


Original Article

The effect of carvacrol on transcription levels of Bcl-2 family proteins in hypertrophied heart of rats

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Abstract

Introduction: Cardiomyocytes apoptosis contributes to the development of left ventricular hypertrophy. The Bcl-2 family members are important regulators of mitochondrial pathway of apoptosis. Monoterpenoid phenol –carvacrol– possesses strong antioxidant properties. The present study aimed to evaluate the effect of carvacrol on transcription level of pro-apoptotic (Bad and Bax) and anti-apoptotic (Bcl-2 and BCL-xL) members of Bcl-2 family in hypertrophied hearts.

Methods: Male Wistar rats (170-200 g) were divided into the following groups: (I) intact animals served as the control (Ctl), (II) un-treated rats subjected to aortic banding to induce left ventricular hypertrophy (H group), (III, IV, V and VI): carvacrol (C)-pretreated rats (5, 10, 25 and 50 mg/kg/day) subjected to aortic banding (H+C5, H+C10, H+C25 and H+C50 groups, respectively). Blood pressure was recorded through the carotid artery cannulation. Fibrosis was assessed by Masson's trichrome staining. Gene expression was evaluated by real time-PCR technique.

Results: In the H+C10, H+C25 and H+C50 groups mean arterial pressure ($P<0.05$, $P<0.001$ and $P<0.001$, respectively) and heart weight to body weight ratio ($P<0.05$, $P<0.01$ and $P<0.001$, respectively) were decreased significantly in comparison with H group. In the H group the Bad mRNA level was increased significantly compared to Ctl ($P<0.001$); while in the H+C10, H+C25 and H+C50 groups Bad mRNA level was decreased significantly ($P<0.05$, $P<0.001$ and $P<0.001$ vs. H). In H+C25 and H+C50 groups Bcl-2 and Bcl-xL mRNA were also up-regulated when compared with Ctl.

Conclusion: Taken together, our results suggest that carvacrol may protect the hypertrophied heart against apoptosis by affecting transcription of Bcl-2 family members.

Keywords:

Cardiac hypertrophy;
Apoptosis;
Carvacrol;
Bcl-2;
Bax;
Bad;
Bcl-xl

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Introduction

Left ventricular hypertrophy (LVH), characterized by marked thickening of left ventricular muscle develops

in response to chronic pressure and volume overload stress in the heart. Hypertension is the most common cause of LVH. Progression of LVH is accompanied with increased risk of myocardial ischemia, arrhythmia, heart failure and eventually sudden death

(Aronow, 2017; Shenasa and Shenasa, 2017). There are emerging evidences that progressive deterioration of the LVH is related to progressive loss of cardiac myocytes due to apoptosis (Narula et al., 1998; van Empel et al., 2005; Wencker et al., 2003).

Although there has been tremendous progress in our understanding of cell death, the physiological and biochemical factors involved in cardiomyocytes apoptosis during progression of LVH remain unclear. Apoptosis is mediated by two distinct evolutionarily conserved pathways: the extrinsic and intrinsic cell death pathways. The extrinsic or death-receptor pathway is activated when death ligands, such as Fas ligand or TNF- α , bind to their cognate receptors at the plasma membrane. The intrinsic or mitochondrial pathway of apoptosis is initiated by intracellular signaling and involves mitochondrial membrane permeabilization and thereby release of pro-apoptotic proteins (Martinou and Youle, 2011). The intrinsic pathway of apoptosis is regulated by anti and pro-apoptotic members of the B-cell lymphoma protein-2 (Bcl-2) family of proteins. This family is composed of proud anti apoptotic proteins (Bcl-2, Bcl-xL, MCL-1, BFL-1, Bcl-W and Bcl2L10) that share up to four conserved regions known as Bcl-2 homology (BH) domains. Anti-apoptotic members such as Bcl-2 and Bcl-extra large (xL) contain all four subtypes of BH domains and promote cell survival by inhibiting the pro-apoptotic members leading to preserve outer mitochondrial membrane integrity. The pro-apoptotic members can be separated into two structurally distinct subfamilies. The "multi domains" proteins (Bax, Bak and BOK) share three BH regions and "BH3-only" proteins (Bnip3, Nix/Bnip3L, Bid, Noxa, Puma, and Bad), share only the BH3 domain and are structurally diverse (Huang and Strasser, 2000).

