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Role of oxidative stress, inflammation, nitric oxide and transforming growth factor-beta in the protective effect of diosgenin in monocrotaline-induced pulmonary hypertension in rats

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ABSTRACT

Pulmonary hypertension is a progressive disease of various origins that is associated with right ventricular dysfunction. In the present study, the protective effect of diosgenin was investigated in monocrotaline-induced pulmonary hypertension in rats. Pulmonary hypertension was induced by a single subcutaneous injection of monocrotaline (60 mg/kg). Diosgenin (100 mg/kg) was given by oral administration once daily for 3 weeks. At the end of the experiment, mean arterial blood pressure, electrocardiography and echocardiography were recorded. Rats were then sacrificed and serum was separated for determination of total nitrate/nitrite level. Right ventricles and lungs were isolated for estimation of oxidative stress markers, tumor necrosis factor-alpha, total nitrate/nitrite and transforming growth factor-beta contents. Myeloperoxidase and caspase-3 activities in addition to endothelial and inducible nitric oxide synthase protein expression were also determined. Moreover, histological analysis of pulmonary arteries and cardiomyocyte cross-sectional area was performed. Diosgenin treatment provided a significant improvement toward preserving hemodynamic changes and alleviating oxidative stress, inflammatory and apoptotic markers induced by monocrotaline in rats. Furthermore, diosgenin therapy prevented monocrotaline-induced changes in nitric oxide production, endothelial and inducible nitric oxide synthase protein expression as well as histological analysis. These findings support the beneficial effect of diosgenin in pulmonary hypertension induced by monocrotaline in rats.

1. Introduction

Pulmonary hypertension (PH) is a life-threatening disease which is characterized by an extensive narrowing of the pulmonary vascular bed with a progressive increase in pulmonary vascular resistance (Umar et al., 2010). When untreated, the disease ultimately results in elevation of pulmonary arterial pressure and right ventricular (RV) hypertrophy with subsequent RV failure and death (Shao et al., 2011). Monocrotaline (MCT)-induced PH is an experimental model that largely mimics human PH regarding hemodynamic disorders, histological changes and high mortality (Henriques-Coelho et al., 2004).

MCT is a pyrrolizidine alkaloid which selectively injures the pulmonary vascular endothelium and induces pulmonary vasculitis (Hessel et al., 2006). MCT-treated rats demonstrated an early endothelial cell injury, followed by progressive pulmonary arterial structural changes which resulted in the development of PH and RV hypertrophy progressing to failure within weeks (Lipke et al., 1993; Aziz et al., 1997).

Oxidative stress plays a pivotal role in the pathogenesis and/or the development of PH by MCT. Increased oxidative stress mediates MCT-induced apoptosis and endothelial dysfunction in the pulmonary vascular endothelial cells (Grobe et al., 2006). Moreover, impaired nitric oxide (NO) synthesis or bioactivity is the main pathological change that is significantly implicated in PH (Ozturk and Uma, 2010). In the presence of oxidative stress, NO reacts with superoxide anion generating peroxynitrite which is a highly toxic molecule leading to more progressive endothelial dysfunction during the development of PH (Oishi et al., 2006). As a result of the lack of available NO and increased oxidative stress, inflammatory and proliferative cascades proceed with further progression of the disease (Bhargava et al., 1999).

Many patients with PH remain symptomatic despite therapy. Current treatments can reduce the severity of hemodynamic disorder; however, gradual deterioration and progression of the disease often necessitate a lung transplant (Umar et al., 2010). The imbalance between NO and oxidative stress plays an important role in the process of many cardiovascular and pulmonary

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diseases. Administration of antioxidants might be beneficial in the treatment of PH.

Estrogen deficiency (i.e. ovariectomy) exacerbates pulmonary hypertension and treatment with estradiol attenuates the disease (Rabinovitch et al., 1981; Resta et al., 2001). Estradiol has shown significant protection against RV hypertrophy and pulmonary arterial medial hypertrophy in pulmonary hypertension in rats (Tofovic et al., 2006). Diosgenin is a plant-derived sapogenin and is a precursor of steroid hormones (Adlercreutz et al., 1991; Au et al., 2004). Diosgenin (as a phytosterogen) is known to possess anti-hyperlipidemic, anti-inflammatory and antioxidant properties (Raju and Mehta, 2009). The beneficial role of diosgenin has been studied in several models of metabolic diseases, inflammation, blood and cerebral disorders, cardiovascular diseases, and cancer (Patel et al., 2012). Moreover, diosgenin has shown to ameliorate palmitate-induced endothelial dysfunction and insulin resistance through improvement of endothelial insulin signaling and enhancement of NO production (Liu et al., 2012). The relaxant response elicited by diosgenin on vascular smooth muscle cells probably occurs due to the activation of cGMP–NO–L-Arginine pathway (Dias et al., 2007). This implicates the possibility of its application in the treatment of many cardiovascular diseases including pulmonary hypertension. Therefore, the goal of the present study was to explore the protective effects of diosgenin on MCT-induced PH through examination of its effects on associated hemodynamic, biochemical and histological alterations.

2. Material and methods

2.1. Animals

Male Wistar rats weighing 180–210 g were obtained from the animal facility of Faculty of Pharmacy, Cairo University. Rats were housed under controlled temperature ($25 \pm 2^{\circ}C$) and constant light cycle (12 h light/dark) and allowed free access to a standard rodent chow diet and water. The investigation complies with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996) and was approved by the Ethics Committee for Animal Experimentation at Faculty of Pharmacy, Cairo University.

2.2. Chemicals

MCT and diosgenin were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All other used chemicals were of analytical grade.

2.3. Experimental design

Rats were randomly divided into three groups, 11 animals each. Group I served as a normal group. Group II received a single subcutaneous injection of MCT (60 mg/kg). MCT was dissolved in 0.1 M HCl and adjusted to pH 7.4 with 0.1 M NaOH (Pullamsetti et al., 2005). Groups III received MCT as in group II followed by daily oral administration of diosgenin (100 mg/kg) for 21 days. Diosgenin was freshly prepared daily in saline. Dose of diosgenin was selected based on its effectiveness and safety as a protective agent in previous experimental studies (Ma et al., 2002; Hamrita et al., 2012).

