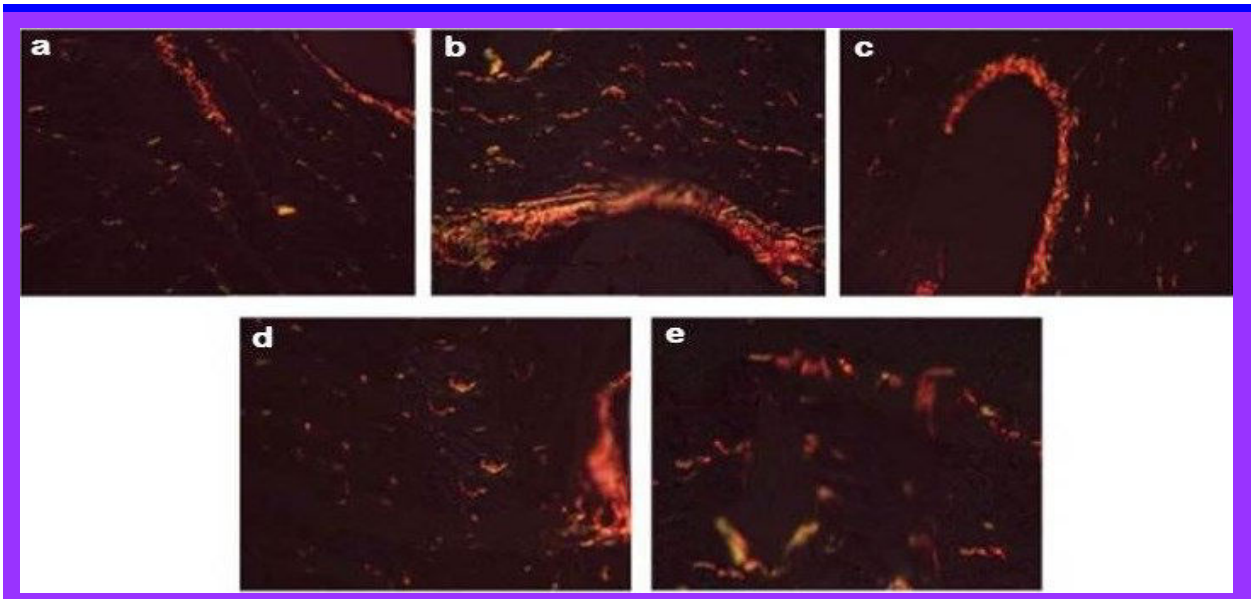

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Research Article

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Protective effects of paeonol on cardiovascular complications in diabetes mellitus involves modulation of PI3K /Akt-GSK-3β signalling, regulation of protease-activated receptor-1 expressions and down-regulation of inflammatory mediators

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Abstract

The present study was taken as an effort to assess the effects of paeonol on diabetic cardiomyopathy. Diabetes was induced in separate groups of Sprague-Dawley rats using streptozotocin. Treatment group animals received paeonol (50, 100 or 200 mg/kg body weight/day; orally) 5 weeks after streptozotocin induction for 6 weeks. Paeonol strikingly reduced myocardial apoptosis and improved cardiac function and myocardial architecture. Serum levels of glucose, reactive oxygen species and inflammatory mediators (TNF-α, IL-6 and IL-1β) were significantly reduced with decreased accumulation of collagen in the cardiac tissue. Paeonol modulated p-Akt, glycogen synthase kinase-3β and glycogen synthase, while significantly down-regulated protease-activated receptor-1, caspase-3, TNF-α, NF-κB p65, and p-Iκ-Bα expressions. Paeonol effectively suppressed diabetic cardiomyopathy by improving myocardial function, regulating the inflammatory responses and Akt signalling.

Introduction

Diabetes mellitus, one of the major health problems worldwide, is increasing alarmingly and by 2030 about 439 million people would be diabetics (Shaw et al., 2010). Diabetic cardiomyopathy, one of the major cardiovascular complications in type-2 diabetes mellitus (Mazzone et al., 2008), is characterized by cardiac dysfunction owing to myocardial structural and functional changes eventually leading to heart failure (Sun et al., 2011).

Accumulating evidences suggest varied mechanisms to be associated with diabetic cardiomyopathy (Wen et al., 2013), including excessive generation of reactive oxygen species (ROS) (Kajstura et al., 2001; Pacher et al., 2007; Wang et al., 2009), activation of inflammatory signals, transcription factors as NF-κB (Aragno et al., 2006; Wang et al., 2009), poly (ADP-ribose) polymerase (Pacher et al., 2002), MAPK cascades (Westermann et al., 2006; Thandavarayan et al., 2009), down-regulation of survival pathways as Akt (Van Linthout et al., 2008), alterations in the extracellular matrix composition (Westermann et al., 2007) and activation of cell death pathways (Frustaci et al., 2000).

ROS-induced oxidative stress, a key factor in diabetic cardiomyopathy etiology (Dewanjee et al., 2009; Bhattacharya et al., 2013) perturbs expressions of several transcription proteins including NF-κB (Bhattacharya et al., 2013). Inflammatory responses critically influence progression of diabetic cardiomyopathy (Lorenzo et al.,

2011; Teixeira-Lemos et al., 2011) and cytokines as TNF-α, IL-6 and IL-1β are reported to potentially cause cardiac injury (Bhattacharya et al., 2013).

Furthermore, protease-activated receptors (PARs) are crucial modulators of inflammation (Cocks and Moffatt, 2000). PAR1, up-regulated by oxidative stress (Nguyen et al., 2001) has been shown to influence Akt signalling (Latha et al., 2015). Glycogen synthase kinase-3β (GSK-3β), a key effector, downstream of Akt, is the chief regulator of glycogen synthase in heart and skeletal muscle (Markou et al., 2008; Patel et al., 2008). In the presence of insulin, activated Akt phosphorylates and inhibits GSK-3β causing activation of glycogen synthase (Muniyappa et al., 2007). In diabetes, GSK-3β is activated by decreased phosphorylation (Montanari et al., 2005; Lajoie et al., 2004) and it negatively regulates glycogen synthase and inhibits glycogen synthesis, indicating the critical role of GSK-3β. Thus, Akt signalling could be a potential target in diabetic cardiomyopathy.

Paeonol, a major phenolic compound of genus *Paeonia*, is used as a nutrient supplement and in Chinese medicine (Deng et al., 2006). It possess bioactive properties as anti-inflammatory (Du et al., 2010; Siu, 2010; Himaya et al., 2012; Lin et al., 2015), neuroprotective (Hsieh et al., 2006; Wu et al., 2008; Su et al., 2010) and cardioprotective effects (Li et al., 2012). Considering these effects we investigated if paeonol regulated inflammatory responses and Akt/GSK-3β signalling in diabetic cardiomyopathy.

Materials and Methods

Animals

Sprague-Dawley rats weighing 180-220 g (Central South University Animal Centre, China) were used for this study. The animals were kept in standard animal cages on a 12 hours/12 hours dark/light cycle with free access to standard rat chow and tap water.

Reagents and antibodies

Antibodies against Akt, p-Akt, GSK-3β, p-GSK-3β,TNF-α, NF-κB p65, p-IκBα, caspase-3 were procured from Santa Cruz Biotechnology, USA. Antibodies against glycogen synthase, p-GS were obtained from Cell Signalling Technology (USA). Paeonol and streptozotocin was obtained from Sigma-Aldrich, MO, USA. All chemicals and reagents used in the study were obtained from Sigma-Aldrich, USA unless otherwise specified.

