Life Sciences 164 (2016) 42–51

Contents lists available at ScienceDirect

Life Sciences

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

Garlic activates SIRT-3 to prevent cardiac oxidative stress and mitochondrial dysfunction in diabetes

Md Razia Sultana [a,1], Pankaj K. Bagul [b,1], Parameshwar B. Katare [b], Soheb Anwar Mohammed [b], Raju Padiya [a], Sanjay K. Banerjee [b,*]

[a] Department of Medicinal Chemistry and Pharmacology, Indian Institute of Chemical Technology, Hyderabad 500007, India
[b] Drug Discovery Research Center (DDRC), Translational Health Science and Technology Institute, Faridabad 122001, Haryana, India

ARTICLE INFO

Article history:
Received 11 April 2016
Received in revised form 28 August 2016
Accepted 29 August 2016
Available online 31 August 2016

Keywords:
SIRT3
MnSOD
Garlic
Acetylation
Mitochondria
Diabetic cardiomyopathy
Sirtuins

ABSTRACT

Background: Cardiac complications are major contributor in the mortality of diabetic people. Mitochondrial dysfunctioning is a crucial contributor for the cardiac complications in diabetes, and SIRT-3 remains the major mitochondrial deacetylase. We hypothesized whether garlic has any role on SIRT-3 to prevent mitochondrial dysfunction in diabetic heart.
Methods: Rats with developed hyperglycemia after STZ injection were divided into two groups; diabetic (Dia) and diabetic + garlic (Dia + Garl). Garlic was administered at a dose of 250 mg/kg/day, orally for four weeks. An additional group was maintained to evaluate the effect of raw garlic administration on control rat heart.
Result: We have observed altered functioning of cardiac mitochondrial enzymes involved in metabolic pathways, and increased levels of cardiac ROS with decreased activity of catalase and SOD in diabetic rats. Cardiac mRNA expression of TFAM, PGC-1α, and CO1 was also altered in diabetes. In addition, reduced levels of electron transport chain complexes that observed in Dia group were normalized with garlic administration. This indicates the presence of increased oxidative stress with mitochondrial dysfunctioning in diabetic heart. We have observed reduced activity of SIRT3 and increased acetylation of MnSOD. Silencing SIRT-3 in cells also revealed the same. However, administration of garlic improved the SIRT-3 and MnSOD activity, by deacetylating MnSOD. Increased SOD activity was correlated with reduced levels of ROS in garlic-administered rat hearts.
Conclusion: Collectively, our results provide an insight into garlic's protection to T1DM heart through activation of SIRT3-MnSOD pathway.

1. Background

Diabetes is a major menace and leading cause of mortality all over the world. One of the many complications of diabetes that are inevitable is cardiovascular complications, which accounts for 80% mortality in diabetic patients [1]. One of the well-known cardiac complications in diabetes is diabetic cardiomyopathy (DCM). DCM is associated with multiple structural and functional abnormalities like, cardiomyocyte hypertrophy, cardiac fibrosis, mitochondrial dysfunction, interstitial accumulation of glycoprotein, systolic and diastolic dysfunction [2]. Moreover, mitochondrial dysfunctioning in diabetic cardiovascular complications remains a crucial contributor and matter of interest [3].

Oxidative stress also contributes equally in the pathogenesis of diabetes and associated complications, which is in part govern by mitochondria [4]. The activity of many mitochondrial proteins is highly influenced by their acetylation status [5]. Sirtuins are a group of histone deacetylases, which deacetylate histone and non-histone proteins. SIRT-3, SIRT-4 and SIRT-5 are mitochondrial sirtuins. Among them, SIRT3 possess robust deacetylating activity and controls the acetylation status of almost 80–90% of mitochondrial proteins [6]. SIRT-3 is an antiaging gene, which is demonstrated to be involved in multiple signaling events including regulation of oxidative stress, metabolic activity, mitochondrial biogenesis, cardiac hypertrophy, and apoptosis [7–9]. Decreased SIRT-3 activity in diabetes may lead to cardiac complications via hyperacetylation of mitochondrial proteins [10]. In addition, SIRT-3 is responsible in regulating whole body energy homeostasis by controlling the metabolic pathways in fuel producing and fuel utilizing organs [11]. In case of diabetic cardiac complications, where the pathology of dysfunctioning is different, the role of SIRT-3 remains to be explored. Progression of cardiac complications in T1DM is faster than in T2DM. Early changes in the heart of T1DM need to be studied to prevent or delay the chronic complications. Based on this fact, in the present

* Corresponding author.
E-mail addresses: mdsultana@gmail.com (M.R. Sultana), pankajbagul2787@gmail.com (P.K. Bagul), pbkatare@gmail.com (P.B. Katare), sohebmsa@gmail.com (S. Anwar Mohammed), rajpadiya@gmail.com (R. Padiya), skbanerjee@thsti.res.in, banerjees74@hotmail.com (S.K. Banerjee).
[1] Authors contributed equally.

http://dx.doi.org/10.1016/j.lfs.2016.08.030
0024-3205/

study we thought to evaluate the early cardiac changes in the heart of diabetic rats.

Garlic, *Allium sativum*, is a widely studied herbal plant for its medicinal use [12]. Several beneficial effects of garlic are evaluated against cardiovascular disease. Previously attenuation of cardiac oxidative stress after garlic administration has also been reported by different investigators [13,14]. Garlic and its metabolites are demonstrated to have multiple beneficial effects not only in disease condition but also in normal physiology [14]. Several studies showed that garlic and its metabolites have promising effect in controlling mitochondrial health [13]. However, the role of garlic on SIRT-3 modulation has not been explored. In the present study, we hypothesized that mitochondrial dysfunction in diabetic heart could be regulated by oral administration of garlic through modulation of SIRT-3 activity. In addition, the effect of garlic and garlic metabolites in modulating SIRT-3 activity is evaluated in in-vitro system.

2. Materials and methods

2.1. Animals study

All animal experimental protocols were approved by the ethical review committee, Institutional Animal Ethical Committee (IAEC) of Indian Institute of Chemical technology (IICT)- Hyderabad and were carried out in accordance with regulations of IAEC and IICT guidelines on the care and welfare of laboratory animals. All animals were treated humanely and with regard for alleviation of suffering. Male Sprague–Dawley rats weighing 200–250 g were purchased from National Institute of Nutrition (NIN), Hyderabad, India. Animals were housed in BIOSAFE, an animal quarantine facility of Indian Institute of Chemical Technology (IICT), Hyderabad, India. Animals had a free access to water and diet. Animals described as fasted were deprived from diet for 12 h.

2.2. Induction of experimental diabetic rat model and treatment schedule

After fasting, rats were administered with a single intraperitoneal injection of streptozotocin (STZ) at a dose of 50 mg/kg, dissolved in 0.1 M ice cold sodium citrate buffer, pH- 4.5. Control rats received same volume of citrate buffer. Animals were then monitored for next seven days for their blood glucose levels. Animals with induced hyperglycemia were then divided in two groups. One group received no treatment except saline orally (Dia group), the second group received 250 mg/kg/day of raw garlic orally, for four weeks (Dia + Garl) (N = 12). Another control group of rats were administered with raw garlic at a dose of 250 mg/kg. Heart samples from these rats were used to evaluate the basal level changes of few parameters. However, the effect of raw garlic administration to control rats was previously well studied and published by our group [14,15]. Dose of garlic was chosen based on our previous study [16]. The garlic homogenate was prepared using fresh garlic by homogenizing in water followed by filtering it and collecting the filtrate. After four weeks of study, animals were sacrificed, heart tissues were collected after dissection of animal, washed in ice cold PBS, blotted dry and put in liquid nitrogen followed by storing at −80 °C for downstream analysis.