2.4. Mean arterial blood pressure, electrocardiographic and echocardiographic measurements

After 3 weeks, animals were weighed. Heart rate (HR) and blood pressure (BP) were measured by the non-invasive tail cuff method using PowerLab data acquisition systems (ADInstruments, Australia). Rats were then anesthetized with thiopental (50 mg/kg, i.p.) and kept warmed with a heating lamp to prevent the incidence of hypothermia. Subcutaneous peripheral limb electrodes were inserted for electrocardiographic recording (HPM 7100, Fukuda Denshi, Tokyo, Japan) to determine QRS amplitude and duration. Spontaneously breathing rats were screened for any RV abnormalities by echocardiography using a Sonosite SonoHeart Elite echo machine (Bothell, USA) with 8-MHz ultrasound probe. Right ventricular anterior wall thickness (RVAWT) was measured in the two-dimensional short-axis parasternal view below the tricuspid valve or in the long-axis parasternal view by M-mode. Right ventricular end-diastolic diameter (RVEDD) was measured in M-Mode of long-axis parasternal view as the distance between interventricular septum and RV anterior wall at the time of left ventricular end diastole. Each parameter was averaged over three cardiac cycles. At the end of the experiment, blood was collected from the retro-orbital sinus using non heparinized capillary tubes for serum separation. Animal was euthanized and lung, RV and left ventricle with septum (LVS) were rapidly excised, washed with ice-cold saline, dried and weighed. For each group, two sets of experiments were conducted; one for biochemical examination and the other ($n=3$) for histological examination.

2.5. Biochemical measurements

Parts of lung and RV were homogenized in ice-cold KCl (1.15%) and NaCl (0.9%), respectively, using a homogenizer (HeidolphDiax 900, Germany) to prepare 10% homogenate. The resultant homogenates were used for determination of the following parameters.

2.5.1. Reduced glutathione

Reduced glutathione (GSH) content was determined using Ellman's reagent according to the method described by Beutler et al. (1963) and expressed as nmol/100 mg protein.

2.5.2. Lipid peroxidation products

Lipid peroxidation products were estimated by determination of the level of thiobarbituric acid reactive substances (TBARS) that were measured according to the assay of Buege and Aust (1978) and expressed as nmol/mg protein.

2.5.3. Myeloperoxidase activity

Myeloperoxidase (MPO) activity was determined kinetically at 460 nm by measuring rate of H_2O_2-dependent oxidation of o-dianisidine that is catalyzed by MPO (Bradley et al., 1982) and expressed as mU/mg protein. One unit of MPO activity is defined as the amount of enzyme that degrades 1 μmol peroxide per min at 25 °C.

2.5.4. Tumor necrosis factor-alpha

Tumor necrosis factor-alpha (TNF-α) content was assessed using rat TNF-α ELISA kit (BD Biosciences, San Diego, USA). The procedure of the used kit was performed according to the manufacturer's instructions and the results were expressed as pg/mg protein.

2.5.5. Serum and tissue total nitrate/nitrite (NO_x)

NO_x was determined spectrophotometrically at 540 nm using Griess reagent after reduction of nitrate to nitrite by vanadium trichloride (Miranda et al., 2001) and expressed in serum as μmol/l and in lung and RV tissues as μmol/g protein.

2.5.6. Transforming growth factor-beta

Transforming growth factor-beta (TGF-β) content was assessed using rat TGF-β ELISA kit (BD Biosciences, San Diego, USA). The procedure of the used kit was performed according to the manufacturer's instructions and the results were expressed as pg/mg protein.

2.5.7. Caspase-3 activity

Caspase-3 activity was estimated using a caspase-3 colorimetric assay kit (R&D Systems Inc., USA). The absorbance was read at 405 nm using a microplate reader (BioTek Instruments, USA). The results were expressed as nmol pNA/h/mg protein. The protein content was determined in all previously mentioned parameters using the method of Lowry et al. (1951).

2.5.8. Western blot analysis of endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS)

Another part of lung or RV was homogenized in lysis HEPES buffer pH 7.4 containing 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 mM leupeptin, 2 mM pepstatin, 0.5 mM phenyl methylsulfonyl fluoride and 1 mM sodium orthovanadate. The tissue lysate was centrifuged at 6000 rpm for 5 min at 4 °C. The lysate was then collected and protein concentration was determined with a BCA protein assay kit (Thermo Fisher Scientific Inc., USA). An aliquot of 20 μg protein from each sample was separated on 8% sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Amersham Bioscience, Piscataway, NJ, USA) using a semidry transfer apparatus (Bio-Rad, Hercules, CA, USA). The membranes were incubated with 5% milk blocking buffer containing 10 mM Tris–HCl (pH 7.4), 150 mM NaCl and Tris-buffered saline with 0.05% Tween-20 (TBST) at 4 °C overnight. The membranes were then washed with TBST and incubated with a 1:2000 dilution of anti-eNOS or anti-iNOS antibodies (Stressgen Biotechnologies, Victoria, British Columbia, Canada) for 1 h at room temperature with constant shaking. The filters were washed and subsequently probed with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Amersham. Life Science Inc., USA). Chemiluminescence detection was performed with the Amersham detection kit according to the manufacturer's protocols and exposed to X-ray film. The amount of eNOS and iNOS protein was quantified by densitometric analysis of the autoradiograms using a scanning laser densitometer (Biomed Instrument Inc., USA). Results were expressed as arbitrary units after normalization for β-actin protein expression.

2.6. Medial wall thickness of pulmonary arteries and cardiomyocyte cross-sectional area

For histological examination, lung and RV free wall from each heart were separated, rinsed in ice-cold saline and immediately fixed in 10% formalin for 24 h. Specimens were processed for paraffin embedding and 5 μm sections were prepared. The sections were stained with haematoxylin and eosin (H&E) and examined microscopically. The thickness of the medial arterial layer was measured in randomly selected small peripheral pulmonary arteries (< 100 μm in external diameter) from the upper and lower lung fields of each rat. Medial thickness was calculated as (external diameter − internal diameter)/external diameter. On the other hand, random areas of RV were examined for each group (3 sections per animal) and cardiomyocyte cross-sectional area was determined. For each section, 100 cardiomyocytes were measured in μm^2 and an average value was calculated. All images were captured and processed using Adobe Photoshop (version 8.0).

2.7. Statistical analysis

All data obtained were presented as mean ± S.E.M. Results were analyzed using one way analysis of variance test (One-way ANOVA) followed by the Student–Newman–Keuls multiple comparison test. Statistical analysis was performed using GraphPad Instat software (version 2.04). For all the statistical tests, the level of significance was fixed at $P < 0.05$.