Experimental and clinical studies have demonstrated that changes in Bcl-2 family expression can contribute to pathophysiology of cardiovascular diseases such as myocardial ischemia (Guo and Li, 2018) and heart failure (Latif et al., 2000). Therefore identification of the novel agents which regulate the expression and function of these proteins may expand the therapeutic implements for prevention of deleterious LVH in hypertensive patients.

Investigations have displayed that plant extracts or their active ingredients (such as monoterpenes) are rich in antioxidants which exert a protective effect on the cardiovascular system (Peixoto-Neves et al.,

2010; Safari et al., 2015). Therefore, there is an increasing interest in the use of natural antioxidants. Carvacrol or cymophenol (2-methyl-5-isopropylphenol) is a main monoterpenicphenol that exists in essential oils of *Labiatae* including *Origanum*, *Satureja*, *Thymbra* and *Thymus revolutus Celak* (Friedman, 2014; Suntres et al., 2015).

Previous studies have demonstrated some of the cardiovascular effects of carvacrol such as fibrinolytic, vasorelaxation and hypotensive effects (Aydin et al., 2007; Lahlou et al., 2000); however recent studies have reported the novel cardioprotective effects for this natural phenol. Carvacrol protects against doxorubicin and cyclophosphamide-induced cardiotoxicity and myocardial ischemia through antioxidant, anti-inflammatory and anti-apoptotic effects (El-Sayed et al., 2016, Cetik et al., 2015, Yu et al., 2013).

Despite the key role of Bcl-2 family in regulation of apoptosis during LVH and discovering the novel cardioprotective effects of carvacrol, there is no report on the effect of this natural phenol on transcriptional profile of Bcl-2 family proteins in hypertrophied hearts. Therefore in the current study we have investigated the effect of carvacrol on hypertrophy markers, as well as Bcl-2-associated X protein (BAX), Bcl-2-associated death promoter (BAD), Bcl-2 and Bcl-xL mRNA levels in a rat model of aortic banding-induced cardiac hypertrophy model.

Materials and methods

Experimental protocol

Male Wistar rats (170-200 g), were obtained from animal house of Shahid Sadoughi University of Medical Sciences, Yazd, Iran. Animal handling and all the related procedures were approved by Shahid Sadoughi University's animal ethics committee. Animals were randomly divided into six groups (n=8 in each group; 5 samples were used for molecular studies and 3 hearts were fixed in paraformaldehyde for fibrosis assessment). In group H, cardiac hypertrophy was induced by the abdominal aortic banding without any treatments. In the treated groups, carvacrol (Merck, Germany) administration was started two weeks prior to the aortic banding and continued until the end of the experiments (four weeks after surgery). Carvacrol (C) was given intraperitoneally at the doses of 5, 10, 25 and 50

mg/kg/day in H+C5, H+C10, H+C25 and H+C50 groups, respectively. Intact animals served as control (Ctl group).

Animal model of cardiac hypertrophy

To induce hypertension and consequently LVH, animals were subjected to abdominal aortic banding. Briefly, rats were anesthetized with intraperitoneal injection of ketamine (70 mg/kg) and xylazine (10 mg/kg). An incision was made in the left flank. After exposing the suprarenal abdominal aorta, a 21-gauge needle was placed beside the artery and a suture was tied around it. After ensuring partial banding of artery and not complete occlusion, the needle was removed. Abdominal wall muscles and skin were sutured by absorbable and non-absorbable sutures, respectively. In the hypertrophic groups, tetracycline was injected to animals intramuscularly for 6 days. Four weeks after aortic banding animals were anesthetized again and arterial blood pressure was measured directly by cannulation of the carotid artery connected to a power lab system. At the end of the intervention, in addition to weighing of rats, the cardiac mass was also measured after being washed with cold saline. From each group, five hearts were collected and kept at -80 °C for molecular studies and three hearts were fixed in paraformaldehyde for histological study (Dorri Mashhadi et al., 2017).