2.3. Measurement of serum biochemical parameters, insulin and glycosylated hemoglobin

Blood glucose levels were measured using OneTouch Horizon glucometer as described before [16]. For the measurement of serum insulin level, a commercially available rat Insulin ELISA kit (Mercodia) was used [16]. Serum glycosylated hemoglobin was measured using kit from Biosystem [16,17]. For the measurement of serum triglycerides, uric acid and cholesterol, we used kits purchased from Simens and measured by Autoblood analyzer [17,18].

2.4. Preparation of heart tissue homogenate

Rat heart tissue homogenate was prepared by homogenizing tissue with ten-time volume of 0.05 M phosphate buffer (pH-7.4) and then centrifuging at 15,000 rpm for 30 min at 4 °C. The resulting supernatant was stored at −80 °C for downstream analysis.

2.5. In-vitro study

Rat cardiomyoblast cells, H9C2, were purchased from ATCC (Manassas, VA), and cultured in Dulbecco's modified Eagle's medium (DMEM). The cultures were supplemented with 10% fetal bovine serum and 100 μg/ml penicillin/streptomycin. When cell populations reached 50–60% confluence, cells were transfected with SIRT-3 cDNA plasmid using X-tremegene-HP DNA transfection reagent, Roche, as per manufacturer's protocol. Cells were maintained for a period of 24 and 48 h after transfection. RNA isolation was carried out after transfection and used for downstream applications. siRNA treatment of cells was carried out using Dhamafect, Dharmacon, USA. Rat SIRT-3 siRNA was ordered from Dharmacon, USA and used at a concentration of 50 nM. Cells were transfected and maintained for 48 h. Cell lysate was prepared and used for downstream application. For SIRT-3 activity analysis, and other experiments, H9C2 cells were treated with freshly prepared raw garlic homogenate and garlic metabolites, allyl methyl sulfide (AMS), allyl methyl sulfoxide (AMSO) and diallyl disulfide (DADS) at a dose used commonly i.e. 100 μM [19,20]. DADS and AMS were purchased from Sigma, USA while AMSO was synthesized in laboratory for the present study (Supplementary file).

2.6. Catalase activity and GSH measurement

The activity of catalase was determined by method described before [14]. The decomposition of hydrogen peroxide (H_2O_2) by catalase is monitored spectrophotometrically at 240 nm.

Myocardial glutathione (GSH) (including total -SH group) content in homogenate was measured by biochemical assay using dithionitrobenzoic acid (DTNB) method as described by Ellman and used by our group before [1,2,21]. Reduced glutathione was taken as reference standard for preparation of Standard graph. To the 2 ml of 0.1 M potassium phosphate buffer (pH 8.4), 0.1 ml of standard or experimental sample (deproteinized with 10%TCA), 0.5 ml of DTNB were added and the volume was made upto 3 ml with double distilled water. Then the mixture was incubated for 10 min at room temperature and absorbance was measured at 412 nm. The GSH content was calculated from standard graph.

2.7. Reactive oxygen species (ROS)

Total Reactive oxygen species in the sample were estimated by using fluorogenic dye 2′,7′-dichlorofluorescein diacetate (DCFDA). The reaction involves the conversion of DCFDA into 2′,7′-dichlorofluorescein (DCF) upon oxidation by ROS. The reaction was initiated by incubating the sample with 100 μM of DCFDA and resultant fluorescence was detected by fluorescence plate reader with a maximum excitation and emission spectra of 488 and 525 nm respectively.

2.8. Gene expression profiling

RNA isolation was carried out from heart tissues of all groups (n = 4) using Trizol reagent. Quantification and quality assessment of RNA was carried out using Nano Drop Spectrophotometer (Thermo Scientific) and running on 1% agarose gel prepared in DEPC treated TBE buffer respectively. The extracted RNA was stored in −80 °C for further use. DNase treatment was carried out to the isolated RNA. cDNA was synthesized using superscript- III reverse transcriptase (Takara, USA). Polymerase chain reaction (PCR) was carried out using VERITI-96well

Thermo cycler (Applied Biosystems Inc., USA) and Emerald GT PCR Master mix (Takara, USA). The data was normalized to expression of reference gene *Ribosomal protein L32* (RPL32) [22,23]. The PCR image density was quantified using Image J software.

2.9. Isolation of mitochondria

Mitochondria were isolated from equal weight of heart tissues using mitochondria isolation kit (Pierce, Thermo scientific, Cat No: 89801). Briefly, heart tissue was cut into small pieces and homogenized using dounce homogenizer and the homogenate was then treated according to protocol provided by manufacturer. The resultant mitochondrial pellet was suspended in MTP buffer containing 110 mM mannitol, 60 mM Tris HCL, 60 mM potassium chloride, 10 mM dibasic potassium phosphate and 0.5 mM EDTA, pH-7.4.

2.10. Mitochondrial respiratory chain complex activity in the diabetic heart

The specific enzymatic activity of mitochondrial electron transport chain (ETC) complex I (NADH-ubiquinone oxidoreductase), complex II (succinate-ubiquinone oxidoreductase), and complex IV (cytochrome *c* oxidase) were measured in the mitochondria isolated from diabetic heart of rats as previously described [24]. Citrate synthase activity was measured according to protocol described before [25].

2.11. SIRT-3 activity assay

SIRT-3 activity was analyzed using fluorometric based assay kit from Cyclex, Japan. The kit is based on generation of fluorescence signal from the deacetylase activity of SIRT-3, which can be monitored at one min interval. SIRT-3 activity in samples was represented as % activity. For measuring SIRT-3 activity in-vitro with garlic homogenate and its metabolites, recombinant SIRT-3 provided with the kit was used and the experiment was carried out as per manufacturer's protocol. Nicotinamide was used as an inhibitor of SIRT-3 to check the assay specificity.

2.12. Immunoblotting

Protein extraction was carried out using Tissue Protein Extraction Reagent (T-PER). After centrifugation at 12,000 rpm for 10 min, supernatant was collected and quantified by Bradford method (Sigma). Protein was resolved in 10–12% SDS-polyacrylamide gel. After electrophoresis, the protein was transferred to polyvinylidine difluoride (PVDF) membrane (GE Healthcare). Blocking of the membrane was performed using 4% western milk in TBS + 0.1% Tween 20 (TBST) at room temperature for 1 h. followed by appropriate primary antibody treatment overnight at 4 °C. The membrane was washed with TBST. After washing, the membrane was incubated with corresponding horseradish peroxidase-labeled secondary antibody at room temperature for 1 h. Membranes were washed with TBST and were visualized using Supersignal west dura chemiluminescent substrate (Thermo Scientific, U.S.A.). SIRT 3 antibody (Cell signaling, dilution 1:1000), Anti-acetylated lysine antibody (Abcam, USA, dilution 1:1000), MnSOD antibody (Abcam, dilution 1:2000), Anti-Rabbit antibody (Cell Signaling, dilution 1:5000), Cocktail of OXOPHOS antibodies (Abcam, dilution 1:400), PGC1-α (Abcam, dilution 1:500) were used for this study.

2.13. Immunoprecipitation

Immunoprecipitation was carried out using Dynabeads Protein G Immunoprecipitation kit (Life Technologies) according to manufacturer's instructions.

2.14. Statistical analysis

All values are expressed as the mean ± standard error. One-way analysis of variance test followed by Bonferroni's correction was first carried out to test for any differences between the mean values of all groups. Significance in group differences was assumed if $p < 0.05$.

3. Results

3.1. Garlic administration attenuated blood glucose and other serum metabolites level

We have observed significant ($p < 0.05$) increase in serum blood glucose and glycated hemoglobin levels in Dia group rats compared to Con group rats. In addition, there was reduced levels of serum insulin in Dia group rats compared to Con. Administration of raw garlic homogenate ameliorated all these changes (Table 1). Moreover, there was significant ($p < 0.05$) increase in the levels of serum triglyceride, uric acid and reduced levels of serum cholesterol in Dia group rats. However, all these parameters were improved after raw garlic administration (Table 1).