3. Results

3.1. Body weight, RV weight, lung weight and percentage of mortality

MCT caused a significant decrease in final body weight (221.75 ± 6.56 vs. 255.83 ± 4.73 g) and 27.27% mortality together with a significant increase in RV/body weight (1.09 ± 0.06 vs. 0.51 ± 0.03 mg/g), RV/LVS (0.44 ± 0.02 vs. 0.22 ± 0.01 g/g) and lung/body weight (10.15 ± 0.70 vs. 6.24 ± 0.17 mg/g) ratios. Treatment with diosgenin significantly decreased RV/body weight, RV/LVS and lung/body weight ratios and caused a decrease in mortality percentage reaching 9.09% (Table 1).

3.2. Mean arterial blood pressure, electrocardiographic and echocardiographic measurements

MCT-treated group showed a significant decrease in heart rate (313.33 ± 5.53 vs. 352.01 ± 9.68 bpm), mean arterial blood pressure (92.45 ± 1.86 vs. 121.42 ± 2.44 mmHg) and QRS amplitude (185.71 ± 15.98 vs. 353.17 ± 14.03 μv) and a significant increase in QRS duration (16.62 ± 0.36 vs. 14.72 ± 0.54 msec) in addition to RVAWT (2.58 ± 0.19 vs. 1.01 ± 0.04 mm) and RVEDD (7.68 ± 0.41 vs. 4.62 ± 0.18 mm) measurements as demonstrated in the echocardiographic images (Fig. 1). Diosgenin treatment resulted in a significant improvement in the aforementioned parameters (Table 1).

Table 1
Effect of diosgenin on monocrotaline (MCT)-induced changes in body, heart and lung weights as well as heart rate, mean arterial blood pressure, electrocardiography and echocardiography.

Parameters	Groups		
	Normal	MCT	Diosgenin
Mortality (%)	0	27.27	9.09
Initial BW (g)	209 ± 2.71	206.66 ± 4.19	208.25 ± 4.32
Final BW (g)	255.83 ± 4.73	221.75 ± 6.56[a]	238.86 ± 3.54[a b]
RV/LVS (g/g)	0.218 ± 0.011	0.443 ± 0.018[a]	0.313 ± 0.011[a b]
RV/BW (mg/g)	0.510 ± 0.030	1.094 ± 0.061[a]	0.787 ± 0.073[a b]
LW (g)	1.55 ± 0.10	2.88 ± 0.29[a]	1.98 ± 0.10[b]
LW/BW (mg/g)	6.24 ± 0.17	10.15 ± 0.70 [a]	8.19 ± 0.22[a b]
BP (mm Hg)	121.42 ± 2.44	92.45 ± 1.86[a]	110.17 ± 3.25[b]
HR (bpm)	352 ± 9.68	313.33 ± 5.53[a]	331.25 ± 7.57[a b]
QRS duration (ms)	14.72 ± 0.54	16.62 ± 0.36[a]	15.47 ± ± 0.25[a b]
QRS amplitude (μV)	353.17 ± 14.03	185.71 ± 15.98[a]	275.83 ± 18.55[a b]
RVAWT (mm)	1.01 ± 0.04	2.58 ± 0.19[a]	1.75 ± 0.16[a b]
RVEDD (mm)	4.62 ± 0.18	7.68 ± 0.41[a]	6.71 ± 0.30[a b]

Each value represents the mean of 5–8 experiments ± S.E.M. BW, Body weight; HW, heart weight; RV, right ventricle; LVS, left ventricle and septum; LW, lung weight; BP, mean arterial blood pressure; HR, heart rate; bpm, beats per min; RVAWT, right ventricular anterior wall thickness; RVEDD, right ventricular end-diastolic diameter.

[a] $P < 0.05$ vs. normal.
[b] $P < 0.05$ vs. MCT.

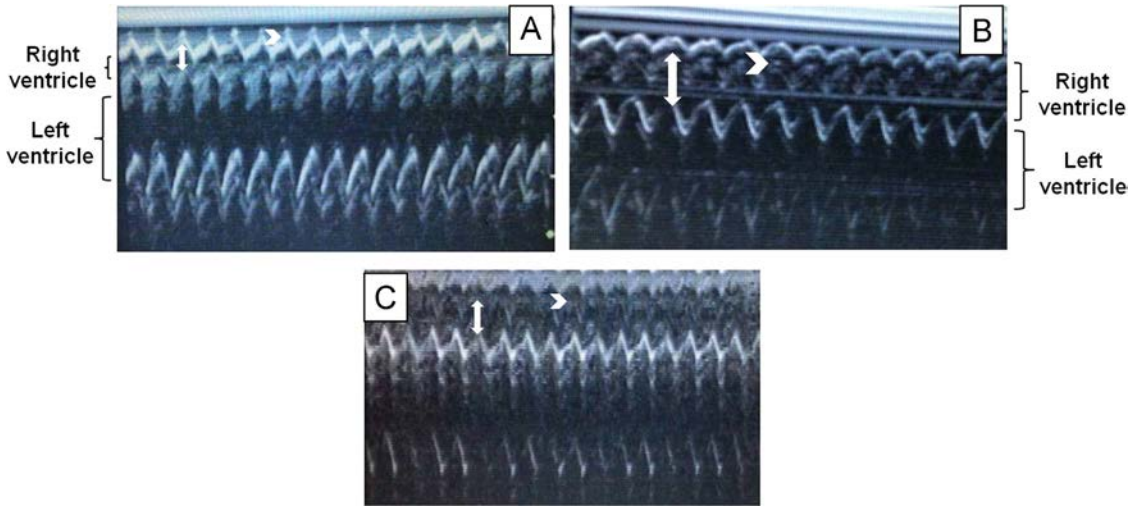

Fig. 1. Effect of diosgenin on monocrotaline (MCT)-induced changes in echocardiography in M-mode in rats revealing right ventricular anterior wall thickness; RVAWT (∑) and right ventricular end-diastolic diameter; RVEDD (⇕). (A) Normal group. (B) MCT group. (C) Diosgenin group.

