

# Alleviation of cardiac mitochondrial dysfunction and oxidative stress underlies the protective effect of vitamin D in chronic stress-induced cardiac dysfunction in rats

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**Abstract.** Chronic stress is associated with oxidative stress and mitochondrial dysfunction. These mechanisms promote adverse cardiovascular events. Though many experimental studies have reported a protective effect of vitamin D (VitD) on cardiovascular system, its effect on cardiovascular system in case of chronic stress is not studied yet. The present study aimed to detect the effects of VitD treatment against chronic immobilization stress (CIS)-induced cardiac dysfunction, focusing mainly on mitochondrial function and oxidative stress in rats. CIS showed cardiac dysfunction as indicated by a significant decrease in the left ventricular end-diastolic and systolic diameters and decrease in ejection fraction and fractional shortening compared to the control group. This was accompanied by a significant decrease in tissue reserves of reduced glutathione (GSH), superoxide dismutase (SOD), ATP and cardiolipin as well as increase in malondialdehyde (MDA) and expression of peroxisome proliferator-activated receptor  $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ). All these effects were accompanied by a significant increase in plasma adrenaline and noradrenaline. Treatment with VitD ameliorated all the aforementioned CIS-induced effects except PGC-1 $\alpha$  expression in a dose-dependent manner. To our knowledge, this is the first study describing the prophylactic cardioprotective effects of VitD against CIS by targeting mitochondrial function.

**Key words:** Chronic stress — Vitamin D — Cardiac dysfunction — Mitochondrial dysfunction

**Abbreviations:** ATP, adenosine triphosphate; CIS, chronic immobilization stress; CL, cardiolipin; EF, ejection fraction; FS, fractional shortening; GSH, reduced glutathione; LVDD, left ventricular end-diastolic diameters; LVSD, left ventricular end-systolic diameters; MDA, malondialdehyde; PGC-1 $\alpha$ , peroxisome proliferator-activated receptor  $\gamma$  coactivator-1 $\alpha$ ; SOD, superoxide dismutase; VitD, vitamin D.

## Introduction

Stress is defined as the effects of psychosocial or environmental factors on physical or mental well-being. The effects of stress that have formed on people are examined on

animals by applying a variety of methods. Immobilization stress method causes both psychological and physiological stress by the restriction of movement, aggression, feeling of distress, and burnout. Consequently, the immobilization stress model is considered as an easy and convenient method (Nayanatara et al. 2012). It has been found that the cardiovascular system is the major target of stress injury (Ippoliti et al. 2013; Liu et al. 2015). Notably, the longstanding association between stress and cardiovascular disease exists despite the underlying mechanisms are not fully understood (Golbidi et al. 2015).

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Mitochondria are mostly responsible for meeting the huge energy demands of the 'fight and flight response' in vital tissues with the help of the stress hormones. Unfortunately, the heart is highly energy-dependent, with about 90% of the energy consumed being supplied by mitochondrial oxidative phosphorylation. Hence, proper functioning of myocardial mitochondria is crucial for cardiac function. Besides that, mitochondria are well known for their contribution to the generation of reactive oxygen species (ROS) and for the balance between the oxidant and antioxidant system (Manoli et al. 2007). So, mitochondrial dysfunction doesn't only produce less ATP but also releases greater amounts of ROS and possess a higher tendency to induce cardiac dysfunction (Marzetti et al. 2013).

Peroxisome proliferator-activated receptor  $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ) is involved in the transcriptional control of cellular energy metabolism, especially in mitochondrial function and biogenesis (Kelly et al. 2004; Leone et al. 2005). It has been reported that marked decrease or increase in expression of PGC-1 $\alpha$  can lead to severe mitochondrial dysfunction and hence, cardiac dysfunction (Russell et al. 2004) in particular circumstances such as: fasting (Rhee et al. 2003), intense physical activity (St-Pierre et al. 2003), cold temperature (Puigserver et al. 1998) and increased sympathetic nervous stimulation as in severe emotional distress or fear (Ananda et al. 2012).

On the other hand, cardiolipin (CL) is a unique phospholipid which is almost exclusively localized in the mitochondrial inner membrane where it is important for maintaining the structural integrity of biological membranes. Also, it is associated with different complexes of the respiratory chain, which are involved in the transduction of electrons and the production of ATP in the mitochondrial inner membrane and hence, normal mitochondrial function (Paradies et al. 2014).

Interestingly, although circulating vitamin D (VitD) is primarily synthesized and catabolized in the kidney, the extrarenal expression of the two most important enzymes responsible for activation or inactivation of VitD, 1 $\alpha$ -hydroxylase and 24-hydroxylase respectively, is thought to be important for local production of VitD. In addition, the cytochromes P450 enzymes, involved in VitD metabolism, and VitD receptor (VDR) have been mapped in the heart suggesting the intracrine or paracrine properties of VitD in cardiovascular system (Bouillon et al. 2008). Several beneficial effects have been attributed to the VDR- VitD endocrine effect on the cardiovascular system. Previous studies suggested that VitD prevents cardiocyte hypertrophy, shows anticoagulant effects in cultured monocytic cells and correlates inversely with the blood pressure level in normotensive as well as hypertensive subjects (Bouillon et al. 2008).