Induction of diabetes and administration of paeonol

Diabetes was induced in the animals by a single injection of streptozotocin, intraperitoneally (50 mg/kg) after 8-12 hours of starvation. Streptozotocin was dissolved in 0.1 M sodium citrate buffer, pH 4.5 (Sun et al., 2011). Control rats were not induced with streptozotocin and were administered equal volumes of citrate buffer. After 72 hours of streptozotocin injection, the blood glucose level was measured by tail vein puncture using glucometer (Accu-Chek, Germany). Animal with a random serum glucose level >200 mg/dL was considered as diabetic. Blood glucose level was monitored periodically. Following 5 weeks of diabetic induction the diabetic rats were grouped separately. Treatment groups received paeonol at 50, 100 or 200 mg/kg orally every day starting from 36th day for a period of 6 weeks. Animals that did not receive paeonol were grouped as diabetic control. At the end of the experimental protocol the cardiac function measurements were obtained, rats were sacrificed under isoflurane anesthesia and their heart tissues were harvested for further analysis. Whole blood from the aorta was collected and serum was separated and used.

Assessment of cardiac function

On the day one before streptozotocin injection, 5 and 11 weeks after streptozotocin induction cardiac function was assessed by echocardiography using GE Vivid 7 ultrasound with 10-MHz transducer (General Electric, USA) as described by Wen et al. (2013) with slight modifications. The rats were exposed to isoflurane anesthesia (3%) and were placed in the supine position and the left ventricular end-diastolic diameter (LVEDD) and left ventricular end-systolic diameter (LVESD) were measured on the parasternal left ventricular long axis view. The measurements taken represent the mean of consecutive 5 cardiac cycles. The LV end-diastolic volume (LVEDV), LV end-systolic volume (LVESV) and LV fractional shortening (LVFS), LV ejection fraction (LVEF) were calculated by use of computer algorithms of the ultrasound systems. All the measurements were taken by an investigator who was blinded to the experimental grouping.

Histopathology and immunohistochemistry

Heart tissue samples were fixed in 4% buffered paraformaldehyde and were embedded in paraffin. Histological architecture was assessed by staining with hematoxylin and eosin. Tissue sections of 3 µm thickness were fixed on slides and were stained with hematoxylin and eosin and then observed by light microscopy (Nikon Eclipse 400). Collagen content of the cardiac tissues (5 µm section) was assessed using sirius red stain.

Content of collagen I and III and expression of cleaved caspase-3 in the heart tissues were examined by immunohistochemistry. The tissue sections were blocked with 5% bovine serum albumin in tris buffered saline (pH 7.4) for about 2 hours. Separate sections were then incubated with primary antibody (PAR1) (Santa Cruz Biotechnology Inc., USA), cleaved caspase-3 antibody (Cell signalling Technology, USA), antibody

against collagen I (Col I; Abcam, USA) or collagen III (Col III; Abcam, USA) overnight at 4°C. Following incubation, the slides were washed and incubated further with biotin-labelled secondary antibody (Vector lab, USA) for 1 hour at room temperature followed by color development with diaminobenzidine solution (DAB) (Vector lab, USA). The positive signals were quantified with Image Pro Plus 6.0 software.

Determination of myocardial apoptosis

Myocardial apoptosis was determined by TUNEL (Terminal deoxyribonucleotidyl transferase-mediated dUTP-biotin nick end labelling) staining. Heart tissue samples were fixed in 4% buffered paraformaldehyde and were embedded in paraffin. The tissue sections were deparrafinized by washing with xylene, rehydrated in graded alcohol series and then immersed with PBS. The assay was carried out according to manufacturer's instructions (R and D systems, USA). In brief, the de-parrafinised tissue sections were incubated with 20 µg/mL proteinase K for 15 min followed by washing with PBS and then incubated with TdT labelling buffer for 5 min. The sections were treated with labelling reaction mix and incubated for about 1 hour. After washing the sections 50 µL of Strep-HRP solution and incubated for 10 min at 37°C. The cells were then stained with 3, 3'-diaminobenzidine (DAB) counter-stained and were visualised.

Western blot analysis

Proteins were isolated from homogenized heart tissue using Trizol reagent (Invitrogen, USA) as previously described (Gao et al., 2008; Wei et al., 2010). Protein concentrations were determined by Bradford assay using protein assay kit from Bio-Rad (Bio-Rad Laboratories, USA). Equal amount of protein (50 µg) from each group were separated elctrophoretically on a SDS-PAGE. The gel was blotted and transferred on to nitrocellulose membrane and incubated with primary antibodies against Akt, p-Akt (ser 473), p-GSK-3β (ser 9), GSK-3β, p-NF-κB p65 (ser 536), p-IκBα, TNF-α, glycogen synthase and p-GS overnight at 4°C. Membranes were washed thrice with TBST buffer and further incubated with secondary antibodies coupled to horseradish peroxidase (1:1000 dilution, Santa Cruz Biotechnology) for 2 hours at room temperature. The immunoreactive bands were visualized with a chemiluminescene system (Amersham Bioscience, UK). The signals were quantified by densitometry.

ELISA

Serum concentrations of TNF-α, IL-1β, IL-6 were measured by enzyme-linked immunosorbent assay (ELISA) (R and D systems, USA) in accordance to the manufacturer's instructions. Serum concentrations of TNF-α, IL-1β, IL-6 were measured by enzyme-linked immunosorbent assay (ELISA) (R and D systems, USA) in accordance to the manufacturer's instructions. In brief 50 µL of the serum sample was added to each well and incubated with the assay diluent for about 2 hours following incubation with specific antibody conjugate and incubated further for 2 hours, after washing 100 µL of the substrate solution was added to each well. The color developed was read at 450 nm.

Determination of ROS

Total ROS in serum were determined using Oxitest™ ROS/RNS assay kit (Cell Biolabs, USA) according to manufacturer's instructions. The assay employs dichlorodihydrofluorescein (DCFH), fluorogenic probe. In the presence of ROS, DCFH is rapidly oxidized to highly fluorescent DCF. Fluorescence is read on a standard fluorometric plate reader at 480 nm excitation/530 nm emission. Briefly, 50 µL of the serum sample 96-well plate and 50 µL of the catalyst was added to each well and incubated for 5 min followed by addition of 100 µL of DCFH and incubated further at room temperature for about 30 min and the fluorescence was measured.

Statistical analysis

The data are presented as mean ± SD from three or six independent experiments. The statistical significance between the groups were analysed by ANOVA (one-way analysis of variance) at $p<0.05$ followed by Duncan's multiple range test (DMRT) for post hoc analysis.

Results

Paeonol reduced blood glucose levels

Diabetic animals presented elevated ($p<0.05$) levels of blood glucose. However rats treated with paeonol exhibited decreased blood glucose levels compared to diabetic control animals (Figure 1). In addition, the trend of decline in the blood glucose levels were more in the rats administered with 100 and 200 mg doses of paeonol than 50 mg. A periodic decrease in the levels was observed on paeonol treatment.

Paeonol significantly reduced the deposition of collagen

Collagen deposition is a feature observed in cardiomyopathy. Collagen deposition affects the mechanical properties of the myocardium and also affects the performance. In our study, marked changes in the deposition of total collagen were observed through sirius red staining (Figure 2). Significantly increased collagen content in the perivascular and as well in interstitial sites in the diabetic control animals was observed. Further immunohistochemistry studies revealed increased deposition of both collagen I and collagen III content in the cardiac tissue of diabetic rats. Paeonol reduced the deposition of collagen content, thus reducing cardiac fibrosis (Figure 3).