Histological study

Paraformaldehyde-fixed and paraffin-embedded heart tissues were sectioned at 6µm and stained with Masson's trichrome for the assessment of cardiac fibrosis. Images were captured by a Nikon microscope equipped with a Sony, Cyber-shot, DSCWX200 camera. (Images were magnified ×200).

Real-time PCR technique

To evaluate the cardiac levels of Bcl-2 family mRNA expression, the heart was rapidly removed and placed on ice. The whole RNA was extracted from the left ventricular tissue using RNx-PLUS solution (Cinna Gen, Iran) according to the manufacturer's protocol. The RNA concentration was determined by measuring its absorbance at 260 nm by a Nano Drop spectrophotometer (Model 2000, Thermo Scientific, Germany). First-strand cDNA synthesis was done, using RevertAid™M-MuLV Reverse transcriptase (Fermentas, USA) in the total volume of 20 µl. cDNA of experimental groups was tested by master mix containing SYBR green (Takara, Japan) with specific primers under real time-PCR reaction (Rotor Gene system -Qiagen, USA). Gene expression was analyzed according to $2^{-\Delta\Delta Ct}$ method. Beta-actin was considered as a reference gene. Sequences of primers are summarized in Table1.

Statistical analysis

The normality of data was assessed by the D'Agostino-Pearson test. Leven's test for equality of variance was performed at 5% significant level. The data of multiple experimental groups were analyzed and compared using one-way ANOVA followed by Tukey post-hoc test. The data were expressed as mean±SEM. $P<0.05$ was considered as the significance level.

Results

Effect of carvedilol on mean arterial pressure and heart weight to body weight ratio

As shown in Figure 1, in the H group the mean arterial pressure was increased significantly compared to Ctl group ($P<0.001$). In the H+C10, H+C25 and H+C50 groups, blood pressure was significantly lower than H group ($P<0.05$, $P<0.001$).

Table 1: Primer sequences for real time RT-PCR of the candidate genes

Gene	Forward primer (5'–3')	Reverse primer (5'–3')
Bad	GGAGCATCGTTCAGCAGCAG	CCATCCCTTCATCTTCCTCAGTC
Bcl-xl	GCTGGTGGTTGACTTTCTCTCC	GGCTTCAGTCCTGTTCTCTTCG
Bcl-2	ATACCTGGGCCACAAGTGAG	TGATTTGACCATTTGCCTGA
Bax	CGAGCTGATCAGAACCATCA	CTCAGCCCATCTTCTCCAG
B-actin	CTGACGTCCACCCTGACT	GGCAGCTATGTGAGAGCC