Furthermore, there are various interventional studies that examined the association between VitD supplementation and prevention of cardiovascular diseases in humans (Zittermann

et al. 2009). Based on the results of these studies, VitD should be a key role for cardiovascular health, although their detailed mechanisms remain unclear, hence the current study may help approximating the knowledge gaps in this field.

Of note, the two most important enzymes responsible for activation or inactivation of 25OHD, 1 $\alpha$ -hydroxylase and 24-hydroxylase, are located in the mitochondria. Moreover, it has been documented that both VDR and its heterodimer partner retinoid X receptor, exist in abundance in mitochondria of platelets and their precursor megakaryocytes (Silvagno et al. 2013). The functional consequences of mitochondrial VDR are not yet clearly understood, however, it indicates a significant role for VitD in mitochondrial function or for tissue-specific functions in platelet or muscle (Psarra et al. 2006).

To the authors' knowledge, the possible cardioprotective effect of VitD to alleviate cardiac dysfunction in relation to mitochondrial dysfunction in chronically stressed rats has not been examined yet. Accordingly, this study was designed to evaluate first; the effect of chronic stress on cardiac function in rats, second; the role of mitochondrial dysfunction in this issue, third; the potential cardioprotective effect of VitD in chronically stressed rats, fourth; the exact mechanisms of VitD involved with the contribution of oxidative stress, stress hormones and mitochondrial function in this event.

## Materials and Methods

### *Drugs and chemicals*

Cholecalciferol was obtained as ampoules from Memphis Co, for Pharm. and Chem. Ind. (Cairo, Egypt), dissolved in olive oil. Other used chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA)

### *Animals*

Forty-eight male Wistar rats weighing 180–220 g were obtained from the Experimental Animal Unit of Moshtohor faculty of agriculture, Benha University. They were acclimatized to standard laboratory conditions for one week. The animals were handled as per the guidelines of (Benha University, Egypt) and in accordance with the National Institute of Health guide for the care and use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). The rats have housed in wire mesh cages under a room temperature of  $24 \pm 1^\circ\text{C}$ . Throughout the experiment, food and tap water were accessible.

### *Experimental design*

Animals were divided into six groups, eight animals each. Group I (Control group): rats received olive oil (0.3 ml/day)

*via* oral gavage for 14 days; Group II (VitD1 group): rats received 6 µg/kg of vitamin D3 dissolved in 0.3 ml of olive oil *via* oral gavage for 14 days; Group III (VitD2 group): rats received 12 µg/kg of vitamin D3 dissolved in 0.3 ml of olive oil *via* oral gavage for 14 days (Detregiachi et al. 2016); Group IV (CIS group; chronic immobilization stress group): rats were exposed to immobilization stress daily for 2 hours (from 9:00 to 11:00 a.m.) for 14 days (Yang et al. 2014); Group V (CIS+VitD1 group): rats were exposed to immobilization stress and received 6 µg/kg of vitamin D3 simultaneously for 14 days; Group VI (CIS+VitD2 group): rats were exposed to immobilization stress and received 12 µg/kg of vitamin D3 simultaneously for 14 days.

#### *Chronic immobilization stress (CIS)*

Rats were exposed to immobilization stress for 2 hours (from 9:00 to 11:00 a.m.) for 14 days (Yang et al. 2014). They were immobilized by taping all the four limbs on a board, after putting them in the supine position using zinc oxide hospital tape. The release was affected by untying the tape after moistening with acetone to minimize discomfort or pain (Sur and Bhattacharyya 1997).

#### *Study procedures*

24 hours after the last immobilization stress regimen and the last dose of VitD treatment of each group, overnight-fasted rats were anesthetized for echocardiography assessment. Then a cranio-caudal incision of about 2 cm was made, parallel and slightly to the left of the sternum through the skin and pectoral muscles to expose the ribs. A blunt curved forceps is then binged between the 5th and the 6th ribs, through the intercostal muscles. The gap is widened so that the rapidly beating heart becomes visible. The intracardiac blood sample was drawn from the right ventricle and centrifuged at  $5000 \times g$  for 15 minutes at room temperature ( $25 \pm 2^\circ\text{C}$ ). The clear supernatant (plasma) was kept at  $-20^\circ\text{C}$  to be used for assessment of adrenaline, and noradrenaline. Finally, the hearts were excised and divided into two halves one stored at  $-80^\circ\text{C}$  for measuring the gene expression of PGC-1 $\alpha$  by real-time PCR. While the other half was homogenized to prepare 10% homogenate to determine cardiac adenosine triphosphate (ATP) content and mitochondrial protein levels of CL. Also, a part of the homogenate was then centrifuged at  $100,000 \times g$  for 1 h at  $4^\circ\text{C}$ , and the supernatant was used to estimate oxidative stress.

#### *Echocardiography*

Echocardiography was done in rats under ketamine (40 mg/kg intraperitoneal) anesthesia, using My lab 30<sup>TM</sup> VET Gold (Esoate co, Italy) equipped with a high-frequency 4–8 MHz

phased array transducer. The rats were placed in the proper posture (semi-left lateral position with upright tilt) after the thoracic walls were shaved clean. Ultrasound gel was placed on the thorax to optimize visibility. Doppler, two-dimensional (2-D) guided M-mode images were recorded from parasternal long-axis and parasternal short-axis and apical four-chamber views. The left ventricular end-diastolic and systolic diameters (LVDD and LVSD) were measured (Wang et al. 2015).