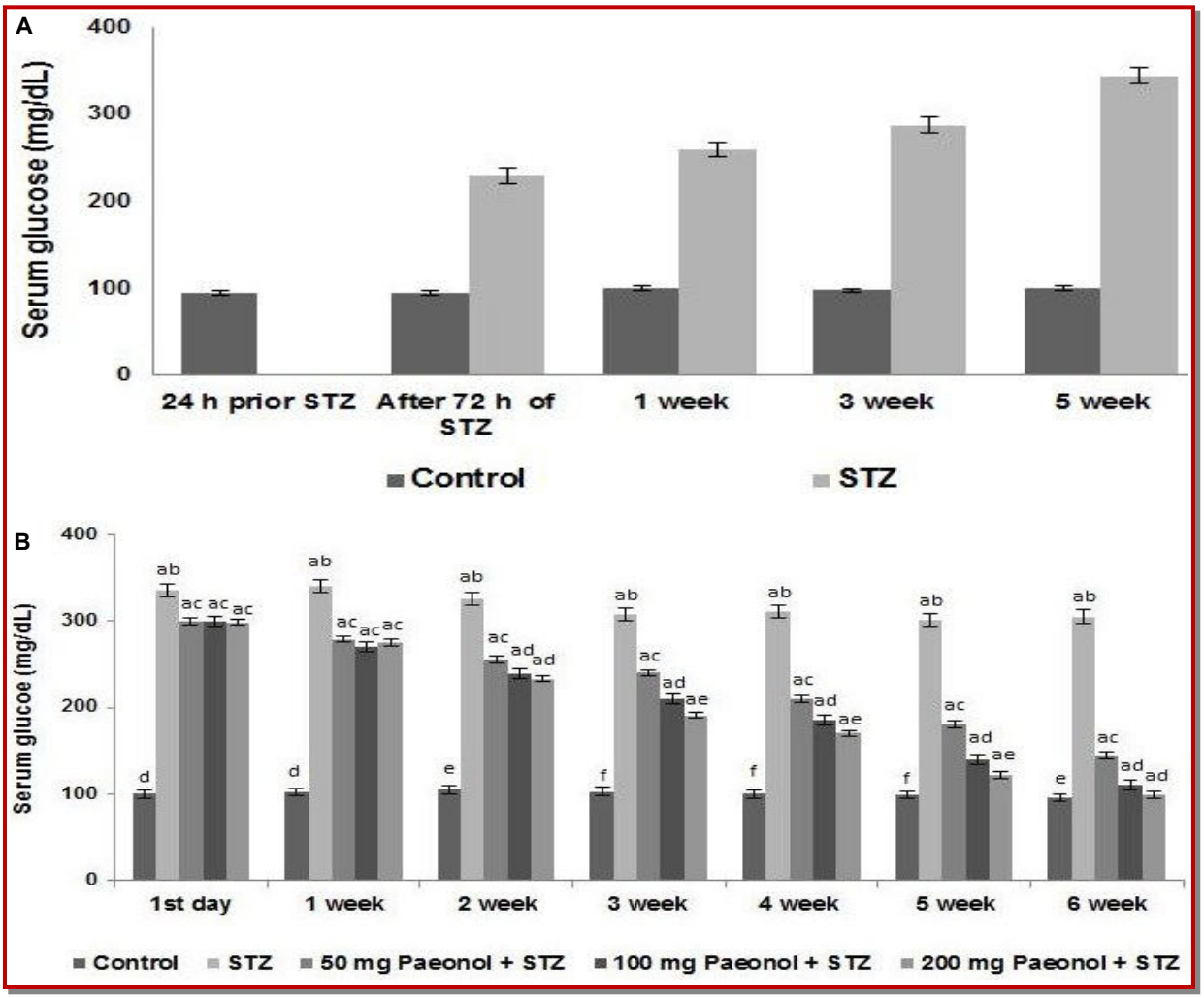

Figure 1: Effect of paeonol on serum glucose levels following streptozotocin induction

Values are represented as mean ± SD, n=6. a represents statistical significance at $p<0.05$ compared against respective controls and b-f represents significant difference ($p<0.05$) between mean values within the groups as determined by one-way ANOVA followed by DMRT analysis

Histological changes

The tissue sectioning of control rats not induced with streptozotocin or treated with paeonol showed normal cardiac tissue with striated branched muscle fibres with homogeneous cytoplasm (Figure 4). Diabetes mellitus lead to a vast alterations in the myocardium. Severe degeneration of cardiac cells with interstitial fibrosis was observed. The sections exhibited irregular orientation of muscle fibres with shruken nuclei and condensed sarcoplasm. However, paeonol treatment substantially reversed the orientation of muscle fibres and improved the architecture of the myocardium. Treatment with paeonol at 200 mg noticeably restored the histology to almost near normal. The results observed were found to be dose-dependent with the highest dose exhibiting the maximum protective effects.

Paeonol improved cardiac functioning

Cardiac functioning was performed at baseline 5 weeks after streptozotocin injection and 6 weeks after paeonol treatment (Table I). The observed results indicated that paeonol improved LVEF and LVFS in diabetic rats compared with the diabetes group. However, paeonol restored the systolic and diastolic diameters (LVESD and LVEDD). It also significantly reduced LVESV and LVEDV. The results suggest that paeonol administration strikingly improved the cardiac function.

Paeonol markedly inhibits apoptosis of cardio-myocytes

As represented in Figure 5, paeonol remarkably reduced apoptotic cell counts in the cardiac tissue. TUNEL positive cells were found to be reduced by paeonol in a dose-dependent manner. Further, immunohistochemistry analysis for activated caspase-3 expressions also supported the results. Increase in caspase 3 positive cell counts ($p<0.05$) in the cardiac tissue of DM control rats were significantly reduced on paeonol treatment, with 200 mg

dose exhibiting the maximum anti-apoptotic effects.

Paeonol modulated the PI3K/Akt/GSK-3β signalling cascade

To further elucidate the possible mechanisms involved in the cardioprotective effects of paeonol, we investigated the effects of paeonol on the PI3K/Akt/GSK-3β signalling. The critical role of Akt in regulating glycogen synthesis is well documented, cell growth and survival. GSK-3β, a critical downstream element of Akt that is inhibited by phosphorylation regulates the activity of glycogen synthase, thereby regulating glycogen synthesis. The level of GSK-3β was enhanced in diabetic rats. However, the levels of Akt and p-Akt were supressed in diabetic rats. Enhanced p-GS expression was in line with GSK-3β level, suggesting the inhibition of glycogen synthase activity and glycogen synthesis contributing to enhanced blood glucose level (Figure 6). Paeonol interestingly caused a multifold increase in the phosphorylated level of Akt and GSK-3β with

