fellowship (TNK) from Council of Scientific and Industrial Research (CSIR) Government of India, and Grant support from Department of Science and Technology (DST) (SR/SO/HS-110/2008) and CSIR (Thunder, BSC0102).

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2015.11.008.

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