Ejection fraction (EF) and fractional shortening (FS) were calculated using the following formulas:

$$\text{FS \%} = (\text{LVDD} - \text{LVSD}) / \text{LVDD} \times 100$$

$$\text{EF \%} = (\text{LVDD}^3 - \text{LVSD}^3) / \text{LVDD}^3 \times 100$$

All parameters were depended on the mean values of three cardiac cycles.

#### *Estimation of plasma adrenaline and noradrenaline*

Plasma adrenaline and noradrenaline were measured using ELISA kits (EIA4776 and EIA4317, DRG International, Inc, USA) according to the method of Burtis and Ashweed (1994).

#### *Measurement of ATP and CL contents*

ATP and CL content were estimated in cardiac tissue homogenates using enzyme-linked immunosorbent assay (ELISA) from (Rat ATPELISA Test Kit, Kamiya Biomedical Company, Seattle, WA, USA) and (Cloud-Clone CoRP Company, Houston, Texas, USA), respectively, in accordance with manufacturer instructions.

#### *Gene expression analysis of PGC 1 $\alpha$ in rat cardiac tissues by real-time PCR*

To study the alterations in the PGC-1 $\alpha$  gene expression, quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis was performed. Total RNA was extracted using RNeasy mini kit (Qiagen, Germany) according to the manufacturer's protocol. Extracted RNA samples were quantified using NanoDrop One spectrophotometer (Thermo Fisher Scientific, USA). mRNA (1 µg) was reverse transcribed using QuantiTect Reverse Transcription Kit (Qiagen, Germany) following manufacturer's instruction (Hoffmann et al. 2015).

PGC-1 $\alpha$  gene was amplified by Step One Plus Real-Time PCR System (Applied Biosystems, Singapore) using QuantiTect SYBR Green PCR Kit (Qiagen, Germany). Temperature cycles were as follows:  $95^\circ\text{C}$  for 30 s followed by 42 cycles of  $95^\circ\text{C}$  for 5 s and  $60^\circ\text{C}$  for 30 s. The SYBR Green fluorescence was detected at the end of each cycle to monitor the amount of PCR product formed during that cycle. Relative quantities of the PGC-1 $\alpha$  gene were normalized against  $\beta$ -actin gene and fold expression changes were calculated

using the 2- $\Delta\Delta$ CT method. Oligonucleotide sequences of the sense and antisense primers as follow: PGC-1 $\alpha$  cDNA (Genbank access No: AB025784), 5'-CCTTTCTGAACTT-GATGTGA-3' (sense), 5'-ATGCTCTTTGCTTTATT-GCT-3' (antisense),  $\beta$ -actin (Genbank access No: J00691), 5'-TCTACAATGAGCTGCGTGTG-3'(sense), 5'-GGTCAG-GATCTTCATGAGGT-3' (antisense) (Ananda et al. 2012).

#### *Assessment of oxidative stress biomarkers in the cardiac homogenate*

Malondialdehyde levels (MDA), the indicator of lipid peroxidation, were measured by using thiobarbituric acid reaction according to Placer et al. (1996). Superoxide dismutase (SOD) activity was estimated according to Marklund (1985). Reduced glutathione (GSH) concentration was measured according to Ellman (1959). Assay commercial kits were provided from (Biodiagnostic, Egypt).

#### *Statistical analysis*

All the data are presented as mean  $\pm$  standard deviation (SD). Comparisons among groups were performed with one-way ANOVA with post hoc test (LSD) using SPSS 19.0 software. A  $p$  value below 0.05 was considered significant.

## Results

#### *Effect of VitD on CIS-induced changes in cardiac functions*

Echocardiography was used to evaluate cardiac function 14 days after chronic stress and VitD supplementation. Compared with control group, chronic stress significantly ( $p < 0.05$ ) decreased LVDD and LVSD. However, stressed rats treated with VitD in a dose of (12  $\mu$ g/kg) showed significant ( $p < 0.05$ ) increase in LVDD and LVSD.

Furthermore, EF and FS of CIS group showed significant ( $p < 0.05$ ) decrease when compared to control group. While, stressed rats treated with VitD in a dose of (12  $\mu$ g/kg) showed significant ( $p < 0.05$ ) increase in EF and FS compared with CIS group (Table1).

On the other hand, VitD supplementation to non-stressed rats could not produce any significant change from that of the control group. Also, VitD treatment to stressed rats in a dose of 6  $\mu$ g/kg resulted in a non-significant effect on cardiac function compared to the CIS group (Table 1).

#### *Effect of VitD on CIS-induced changes in cardiac tissue antioxidant and lipid peroxidation parameters*

Data revealed that CIS significantly increased ( $p < 0.05$ ) MDA level in cardiac tissues as compared with the control group. VitD supplementation to stressed rats in a dose of 6  $\mu$ g/kg significantly decrease MDA level compared to the CIS group, but the values still significantly higher ( $p < 0.05$ ) than in the controls. While VitD supplementation in a dose of 12  $\mu$ g/kg partially reversed the CIS-induced elevation of MDA activity in cardiac tissues compared to that of the control group.