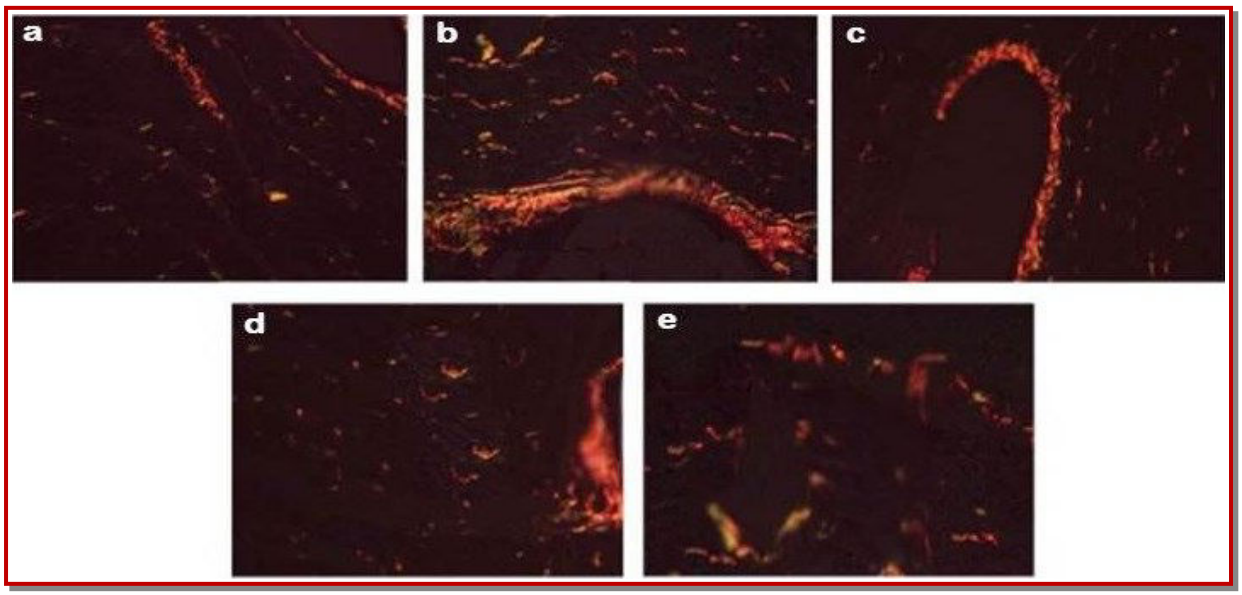

Figure 2: Sirius red staining showing the effect of paeonol on collagen deposition in cardiac tissues. The myocardium of the diabetic control rats presents with raised collagen accumulation (b) as compared against normal control rats (a) Paeonol treatment caused marked decreased collagen deposition in the myocardium as seen by sirius red staining (c-e); (a) Control; b) streptozotocin; c) 50 mg paeonol + streptozotocin; d) 100 mg paeonol + streptozotocin; e) 200 mg paeonol + streptozotocin)

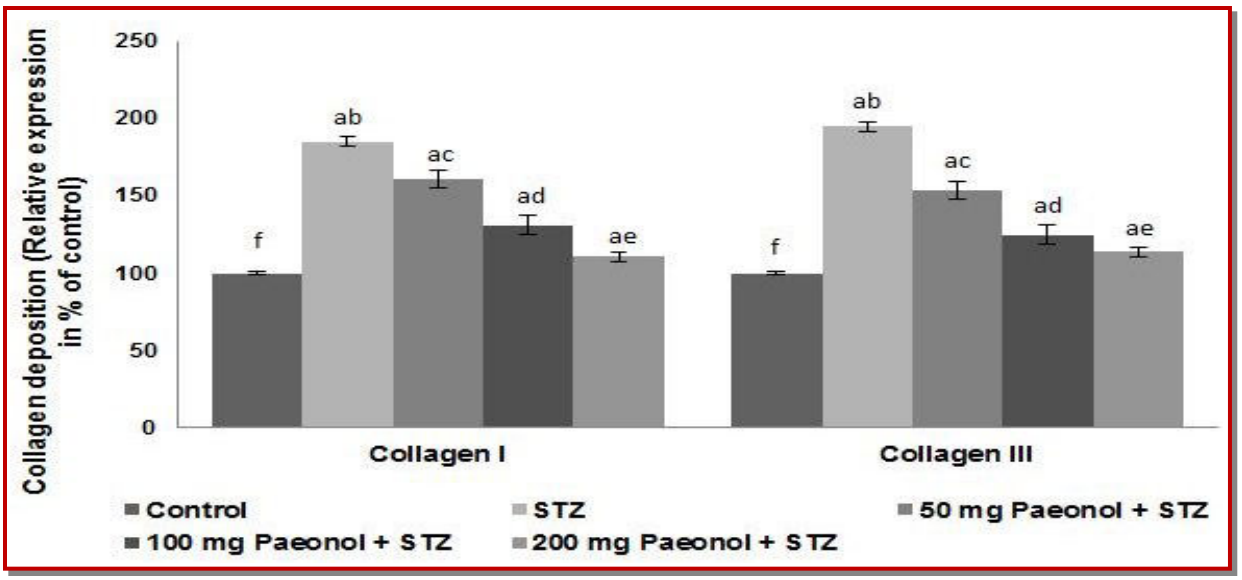

Figure 3: Effect of paeonol on collagen deposition in cardiac tissues

Values are represented as mean ± SD; n=6; a represents statistical significance at $p<0.05$ compared against respective controls and b-f represents significant difference ($p<0.05$) between mean values within the groups as determined by one-way ANOVA followed by DMRT analysis

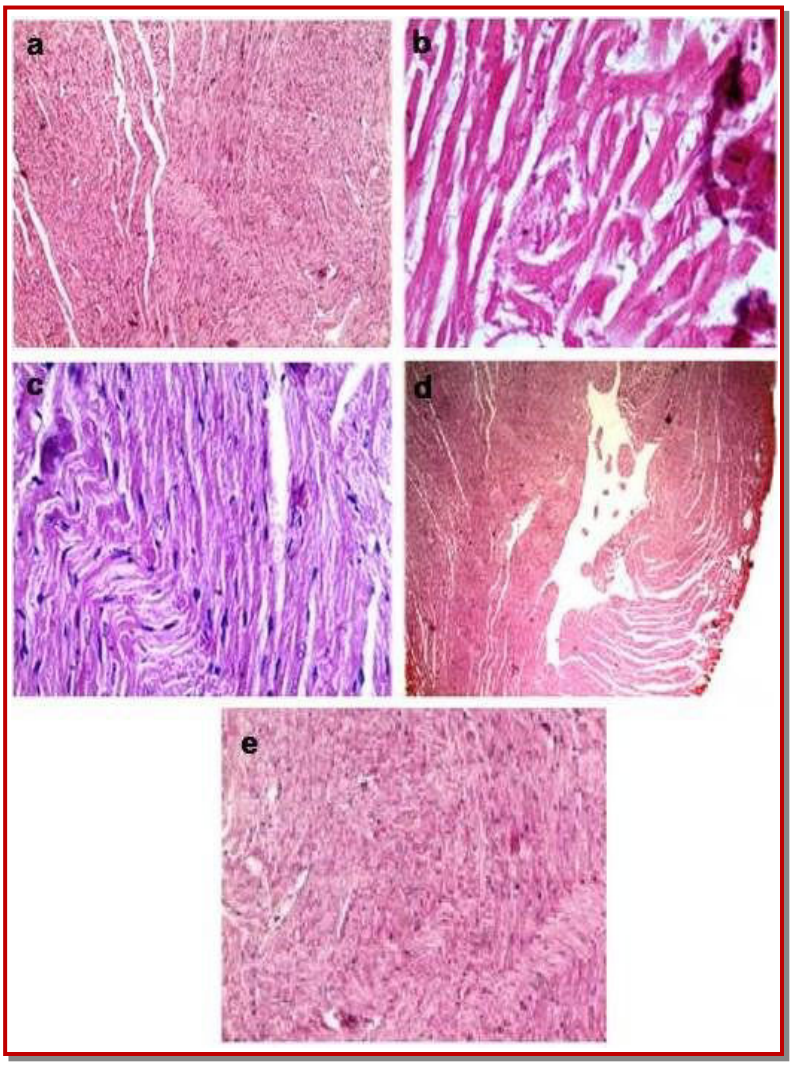

Figure 4: Histological section showing the effect of paeonol restored the normal myocardial architecture. Marked alterations in the myocytes and shrunken nuclei observed in cardiac tissues of diabetes-induced rats (b) as compared to normal control (a). These aberrant alterations were effectively restored on paeonol treatment (c-e). Paeonol treatment normalised the orientation of the cardiac fibrils and reduced fibrosis of the myocardium. (a) Control; b) streptozotocin; c) 50 mg paeonol + streptozotocin; d) 100 mg paeonol + streptozotocin; e) 200 mg paeonol + streptozotocin)