3.2. Effect of garlic on myocardial expression of collagen and β-MHC mRNA

mRNA expression of myocardial β-MHC and collagen mRNA were increased in Dia group rats compared to Con group ($p < 0.01$). Garlic administration decreased the mRNA expression levels of myocardial collagen ($p < 0.05$). However, the levels of β-MHC were not significantly decreased by garlic (Fig. 1).

3.3. Garlic administration reduced ROS levels and increased SOD, GSH and catalase activity in diabetic heart

Administration of raw garlic homogenate to diabetic rats significantly ($p < 0.05$) reduced the increased ROS levels that were observed in Dia group rat heart (Fig. 2A). Decreased myocardial activity of catalase and SOD in diabetic rat heart was normalized with raw garlic administration (Fig. 2B and C). In addition, the reduced glutathione level in Dia group was normalized with garlic administration (Fig. 2D).

3.4. Raw garlic improves mitochondrial citrate synthase and enzyme activities of electron transport chain (ETC) complex assembly

Activity of citrate synthase was reduced in diabetic rat heart ($p < 0.05$). However, administration of raw garlic improved the citrate synthase activity significantly ($p < 0.05$) (Fig. 3A). Activity of mitochondrial Complex I, i.e. NADH dehydrogenase enzyme was decreased ($p < 0.01$) in Dia group compared with Con group. Garlic has significantly ($p < 0.05$) increased the complex I activity compared to Dia group (Fig. 3B). Significant ($p < 0.05$) reduction in the activity was observed for Complex-II i.e. succinate dehydrogenase, which was normalized with raw garlic administration ($p < 0.05$) (Fig. 3C). Myocardial cytochrome *c* oxidase (Complex-IV) activity found to be significantly ($p < 0.05$) increased in Dia group compared to Con group. Garlic has significantly ($p < 0.05$) decreased cytochrome *c* oxidase levels (Fig. 3D).

Table 1
Serum parameters of animals in all groups.

Parameter	Control	Diabetic	Diabetic + garlic
Blood glucose (mg/dl)	99.2 ± 1.4	355.2 ± 64.6*	288.6 ± 36.7#
Glycated hemoglobin (%)	5.72 ± 0.32	8.96 ± 0.35*	6.27 ± 0.27#
Serum insulin (pmol/l)	50.6 ± 8.1	31 ± 2.6*	44.3 ± 5.2#
Serum triglycerides (mg/dl)	99.5 ± 7.9	154.2 ± 17.1*	68.6 ± 8.5#
Serum cholesterol (mg/dl)	62 ± 2.28	59.83 ± 5.46*	83.71 ± 2.71#
Serum uric acid (mg/dl)	1.3 ± 0.035	1.7 ± 0.049*	1.39 ± 0.085#

The values are means ± SEM. *, #, $p < 0.05$, * vs. Con, # vs. Dia, n = 8/group.

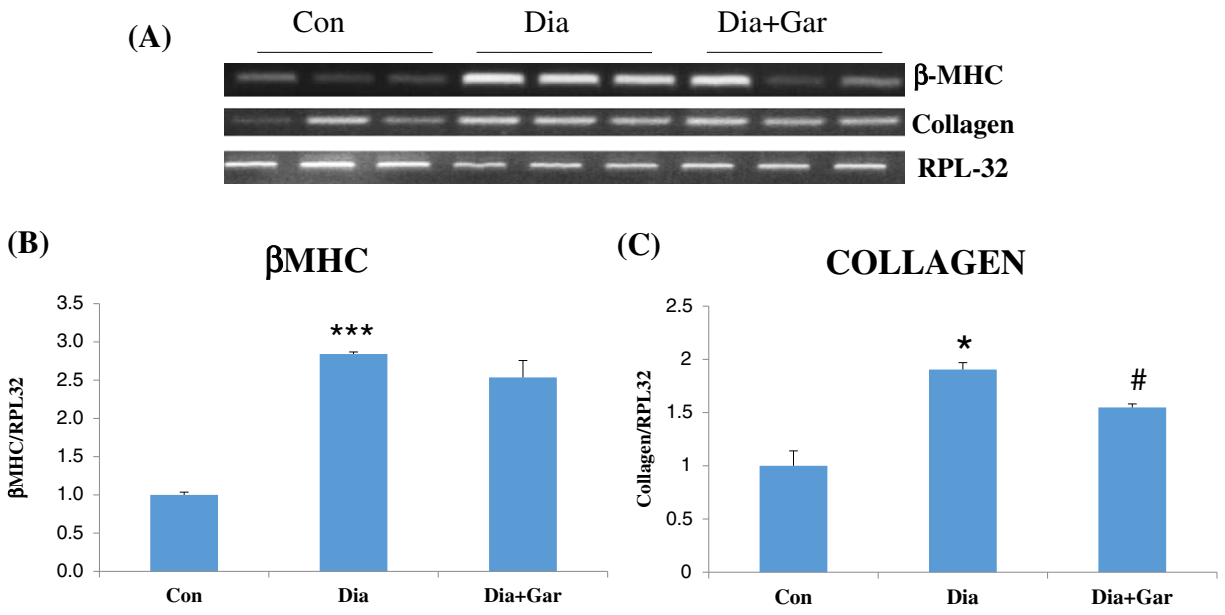

Fig. 1. Myocardial mRNA expression of β-MHC and collagen. Effect of garlic administration on β-MHC mRNA expression (A, B). Collagen mRNA expression (A, C). The values are means ± SEM. ***p < 0.001, *, #, p < 0.05, * vs. Con, # vs. Dia, n = 4/group.

3.5. Garlic normalized the expression of genes responsible for mitochondrial biogenesis

Gene expression analysis of mitochondrial transcription factor (TFAM) was reduced in Dia group. Administration of raw garlic increased the expression of TFAM (Fig. 4A,C). mRNA expression of PGC1-α, a coactivator of mitochondrial biogenesis, was increased in diabetic heart. However, increased PGC1-α in heart was reduced with raw garlic administration (Fig. 4A,D). Similarly, mRNA expression of MT-CO1 was analyzed in the heart of diabetic rats. There was no change in the mRNA expression of MT-CO1 in diabetic heart. However, administration of raw garlic increased MT-CO1 expression (Fig. 4A,B).

