Original Article

Effect of allopurinol and benzbromarone on diabetic cardiomyopathy and vasculopathy in streptozotocin-induced diabetic rats

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Abstract

Introduction: Allopurinol, a xanthine oxidase inhibitor, reduces both plasma uric acid (UA) and oxidative stress, and benzbromarone, a uricosuric agent, reduces the level of plasma UA. This study was designed to evaluate cardiac mechanical and endothelial functions of the allopurinol- and benzbromarone-treated diabetic rats, and to investigate the underlying mechanism (antioxidant or UA lowering activity) of allopurinol beneficial effects.

Methods: Diabetes was induced by injecting streptozotocin to male *Spargue-Dawley* rats. Diabetic animals were treated with allopurinol and benzbromarone. After six weeks of treatment, left ventricular systolic/diastolic functions of hearts, contraction/relaxation responses to phenylephrine and acetylcholine of aortae, and serum levels of malondialdehyde, 8-isoprostane-2α and UA were measured.

Results: Diabetic cardiomyopathy and vasculopathy were characterized by reduced myocardial performance and decreased aortic endothelial response to the vasorelaxation effect of acetylcholine. The serum levels of malondialdehyde and 8-isoprostane-2α levels were elevated in diabetic animals. Allopurinol attenuated the diabetes-induced diastolic impairment of the hearts, endothelial dysfunction of the aortae and decreased oxidative stress parameters in serum; however, benzbromarone had none of these effects. Both, allopurinol and benzbromarone, diminished the elevated levels of UA in diabetic animals.

Conclusion: Allopurinol improved diabetic cardiomyopathy and aortic endothelial cell dysfunction in diabetic animals through antioxidant effects.

Introduction

Diabetes mellitus (DM) is one of the most important health problem in modern societies. Its prevalence is increasing and in 2015 there were 415 million diabetic patient around the world. This number is expected to reach 642 million by 2040 (Lau et al., 2013). DM microvascular (retinopathy, nephropathy and neuropathy) and macrovascular (coronary arteriopathy, cerebral vasculopathy and peripheral vasculopathy) complications are the leading cause of

dysfunction, reduced life expectancy and increased health-care costs (Brownlee, 2001).

Diabetic cardiomyopathy (DCM) is structural and functional disturbances of the myocardium, which is not directly related to other confounding factors, such as atherosclerosis or hypertension (Boudina and Abel, 2010). DM-induced endothelial dysfunction (ED) is a condition in which the endothelium loses its physiological properties, particularly reduced production and bioavailability of nitric oxide (NO) (Sena et al., 2013). ED is a critical and initiating cause of pathogenesis of vascular complications (Tabit et al., 2010).

Several lines of evidence show that oxidative stress plays an important role in cardiovascular complications of DM, especially ED and DCM (Rochette et al., 2014; Asmat et al., 2016). Xanthine oxidase (XO) is a putative source of reactive oxygen species (ROS) and contributes to the initiation and progression of diabetic complications. Moreover, XO plays an important role in the oxidation of hypoxanthine and xanthine in order to produce uric acid (UA).

Allopurinol (ALP), a purine analog, inhibits XO and reduces UA synthesis and ROS production (George et al., 2006). ALP prevents the progression of diabetic cardiovascular complications, but in this regard there are controversial results. Some studies showed the beneficial effects of ALP on different type of cardiomyopathy (Rajesh et al., 2009) and ED (Inkster et al., 2007; Kanbay et al., 2014) that were due to inhibition of XO and reduction of oxidative stress. In contrast, some proposed that the protective effect of ALP in diabetic nephropathy might be related to mechanisms other than the reduction of oxidative stress (Kosugi et al., 2009). This conclude that the nephroprotective effect of ALP was associated with the lowering plasma UA level and not the oxidative stress. In addition, the anti-oxidative effect of ALP in clinical study is still questionable (Afshari et al., 2004). Recently, several studies have shown the independent predictive role of serum UA in cardiovascular diseases in diabetic patients (Zoppini et al., 2009; Zoppini et al., 2011).