3.3. Biochemical measurements

MCT treatment induced a state of oxidative stress as indicated by a significant increase in lipid peroxidation (1.17 ± 0.11 vs. 0.72 ± 0.07 nmol/mg protein in lung and 5.20 ± 0.16 vs. 4.17 ± 0.21 nmol/mg protein in RV) and a significant decrease in GSH content (18.05 ± 1.07 vs. 41.25 ± 2.72 nmol/100 mg protein in lung and 34.99 ± 3.30 vs. 57.5 ± 2.05 nmol/100 mg protein in RV) (Fig. 2). Treatment with diosgenin normalized TBARS and GSH in both tissues. Interestingly, diosgenin administration to MCT-treated rats significantly increased lung GSH content above normal value (Fig. 2).

Furthermore, oxidative stress status was associated with a state of inflammation as indicated by a significant elevation of MPO activity (90.64 ± 7.92 vs. 50.96 ± 4.72 mU/mg protein in lung and 19.76 ± 0.64 vs. 13.64 ± 0.48 mU/mg protein in RV) and TNF-α content (766.50 ± 67.21 vs. 451.52 ± 37.91 pg/mg protein in lung and 1116.01 ± 105.03 vs. 615.02 ± 48.11 pg/mg protein in RV) (Fig. 3). Diosgenin significantly ameliorated MPO activity and TNF-α content in both tissues.

The elevation of oxidative stress and inflammatory markers in MCT-treated group was accompanied by about 5 and 7 fold increase in iNOS protein expression in lung and RV, respectively. These results were associated with a decrease in eNOS protein expression to approximately 1/2 and 1/4 of their original values in lung and RV, respectively (Fig. 4). The aforementioned changes were associated with a significant decrease in serum NO_x level (30.8 ± 1.66 vs. 52.01 ± 4.39 μmol/l) and a significant increase in NO_x (9.88 ± 1.08 vs. 5.68 ± 0.17 μmol/g protein in lung and 15.88 ± 1.32 vs. 9.32 ± 1.02 μmol/g protein in RV) (Fig. 5). Treatment with diosgenin significantly decreased iNOS and significantly increased eNOS protein expression. Diosgenin therapy also completely normalized tissue NO_x whereas serum NO_x was significantly increased compared to MCT group (Figs. 4 and5).

The elevation of inflammatory markers was also correlated with a significant increase in caspase-3 activity (52.6 ± 3.68 vs. 22.8 ± 2.21 nmol pNA/h/mg protein in lung and 14.36 ± 0.12 vs. 6.32 ± 0.62 nmol pNA/h/mg protein in RV) and TGF-β content (343.00 ± 29.50 vs. 175.03 ± 15.81 pg/mg protein in lung and 592.02 ± 56.00 vs. 330.11 ± 32.01 pg/mg protein in RV), indicating the incidence of apoptosis and hypertrophy of both tissues, respectively. Treatment with diosgenin caused a significant decrease in caspase-3 activity in both tissues. On the other hand, diosgenin administration completely normalized TGF-β content in lung and RV tissues (Fig. 6).

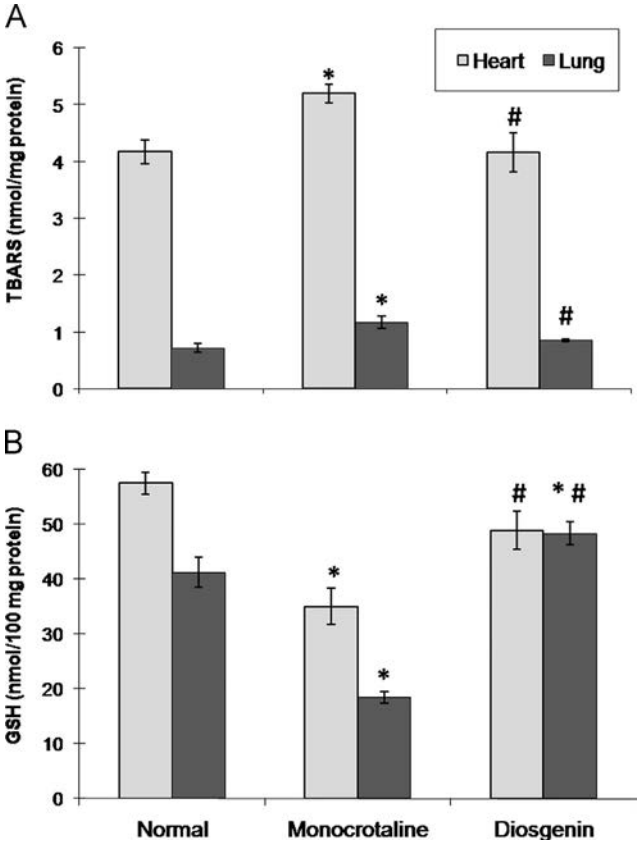

Fig. 2. Effect of diosgenin on monocrotaline (MCT)-induced changes in oxidative stress markers in right ventricular and lung tissues of rats. (A) Thiobarbituric acid reactive substances (TBARS). (B) Reduced glutathione (GSH). Each value represents the mean of 5–8 experiments ± S.E.M. $^{*}P < 0.05$ vs. normal, $^{\#}P < 0.05$ vs. MCT.

3.4. Medial wall thickness of pulmonary arteries and cardiomyocyte cross-sectional area

In MCT group, the percentage of the medial wall thickness of pulmonary arteries significantly increased from $18.13 \pm 1.38\%$ (normal) to $47.25 \pm 3.18\%$ (Fig. 7) and the cardiomyocyte cross-sectional area significantly increased from 173.25 ± 7.10 μm^2 (normal) to 353.75 ± 34.89 μm^2 (Fig. 8). Treatment with diosgenin significantly decreased the medial wall thickness and the