enhanced glycogen synthase expression, thus promoting glycogen synthesis and reduction of blood glucose level. Increased p-Akt caused increased p-GSK-3β, which further resulted in activation of glycogen synthase. These observations suggest the capacity of paeonol in promoting glycogen synthesis and also reducing blood glucose level as well.

Anti-oxidant and anti-inflammatory effects of paeonol

Hyperglycemia increases glucose oxidation and generation of ROS. ROS has been suggested to be involved in cardiac dysfunctions in diabetic conditions (Wang et al., 2011). Further, oxidative stress is associated with inflammation (Zhang et al., 2010). Inflammatory responses have been known to play significant influence on the cardiovascular complications in diabetes. We assessed the influence of paeonol on inflammatory markers and ROS levels. Significant ($p<0.05$) elevation in the expression levels of NF-κB p65 and p-IκBα was observed in diabetic control rats (Figure 7). The transcription factor, NF-κB is well known to play a crucial role in the regulation of various genes involved in inflammatory responses (Sun et al., 2011). Increase in p-IκBα contributes to release of active NF-κB. The serum levels of inflammatory mediators - IL -6, IL-1β and TNF-α were found to be increased several fold in diabetic control rats (Figure 7). Paeonol treatment decreased the expression of NF-κB p65 and p-IκBα and serum levels of inflammatory mediators and raised the level of ROS. Expression of TNF-α protein correlated with the serum levels. TNF-α is an important pro-inflammatory cytokine and also a valuable marker in heart failure (Miettinen et al., 2008; Vaz Perez et al., 2010). Thus, significant reduction in the levels of TNF-α

Table I

Influence of paeonol on cardiac functioning

	LVEDD (mm)			LVESD (mm)		
Groups	Baseline	5 weeks after STZ and prior paeonol treatment	6 weeks after Paeonol treatment	Baseline	5 weeks after STZ and prior paeonol treatment	6 weeks after Paeonol treatment
Control	2.8 ± 0.1[a]	2.8 ± 0.0[b]	2.8 ± 0.1[c]	1.6 ± 0.1[a]	1.6 ± 0.1[b]	1.6 ± 0.1[d]
STZ Control	2.7 ± 0.1[a]	3.4 ± 0.2[a]	3.4 ± 0.2[a]	1.5 ± 0.1[a]	2.3 ± 0.1[a]	2.3 ± 0.2[a]
50 mg Paeonol + STZ	2.7 ± 0.1[a]	3.4 ± 0.2[a]	3.0 ± 0.1[b]	1.5 ± 0.1[a]	2.2 ± 0.1[a]	1.9 ± 0.1[b]
100 mg Paeonol + STZ	2.7 ± 0.1[a]	3.3 ± 0.2[a]	2.9 ± 0.2[b]	1.6 ± 0.1[a]	2.2 ± 0.2[a]	1.8 ± 0.1[c]
200 mg Paeonol + STZ	2.8 ± 0.2[a]	3.4 ± 0.1[a]	2.8 ± 0.3[c]	1.6 ± 0.2[a]	2.3 ± 0.1[a]	1.8 ± 0.2[c]
	LVESV (mL)			LVEDV (mL)		
Groups	Baseline	5 weeks after STZ and prior paeonol treatment	6 weeks after Paeonol treatment	Baseline	5 weeks after STZ and prior paeonol treatment	6 weeks after Paeonol treatment
Control	0.3 ± 0.1[a]	0.3 ± 0.1[b]	0.2 ± 0.0[c]	1.1 ± 0.1[a]	1.1 ± 0.1[c]	1.1 ± 0.0[c]
STZ Control	0.2 ± 0.1[a]	0.4 ± 0.0[a]	0.4 ± 0.0[a]	1.0 ± 0.0[a]	1.2 ± 0.1[a]	1.2 ± 0.1[a]
50 mg Paeonol + STZ	0.3 ± 0.0[a]	0.4 ± 0.0[a]	0.3 ± 0.0[a]	1.1 ± 0.1[a]	1.2 ± 0.0[a]	1.2 ± 0.1[a]
100 mg Paeonol + STZ	0.3 ± 0.0[a]	0.4 ± 0.0[a]	0.3 ± 0.1[b]	1.1 ± 0.1[a]	1.2 ± 0.1[a]	1.1 ± 0.0[b]
200 mg Paeonol + STZ	0.2 ± 0.0[a]	0.4 ± 0.1[a]	0.2 ± 0.0[c]	1.1 ± 0.1[a]	1.3 ± 0.1[b]	1.0 ± 0.1[c]
	LVEF (%)			LVFS (%)		
Groups	Baseline	5 weeks after STZ and prior paeonol treatment	6 weeks after Paeonol treatment	Baseline	5 weeks after STZ and prior paeonol treatment	6 weeks after Paeonol treatment
Control	76.5 ± 3.4[a]	75.3 ± 5.8[a]	76.7 ± 3.5[a]	45.2 ± 1.5[a]	45.2 ± 2.5[a]	46.0 ± 1.0[a]
STZ Control	78.7 ± 4.8[a]	68.3 ± 4.5[b]	69.1 ± 4.2[b]	44.5 ± 2.1[a]	32.8 ± 3.1[b]	33.2 ± 3.5[c]
50 mg Paeonol + STZ	77.1 ± 2.2[a]	66.1 ± 3.0[b]	71.2 ± 2.2[b]	43.3 ± 1.2[a]	33.7 ± 2.1[b]	38.2 ± 1.9[b]
100 mg Paeonol + STZ	76.3 ± 1.4[a]	64.1 ± 2.1[b]	73.0 ± 6.5[b]	44.3 ± 1.9[a]	33.5 ± 2.0[b]	41.2 ± 2.7[b]
200 mg Paeonol + STZ	76.1 ± 4.5[a]	65.9 ± 6.1[b]	78.9 ± 7.6[a]	45.1 ±2.2[a]	32.7 ± 1.5[b]	46.0 ± 1.1[a]

LVEDD- Left-ventricular end diastolic diameter; LVESD- Left-ventricular end systolic diameter; LVEDV- Left-ventricular end diastolic volume; LVESV- Left-ventricular end systolic volume; LVFS- Left ventricular fractional shortening; LVEF- Left ventricular ejection fraction; Paeonol effectively improved the cardiac functioning following streptozotocin (STZ) induction; Values are represented as mean ± SD; n=6; Values within the same column not sharing a common alphabet differ significantly at $p<0.05$ as determined by one-way ANOVA followed by DMRT analysis

and also ROS levels following paeonol treatment indicates the protective effects of paeonol on cardiac tissues in diabetes.