Benzbromarone (BZB), a uricosuric agent, inhibits UA reabsorption from renal tubules and reduces serum UA. There are no consistency about the antioxidant action of BZB. In most studies it has been shown that BZB has no significant effect on oxidative stress

(Kaufmann et al., 2005; Goharinia et al., 2017). However, in some studies it has shown direct and indirect antioxidant effect (Muraya et al., 2018). In contrast in some studies it is claims that it aggravates the oxidative stress (Sun et al., 2018).

In the present study, endothelial responses and cardiac mechanical functions of the ALP-treated diabetic rats were investigated. To recognize which ALP actions (antioxidant or UA lowering activity) is involved in its potentially useful effect, BZB was used as a comparator.

Materials and methods

Animals

Male mature *Spargue-Dawley* rats (200-220g) were purchased from the Center of Comparative and Experimental Medicine, Shiraz University of Medical Sciences, Shiraz, Iran. All animals were kept in standard condition. The rats were randomly divided into normal and diabetic groups. The study protocols were approved by the local Ethics Committee of Shiraz University of Medical Sciences (Ethics committee approval number; ec-p-91-5317).

To induce type 1 diabetes, streptozotocin (STZ, 60mg/kg, ip) was administered. Five days after STZ injection, fasting plasma glucose (FPG) level was checked and rats with FPG greater than 300mg/dl were considered as diabetic rats. Experimental animals were divided into four groups: 1) normal group including non-diabetic normal rats received distilled water; 2) diabetic rats received the same volume of distilled water (Diabetic group); 3) diabetic rats received 50mg/kg/day of ALP by gavage (Diabetic+ALP group) and 4) diabetic rats received 10mg/kg/day of BZB by gavage (Diabetic+BZB group). ALP and BZD were dissolved in distilled water and their doses were selected based on previous studies (Goharinia et al., 2017). All diabetic and normal rats had free access to rodent chow and tap water *ab libitum*.

Isolated heart study

After six weeks of treatment, the heparinized subjects (1000IU/kg) were anesthetized with a mixture of ketamine (80mg/kg) and xylazine (10mg/kg). Peripheral blood samples were drawn to determine biochemical parameters. Then, the thorax was opened by a midline incision and the heart was

rapidly excised. Thereafter, aorta was cannulated by a 21-gauge needle, and heart was perfused with Krebs solution in a Langendorff apparatus (ML176-V; ADInstruments, New South Wales, Australia) at a constant flow (12ml/min). This process was done swiftly to prevent ischemia.

The heart was allowed to be stabilized for 30min and after that the heart rate (HR), left ventricular end-diastolic pressure, perfusion pressure (PP), developed pressure (DP), the velocity of systolic contraction (+dP/dt) and the velocity of diastolic relaxation (-dP/dt) were recorded using a PowerLab recorder and LabChart 5.0 software (ADInstruments, New South Wales, Australia).

Krebs solution contained (nM): NaCl 118, $CaCl_2$ 3.3, KH_2PO_4 1.2, $MgSO_4$ 2.4, KCl 4, $NaHCO_3$ 25, and D-glucose 11 was passed through 4μM filter and aerated with O_2 (95%) and CO_2 (5%) at 37°C. Left ventricular developed pressure (LVDP) was calculated as the difference between maximal systolic and end-diastolic pressures.

Isolated aorta study

After removing the heart, thoracic aorta was dissected, the connective tissue was removed and divided into 4-mm rings. In a bath filled with physiological salt solution, each ring was connected to a triangular-shaped hook and a pressure transducer (K30, Hugo Saches Electronik). The resting tension was set at 1g. The composition of physiological salt solution was (nM): NaCl 118, $CaCl_2$ 3.3, KH_2PO_4 1.2, $MgSO_4$ 2.4, KCl 4, $NaHCO_3$ 25, and D-glucose 11 that was passed from 4μM filter and aerated with O_2 (95%) and CO_2 (5%) at 37°C.