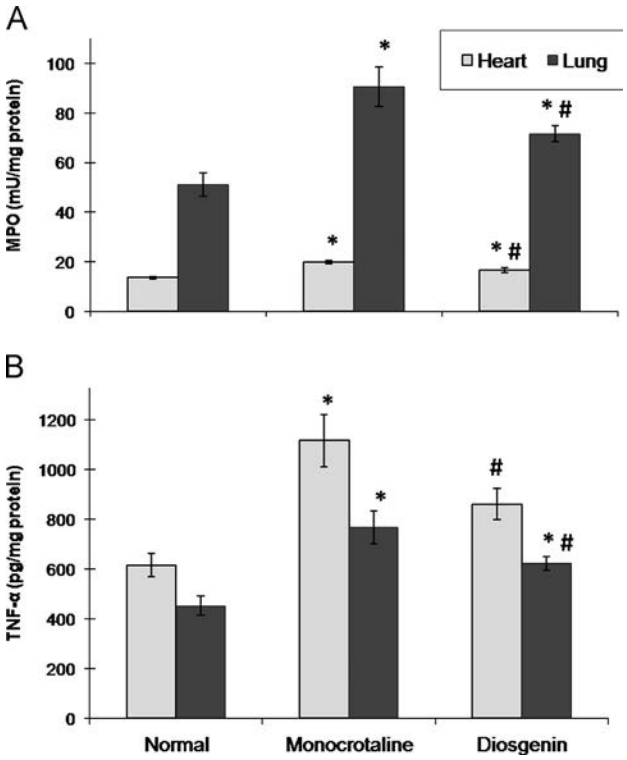

Fig. 3. Effect of diosgenin on monocrotaline (MCT)-induced changes in inflammatory markers in right ventricular and lung tissues of rats. (A) Myeloperoxidase (MPO) activity. (B) Tumor necrosis factor-alpha (TNF-α). Each value represents the mean of 5–8 experiments ± S.E.M. $^{*}P < 0.05$ vs. normal, $^{\#}P < 0.05$ vs. MCT.

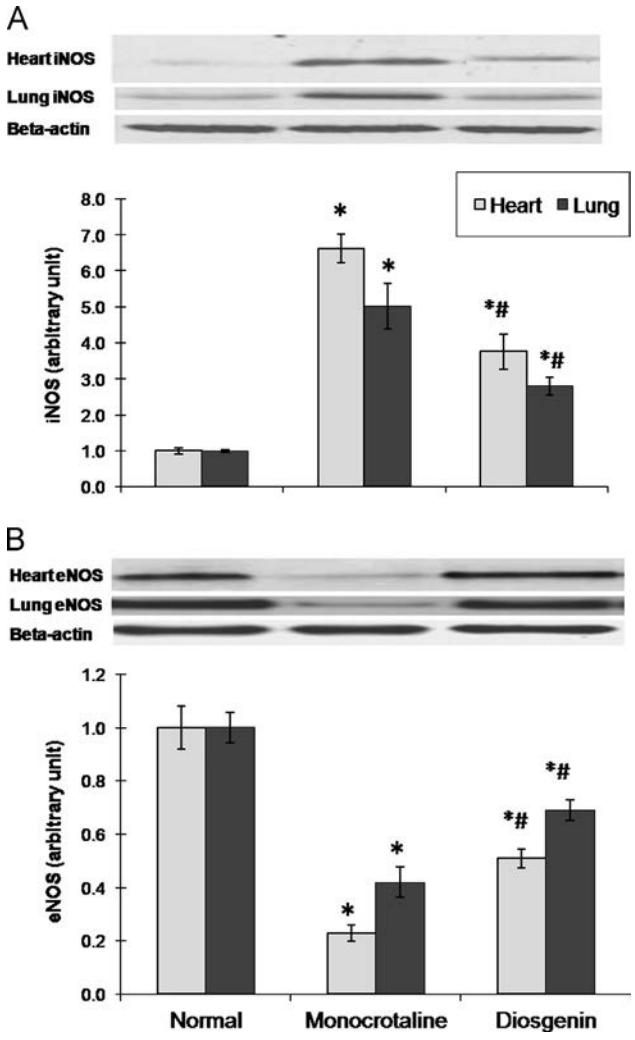

Fig. 4. Effect of diosgenin on monocrotaline (MCT)-induced changes in inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) protein expression in right ventricular and lung tissues in rats. (A) iNOS expression. (B) eNOS expression. Each value represents the mean of 5–8 experiments ± S.E.M. $^{*}P < 0.05$ vs. normal, $^{\#}P < 0.05$ vs. MCT.

cardiomyocyte cross-sectional area compared to MCT group (Figs. 7 and 8).

4. Discussion

The present study was directed to examine the possible protective effects of diosgenin on MCT-induced PH in rats through examination of its effects on associated hemodynamic, biochemical and histological alterations. In the present study, MCT caused a significant decrease in final body weight where rats treated with MCT have previously been reported to be associated with growth restriction and severe anorexia (Molteni et al., 1989; Steffen et al., 2008). The decrease in body weight was accompanied by 27.27% mortality together with a significant increase in RV/body weight and RV/LVS ratios indicating RV hypertrophy and a significant increase in lung/body weight ratio demonstrating the hyperplasia of lung cells and the presence of an extensive proliferative pulmonary response to MCT treatment (Koo et al., 2011).

Furthermore, MCT-treated group showed a significant decrease in heart rate, blood pressure and QRS amplitude together with a significant increase in RVAWT, RVEDD and QRS duration measurements. Electrocardiography and echocardiography are simple noninvasive diagnostic tests for the detection of PH (Henkens et al., 2007). A direct cardiotoxic action of MCT and its associated coronary medial wall thickening may further account for depression of ventricular function (Akhavein et al., 2007). RV hypertrophy and down-regulation of β_1-adrenoceptor may also contribute to impairment of electromotive forces as demonstrated by a decrease in mean QRS vector magnitude (Leineweber et al., 2003; Henkens et al., 2007).

Treatment with diosgenin significantly decreased RV/body weight, RV/LVS and lung/body weight ratios and caused a decrease in mortality percentage reaching 9.09%. Diosgenin therapy also significantly decreased QRS duration, RVEDD and RVAWT compared to MCT group. Diosgenin restored altered electrocardiograms (ECG) in a previous study of isoproterenol-induced myocardial infarction in rats (Salimeh et al., 2011). The improvement in the aforementioned parameters was correlated with the observed amelioration of biochemical parameters and histological changes of pulmonary arteries and RV in the present study.