On the other hand, CIS caused a significant reduction in GSH level and SOD activity compared to the control group ( $p < 0.05$ ). VitD supplementation to stressed rats in a dose of 6 or 12  $\mu$ g/kg showed a significant increase in cardiac tissue level of GSH and SOD activity compared to the CIS group ( $p < 0.05$ ). VitD supplementation in a dose of 12  $\mu$ g/kg showed significant ( $p < 0.05$ ) increase in cardiac tissue GSH level and activity of SOD as compared to the stressed rats treated with VitD in a dose of 6  $\mu$ g/kg (Table 2).

#### *Effect of VitD on CIS-induced changes of mitochondrial dysfunction markers*

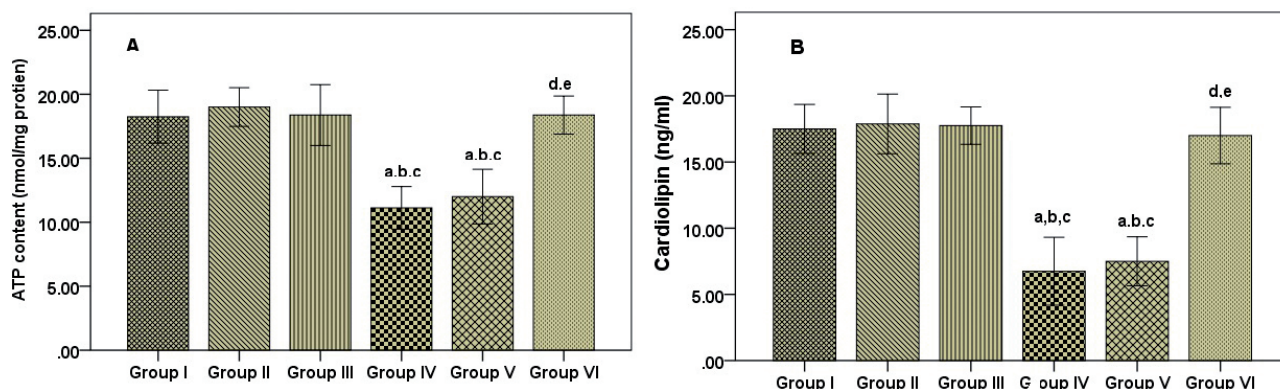
CIS induced a significant decrease in ATP and CL contents compared to the control group ( $p < 0.05$ ). VitD treatment to

**Table 1.** Echocardiography results in different study groups

Group	LVDD (nm)	LVSD (nm)	EF (%)	FS (%)
I	7.10 $\pm$ 0.26	4.07 $\pm$ 0.19	81.13 $\pm$ 1.18	42.66 $\pm$ 1.16
II	7.16 $\pm$ 0.26	4.07 $\pm$ 0.19	80.64 $\pm$ 0.98	42.16 $\pm$ 0.99
III	7.04 $\pm$ 0.25	4.03 $\pm$ 0.18	81.23 $\pm$ 0.60	42.75 $\pm$ 0.61
IV	5.25 $\pm$ 0.17 <sup>a,b,c</sup>	3.30 $\pm$ 0.10 <sup>a,b,c</sup>	73.91 $\pm$ 4.01 <sup>a,b,c</sup>	37.13 $\pm$ 2.64 <sup>a,b,c</sup>
V	5.08 $\pm$ 0.70 <sup>a,b,c</sup>	3.32 $\pm$ 0.13 <sup>a,b,c</sup>	75.75 $\pm$ 1.73 <sup>a,b,c</sup>	37.67 $\pm$ 1.50 <sup>a,b,c</sup>
VI	6.73 $\pm$ 0.14 <sup>d,e</sup>	3.97 $\pm$ 0.09 <sup>d,e</sup>	79.41 $\pm$ 0.81 <sup>d,e</sup>	40.96 $\pm$ 0.78 <sup>d,e</sup>

Values are expressed as means  $\pm$  SD,  $n = 8$ . LVDD, left ventricular diastolic dimension; LVSD, left ventricular systolic dimension; EF, ejection fraction; FS, fractional shortening. Value  $p < 0.05$  is significant tested by using One-way analysis of variance (ANOVA) and *post hoc* multiple comparisons. <sup>a</sup>  $p < 0.05$  compared with Group I (control group); <sup>b</sup>  $p < 0.05$  compared with Group II (VitD1 group); <sup>c</sup>  $p < 0.05$  compared with Group III (VitD2 group); <sup>d</sup>  $p < 0.05$  compared with Group IV (CIS group); <sup>e</sup>  $p < 0.05$  compared with Group V (CIS+VitD1 group). Group VI: CIS+VitD2 group. For more information, see Materials and Methods.





**Figure 1.** Effect of VitD on CIS-induced changes in cardiac muscle ATP content (A) and cardiolipin content (B) in experimental groups. Data represent means  $\pm$  SD of 8 rats in each group.  $p < 0.05$  is significant tested by using One-way analysis of variance (ANOVA) and *post hoc* multiple comparisons. <sup>a</sup>  $p < 0.05$  compared with Group I (control group); <sup>b</sup>  $p < 0.05$  compared with Group II (VitD1 group); <sup>c</sup>  $p < 0.05$  compared with Group III (VitD2 group); <sup>d</sup>  $p < 0.05$  compared with Group IV (CIS group); <sup>e</sup>  $p < 0.05$  compared with Group V (CIS+VitD1 group). Group VI, CIS+VitD2 group.

non-stressed rats could not produce any significant change from that of the control group. Also, VitD supplementation to stressed rats in a dose of 6  $\mu\text{g}/\text{kg}$  resulted in a non-significant increase in ATP (Figure 1A) and CL (Figure 1B) contents compared to the CIS group. While, VitD supplementation in a dose of 12  $\mu\text{g}/\text{kg}$  resulted in a significant increase in ATP and CL contents compared to the CIS group ( $p < 0.05$ ).