Paeonol reduces the expressions of PAR1

As presented in Figure 8, diabetes mellitus-induced rats presented diabetic cardiomyopathy and expressed elevated number of stained PAR1 positive cells ($p<0.05$) as compared to control rats. Treatment with paeonol considerably reduced the PAR1 expression in a dose-dependent manner. Activation of PAR1 has been reported to mediate inflammatory responses and in contractility (Steinberg, 2005). Thus, down-regulation of PAR1 in paeonol treatment suggests the anti-inflammatory effects.

Discussion

Paeonol effectively reduced blood glucose levels over the period of treatment, suggesting the anti-hyperglycemic effects. In addition paeonol significantly attenuated oxidative stress which was evidenced by the observed striking decrease in ROS level and also reduced apoptosis of cardiomyocytes. TUNEL positive cell counts drastically reduced on paeonol treatment. Anti-apoptotic effect of paeonol was also revealed by substantial reduction in cleaved caspase-3 positive cell counts.

Diabetic cardiomyopathy exhibits ventricular dysfunction with increased risk of heart failure (Tarquini et al., 2012). Consistently with previous reports (Sun et al.,

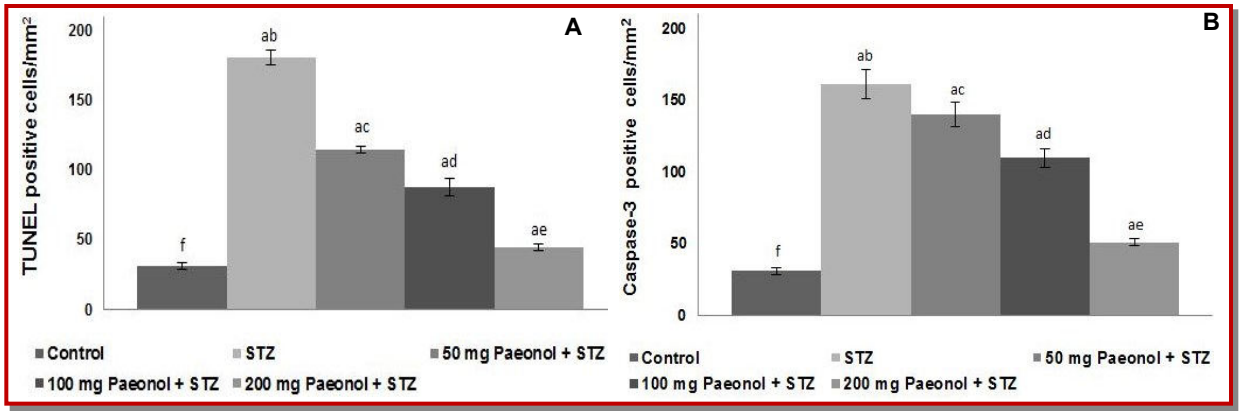

Figure 5: Paeonol reduces the intensive apoptosis in diabetic cardiomyopathy following streptozotocin (STZ) induction. Paeonol reduced the apoptotic cell counts markedly as determined by decrease in TUNEL positive cells (A) and also decreased the expression of cleaved caspase-3 (B)

Values are represented as mean ± SD, n=6. a represents statistical significance at $p<0.05$ compared against respective controls and b-f represents significant difference ($p<0.05$) between mean values within the groups as determined by one-way ANOVA followed by DMRT analysis

2011; Yu et al., 2012), streptozotocin-induced rats exhibited features of diabetic cardiomyopathy with declined diastolic and systolic myocardial performance and excessive accumulation of collagen and severe alteration in cardiac tissue architecture. Perinuclear vacuolization, destruction and loss of myofibrils, shrunken nuceli, irregular orientation of fibrils, swollen mitochondria were observed in the cardiac tissue of streptozotocin-induced diabetes mellitus control rats. Paeonol markedly reversed the orientation and normalized alterations in myofilaments.

Many phytochemicals have the capacity to neutralize free radicals in diabetes (Jovanovic et al., 2001; Rajesh et al., 2010) and also exhibit anti-apoptotic effects (Rajesh et al., 2010; Sun et al., 2011). Oxidative stress due to excessive free radicals has been reported to play crucial roles in the development of cardiac failure and left ventricular dysfunctioning and remodelling in diabetic cardiomyopathy (Rajesh et al., 2010). Hyperglycemia-induced oxidative stress is considered as a major risk factor contributing to the vascular pathogenesis in the diabetic myocardium, that leads to fibrosis, hypertrophy of the cardiac tissue, myocardial cell death and endothelial dysfunction (Cai and Kang, 2003; Fang et al., 2004; Li et al., 2005). Wang et al. (2011) reported exacerbated glucose oxidation in hyperglycemia that leads to generation of ROS, which subsequently causes DNA damage and apoptosis. Earlier reports have demonstrated myocardial apoptosis in diabetic patients (Frustaci et al., 2000) as well in diabetic animal models (Kajstura et al., 2001; Cai et al., 2002). Furthermore, apoptosis associated with raised level of oxidative stress in multiple organ systems in diabetes mellitus has been well documented (Alici et al., 2000; Cai et al., 2000). Thus, striking suppression of ROS levels could have attributed to the decrease in the apoptotic counts suggests the protective effect of paeonol on cardiac tissue.

Immunohistochemical analysis revealed accumulation of collagen I and III, which account for major collagen in the cardiac tissue, resulting in diabetes- induced cardiac fibrosis. Previous studies have shown alterations in the extracellular matrix associated with cardiac fibrosis (Tschope et al., 2004; Westermann et al., 2007; Zhang et al., 2008). Thus, paeonol-induced striking decreases in collagen accumulation of the cardiac tissues, suggests its capacity in inhibiting cardiac fibrosis. In our study, paeonol significantly attenuated the cardiac systolic and diastolic dysfunction in experimental diabetic cardiomyopathy. Paeonol improved left ventricular functioning and restored the systolic and diastolic volume.

Hyperglycemia-induced-ROS generation has been reported to impair vital cell survival pathways as Akt signalling cascades (Van Linthout et al., 2008). In our study, marked decline in active Akt and glycogen synthase levels were observed with increased expression of GSK-3β. Paenol interestingly down-regulated GSK-3β and increased expressions of p-Akt, p -GSK-3β and glycogen synthase. PI3K/Akt signalling is involved in regulating cell survival and apoptosis. Akt promotes cell survival, inhibits proteins involved in apoptotic cascade and additionally regulates cardiovascular functions (Katare et al., 2010). GSK-3β, a major substrate of Akt functions as negative regulator of glycogen synthesis and glucose metabolism and also plays a crucial role in the apoptotic signalling cascades (Muniyappa et al., 2007; Wang et al., 2009; Liu et al., 2010). Our results demonstrated that paeonol possibly exerts its protective effects through modulation of Akt/ GSK-3β pathways.