The tissues were allowed to stabilize for an hour. The bath solution was changed every 20min. At the end of this period, cumulative concentrations of phenylephrine (PE) were added to the organ bath and the pEC_{50} (negative logarithm of the required concentration to achieve half-maximal contraction) value and maximal induced contraction of PE (C_{max}) were calculated. Thereafter, each tissue was washed out and after relaxation and reaching the basic tone, it was contracted once again with the EC_{50} of PE. After reaching the maximum and constant contraction, cumulative concentrations of acetylcholine (ACh) were added to the organ bath and its pEC_{50} (negative logarithm of the required concentration to achieve half-maximal relaxation),

and maximum induced relaxation (R_{max}) were calculated.

Biochemical assays

Peripheral blood samples were centrifuged at 3000rpm for 20min at room temperature to obtain serum for assessment of FPG, UA, malondialdehyde (MDA) and 8-isoprostane-2α (IP) levels. The serum were kept at -80°C until assessment. UA levels were measured using the automated hematology analyzer Sysmex XS-800i device (Norderstedt, Germany).

MDA as a marker of lipid peroxidation, was measured using thiobarbituric acid (TBA)-malondialdehyde method. MDA level was assessed by utilizing tetraethoxypropane (TEP) standard curve. TEP standard curve was plotted using standard solutions (0, 1, 2, 2.5, 5 and 10 μM). The 0.5ml of serum or standard solutions was taken in a test tube and 2ml of the TBA-TCA reagent (0.25N HCl, 0.375% w/v TBA and 15% w/v trichloroacetic acid) was added. This mixture was heated in a water bath (15min), cooled in a cold water bath (10min) and then centrifuged at 2000g (15min). The optical density of solution was read spectrophotometrically at 535nm (Talebianpoor and Mirkhani, 2012). IP was measured by an enzyme immunoassay kit according to the manufacturer's instructions (8-iPF2α ELISA Kit, Enzo Life Sciences Inc., USA).

Statistical analysis

Data are shown as mean±SEM. Statistical evaluation was determined using SPSS software (Version 18). Results were compared by One-way ANOVA test with Tukey post-test. $P<0.05$ was considered to be statistically significant.

Results

Diabetes induction and body weight changes

Diabetes induction success rate was approximately 80% and the mortality rate of diabetic animals was about 15% at the end of sixth week. After four and six weeks, the body weight of the diabetic rats was reduced significantly, while body weight of diabetic animals treated with ALP and BZB groups did not change significantly (Table 1).

The effect of treatments on cardiac function

Type 1 diabetes caused a significant reduction in HR,

Table 1: Body weight of animals

Groups	Body weight (g)		
	Day-0	Day-28	Day-42
Normal	203 ± 3	241 ± 6	269 ± 7
Diabetic	201 ± 2	202 ± 7 *	183 ± 6 ***
Diabetic+ALP	204 ± 2	200 ± 7	201 ± 9
Diabetic+BZB	199 ± 4	202 ± 9	193 ± 6

Values are expressed as the mean±SEM (n=7–10/group). ALP: allopurinol; BZB: benzbromarone. Differences were tested by one-way ANOVA and post-hoc test of Tukey. *$P<0.05$ and ***$P<0.001$ represent the difference between normal and diabetic groups.