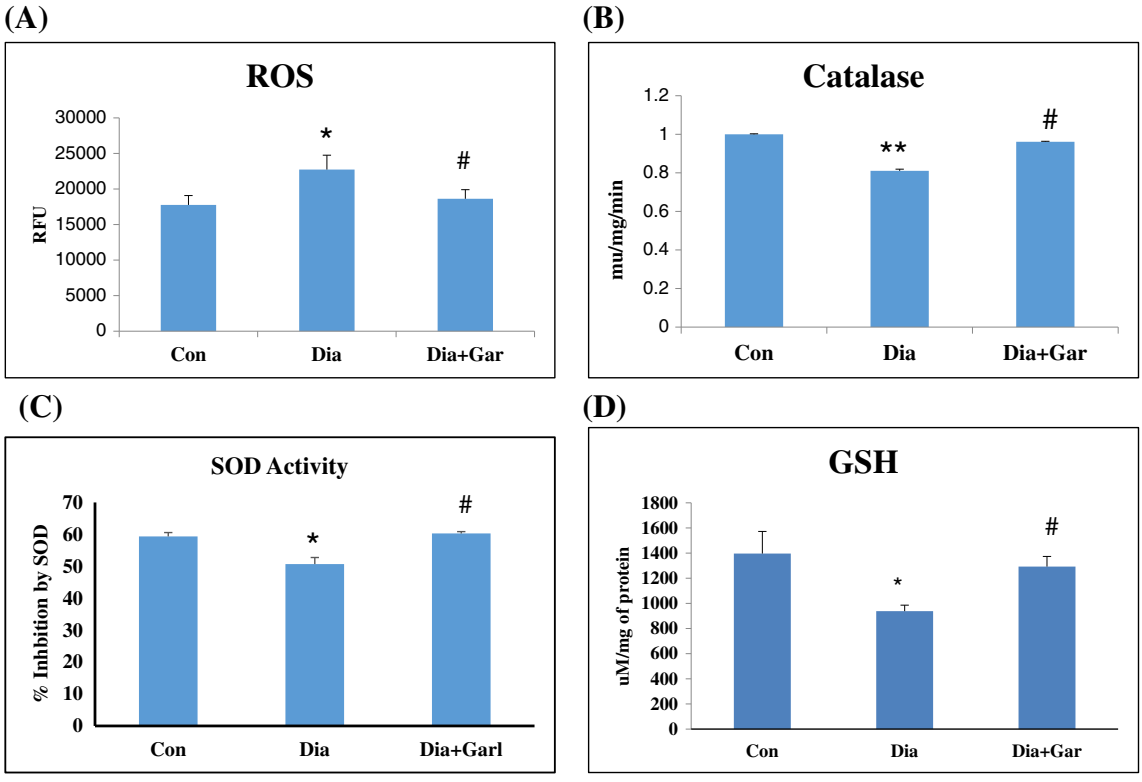

Fig. 2. Myocardial ROS generation, GSH, SOD and catalase activity. Effect of garlic administration on myocardial (A) ROS generation. (B) Catalase activity, (C) total SOD activity, (D) GSH levels. The values are means ± SEM. *, **p < 0.05, 0.01 vs. control group. #, p < 0.05 vs. diabetic group, n = 6/group.

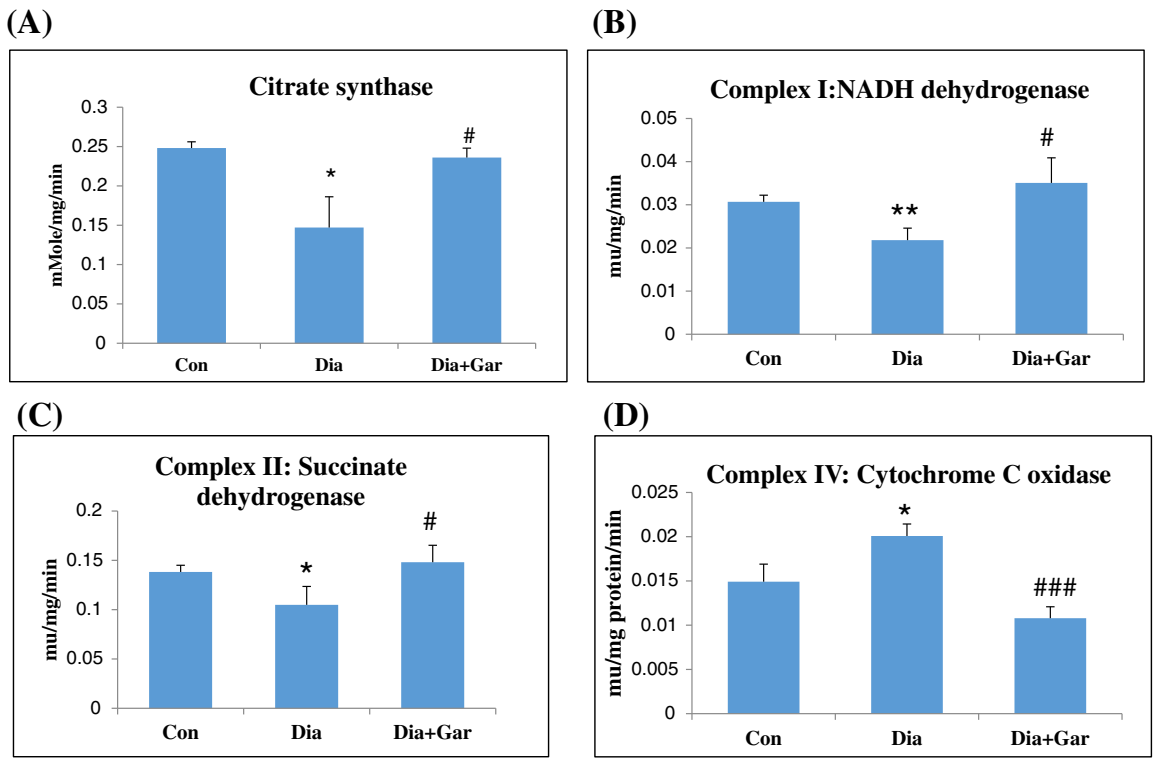

Fig. 3. Mitochondrial enzyme activity. (A) Citrate synthase activity. (B) Complex-I: NADH dehydrogenase activity. (C) Complex-II: succinate dehydrogenase activity. (D) Complex-IV: cytochrome *c* oxidase activity. The values are means ± SEM. *, $p < 0.05$ vs. control group. #, $p < 0.05$ vs. diabetic group, n = 6/group.

3.6. Garlic elevated myocardial SIRT3 and MnSOD expression

We have analyzed the effect of garlic treatment on SIRT3 mRNA and protein expression. As shown in Fig. 5, mRNA expression of SIRT 3 was significantly decreased in diabetic heart, which was significantly increased after garlic administration. Interestingly, there was no change in SIRT-3 protein expression in diabetic heart but was significantly increased after garlic administration. We further analyzed SIRT-3 activity and found reduced activity of SIRT-3 in diabetic rat heart and increased with administration of raw garlic (Fig. 5). To look the effect of SIRT-3 on mitochondrial superoxide dismutase (Mn-SOD), we measured MnSOD gene and protein expression in rat heart in all groups. We found that there was a decrease in myocardial MnSOD gene and protein expression in diabetic heart compared to Con group. However, administration of raw garlic increased both gene and protein expression of MnSOD in diabetic heart (Fig. 5).

3.7. SIRT-3 deacetylates Mn-SOD to increase its activity

To find out the effect of SIRT-3 on MnSOD activity, we transfected H9C2 cells with SIRT-3. SIRT-3 transfection in H9C2 cells for 24 and 48 h showed no change in mRNA expression of Mn-SOD at 24 h but a slight increase at 48 h (Fig. 6A). In-vivo data showed that the acetylation status of Mn-SOD was increased in diabetic rat heart, which was reduced after raw garlic administration (Fig. 6B). We found increased acetylation status of MnSOD in SIRT-3 silenced H9C2 cells (Fig. 6C), indicating the specificity of SIRT-3 in regulating MnSOD acetylation rather than expression.

3.8. Garlic and its metabolites activates SIRT-3 in-vitro

We have analyzed the activity of SIRT-3 in H9C2 cell lysate treated with garlic homogenate and metabolites of garlic. We found increased SIRT-3 activity in garlic treated and AMS, AMSO, DADS treated cells (Fig. 7A). In addition, in-vitro analysis of recombinant SIRT-3 activation by garlic homogenate and its metabolites also revealed similar outcomes (Fig. 7B).

Our data indicate that reduced activity of mitochondrial electron transport chain in diabetic heart increases the ROS production. In addition, activity of MnSOD is reduced due to hyperacetylation of the same protein in diabetic heart. As SIRT-3 activity was reduced in diabetic heart, SIRT3 is unable to deacetylate and activate MnSOD. Therefore, the increased levels of ROS affect the mitochondrial health and function leading to reduced expression of mitochondrial health markers. Administration of raw garlic homogenate activates SIRT-3 and prevented the mitochondrial dysfunctioning in diabetic heart by activating MnSOD.

3.9. Garlic does not alter SIRT-3 and ETC protein expression in control rats

We have not observed any change in the protein expression of SIRT-3 as well as mitochondrial ETC complexes except Complex-I and IV, which are increased with raw garlic administration to control rats. However, the reduced expression of SIRT-3 as well as ETC complexes in Dia group rat heart are normalized with raw garlic administration in Dia + Gar group (Fig. 8).

4. Discussion

Despite understanding the molecular aspects of disease regulation, diabetes has been a major problem in the world. The complications are indeed a matter of concern for all diabetic population. These complications consist of microvascular and macrovascular disease, which accounts for the major mortality rate [26]. Genetic predisposition explains only to an extent of etiology of diabetes [27]. Recent studies are exploring the role of epigenetic factors in pathophysiology of diabetes [28].