As shown in Figure 2, the mRNA of PGC-1 $\alpha$ , known as a master regulator of mitochondrial biogenesis, increased in response to CIS compared with the control ( $p < 0.05$ ). However, VitD pretreatment in doses of 6 and 12  $\mu\text{g}/\text{kg}$  led to a significant further induction of PGC-1 $\alpha$  expression in comparison to

CIS group ( $p < 0.05$ ). It should be considered that the results regarding PGC-1 $\alpha$  in non-stressed treated groups did not show a statistically significant difference in comparison to the control.

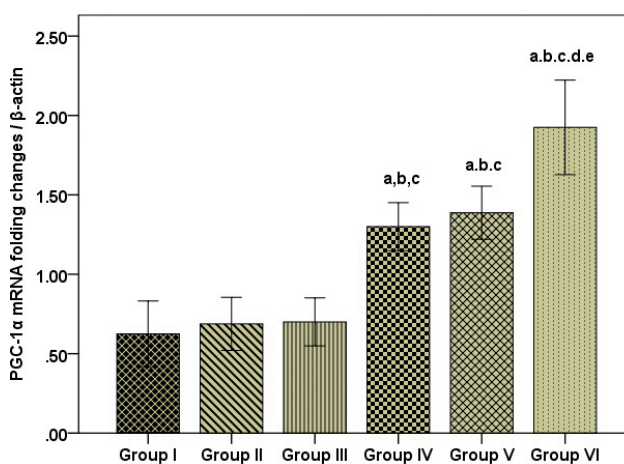
*Effect of VitD on CIS-induced changes of plasma adrenaline and noradrenaline levels.*

Treatment of VitD at the dose of 12  $\mu\text{g}/\text{kg}$  resulted in a significant decline of plasma adrenaline and noradrenaline levels

**Table 2.** Statistical comparison of antioxidant parameters (MDA, GSH and SOD) in cardiac tissues in different study groups

Group	MDA (nmol/g tissue)	GSH (nmol/g tissue)	SOD (U/g protein)
I	29.22 $\pm$ 2.98	70.81 $\pm$ 1.44	86.51 $\pm$ 4.49
II	29.53 $\pm$ 2.94	71.20 $\pm$ 1.06	87.95 $\pm$ 3.52
III	30.17 $\pm$ 3.37	71.58 $\pm$ 1.43	85.47 $\pm$ 4.66
IV	48.67 $\pm$ 2.69 <sup>a,b,c</sup>	51.99 $\pm$ 2.27 <sup>a,b,c</sup>	55.84 $\pm$ 0.88 <sup>a,b,c</sup>
V	35.51 $\pm$ 2.06 <sup>a,b,c,d</sup>	68.75 $\pm$ 1.13 <sup>a,b,c,d</sup>	78.48 $\pm$ 1.39 <sup>a,b,c,d</sup>
VI	30.13 $\pm$ 2.08 <sup>d,e</sup>	71.32 $\pm$ 1.28 <sup>d,e</sup>	86.12 $\pm$ 2.64 <sup>d,e</sup>

Values are expressed as means  $\pm$  SD,  $n = 8$ . MDA, malondialdehyde; GSH, reduced glutathione; SOD, superoxide dismutase. Value  $p < 0.05$  is significant tested by using One-way analysis of variance (ANOVA) and *post hoc* multiple comparisons. <sup>a</sup>  $p < 0.05$  compared with Group I (control group); <sup>b</sup>  $p < 0.05$  compared with Group II (VitD1 group); <sup>c</sup>  $p < 0.05$  compared with Group III (VitD2 group); <sup>d</sup>  $p < 0.05$  compared with Group IV (CIS group); <sup>e</sup>  $p < 0.05$  compared with Group V (CIS+VitD1 group). Group VI: CIS+VitD2 group. For more information, see Materials and Methods.



**Figure 2.** Effect of vitamin D on CIS-induced changes in PGC-1 $\alpha$  mRNA expression in cardiac tissues. Data represent means  $\pm$  SD of 8 rats in each group.  $p < 0.05$  is significant tested by using ANOVA and *post hoc* multiple comparisons. <sup>a</sup>  $p < 0.05$  compared with Group I (control group); <sup>b</sup>  $p < 0.05$  compared with Group II (VitD1 group); <sup>c</sup>  $p < 0.05$  compared with Group III (VitD2 group); <sup>d</sup>  $p < 0.05$  compared with Group IV (CIS group); <sup>e</sup>  $p < 0.05$  compared with Group V (CIS+VitD1 group). Group VI, CIS+VitD2 group.