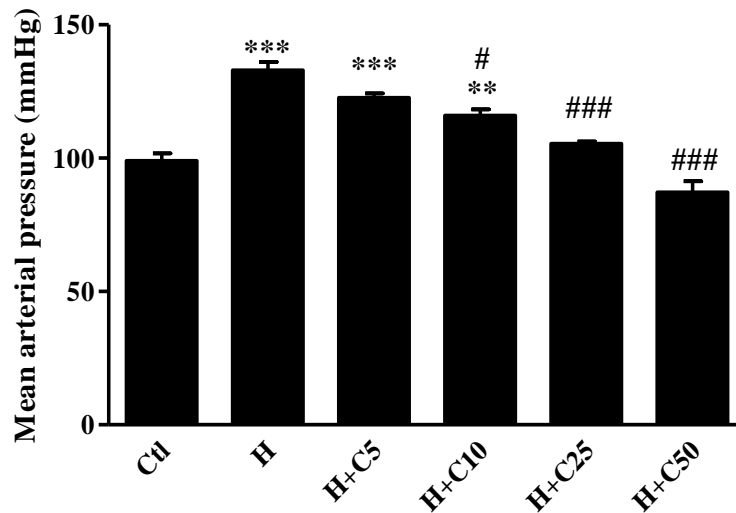


Fig.1. Mean arterial pressure in experimental groups. Blood pressure was recorded directly through carotid artery cannulation in the intact rats (Ctl), un-treated rats subjected to cardiac hypertrophy (H) and rats pretreated with carvedilol (C) at doses of 5, 10, 25 and 50 mg/kg/day before induction of hypertrophy. Data are presented as mean±SEM (n=8). ** $P<0.01$, *** $P<0.001$ vs Ctl. # $P<0.05$ and ### $P<0.001$ vs H.

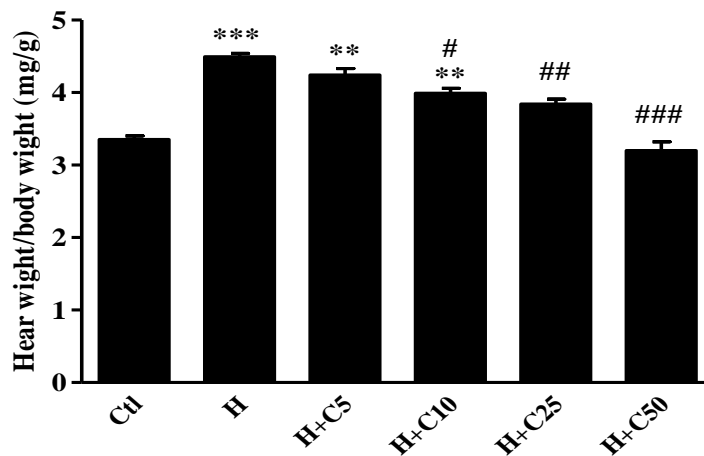
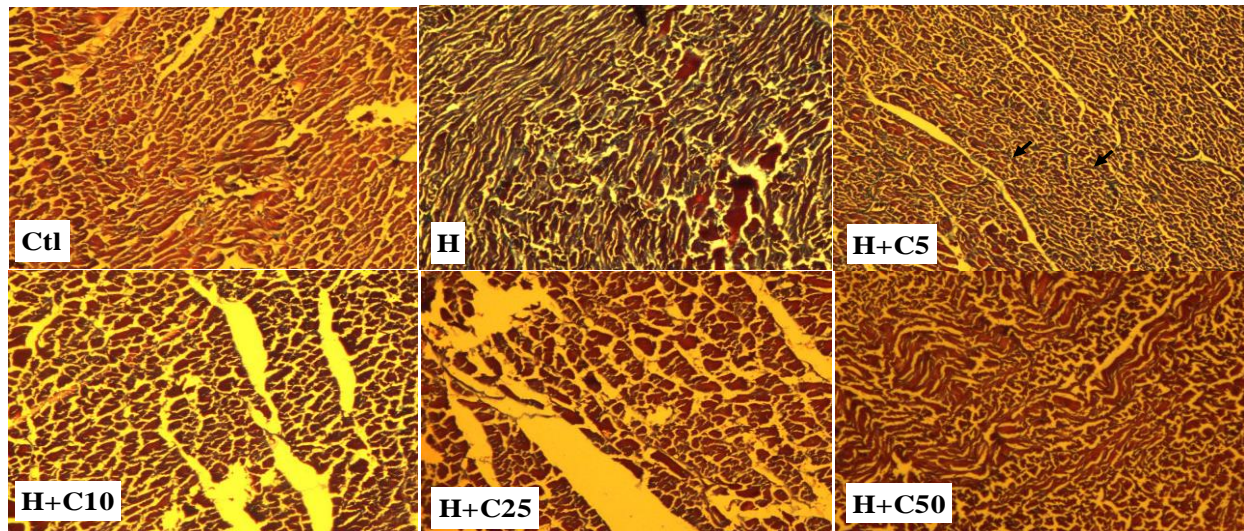