Garlic was proven to possess antioxidant [16] and cardio-protective effects [13]. However, its role in diabetic heart is yet to be explored. Garlic is widely used in its common raw vegetable form. Therefore, in the

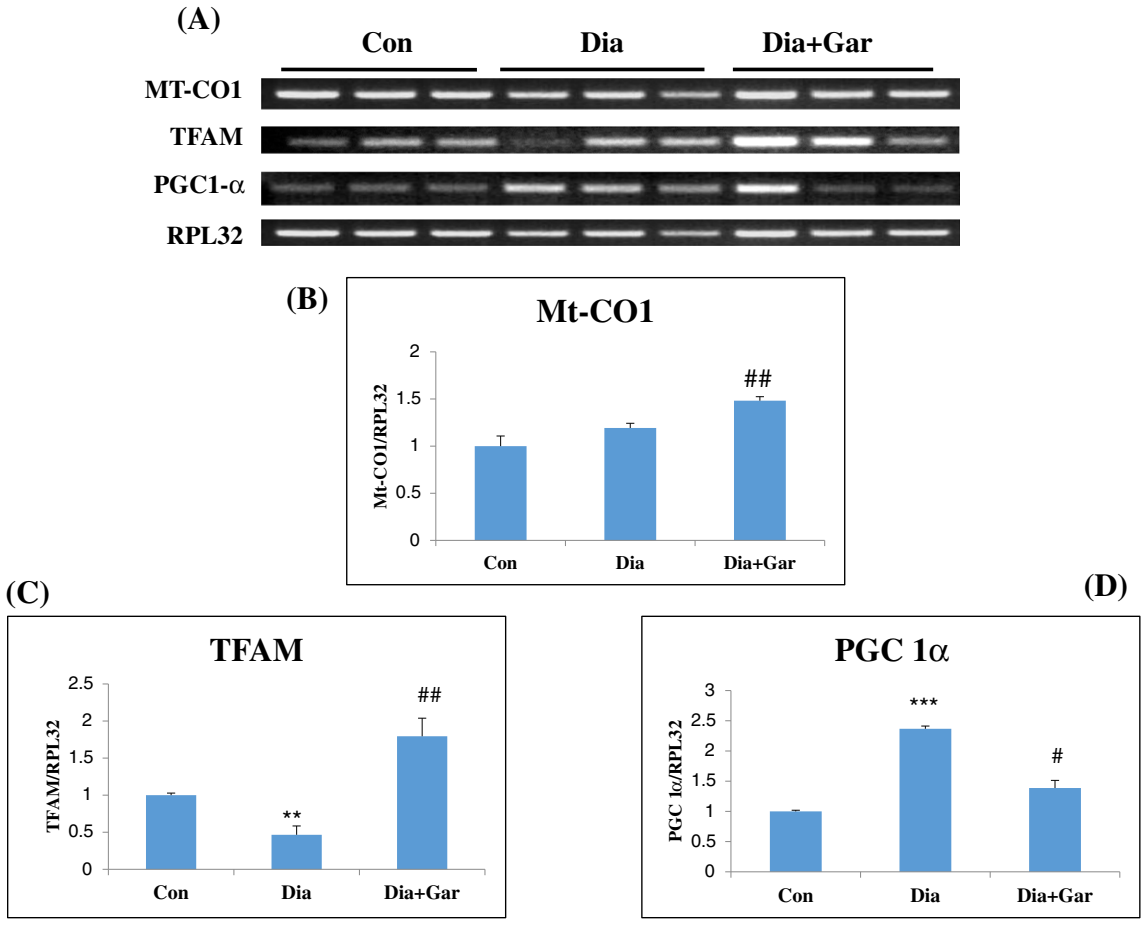

Fig. 4. Myocardial mRNA expression of MT-CO1, TFAM and PGC1-α. Effect of garlic administration on (A) MT-CO1 expression. (B) TFAM expression. (C) PGC1-α expression. The values are means ± SEM. *, $p < 0.05$, **, $p < 0.01$. * vs. Con, # vs. Dia, n = 4/group.

present study, we chose raw garlic to study its effect on diabetic heart of STZ induced diabetic rats. Several studies showed that metabolic disturbances in diabetic patients are associated with cardiac complications [29]. Similar to other studies, we observed increased blood glucose levels, glycated hemoglobin, uric acid, triglyceride, and decreased serum insulin levels in STZ treated rats. Administration of garlic attenuated the metabolic alterations. β-MHC and collagen, two important markers were measured to find the initiation of cardiac complications. mRNA expression of both genes were increased in diabetic rats. Garlic reduced the expression of collagen significantly but failed to improve β-MHC gene expression.

In the present study we have analyzed cardiac mRNA expression of three important mitochondrial genes i.e., PGC1α, TFAM and Mt.-CO1. These genes regulate the mitochondrial biogenesis and function in disease conditions [30,31]. Our data revealed that, although the mRNA expression of PGC1-α is increased, the expression of TFAM was reduced in diabetic heart with no change in MTCO-1 expression. Data indicates that although the nuclear trigger for mitochondrial biogenesis has been initiated, problem persists at the mitochondrial level. Administration of raw garlic improved the expression of these genes.

To further confirm the role of garlic on mitochondrial function and efficiency in diabetic heart, we measured the activity of mitochondrial enzymes. Activities of myocardial citrate synthase, NADH dehydrogenase and succinate dehydrogenase were significantly decreased in diabetic group and significantly increased after garlic administration. Interestingly, myocardial cytochrome *c* oxidase activity was significantly increased in diabetic group, which was decreased after raw garlic administration. These altered activity of mitochondrial enzymes, specifically those of electron transport chain (ETC) gives a strong indication for the altered redox status of diabetic heart.

To analyze the redox status of diabetic and garlic treated diabetic heart, we measured the levels of ROS and endogenous antioxidants activity. We found increased levels of ROS with decreased activity of SOD and catalase in diabetic heart. Administration of garlic prevented the development of oxidative stress by modulating all these parameters. The above data demonstrate that, STZ induces hyperglycemia which leads to mitochondrial dysfunction that generates excessive ROS with diminished antioxidant defense system in heart.

To find out the mechanism behind increased oxidative stress in diabetic heart and improvement after garlic administration, we have analyzed mRNA expression and activity of SIRT-3 in diabetic rat heart. As SIRT3 is the major protein deacetylase in mitochondria [32], we focused to find out whether SIRT3 regulates oxidative stress in diabetic heart through its deacetylating action. In the present study, decreased expression and activity of SIRT-3 was observed in the diabetic heart. This decrease in mRNA expression and activity of SIRT-3 was normalized after garlic administration. We then checked the mRNA and protein expression of MnSOD, a major antioxidant in mitochondria. A reduced level of MnSOD in diabetic heart was normalized after raw garlic administration.