( $p < 0.05$ ), which recorded a significant rise in response to CIS, compared to the control group ( $p < 0.05$ ) (Figure 3A,B). It should be noted that the results regarding adrenaline and noradrenaline in non-stressed treated groups were statistically insignificant in comparison to the control.

## Discussion

Death following circumstances of chronic stress has been linked to the cardiac dysfunction, whose pathomechanism is still unclear. In this study, we sought to determine whether mitochondrial dysfunction and oxidative stress play a role in the pathogenesis of this cardiac entity. Also, we aimed to investigate the effect and to elucidate the mechanism of action of VitD against chronic stress-induced cardiac dysfunction, hoping to provide evidence for its therapeutic potential through protection against or reversal of chronic stress-induced cardiac dysfunction.

### Chronic stress induces cardiac dysfunction

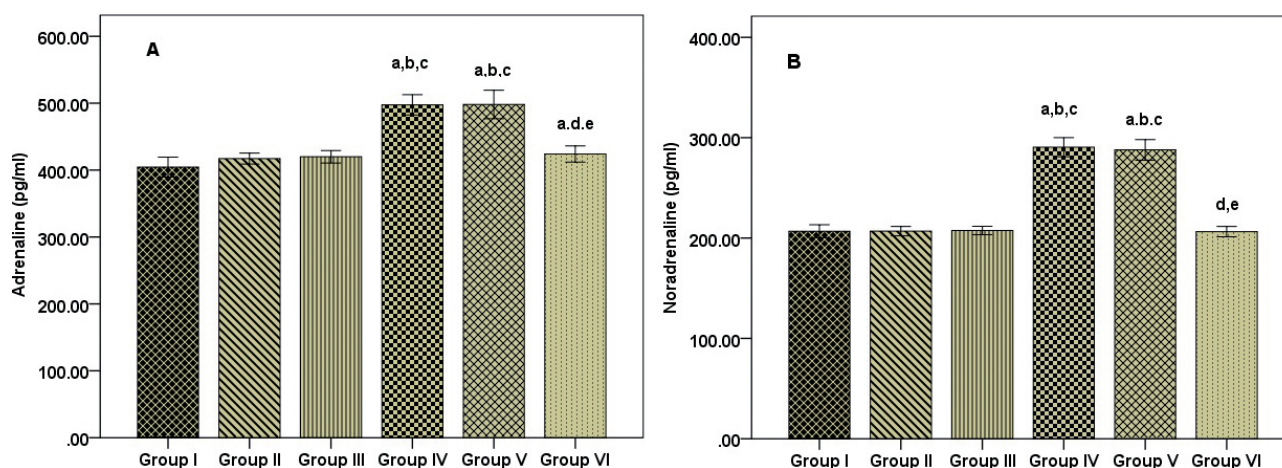
In the current study, it has been demonstrated that CIS 2 hours/day for 14 days significantly resulted in increased left ventricular dysfunction and decreased the FS and EF in the echocardiographic assessment. These findings are similar to that reported by others (Bin-Jaliah 2017; Demircit and Sahin 2017). These studies explained the cardiac dysfunction in chronic stress by impaired myocardial metabolism, overactivity of the sympathetic nervous system, abnormal coronary vessel reactivity that reduces myocardial microperfusion and

by endothelial dysfunction. Another study has shown that social stress leads to cardiomyocyte contractile dysfunction and myocardial pathologies by causing intracellular  $Ca^{2+}$  dysregulation and oxidative stress (Turdi et al. 2012).

### Mechanisms of CIS-induced cardiac dysfunction:

The cardiac dysfunction in chronic stress may be explained by many mechanisms: First, decreased cellular CL and ATP content, which represent a sensitive readout of mitochondrial function. Our results clearly indicate that CL and ATP contents were decreased in cardiac tissues of rats subjected to CIS when compared to the control group. The decrease in ATP may be attributed to the decreased CL level, as CL plays a pivotal role in electron/proton flux and hence, ATP synthesis. As well, it interacts with a number of proteins and enzymes involved in fundamental mitochondrial bioenergetic processes, such as respiratory chain complexes and mitochondrial substrate carriers. More to the point; it is required for their optimal activity (Paradies et al. 2014). Abnormal CL profile in cardiovascular disorders may result from depletion due to decreased CL synthase activity; changes in acyl chain composition due to altered activity of enzymes for CL remodeling and CL oxidation due to attack of its unsaturated fatty acyl chains by ROS (Paradies et al. 2015).

Second, increased ROS generation and oxidative stress, which in turn aggravates the decreased ATP level and mitochondrial dysfunction. In the current study, we observed signs of oxidative stress exhibited by significant elevation of lipid peroxides (MDA) accompanied with a significant



**Figure 3.** Effect of vitamin D on CIS-induced changes in plasma adrenaline (A) and nor-adrenaline levels in experimental groups. Data represent means  $\pm$  SD of 8 rats in each group.  $p < 0.05$  is significant tested by using ANOVA and *post hoc* multiple comparisons. <sup>a</sup>  $p < 0.05$  compared with Group I (control group); <sup>b</sup>  $p < 0.05$  compared with Group II (VitD1 group); <sup>c</sup>  $p < 0.05$  compared with Group III (VitD2 group); <sup>d</sup>  $p < 0.05$  compared with Group IV (CIS group); <sup>e</sup>  $p < 0.05$  compared with Group V (CIS+VitD1 group). Group VI, CIS+VitD2 group.

decrease in heart tissue antioxidant enzyme (SOD) and the levels of non-enzymatic antioxidant (GSH), which were decreased in stressed rats as compared to the control group. The decreased antioxidant activity in stress-induced cardiac dysfunction can be explained by other studies (Sebastiani et al. 2007; Ananda et al. 2012) which showed that long-term exposure to stress concentrations of glucocorticoids is associated with respiratory chain dysfunction, increased ROS generation and mitochondrial structural aberrations. Sebastiani et al. (2007) and Ananda et al. (2012) also revealed that mitochondrial dysfunction can contribute to increased oxidative stress both by reduction of ATP/adenosine monophosphate ratio and by mitochondrial-derived oxygen radicals.