MCT treatment induced a state of oxidative stress as indicated by a significant increase in lipid peroxidation and a significant decrease in GSH content in lung and RV tissues. Induction of oxidative stress may comprise an obligatory link between MCT treatment and the incidence of cytotoxicity in pulmonary vascular endothelial cells. Increased oxidative stress and reactive oxygen species production in lung and RV tissues play a crucial role in the pathogenesis of MCT-induced PH (Aziz et al., 1997; Kamezaki et al., 2008). Furthermore, this status of oxidative stress was associated with a state of inflammation as indicated by a significant elevation of TNF-α content and MPO activity in both tissues. Inflammation is the main feature of MCT-induced PH as demonstrated by early inflammatory cells recruitment and cytokine activation (Dorfmuller et al., 2003). Oxidative stress is known to regulate the expression of several genes that are involved in the production of inflammatory cytokines, including TNF-α to initiate the proliferative responses (Baeuerle,

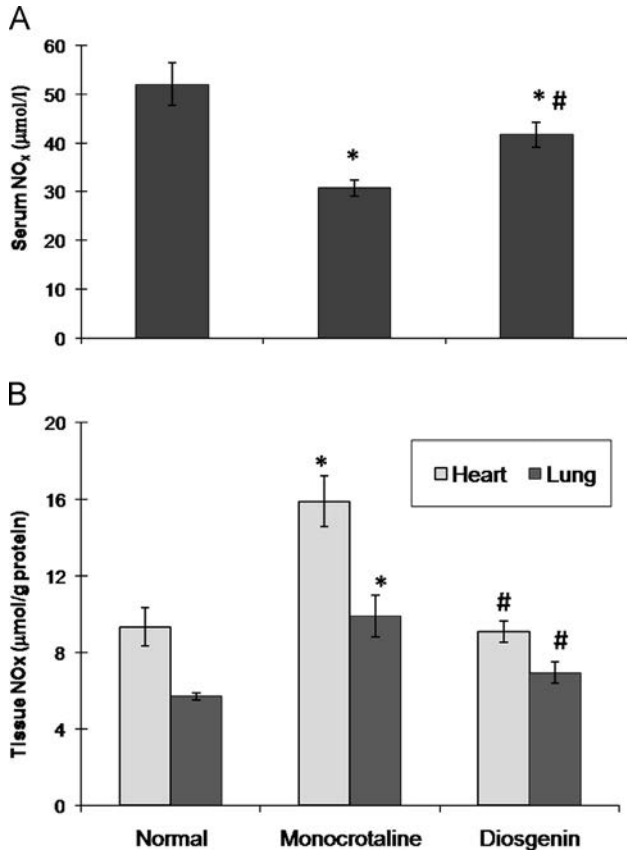

Fig. 5. Effect of diosgenin on monocrotaline (MCT)-induced changes in total nitrate/nitrite (NO_x) in rats. (A) Serum level. (B) Right ventricular and lung tissues contents. Each value represents the mean of 5–8 experiments ± S.E.M. $^{*}P < 0.05$ vs. normal, $^{\#}P < 0.05$ vs. MCT.

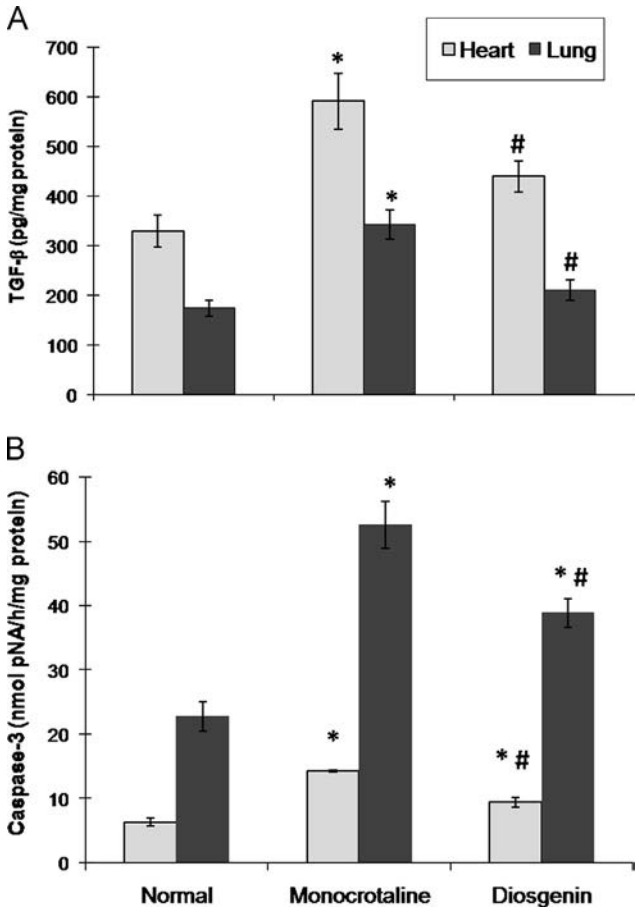

Fig. 6. Effect of diosgenin on monocrotaline (MCT)-induced changes in transforming growth factor-beta (TGF-β) content and caspase-3 activity in right ventricular and lung tissues in rats. (A) TGF-β content (B) caspase-3 activity. Each value represents the mean of 5–8 experiments ± S.E.M. $^{*}P < 0.05$ vs. normal, $^{\#}P < 0.05$ vs. MCT.

1998). The accumulation of perivascular inflammatory cell infiltrates such as neutrophils contributes more to reactive oxygen species production that triggers the destruction of lung tissues and promotes intravascular platelet aggregation (Hassoun et al., 2009). Moreover, the lack of the available NO activates the inflammatory and the proliferative cascades in PH (Chan and Loscalzo, 2008).

Treatment with diosgenin normalized TBARS and GSH contents in both tissues. Interestingly, diosgenin administration to MCT-treated rats significantly increased lung GSH content above normal value. The antioxidant effect of diosgenin was previously reported in experimental models of high-cholesterol fed rats and isoproterenol-induced myocardial infarction (Son et al., 2007; Jayachandran et al., 2009). The molecular mechanism of diosgenin is directly related to its notable ability to scavenge most of the radical species owing to the presence of hydroxyl group in its structure (Hamrita et al., 2012). In addition, diosgenin has been shown to strengthen and up-regulate anti-oxidative defense against free radicals in models of induced oxidative stress (Chiu et al., 2011). The elevated content of GSH by diosgenin treatment is one of the major events in its antioxidant mediated vascular protection (Manivannan et al., 2013).

In the present study, diosgenin significantly ameliorated TNF-α content and MPO activity in both tissues. Diosgenin dose-dependently attenuated sub-acute intestinal inflammation and normalized bile secretion in a previous study of indomethacin-induced intestinal inflammation in rats (Yamada et al., 1997). Another study demonstrated the anti-inflammatory effect of diosgenin in palmitate-induced endothelial dysfunction and insulin resistance. Diosgenin was found to exert a suppressed action on NF-κB gene, which has been identified in the regulation of pro-inflammatory cytokines production (Shishodia and Aggarwal, 2006; Liu et al., 2012).