It was previously reported by one group that increased expression of MnSOD after garlic treatment is due to activation of Akt-NRF-2 pathway [33]. Thus, it could be possible that SIRT-3 might have some additional benefit by modulating MnSOD activity. We then demonstrated whether the action of SIRT-3 on SOD activity is through transcription level or because of post-translational modifications. To demonstrate the same, we have overexpressed SIRT-3 in rat cardiomyoblast cells (H9C2). We

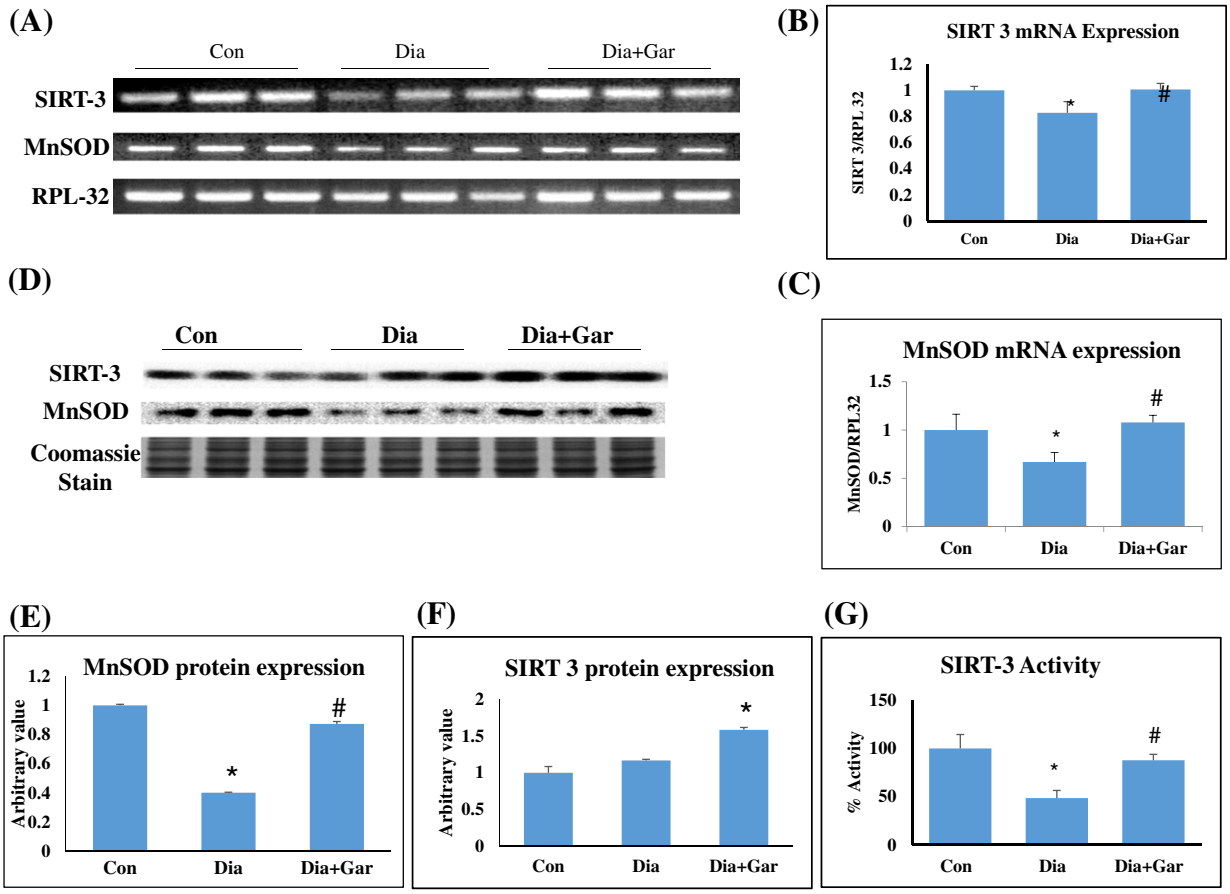

Fig. 5. Myocardial expression of SIRT 3 and MnSOD. Effect of garlic on SIRT-3 gene expression (A, B) MnSOD mRNA expression (A, C) SIRT-3 and MnSOD protein expression (D, E, F) and SIRT-3 activity (G). The values are means ± SEM. *, #, $p < 0.05$, * vs. Con, # vs. Dia, n = 4/group.

found that increased expression of SIRT-3 was not associated with increased transcription. However, prolong overexpression leads to slight increase in Mn-SOD mRNA expression. In addition, silencing SIRT-3 in cells leads to increased acetylation status of MnSOD, which indicates that SIRT-3 is crucial in regulating the acetylation of MnSOD. To confirm the effects of SIRT-3, we have analyzed the acetylation status of Mn-SOD

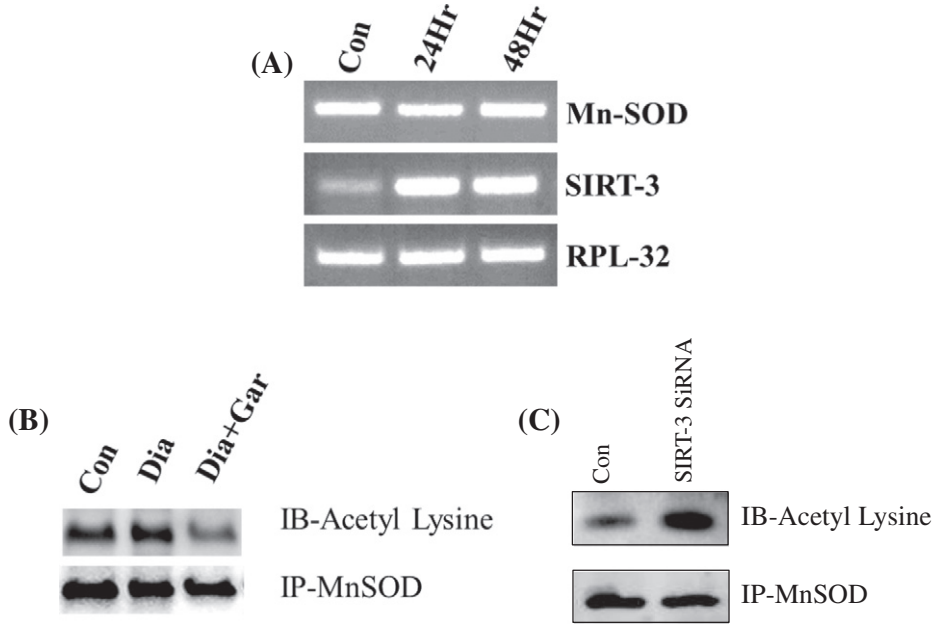

Fig. 6. Effect of SIRT-3 on MnSOD expression and acetylation. A) Effect of SIRT-3 overexpression on MnSOD mRNA expression after 24 and 48 h. in H9C2 cells. B) Acetylation status of MnSOD measured after MnSOD immunoprecipitation.

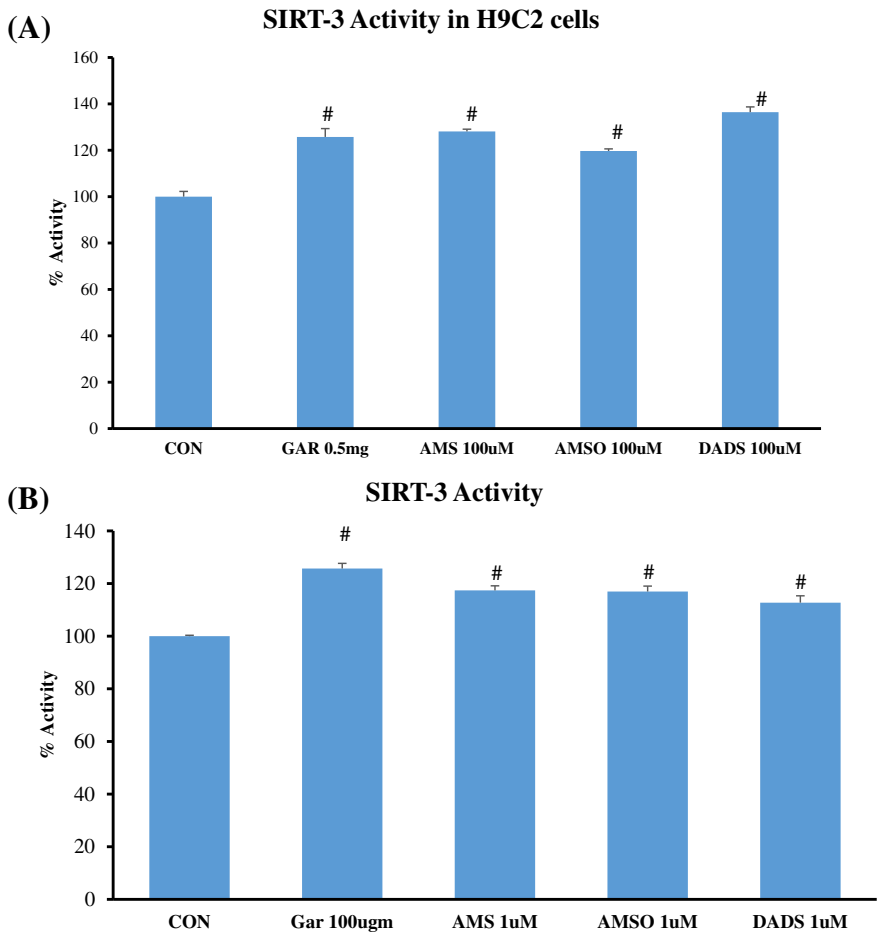

Fig. 7. SIRT-3 activity analysis by garlic homogenate and garlic metabolites. A) SIRT-3 activity in H9C2 cell after treatment with garlic homogenate and garlic metabolites. B) The effect of garlic homogenate and garlic metabolites on SIRT-3 enzyme activity in-situ. The values are means ± SEM. #, $p < 0.05$ vs. Con.