Fig.2. Heart weight to body weight ratio (HW/BW) in experimental groups. As a main marker of cardiac hypertrophy, HW/BW was assessed in the intact rats (Ctl), un-treated rats subjected to cardiac hypertrophy (H) and rats pretreated with carvedilol (C) at doses of 5, 10, 25 and 50 mg/kg/day before induction of hypertrophy. Left ventricular hypertrophy was accompanied by excessive collagen deposition as shown by the arrow in photomicrographs of Masson's Trichrome-stained heart sections (x200). Data are presented as mean±SEM (n=8). ** $P<0.01$, *** $P<0.001$ vs Ctl. # $P<0.05$, ## $P<0.01$ and ### $P<0.001$ vs H.

and $P < 0.001$, respectively).

In H group the heart weight to body weight ratio (HW/BW) was also increased significantly in comparison with Ctl group ($P < 0.001$); however in the H+C10, H+C25 and H+C50 groups which were pretreated with carvacrol, the HW/BW was significantly different from the H group ($P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively) (Fig. 2).

Effect of carvacrol on transcription level of pro-apoptotic members of Bcl-2 family

As displayed in Figure 3, the changes of Bax mRNA levels were not proved to be statistically significant

among the experimental groups; however, in the H group the Bad mRNA level was increased by $96.2 \pm 6.7\%$ indicating a significant difference compared to the Ctl group ($P < 0.001$). While in the H+C10, H+C25 and H+C50 groups, the Bad mRNA decreased by $56 \pm 7.8\%$, $28 \pm 3.7\%$ and $34 \pm 8.3\%$, respectively which shows a significant difference compared with the H group ($P < 0.05$, $P < 0.001$ and $P < 0.001$, respectively).

Effect of carvacrol on transcription level of anti-apoptotic members of Bcl-2 family