The elevation of oxidative stress and inflammatory markers in MCT-treated group was associated with up-regulation of iNOS together with down-regulation of eNOS protein expression. The aforementioned changes were associated with a significant decrease in serum NO_x level and a significant increase in NO_x content in both lung and RV tissues. Serum NO_x level gives an indication of NO generation and eNOS activity. eNOS expression and NO production have been reported to decrease in PH (Girgis et al., 2005). This could be related to alteration in L-arginine metabolism and/or decreased bioavailability (Böger and Ron, 2005). Moreover, increased oxidative stress and inflammation could afford an additional contribution to endothelial dysfunction. On the other hand, the increased tissue NO_x content in MCT group revealed the severe inflammatory changes in lung and RV tissues and the contribution of iNOS to the damaging effect through increased production of peroxynitrite in the presence of oxidative stress (Sasaki et al., 2004).The primary target for nitrosative stress is GSH depletion by its nitrosylation which exacerbates oxidative stress and makes the cells more susceptible to toxicity (Kannappan et al., 2010). Therefore, the imbalance in eNOS and iNOS activities and the associated increase in oxidative stress may play an important role in the deleterious effect of PH.

Administration of diosgenin significantly decreased iNOS and significantly increased eNOS protein expression. Diosgenin therapy also completely normalized tissue NO_x content whereas serum NO_x level was significantly increased compared to MCT group.

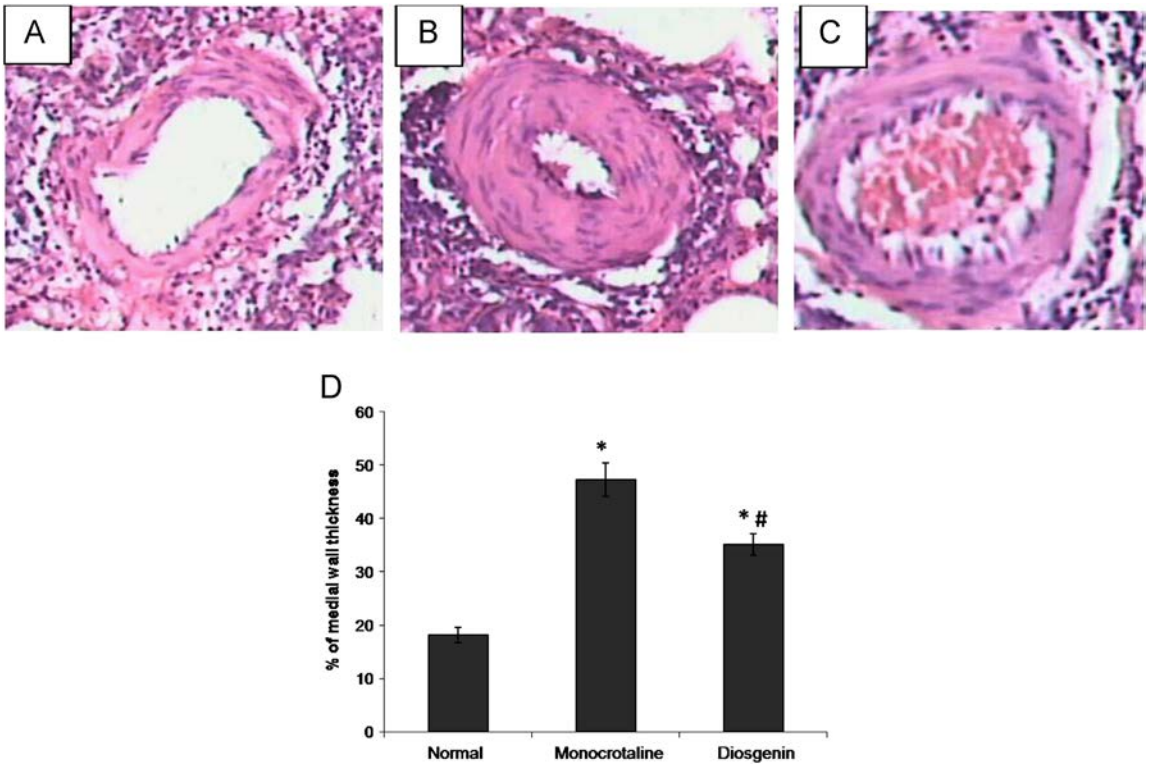

Fig. 7. Effect of diosgenin on monocrotaline (MCT)-induced changes in medial wall thickness of pulmonary arteries in rats. (A) Normal group. (B) MCT group. (C) Diosgenin group (H&E $\times 200$). (D) Percentage of the medial thickness of pulmonary arteries. Each value represents the mean of 3 experiments $\pm$ S.E.M. $^{*}P < 0.05$ vs. normal, $^{\#}P < 0.05$ vs. MCT.

Diosgenin previously improved vascular function by increasing aortic eNOS expression in chronic renal failure rats (Manivannan et al., 2013). Hence, the protective action of diosgenin might be mediated by its ability to increase NO bioavailability through its antioxidant potential. Phytoestrogens also possess protective actions that depend on relaxation of many vascular beds where oestradiol has previously shown to inhibit medial thickening of pulmonary arteries and neomuscularisation in an experimental model of pulmonary hypertension (Farhat et al., 1993; Parker et al., 2000). The protective effect of oestradiol involves an increase in endothelial NO synthase in vascular smooth muscle cells, leading to increased cGMP or reduced endothelin-1 (Parker et al., 2000; Dias et al., 2007).

The elevation of oxidative stress and inflammatory markers was also correlated with a significant increase in TGF-β content and caspase-3 activity in lung and RV tissues indicating hypertrophied tissues and demonstrating the incidence of apoptosis, respectively. Elevations of a variety of growth factors and/or their mRNA have been reported in PH (Arcot et al., 1993; Berg et al., 1998). TGF-β signaling pathways have been implicated in the pathogenesis of PH and the control of many cellular functions including proliferation, differentiation and extracellular matrix secretion and deposition (Schermuly et al., 2011). Moreover, endothelial cell apoptosis in pulmonary vasculature might trigger the pathological vascular remodeling and cellular hyperproliferation which are the hallmarks leading to the progression of PH (Jurasz et al., 2010). Previous study has reported an increased caspase-3 mRNA expression in both lung and RV tissues by MCT treatment (Sun et al., 2009). Increased apoptotic cell death could be related to increased oxidative stress and peroxynitrite generation. The pro-apoptotic mechanisms of peroxynitrite include protein and DNA oxidation, lipid peroxidation, protein nitration and endoplasmic reticulum stress with subsequent increase in caspase activity (Virág et al., 1998; Oyadomari et al., 2001).