in diabetic heart. We found increased acetylation of Mn-SOD in diabetic heart. This correlates with decreased myocardial SOD activity as observed in diabetic group. However, this increased acetylation of Mn-SOD was reduced with garlic administration. We then looked whether it is a direct or indirect activating action of garlic on SIRT-3. We first demonstrated SIRT-3 activity in H9C2 cells treated with raw garlic homogenate and garlic metabolites, and found increased activity of SIRT-3. Increased SIRT3 activity in cells may also be possible due to increase in intracellular NAD/NADH ratio. Any natural compounds and antioxidants may also have the similar effect, which indirectly affect SIRT3 activity. To confirm whether this enhanced SIRT3 activity is due to direct interaction of SIRT3 with garlic metabolites, we did enzymatic activity of SIRT-3 in-vitro in presence of garlic homogenate and its metabolites. Similar to H9C2 cell treatment, we have also observed increased enzymatic activity of SIRT-3 by these compounds. Our data indicate that garlic has potential to activate SIRT-3 and regulating mitochondrial function.

5. Conclusion

The present study shows that garlic protected diabetic heart from oxidative stress by enhancing SIRT3 activity and attenuating mitochondria dysfunction.

Abbreviations

T1DM	type I diabetes mellitus
T2DM	type II diabetes mellitus
SIRT	silent information regulator type 2
DCM	diabetic cardiomyopathy
ROS	reactive oxygen species
MnSOD	manganese/mitochondrial superoxide dismutase
Dia	diabetic
Dia + Garl	diabetic + garlic
TFAM	mitochondrial transcription factor
PGC1-α	peroxisome proliferator activator receptor co-activator alpha
CO-1	cytochrome *c* oxidase

Conflicts of interests

The authors declare that they have no competing interests.

Authors' contribution

PKB, RS, RP and PK carried out animal experimentation, biochemical and molecular estimation and statistical analysis of results. SKB and PKB conceived the study, and participated in its design, coordination and drafted the manuscript. The authors read and approved the manuscript.

Acknowledgments

Financial assistance was provided by grant support (SB/SO/AS18/2011) from Department of Science and Technology (DST) and (BSC0103 UNDO) from Council of Scientific and Industrial Research (CSIR) network project. SKB is thankful to DBT for providing Ramalingaswami Fellowship, PKB is thankful to CSIR and PBK, SAM are thankful to ICMR for providing Senior Research Fellowship (SRF).

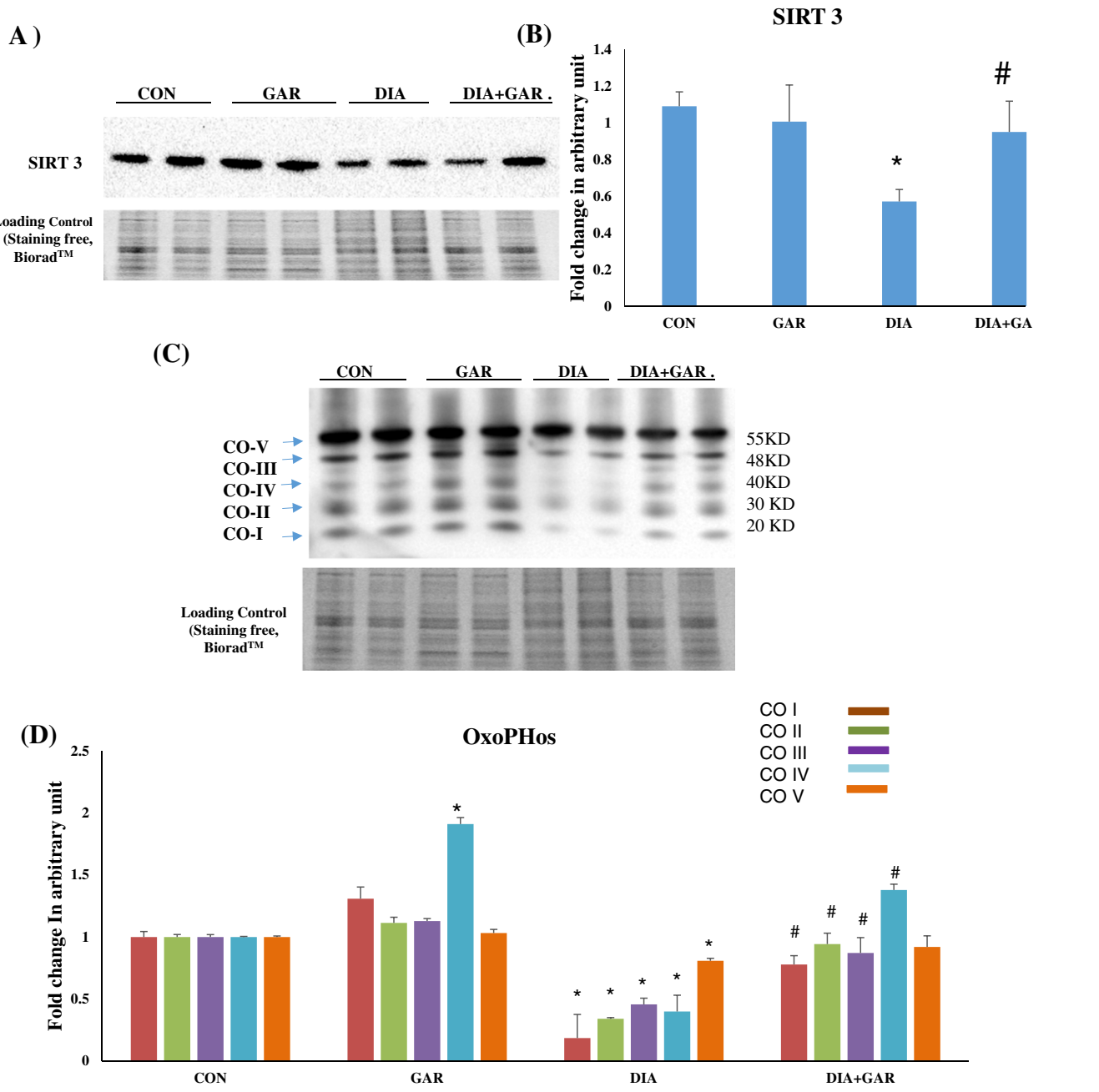

Fig. 8. Effect of garlic on SIRT-3 and mitochondrial electron transport chain complex expression. A) SIRT-3 immunoblots, B) expression changes of SIRT-3, C) ETC immunoblots, D) expression changes of ETC complexes. The values are means ± SEM. *, #, $p < 0.05$, * vs. Con, # vs. Dia, n = 4/group.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.lfs.2016.08.030.