As displayed in Figure 4, in the H group the Bcl-2

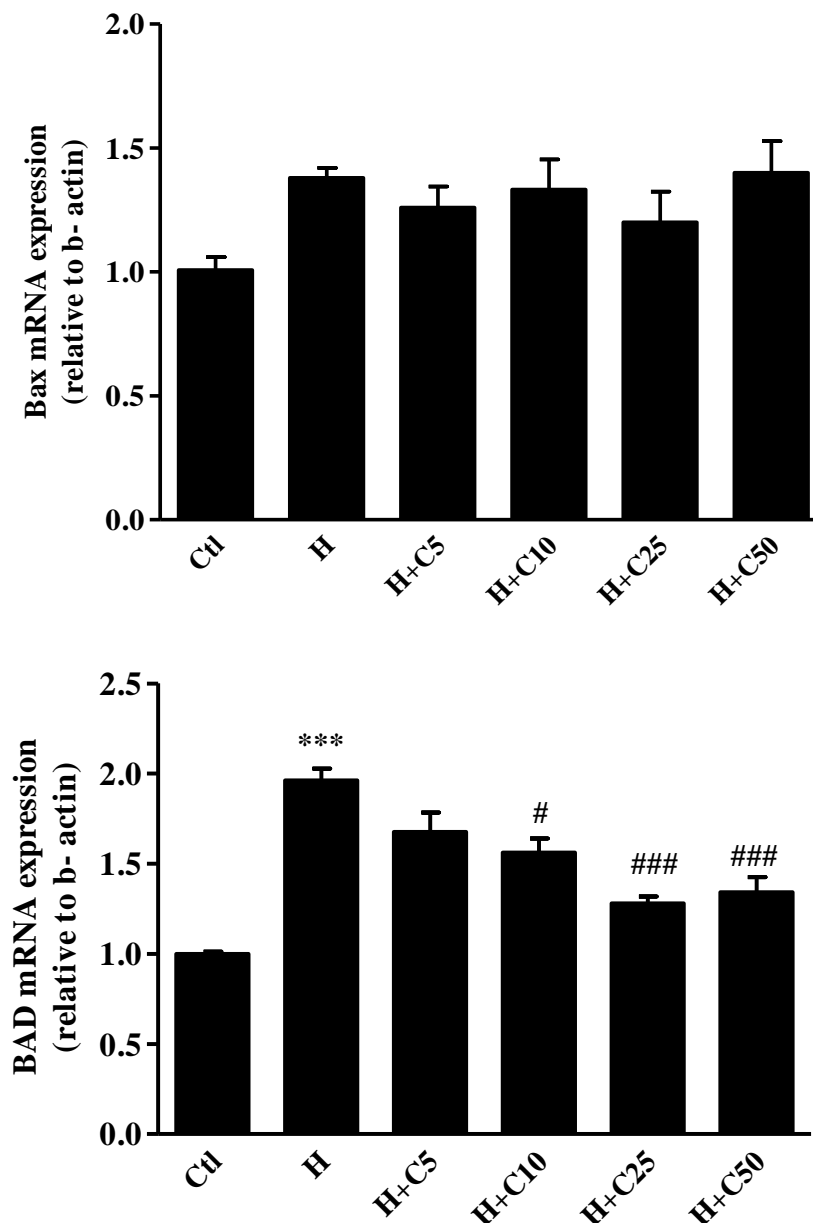


Fig. 3. The transcription levels of pro-apoptotic proteins of Bcl-2 family. Cardiac Bax and BAD mRNA levels were evaluated in the intact rats (Ctl), un-treated rats subjected to cardiac hypertrophy (H) and rats pretreated with carvacrol (C) at doses of 5, 10, 25 and 50 mg/kg/day before induction of hypertrophy. Data are presented as mean \pm SEM ($n=5$). *** $P < 0.001$ vs. Ctl. # $P < 0.001$ and ### $P < 0.001$ vs. H.