Treatment with diosgenin caused a significant decrease in caspase-3 activity in both tissues. On the other hand, diosgenin administration completely normalized TGF-β content in lung and RV tissues. Diosgenin may inhibit proliferation and TGF-β content through the partial preservation of NO. Moreover, inhibition of reactive oxygen species toxicity by diosgenin could be effective in preventing caspase activation and consequent apoptosis. Enhancement and preservation of NO bioavailability may also inhibit neutrophil accumulation in injured tissue and thus neutrophil-induced apoptotic death (Liang et al., 2004). Stimulation of eNOS protein expression as demonstrated by diosgenin therapy could reduce nitrosative stress and decrease apoptosis. Diosgenin has been reported to inhibit hydrogen peroxide-induced human vascular endothelial apoptosis partly through regulating NO pathway (Gong et al., 2010). The anti-apoptotic effects of NO might be related to the ability of NO, through cGMP and cGMP dependent protein kinase G, to increase bcl-2 and heat-shock protein-70 and -32 expressions, thus inhibiting the release of mitochondrial cytochrome c and apoptosis inducing factor (Chung et al., 2001; Liang et al., 2004).

Finally, the developed pulmonary arterial changes and the cardiomyocyte cross-sectional area were correlated with hemodynamic and biochemical changes that were observed in the present study. The percentage of the medial wall thickness of pulmonary arteries and the cardiomyocyte cross-sectional area were significantly increased in MCT group. MCT has been reported to produce vascular endothelial damage, medial-wall thickening and a progressive decrease in lumen diameter in pulmonary arteries (Meyrick et al., 1980). Muscularization and hypertrophy of pulmonary arteries lead to increased vascular resistance, increased pulmonary arterial pressure and subsequent RV hypertrophy (Mitani et al., 1997; Hessel et al., 2006). Treatment with diosgenin significantly decreased the medial wall thickness and the cardiomyocyte cross-sectional area compared to MCT group. Growth

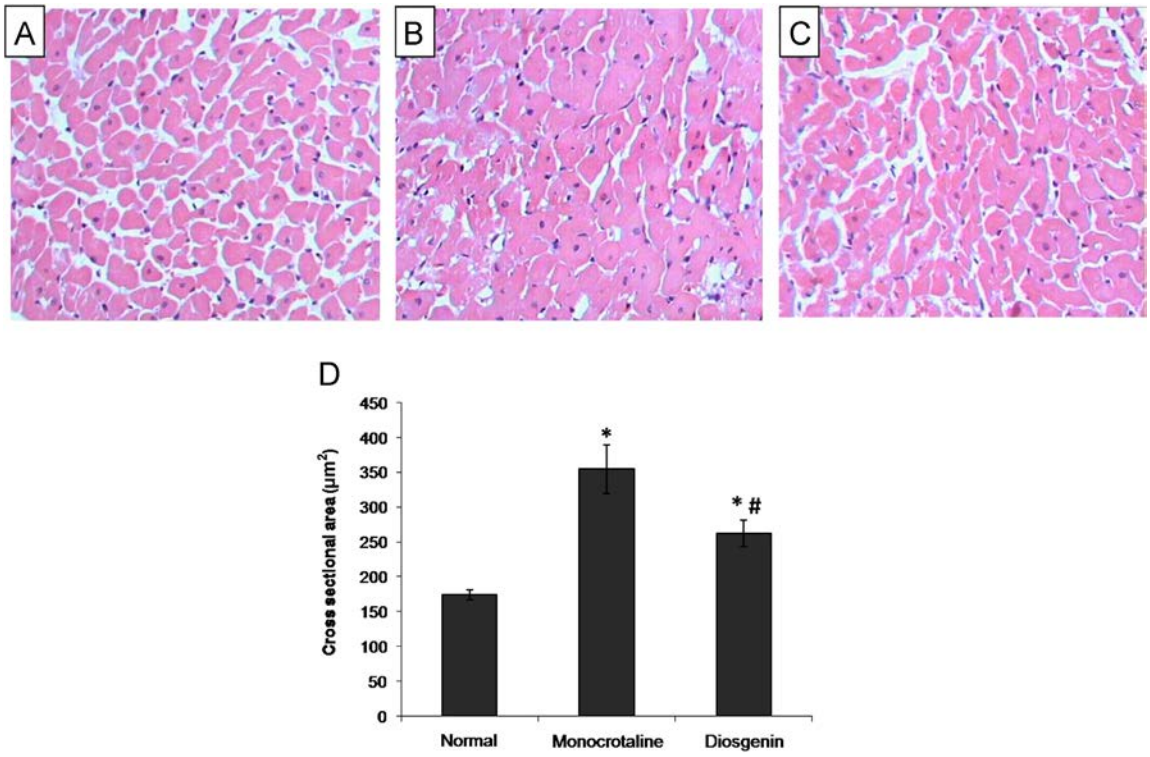

Fig. 8. Effect of diosgenin on monocrotaline (MCT)-induced changes in cardiomyocyte cross-sectional area in RV in rats. (A) Normal group. (B) MCT group. (C) Diosgenin (H&E ×200). (D) Cardiomyocyte cross sectional area in μm^2. Each value represents the mean of 3 experiments ± S.E.M. $^{*}P < 0.05$ vs. normal, $^{\#}P < 0.05$ vs. MCT.

factors and reactive oxygen species have been reported to play an important role in the remodeling of pulmonary arteries in PH (Jeffery and Wanstall, 2001; Aggarwal et al., 2013). Hence, the aforementioned improvement in RV hypertrophy by diosgenin therapy, as indicated by amelioration of the cardiomyocyte cross-sectional area, could be secondary to reduced medial wall thickness of pulmonary arteries and inhibition of reactive oxygen species and TGF-β-induced proliferation.

5. Conclusion

Administration of diosgenin provided a significant protection in MCT-induced PH in rats. Diosgenin therapy was effective toward alleviating oxidative stress, inflammation, apoptosis and histological changes. The protective effect of diosgenin could be mediated through preserving eNOS expression together with inhibiting the deleterious iNOS overexpression and its associated elevation in inflammatory markers. Further studies are necessary to examine the effect of diosgenin intervention in the treatment rather than in the prevention of MCT-induced PH. Moreover, clinical studies are required to establish the effectiveness of this intervention in patients suffering from PH.

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