Table 2: Biomechanical measurements of cardiac function

Group	HR (beats/min)	PP (mmHg)	LVDP (mmHg)	+dp/dt	-dp/dt
Normal	216.0 ± 8.5	63.6 ± 2.4	77.6 ± 5.0	2606.9 ± 186.6	-1467.3 ± 25.3
Diabetic	174.7 ± 8.8 *	64.9 ± 6.6	63.8 ± 5.2	1414.8 ± 134.9 ***	-784.9 ± 76.0 ***
Diabetic+ALP	188.0 ± 5.8	68.9 ± 6.9	82.5 ± 5.0	1853.2 ± 91.8	-1073.5 ± 70.1 #
Diabetic+BZB	170.5 ± 7.3	70.2 ± 4.7	74.8 ± 5.9	1641.1 ± 128.8	-824.1 ± 53.3

Values are expressed as the mean±SEM (n=7–10/group). HR: heart rate; PP: perfused pressure; LVDP: left ventricular developed pressure (the difference between maximal systolic and end-diastolic pressures); +dp/dt: the rate of left ventricle pressure rise in early systole; -dp/dt: the rate of left ventricle pressure decrease in early diastole; ALP: allopurinol; BZB: benzbromarone. Differences were tested by one-way ANOVA and post-hoc test of Tukey. *$P<0.05$ and ***$P<0.001$ represent the difference between normal and diabetic groups. #$P<0.05$ represents the difference between treated- diabetic and diabetic groups.

Table 3: Biomechanical measurements of aortic function

Group	PE		ACh	
	pEC_{50}	C_{max} (mg)	pEC_{50}	R_{max} (%)
Normal	6.98 ± 0.07	353.4 ± 35.4	7.99 ± 0.11	96.6 ± 2.0
Diabetic	6.79 ± 0.06	356.3 ± 35.7	6.81 ± 0.18 *	78.5 ± 3.0 *
Diabetic+ALP	7.11 ± 0.08 #	318.7 ± 41.7	7.54 ± 0.17 #	90.1 ± 2.2 #
Diabetic+BZB	6.96 ± 0.09	365.6 ± 34.8	6.96 ± 0.27	83.3 ± 4.3

Values are expressed as the mean±SEM (n=7–10/group). PE: phenylephrine; Ach: acetylcholine; pEC_{50}: -log of EC_{50} (the required concentration to achieve half-maximal contraction); C_{max}: phenylephrine induced maximal contraction; R_{max}: acetylcholine induced maximum relaxation; ALP: allopurinol; BZB: benzbromarone. Differences were tested by one-way ANOVA and post-hoc test of Tukey. *$P<0.05$ represents the difference between normal and diabetic groups. #$P<0.05$ represents the difference between treated-diabetic and diabetic groups.

+dp/dt and -dp/dt in isolated rat heart ($P<0.05$, $P<0.001$ and $P<0.001$, respectively). Meanwhile, it had no significant effect on PP and LVDP. In ALP-treated groups, +dp/dt had increased, but not statistically significant ($P=0.074$) and -dp/dt were increased significantly ($P=0.017$). The +dp/dt ($P=0.502$) and -dp/dt ($P=0.966$) had not changed in BZB-treated groups. Other biomechanical parameters of cardiac function had not significantly changed in

the treated groups (Table 2).