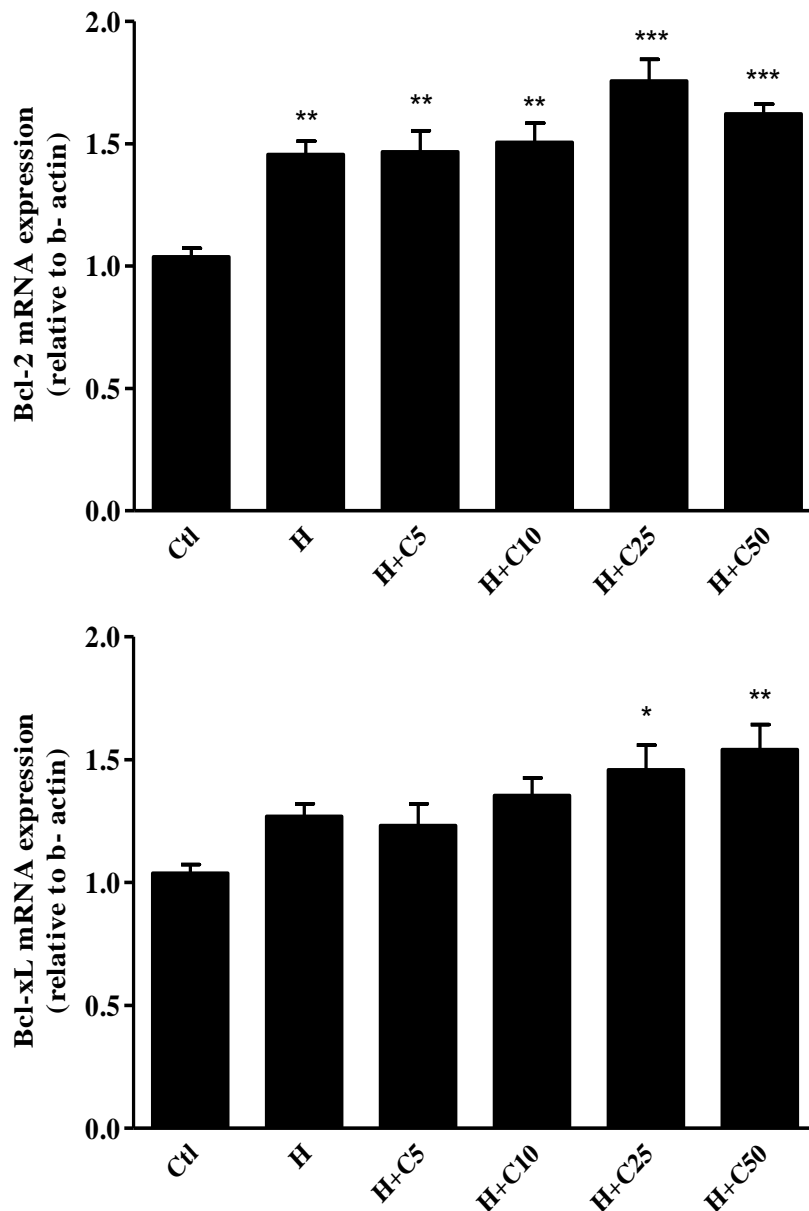


Fig. 4. The transcription levels of anti-apoptotic proteins of Bcl-2 family. Cardiac Bcl-2 and Bcl-XI mRNA levels were evaluated in the intact rats (Ctl), un-treated rats subjected to cardiac hypertrophy (H) and rats pretreated with carvedilol (C) at doses of 5, 10, 25 and 50 mg/kg/day before induction of hypertrophy. Data are presented as mean±SEM (n=5). * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ vs. Ctl.

mRNA level was increased by $45.6\pm 5.3\%$ indicating a significant difference compared to the Ctl group ($P<0.05$). Also, in the H+C5, H+C10, H+C25 and H+C50 groups, the Bcl-2 mRNA reached to $46.7\pm 8\%$, $50\pm 7.8\%$, $75\pm 8\%$ and $62\pm 7.2\%$, respectively, which show significant increase in comparison with Ctl ($P<0.01$, $P<0.01$, $P<0.001$ and $P<0.001$, respectively); however the changes were not significant when compared with H group.

Regarding the transcription level of Bcl-xL, our data showed that in the H+C25 and H+C50 groups, the Bcl-xL mRNA increased by $45.8\pm 9\%$ and $54.1\pm 10\%$, respectively which shows a significant increase

compared to the Ctl ($P<0.05$ and $P<0.01$, respectively). In other experimental groups, the mRNA change was not statistically significant (Fig. 4).

Discussion

The first part of the present study revealed that four weeks after abdominal aorta banding, blood pressure and heart weight to body weight ratio were increased. Our data is in agreement with previous studies used this model to induce left ventricular hypertrophy in rats (Jahanbakhsh et al., 2012; Juric et al., 2007; Németh et al., 2016). In addition, in hypertrophied left

ventricles pro-apoptotic protein BAD and anti-apoptotic protein Bcl-2 were up-regulated at mRNA levels. Increased expression of pro-apoptotic proteins of Bcl-2 family has been shown to contribute in the pathogenesis of diverse cardiovascular diseases including pressure overload-induced hypertrophy (Condorelli et al., 1999).