References

[1] A. Nicolucci, G. De Berardis, M. Sacco, G. Tognoni, Primary prevention of cardiovascular diseases in people with diabetes mellitus: a scientific statement from the American Heart Association and the American Diabetes Association: response to Buse et al. Diabetes Care 30 (6) (2007) e57 author reply e8.
[2] Z.Y. Fang, J.B. Prins, T.H. Marwick, Diabetic cardiomyopathy: evidence, mechanisms, and therapeutic implications, Endocr. Rev. 25 (4) (2004) 543–567.
[3] T.A. Ajith, T.G. Jayakumar, Mitochondria-targeted agents: future perspectives of mitochondrial pharmaceutics in cardiovascular diseases, World J. Cardiol. 6 (10) (2014) 1091–1099.
[4] S. Tangvarasittichai, Oxidative stress, insulin resistance, dyslipidemia and type 2 diabetes mellitus, World J. Diabetes 6 (3) (2015) 456–480.
[5] K.A. Anderson, M.D. Hirschey, Mitochondrial protein acetylation regulates metabolism, Essays Biochem. 52 (2012) 23–35.
[6] P.K. Bagul, S.K. Banerjee, Insulin resistance, oxidative stress and cardiovascular complications: role of sirtuins, Curr. Pharm. Des. 19 (32) (2013) 5663–5677.
[7] M.D. Hirschey, T. Shimazu, J.Y. Huang, B. Schwer, E. Verdin, SIRT3 regulates mitochondrial protein acetylation and intermediary metabolism, Cold Spring Harb. Symp. Quant. Biol. 76 (2011) 267–277.
[8] S. Matsushima, J. Sadoshima, The role of sirtuins in cardiac disease, Am. J. Phys. Heart Circ. Phys. (2015) ajpheart 00053 2015.
[9] C. Koentges, K. Pfeil, T. Schnick, S. Wiese, R. Dahlbock, M.C. Cimolai, et al., SIRT3 deficiency impairs mitochondrial and contractile function in the heart, Basic Res. Cardiol. 110 (4) (2015) 36.
[10] M.D. Hirschey, T. Shimazu, E. Jing, C.A. Grueter, A.M. Collins, B. Aouizerat, et al., SIRT3 deficiency and mitochondrial protein hyperacetylation accelerate the development of the metabolic syndrome, Mol. Cell 44 (2) (2011) 177–190.

[11] K.E. Dittenhafer-Reed, A.L. Richards, J. Fan, M.J. Smallegan, A. Fotuhi Siahpirani, Z.A. Kemmerer, et al., SIRT3 mediates multi-tissue coupling for metabolic fuel switching, Cell Metab. 21 (4) (2015) 637–646.
[12] L. Bayan, P.H. Koulivand, A. Gorji, Garlic: a review of potential therapeutic effects, Avicenna J. Phytomed. 4 (1) (2014) 1–14.
[13] T.N. Khatua, R. Adela, S.K. Banerjee, Garlic and cardioprotection: insights into the molecular mechanisms, Can. J. Physiol. Pharmacol. 91 (6) (2013) 448–458.
[14] S.K. Banerjee, A.K. Dinda, S.C. Manchanda, S.K. Maulik, Chronic garlic administration protects rat heart against oxidative stress induced by ischemic reperfusion injury, BMC Pharmacol. 2 (2002) 16.
[15] S.K. Banerjee, M. Maulik, S.C. Mancahanda, A.K. Dinda, S.K. Gupta, S.K. Maulik, Dose-dependent induction of endogenous antioxidants in rat heart by chronic administration of garlic, Life Sci. 70 (13) (2002) 1509–1518.
[16] R. Padiya, T.N. Khatua, P.K. Bagul, M. Kuncha, S.K. Banerjee, Garlic improves insulin sensitivity and associated metabolic syndromes in fructose fed rats, Nutr. Metab. 8 (2011) 53.
[17] P.K. Bagul, H. Middela, S. Matapally, R. Padiya, T. Bastia, K. Madhusudana, et al., Attenuation of insulin resistance, metabolic syndrome and hepatic oxidative stress by resveratrol in fructose-fed rats, Pharmacol. Res. 66 (3) (2012) 260–268.
[18] P.K. Bagul, A.K. Dinda, S.K. Banerjee, Effect of resveratrol on sirtuins expression and cardiac complications in diabetes, Biochem. Biophys. Res. Commun. 468 (1–2) (2015) 221–227.
[19] G.A. Benavides, G.L. Squadrito, R.W. Mills, H.D. Patel, T.S. Isbell, R.P. Patel, et al., Hydrogen sulfide mediates the vasoactivity of garlic, Proc. Natl. Acad. Sci. U. S. A. 104 (46) (2007) 17977–17982.
[20] T.N. Khatua, A.K. Dinda, U.K. Putcha, S.K. Banerje, et al., Biochem. Biophys. Rep. 5 (2016) 77–88.
[21] G.L. Ellman, Tissue sulfhydryl groups, Arch. Biochem. Biophys. 82 (1) (1959) 70–77.
[22] D. Chowdhury, A.D. Tangutur, T.N. Khatua, P. Saxena, S.K. Banerjee, M.P. Bhadra, A proteomic view of isoproterenol induced cardiac hypertrophy: prohibitin identified as a potential biomarker in rats, J. Transl. Med. 11 (2013) 130.
[23] P.K. Bagul, N. Deepthi, R. Sultana, S.K. Banerjee, Resveratrol ameliorates cardiac oxidative stress in diabetes through deacetylation of NFkB-p65 and histone 3, J. Nutr. Biochem. (2015).
[24] I.A. Trounce, Y.L. Kim, A.S. Jun, D.C. Wallace, Assessment of mitochondrial oxidative phosphorylation in patient muscle biopsies, lymphoblasts, and transmitochondrial cell lines, Methods Enzymol. 264 (1996) 484–509.
[25] D. Shepherd, P.B. Garland, The kinetic properties of citrate synthase from rat liver mitochondria, Biochem. J. 114 (3) (1969) 597–610.
[26] A.M. Balkhi, A.M. Reid, S.C. Westen, B. Olsen, D.M. Janicke, G.R. Geffken, Telehealth interventions to reduce management complications in type 1 diabetes: a review, World J. Diabetes 6 (3) (2015) 371–379.
[27] E. Ahlqvist, N.R. van Zuydam, L.C. Groop, M.I. McCarthy, The genetics of diabetic complications, Nat. Rev. Nephrol. 11 (5) (2015) 277–287.
[28] L.M. Villeneuve, M.A. Reddy, R. Natarajan, Epigenetics: deciphering its role in diabetes and its chronic complications, Clin. Exp. Pharmacol. Physiol. 38 (7) (2011) 451–459.
[29] R. Fontes-Carvalho, R. Ladeiras-Lopes, P. Bettencourt, A. Leite-Moreira, A. Azevedo, Diastolic dysfunction in the diabetic continuum: association with insulin resistance, metabolic syndrome and type 2 diabetes, Cardiovasc. Diabetol. 14 (2015) 4.
[30] S. Baldelli, K. Aquilano, M.R. Ciriolo, PGC-1alpha buffers ROS-mediated removal of mitochondria during myogenesis, Cell Death Dis. 5 (2014), e1515.
[31] M. Pejznochova, M. Tesarova, H. Hansikova, M. Magner, T. Honzik, K. Vinsova, et al., Mitochondrial DNA content and expression of genes involved in mtDNA transcription, regulation and maintenance during human fetal development, Mitochondrion 10 (4) (2010) 321–329.
[32] M.F. Green, M.D. Hirschey, SIRT3 weighs heavily in the metabolic balance: a new role for SIRT3 in metabolic syndrome, J. Gerontol. A Biol. Sci. Med. Sci. 68 (2) (2013) 105–107.
[33] R. Padiya, D. Chowdhury, R. Borkar, R. Srinivas, M. Pal Bhadra, S.K. Banerjee, Garlic attenuates cardiac oxidative stress via activation of PI3K/AKT/Nrf2-Keap1 pathway in fructose-fed diabetic rat, PLoS One 9 (5) (2014), e94228.