Further, paeonol exerted anti-inflammatory effects by significantly blocking the expression of NF-κBp65, TNFα and p-Iκ-Bα in the cardiac tissues. In streptozotocin-induced diabetic control rats, increased expression of PAR1 also could have contributed to

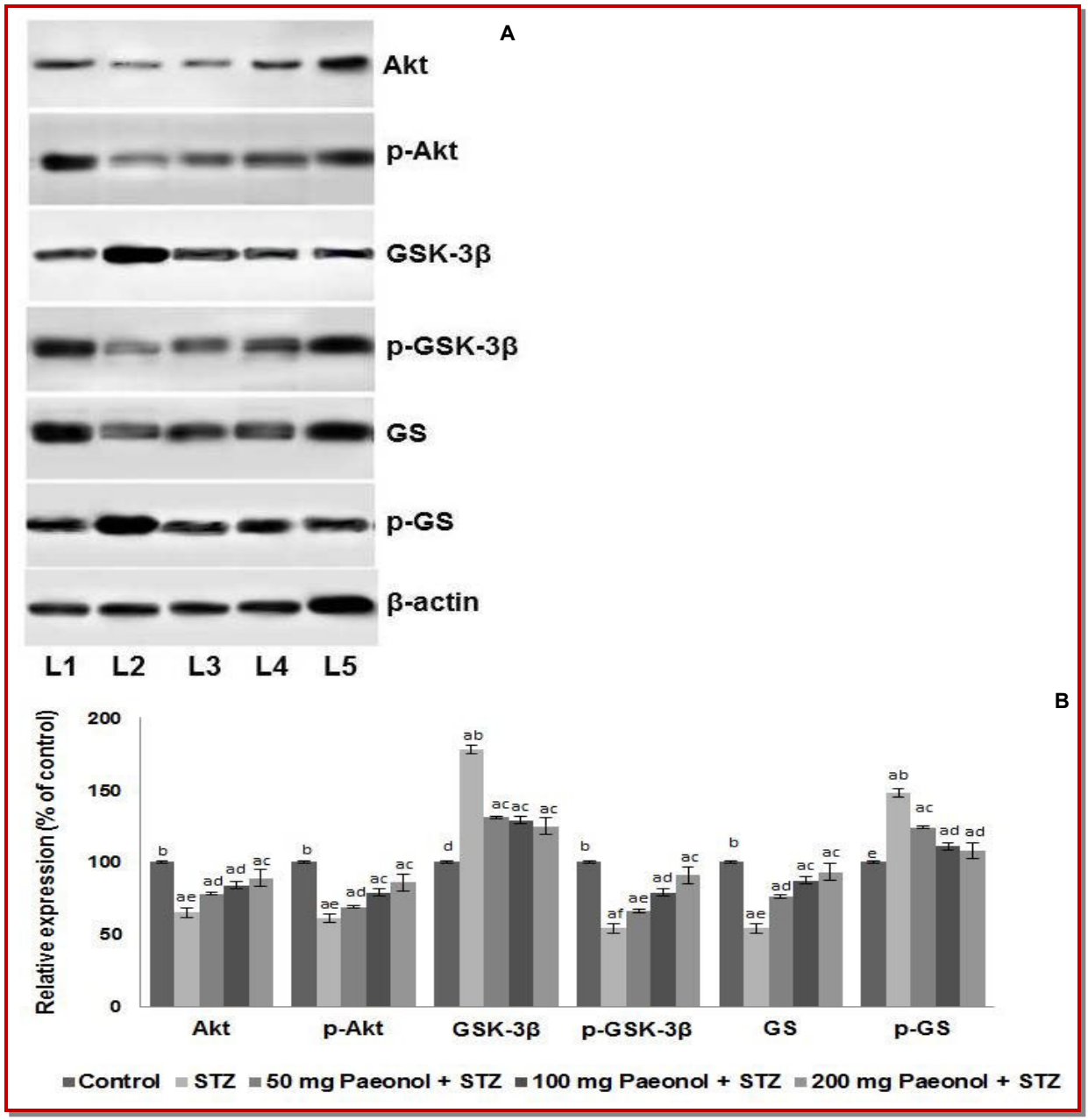

Figure 6: Influence of paeonol on the expressions of PI3K/Akt signalling pathway proteins. Paeonol markedly down-regulated active GSK-3β and increased phosphorylated levels of Akt, GS and GSK-3β (A). Relative expressions of proteins (B)

Values are represented as mean ± SD, n=6. a represents statistical significance at $p<0.05$ compared against respective controls and b-f represents significant difference ($p<0.05$) between mean values within the groups as determined by one-way ANOVA followed by DMRT analysis (L1-Control; L2-streptozotocin; L3-50 mg paeonol + streptozotocin; L4-100 mg paeonol + streptozotocin; L5-200 mg paeonol + streptozotocin)

diabetic cardiomyopathy and inflammation. Expression of cardiac inflammatory markers such as TNF-α, IL-1β, and IL-6 are implicated in diabetic cardiomyopathy (Westermann et al., 2006; Thandavarayan et al., 2009). Studies have demonstrated that excessive ROS activate NF-κB (Aragno et al., 2006; Mariappan et al., 2010) which further cause the expression of important pro-inflammatory cytokines. TNF-α has been reported as a strong mediator in cardiac dysfunctioning and failure. TNF-α acts as a signal amplifier and intensifies inflammatory responses and also contributes to myocardial hypertrophy and fibrosis, leading to LV remodelling and dysfunction as well (Sun et al., 2007). Paeonol, administration to the rats for 6 weeks caused a remarkable decrease in the expression of TNF-α, NF-κBp65 and p-Iκ-Bα in the myocardium and also levels of serum IL-6, IL-1β and TNF-α. Thus, paeonol may serve as a potential candidate in preventing LV dysfunction.