In agreement with our findings, in a study by Latif et al. (2000) it has been shown that in the heart of patient with end stage of heart failure the number of apoptotic cardiomyocytes, BAD and BAK as well as Bcl-2 and Bcl-xL proteins expression were increased. In our study Bcl-2 transcription level was up-regulated in hypertrophied hearts suggesting a compensatory response of the heart to hypertrophy stimuli. Our data in this issue is in agreed with study of Latif et al. In response to acute ischemia reperfusion injury in rats, Bcl-2 expression decreased, suggesting the different response of heart to acute and chronic stress conditions (Liu, 2018). Precisely how the Bcl-2 family proteins regulate apoptosis is still vague; however most studies revealed that the pro-apoptotic Bax and Bak are maintained in an inactive conformation through direct interactions with one or two different anti-apoptotic Bcl-2 proteins. In response to an apoptotic stimulus, BH3-only proteins bind to and deactivate the anti-apoptotic Bcl-2 proteins, thereby releasing Bax and Bak. Over expression of Bcl-2 or Bcl-xL has been reported to prevent Bax translocation and activation (Van Laethem et al., 2004).

The second part of our study showed that carvedilol prevented aortic banding-induced hypertension and decreases HW/BW. These protective effect was accompanied by decrease of pro-apoptotic factor-BAD- and up-regulation of anti-apoptotic factors, Bcl-2 and Bcl-xL in left ventricular tissue. There are few reports on cardioprotective effects of carvedilol and the mechanisms responsible for these effects of carvedilol have not been fully understand. Aydin et al. (2007) have shown that carvedilol at a dose of 100 mg/kg reduced blood pressure as well as heart rate and prevented the hypertension induced by L-NAME (N[omega]-nitro-L-arginine methyl ester) in normotensive rats.

Carvedilol inhibits voltage dependent calcium channel and transient receptor potential channels and thereby exhibits hypotensive and bradycardia effect (Dantas et al., 2015). Recently it has been demonstrated that

administration of carvedilol (25 mg/kg/day) for 14 days ameliorated doxorubicin-induced cardiotoxicity in rats. This effect was accompanied by antioxidant, anti-inflammatory and anti-apoptotic effects of carvedilol (El-Sayed et al., 2016). It has been also shown that treatment of rats with 5 and 10 mg/kg of carvedilol protects the heart against cyclophosphamide-induced heart injury through augmentation of cardiac antioxidant capacity and decrease of inflammation (Cetik et al., 2015).

The effect of carvedilol on Bax and Bcl-2 expression has been shown in a study by Yu et al. (2013) who found that carvedilol decreased caspase-3 and Bax and increased Bcl-2 protein level in infarcted heart of rats. Increase of total antioxidant capacity in human lymphocytes (Türkez and Aydın, 2016) and neurons (Aydın et al., 2014) by carvedilol as well as the neuroprotective effect of carvedilol (12.5 and 25 mg/kg/day) in a rat model of Parkinsonism have also been shown (Lins et al., 2018).

In our study carvedilol reversed the hypertrophy-induced up-regulation of BAD mRNA suggesting that carvedilol may protect the heart against deleterious effects of pathological hypertrophy at least, in part by decrease of pro-apoptotic factors. Chen et al. (2017) in a recent study has shown that carvedilol protected the heart against ischemia reperfusion injury by increase of superoxide dismutase and catalase as well as decrease of cardiomyocytes apoptosis through involvement of the MAPK/ERK and Akt/eNOS signaling pathways.

Conclusion

Taken together, the results of the present study revealed that carvedilol increases Bcl-2 and Bcl-xL mRNA level and decreases Bad mRNA level in the hypertrophied left ventricles. Augmentation of anti-apoptotic expression and attenuation of pro-apoptotic expression can be taken as a novel mechanism into consideration in order to protect the heart against hypertrophy. Assessing the effects of this monoterpene in the heart can lead us toward using it in treatment of cardiovascular diseases such as left ventricular hypertrophy.

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Conflict of interest

The authors confirm that this article content has no conflict of interest.

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