The effect of treatments on aortic endothelial function

The potency of PE had not changed in the diabetic group. Although PE potency had increased significantly in ALP-treated group, BZB did not significantly affect this parameter. There were no significant differences in the maximal efficacy of PE-
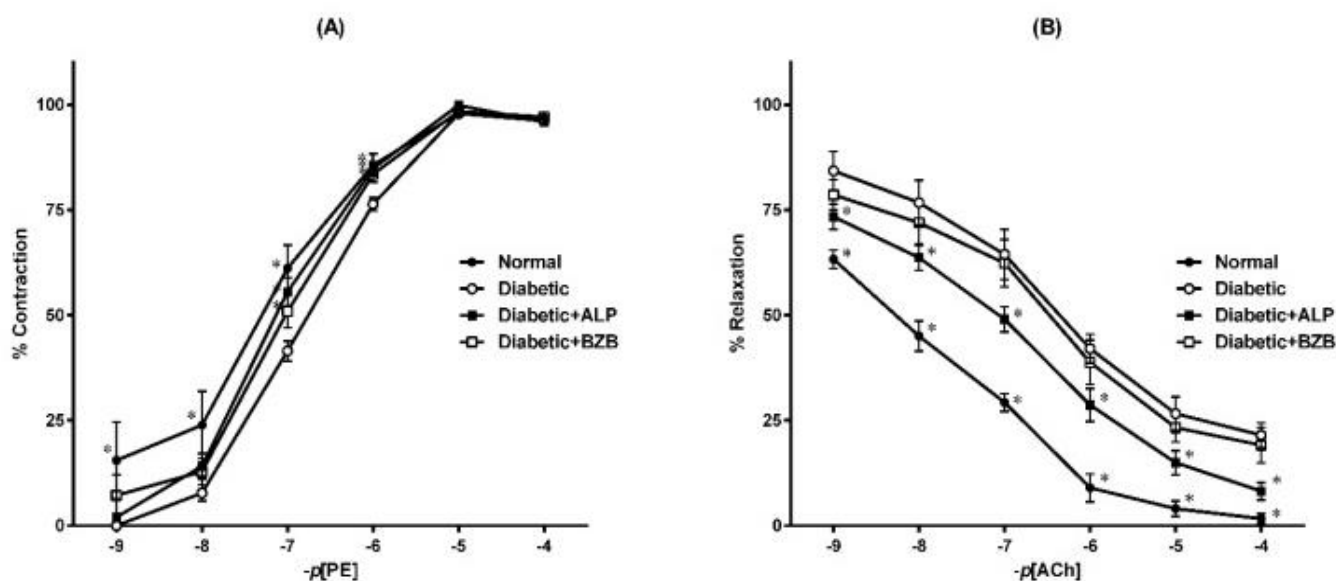
Fig.1. Vascular response to cumulative concentration of phenylephrine (A) and acetylcholine (B) in all the studied groups. ALP: allopurinol; BZB: benzbromarone. Each point represents the mean±SEM (n=7–10/group). Differences were tested by one-way ANOVA and post-hoc test of Tukey. $^*P<0.05$ versus diabetic group at the same time points.

Table 4: Biochemical assessment results

Groups	FPG (mg/dl)			UA (mg/dl)	Plasma MDA (nmol/g)	Plasma IP (pg/ml)
	Day-0	Day-28	Day-42			
Normal	115 ± 5	107 ± 5	112 ± 3	1.7 ± 0.2	2.33 ± 0.29	229.20 ± 38.82
Diabetic	429 ± 22***	451 ± 24***	430 ± 22***	3.7 ± 0.4***	8.54 ± 1.03***	558.03 ± 87.38*
Diabetic+ALP	406 ± 10	414 ± 15	365 ± 16$^{#}$	2.0 ± 0.2$^{###}$	3.73 ± 0.75$^{##}$	252.19 ± 33.53$^{#}$
Diabetic+BZB	418 ± 17	447 ± 34	399 ± 20	2.3 ± 0.3$^{##}$	7.48 ± 1.21	626.07 ± 83.14

Values are expressed as the mean±SEM (n=7–10/group). FPG: fasting plasma glucose; UA: uric acid; Plasma MDA: plasma malondialdehyde amounts; Plasma IP: plasma 8-isoprostane-2α; ALP: allopurinol; BZB: benzbromarone. Differences were tested by one-way ANOVA and post-hoc test of Tukey. $^*P<0.05$ and $^{***}P<0.001$ represent the difference between normal and diabetic groups. $^{#}P<0.05$, $^{##}P<0.01$ and $^{###}P<0.001$ represent the difference between treated-diabetic and diabetic groups.

induced contraction among the normal, diabetic, ALP and BZB groups (Table 3).

Cumulative concentrations of ACh on PE-incubated aortic rings induced relaxation in a concentration-dependent manner (Fig. 1). Diabetes caused a significant reduction in ACh potency and maximal efficacy of ACh-induced relaxation. In ALP-treated group, the ACh potency and maximal efficacy improved (P=0.019 and P=0.004, respectively), but in BZB-treated group, these parameters did not change significantly (Table 3).