PARs have been demonstrated to be linked with

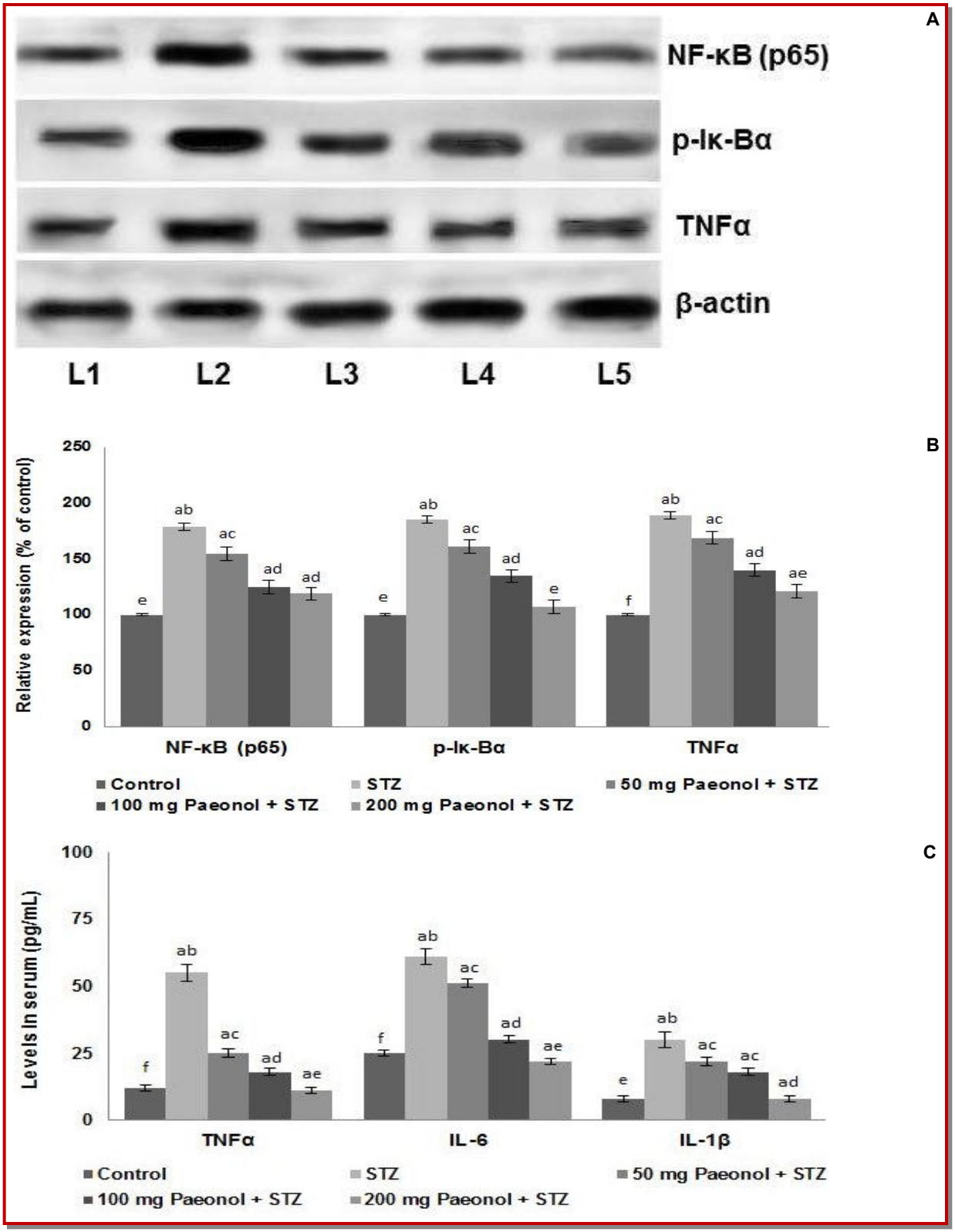

Figure 7: Antioxidant and anti-inflammatory effects of paeonol. Paeonol effectively down-regulated expressions of NF-κBp65 and p-Iκ-Bα in the cardiac tissues (A and B), reduced serum levels of inflammatory mediators (C) and decreased serum ROS levels (D)

Values are represented as mean ± SD, n=6. a represents statistical significance at $p<0.05$ compared against respective controls and b-f represents significant difference ($p<0.05$) between mean values within the groups as determined by one-way ANOVA followed by DMRT analysis (L1-Control; L2-streptozotocin; L3-50 mg paeonol + streptozotocin; L4-100 mg paeonol + streptozotocin; L5-200 mg paeonol + streptozotocin)

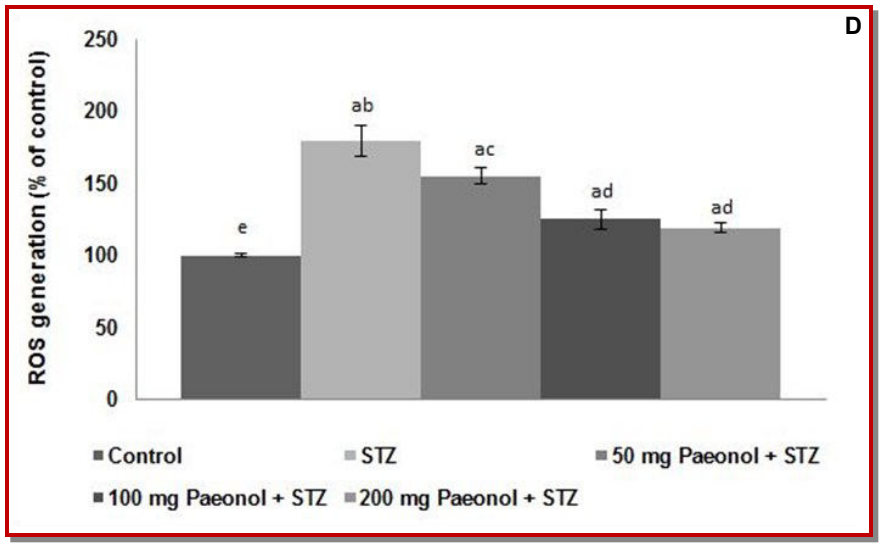

Figure 7 (Cont.): Antioxidant and anti-inflammatory effects of paeonol. Paeonol effectively down-regulated expressions of NF-κBp65 and p-Iκ-Bα in the cardiac tissues (A and B), reduced serum levels of inflammatory mediators (C) and decreased serum ROS levels (D)

Values are represented as mean ± SD, n=6. a represents statistical significance at p<0.05 compared against respective controls and b-f represents significant difference (p<0.05) between mean values within the groups as determined by one-way ANOVA followed by DMRT analysis (L1-Control; L2-streptozotocin; L3-50 mg paeonol + streptozotocin; L4-100 mg paeonol + streptozotocin; L5-200 mg paeonol + streptozotocin)

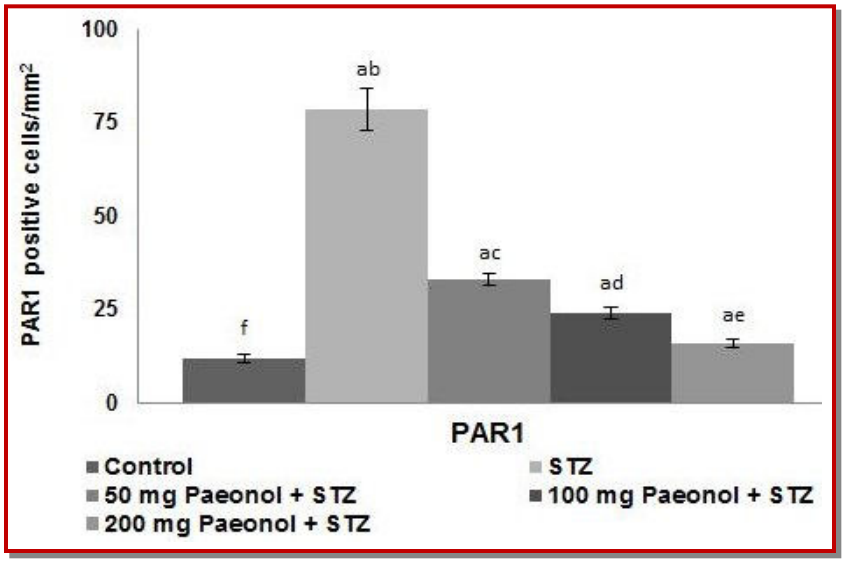

Figure 8: Effect of paeonol on the expressions of PAR1. Paoenol significantly down-regulated the expressions of PARI in the myocardial tissues

Values are represented as mean ± SD, n=6. a represents statistical significance at p<0.05 compared against respective controls and b-f represents significant difference (p<0.05) between mean values within the groups of same cell line as determined by one-way ANOVA followed by DMRT analysis

regulation of various cellular functions in the cardiovascular system (Shah, 2009). PARs are crucial for normal homeostasis and also are implicated in vascular disorders that are associated with chronic inflammation (Steinberg, 2005). Activation of PAR1 has been shown to mediate inflammatory responses and contractility (Steinberg, 2005). Down-regulation of PAR1 by paeonol appears to be a promising strategy to treat DCM in DM.

Collectively, the results of the study indicate the efficacy of paeonol in improving cardiac function and architecture. Paeonol also exerts cardioprotective effects via inhibiting inflammatory responses, oxidative stress and modulating Akt/GSK-3β signalling.

Ethical Issue

The experimental procedures involving rats were

carried out in accordance to the guidelines issued by the National Institutes of Health on the Use of Laboratory Animals.

Conflict of Interest

The authors declare no conflict of interest.

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