It has also been reported that overproduction of ROS consumes ATP and damages the ATP synthase so that ATP content within cells decreases, leading to mitochondria dysfunction. Such a disturbance of mitochondrial respiratory chain function could lead to decay in mitochondria energy production and, sooner or later, to cellular injury or dysfunction, particularly in 'high metabolic rate' tissues as heart muscle (Carreté et al. 2002).

Third, overexpression of *PGC-1 $\alpha$* , which aggravates the mitochondrial impairment, worsens the oxidative stress and further lowers ATP generation. Our results indicate that *PGC-1 $\alpha$*  expression was significantly increased in cardiac tissues of rats subjected to CIS with concomitant significant elevation of plasma catecholamine levels when compared to the control group.

These results are in agreement with previous studies (Finck and Kelly 2007; Ananda et al. 2012) which found that *PGC-1 $\alpha$*  expression was significantly increased in cardiac tissues of rats subjected to stressors. Ananda et al. (2012) explained the overexpression of *PGC-1 $\alpha$*  in cardiac tissue of stressed rats by the increase in catecholamine, the primary hormonal mediators of the stress response, which affect mitochondrial metabolism by mobilizing energy substrates from body reserves for oxidation. Adrenaline acts through the Gs-protein-coupled  $\beta$ 2-adrenergic receptor to stimulate adenylyl cyclase activity and cAMP production, activating protein kinase A. This signaling pathway activates *PGC-1 $\alpha$* , to exert downstream effects that stimulate mitochondrial biogenesis and oxidative phosphorylation. Another study documented that overexpression of *PGC-1 $\alpha$*  could lead to a mitochondrial biogenic response and cardiomyopathy due to mitochondrial ultrastructural changes and finally dysregulated mitochondrial metabolism (Russell et al. 2004).

Of note, we speculated that the mitochondrial abnormalities caused by extreme production of *PGC-1 $\alpha$*  in the heart of stressed rats could cause cardiomyocyte injury *via* oxidative stress and so cardiac dysfunction. Previous studies (Lehman et al. 2000; Finck and Kelly 2007) found that during periods of stress, catecholamine level is markedly elevated, as shown in our study. Such increase could stimulate the production

of ROS most likely through prolonged *PGC-1 $\alpha$*  upregulation that play a part in oxidative stress. ROS could injure cardiac cells directly and begin a series of local chemical reactions and genetic modifications that intensifies the cardiomyocyte dysfunction (Chalkias and Xanthos 2012).

Of interest, we found that ATP production was decreased in spite of the overexpression of *PGC-1 $\alpha$* . A finding that may be explained by previous studies (Russell et al. 2004; Bilsen et al. 2008), which reported that *PGC-1 $\alpha$*  overexpression in cardiac tissues stimulates extra-mitochondrial oxygen consumption. Hence, causing the shift to anaerobic glycolytic pathways and with time this metabolic shift turns out to be maladaptive, resulting in a circumstance of decreased mitochondrial ATP production in the cardiac myocytes. Following this line of elucidation, it would be possible to suppose that overexpression of *PGC-1 $\alpha$*  leads to mitochondrial dysfunction, which worsens cardiac function in stressed rats as well (Cocco and Chu 2007).

To study the effect of VitD supplementation on CIS-induced cardiac dysfunction, we opt to test VitD3 in two doses. The supplement and doses of VitD3 were chosen based upon previous studies of vitD supplementation (Dregiachi et al. 2016; Hataikarn et al. 2013). These studies showed that VitD3 tended to increase serum total 25(OH) D levels more when compared with the same dosage of VitD2. The underlying basis for this finding appears to be a concurrent decrease in 25(OH) D3 after supplementation with VitD2. They speculated that the greater affinity of VitD3 for the VitD binding protein and less clearance of 25(OH) D3 would maintain its serum levels in the case of VitD3 supplementation, not VitD2.

#### *Effect of VitD on CIS-induced cardiomyopathy*

Interestingly, treatment with VitD significantly reversed the effects of CIS on left ventricular function, EF, and FS, in a dose-dependent manner as compared to CIS group. To the best of our knowledge, this is the first study demonstrating the effect of VitD therapy on cardiac dysfunction induced by CIS, though; previous experimental studies have demonstrated the protective effect of VitD on cardiac and endothelial functions (Bodyak et al. 2007; Simpson 2011). As well, other studies suggested a close association between VitD deficiency and cardiovascular diseases (O'Connell et al. 1997; Mancuso et al. 2008). Moreover, previous reports demonstrated that the rate and magnitude of cardiomyocyte sarcomere contraction are adjusted by VitD *via* interaction with caveolin-3 in t-tubules (Zhao and Simpson 2010). This rapid nongenomic process is thought to be mediated by membrane-bound VDR. Cardiomyocyte relaxation was shown to be accelerated by VitD, which may improve coronary perfusion during diastole. Intracellular calcium influx into cardiomyocytes was also reported to increase



by VitD *via* modulation of  $\beta$ -adrenergic pathways (Santillán et al. 1999).