The effect of treatments on biochemical parameters

After the intervention period, the STZ-treated rats had

a FPG levels of 430±22 mg/dl, while this level was 112±3 mg/dl in the normal group (P<0.001). All FPG levels data are reported in Table 4. Serum UA level was increased in diabetic group and treatment with ALP and BZB caused a significant reduction in serum UA levels in comparison with the diabetic group (P<0.001 and P<0.01 respectively). Diabetes caused a significant increase in serum MDA and IP levels. These oxidative stress indices had significantly improved by ALP treatment, while there were no significant changes in BZB-treated group (Table 4).

Discussion

Oxidative stress is a well-known and critical factor in

micro- and macrovascular complications of DM. ALP inhibits XO activity and reduces oxidative stress, which also lowers UA. In studies that ALP was shown to have beneficial effects on diabetic complications, the mechanism was attributed to its antioxidant effect. Nevertheless, some consider ALP's UA lowering effect as the main involved mechanism. The present study was designed to evaluate the effect of ALP on diabetic cardiomyopathy and vasculopathy, and to investigate which of the antioxidant and/or UA lowering activities were involved, and BZB as a uricosuric agent without any antioxidant activity was used as a control.

STZ induced a significant increase in FPG and severe DM in treated animals (Table 4). Diabetic rats showed a significant increase in serum UA, as well as MDA and IP (as oxidative stress markers) (Table 4). STZ accumulates in pancreatic B cells by glucose transporter type 2, and causes DNA alkylation and poly ADP-ribosylation, a process which depletes cellular NAD^+ and ATP. Augmented ATP dephosphorylation enhances supply of substrate for XO, and increases the ROS generation and UA. Furthermore, STZ produces toxic amounts of NO, which inhibits aconitase activity and potentiates DNA damage and destruction of B cells (Szkudelski, 2001).

Serum UA were significantly reduced in diabetic rats treated with both ALP and BZB, and UA level showed no significant difference between these groups in the administered doses (ALP, 50 mg/kg/day and BZB 10 mg/kg/day) (Table 4). Oxidative stress markers in the ALP-treated diabetic group were significantly less than the diabetic group, but BZB did not affect oxidative stress in DM. After six weeks of treatment, ALP induced a slight but significant decrease in FPG, while BZB did not have this property. Possibly, it is related to antioxidant properties of ALP, because the cytotoxic mechanism of STZ on the B cells of pancreas is intensively mediated by ROS.

In Langendorff preparation, the maximum velocity of systolic contraction (+dP/dt) and diastolic relaxation (-dP/dt) decreased significantly in diabetic group, while, PP did not change significantly. These observations confirm the induction of DCM (Table 2). ALP administration improved the +dP/dt in diabetic animal; however, the calculated P value did not reach a significant level (P=0.074), and ALP improved -dP/dt significantly. BZB had no significant positive impacts

on the mentioned parameters of cardiac function (Table 2).

DCM constitutes of structural and functional changes of the myocardium. It has been suggested that hyperglycemia-induced oxidative stress has a crucial role in the initiation and progression of DCM. ROS over-production results in disrupted calcium handling, including decreased sarcoplasmic reticulum calcium storage, attenuated ryanodine receptors function, and diminished sarco/endoplasmic reticulum calcium-ATPase activity in diabetic cardiomyocytes (Choi et al., 2002; op den Buijs et al., 2005). Hyperglycemia-induced cardiac ED (see below) particularly by the changes in Ca^{2+} homeostasis has a crucial role in DCM (Sheikh et al., 2012; Joshi et al., 2014). These include decreased in sarco/endoplasmic reticulum calcium-ATPase activity, and reduction in sodium-calcium exchanger expression and activity. Additionally, cardiac cell apoptosis and necrosis in DM is related to increased oxidative stress, inflammation and matrix metalloproteinase activity (Frustaci et al., 2000). Recent studies have shown oxidative stress-induced autophagy and mitophagy defects are involved in pathogenesis of DCM (Kobayashi and Liang, 2015).

Based on the obtained results it can concluded that ALP can improve DCM by reducing oxidative stress. In previous studies, ALP improved diabetic cardiomyopathy in animal models of diabetes and these beneficial effects were associated with decreasing oxidative/nitrosative stress and fibrosis (Rajesh et al., 2009). It should be noted that ALP-treated animals had a lower (but statistically significant) serum FPG levels at the end of intervention period in comparison with the diabetic group (Table 4). However, decrease in PFG was very low, but it is unlikely to have a prominent role in beneficial effects of ALP. Using BZB as a control revealed that UA lowering effect did not contribute to the obtained results.

In the current study, the potency and efficacy of PE did not change in the diabetic group. ALP-treated group had greater potency in contraction responses to PE in comparison with the diabetic animals. We have no convincing explanation for this observation. As expected, the potency and efficacy of ACh to relax diabetic aortic rings were lower in the diabetic group. A finding that was reported in many other studies (Gokce and Haznedaroglu 2008; Haznedaroglu and

Gokce 2014).

ED is a condition in which the endothelium loses its physiological properties. The most important features of ED are reduced production/bioavailability of NO and decreased the responsiveness of target tissues to NO (Tabit et al., 2010; Sena et al., 2013). oxidative stress is an important factor involved in pathogenesis of diabetic ED. Hyperglycemia induces four major mechanisms, including increased hexosamine pathway flux, activated several isoforms of protein kinase C, increased polyol pathway flux and increased advanced glycation end-product formation. ROS overproduction is common link between these pathogenic mechanisms and hyperglycemia-induced damages (Brownlee, 2001). Intracellular sources of ROS are enzymes, such as XO, NAD(P)H oxidase and the components of the mitochondrial electron-transport system (Brownlee, 2001). The increased over-expression or activity of XO, NAD(P)H oxidase, and NAD(P)H oxidase potentiate the ROS-induced cellular damages (Rajesh et al., 2009). ROS oxidizes crucial biomolecules including DNA, RNA, proteins and lipids resulting in alternation of cell signaling, modification of gene expression and apoptosis (Joshi et al., 2014), as well as activation of redox-sensitive transcription factors, NF-κβ, leading to the progression of various DM complications (Tabit et al., 2010).

ROS can react with NO to form peroxynitrite, which damages some vascular enzymes function, including endothelial NO synthase (eNOS) in endothelial cells and guanylyl cyclase in vascular smooth muscle cells (Tabit et al., 2010). ROS increases lipid peroxidation products and impairs receptor (acetylcholine and serotonin)-dependent activation of eNOS. Furthermore eNOS expression was downregulated under oxidative stress and inflammatory condition (Tabit et al., 2010).

Treatment with ALP increased the potency and maximum efficacy of ACh compared to diabetic animals. It seems that the beneficial effects of ALP on endothelial cells are related to the XO-inhibition property of the agent. As it was mentioned in other studies, oxidative stress has a key role in the initiation and progression of ED (Zhu et al., 2011; Roghani et al., 2013). Since, treatment with BZB had no significant effect on the potency and efficacy of PE or Ach, it was concluded that lowering UA level had no significant role in the obtained results of ALP. In a previous study, ALP was marked as an endothelial protective agent in diabetic rats due to augmentation of antioxidant defenses (Butler et al., 2000). Some other studies also showed that the protective effect of ALP on ED in animal model of type 1 diabetes was related to decrease ROS formation (Desco et al., 2002). The same result was obtained using febuxostat, another XO inhibitor. (Hwang et al., 2014). Although, in some studies the useful effects of ALP on diabetic complications, such as ED and diabetic nephropathy was attributed to its UA lowering effect; however, we did not find any evidence of this claim.

Conclusion

ALP improved DCM and ED in diabetic animals. It seems that these effects are produced by its antioxidant action.

Acknowledgments

Conflict of interest

The authors declare that they have no conflict of interest.

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