In fact, experimental VitD deficiency is accompanied by increased renin expression and angiotensin II concentration, high blood pressure and cardiac hypertrophy, whereas VitD analogues improve left ventricular hypertrophy and diastolic function in the spontaneously hypertensive rat (Ferder et al. 2013). As well, a previous study reported that taking VitD supplements may improve cardiac function in chronic heart failure patients and increase left ventricular ejection fraction. After 12 months; patients who took vitamin D had greater improvement in echocardiographic measures of LV function, LV dimensions and volumes than patients who took a placebo (Klaus et al. 2016)

#### *VitD alleviates cardiac dysfunction via different mechanisms:*

The improvement of cardiac functions in VitD-treated groups may be attributed to different mechanisms. First, enhancement of mitochondrial function reflected as elevation of ATP and CL contents. Our study revealed that treatment of stressed rats with vitD significantly improved mitochondrial function, in a dose-dependent manner, as evident from the increase in ATP and CL compared to non-treated stressed rats. A growing body of evidence has linked abnormalities in CL content, structure and acyl chains composition to mitochondrial dysfunction in various physiopathological states and disorders, including cardiovascular diseases (Paradies et al. 2015). To the best of our knowledge this work is the first to show rise of CL content after VitD supplement to stressed rats. A finding that might be explained by enhanced CL synthase activity and diminished CL oxidation by ROS attacks. Importantly, CL elevation increases ATP generation, as CL is essential for the assembly and function of the mitochondrial electron transport chain complexes (Paradies et al. 2015).

Second, VitD plays a key role in regulating ROS levels and maintaining the stability of cell signaling pathways. The current results showed that VitD treatment to stressed rats, in a dose-dependent manner, caused a significant decrease in oxidative stress when compared to CIS group. VitD can conserve mitochondrial function by regulating the expression and/or activity of antioxidant enzymes, including glutathione reductase and SOD as well as, decreasing lipid peroxidation (Berridge 2017). These results similar to that of Farhang et al. (2017) who revealed that VitD ameliorates oxidative stress by raising the antioxidant enzymes activity in the cardiac tissue of high-fat diet induced obese rats. Also Tarcin et al. (2009) attributed the improvement in cardiovascular health by VitD to its protective effects against oxidative stress.

Third, VitD affects plasma catecholamine level, which is evident in the present study by a dose-dependent significant decrease of their levels in stressed rats treated with VitD, when compared to CIS group. Nevertheless, few studies have

investigated the effect of VitD on autonomic nervous system activity. One study reported that VitD was negatively correlated with plasma metanephrine, a measure of catecholamine synthesis in the adrenal medulla (Burt et al. 2016). In contrast, an *in vitro* study reported that VitD increased tyrosine hydroxylase mRNA, suggesting that VitD might stimulate the rate-limiting step in catecholamine biosynthesis (Puchacz et al. 1996). With such conflicting results, we advertise the need for further investigation into the relationship between VitD and catecholamine levels during stress.

Surprisingly, the current study showed a significant further induction of *PGC-1 $\alpha$*  expression when compared to the CIS group. In line with this finding Peng et al. (2017), reported that VitD binds to VDR at *PGC-1 $\alpha$*  promoter and thus induces its expression, in zebrafish visceral adipose tissue. Moreover, results of Ryan et al. (2016) strengthen our findings of the mitochondria-protective role of VitD, as they reported that VitD has important effects on mitochondrial morphology, physiology, and expression of key mitochondrial proteins. Mitochondrial oxygen consumption rate raises in skeletal muscle cells treated with VitD due to increasing in mitochondrial volume fraction and branching steady with mitochondrial biogenesis. In particular, respiration coupled to the generation of ATP is increased, suggesting that VitD increases energy production in muscle.

Our findings of increased ATP and *PGC-1 $\alpha$*  expression following treatment with VitD are consistent with the report of Sinha et al. (2013), which showed that treatment of VitD-deficient humans with cholecalciferol improves muscle mitochondrial function. We suggested that all above-mentioned mechanisms together with increased *PGC-1 $\alpha$*  expression, in VitD-treated group may underlie the improvement of mitochondrial function and ATP elevation (Silvagno et al. 2013).

There is a limitation to our study: We did not assess the protein level of PGC1- $\alpha$  in stressed or VitD treated rats, however, our data open the gate for further studies in this area.

## Conclusion

The present findings have demonstrated that improved mitochondrial function and oxidative stress and decreased catecholamine levels may participate to the VitD-mediated protection against cardiac dysfunction induced by CIS in rats.

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**Ethical approval.** All applicable national, international, and/or institutional guidelines for the care and use of animals were followed.

**Author contribution declaration.** All authors have contributed sufficiently in the formation and design of this work or the analysis and explanation of the data, as well as the inscription and reviewing of the final version of the document.

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