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Original Article

Protective effects of betaine on isoprenaline-induced myocardial infraction mediate via *apoE*, *Bcl-2* and anti-oxidative agents in rat model

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ABSTRACT

Betaine, a multifunction molecule, has a cardio protective feature but the mechanism is not well-understood. Hence, we evaluated the effect of betaine on cardiac tissue and serum biomarkers. Betaine administered to Albino rats at 50, 150 and 250 mg/kg, and sham group received deionized water. Myocardial infarction was induced by isoproterenol (100 mg/kg). The tunnel test and immunohistochemistry were performed on heart tissue to detect apoptosis and nitric oxide levels respectively. Serum levels of lipid peroxidation (LPO) and superoxide dismutase (SOD) were measured with colorimetric methods. The *apoE* and *Bcl-2* gene expression were measured by RT-PCR in heart tissues and peripheral blood mononuclear cells (PBMCs). The results showed that betaine pretreatment at 250mg/kg for 60 days which reduces the apoptotic cells in the heart tissue after isoproterenol-induced myocardial infraction (P= 0.038). Cardiac *Bcl-2* levels were upregulated by betaine, in which the maximum levels were observed in 150 mg/kg dosage. Similar finding was observed in the *apoE* expression in PBMCs. The tissue NOS levels were not change by betaine pretreatment significantly. Both superoxide dismutase and LPO levels were reduced by betaine pretreatment dose dependently. Our results confirm the protective effect of betaine pretreatment that is mediated by *Bcl-2* and anti-oxidative properties.

اثرات محافظتی بتائین بر سکته قلبی ناشی از ایزوپرنالین از طریق *apoE*، *Bcl-2* و عوامل آنتی اکسیداتیو در مدل موش صحرایی

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چکیده

بتائین، یک مولکول چند عملکردی، دارای ویژگی حفاظت قلبی است ولی مکانیسم آن به خوبی مشخص نیست. لذا در این مطالعه ما به بررسی اثر بتائین بر بافت قلب و بیومارکرهای سرمی پرداختیم. بتائین در دوز های ۵۰، ۱۵۰ و ۲۵۰ میلی گرم بر کیلوگرم بدن به موش های صحرایی آلبینو داده شد. سکته قلبی با تزریق ۱۰۰ میلی گرم بر وزن رت القا شد. تست تانل و تست ایمنوهیستوشیمی به ترتیب برای بررسی آپوپتوز و سطح پروتئین نیتریک اکسید انجام شد. پراکسیداسیون لیپیدی و مقادیر آنزیم سوپر اکسید دیسموتاز با روش کالریمتری انجام شد. بیان ژن های *Bcl-2* و *آپوE* در سلول های تک هسته ای خون محیطی با روش RT-PCR تعیین شد. نتایج نشان داد که تجویز بتائین در دوز ۲۵۰ میلی گرم بر وزن بدن به مدت ۶۰ روز سبب کاهش آپوپتوز پس از سکته قلبی می شود (P=۰/۰۳۸). ژن *Bcl-2* در بافت قلب در دوز ۱۵۰ میلی گرم بر وزن بدن القا شده و نتایج مشابهی برای *آپوE* مشاهده شد. در حالیکه تغیراتی در مقادیر نیتریک اکسید مشاهده نشد اما پراکسیداسیون لیپیدی و سطوح آنزیم سوپر اکسید دیسموتاز کاهش یافت. نتایج مطالعه ما حاکی از آن است که بتائین از طریق *Bcl-2* و ویژگی ضد استرس اکسیداتیو، سبب حفاظت قلب می شود.

واژه های کلیدی: بتائین، ایزوپرنالین، سکته قلبی، آپوپتوز

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INTRODUCTION

In addition to methyl donor properties, betaine works as an osmoprotective molecule. Both methyl metabolism and osmosis are important in the myocardial infarction metabolism [1]. The protective effect of betaine on cardiovascular disease has a long history [2]. Betaine is a precursor of S-adenosylmethionine and its reduced levels are associated with homocystinuria and hyperhomocysteinemia [3]. Betaine supplementation could prevent nitric oxide metabolism disturbance and lipid disorder [4]. Some studies showed that betaine modulates living cells against apoptosis [5]. Almost all cardiovascular risk factors have a relationship with betaine. Apoptosis plays a key role in the pathogenesis in myocardial infarction [6]. Affecting intracellular signaling, a methyl-donor treatment can either increase and reduce the apoptosis [7]. The role of *Bcl-2* family proteins made cells committed to apoptosis by different mechanisms. *Bcl-2* regulates apoptotic process after myocardial infraction. Some studies showed that betaine can affect the apoptosis in cancer cell lines via Bcl-2 expression [8]. A well-documented role of nitric oxide has been defined as the result of blood pressure, vascular function, and prevention smooth muscle cell proliferation [9]. Among the three nitric oxide synthesis enzymes, iNOS (NOS2) is activated by post myocardial infraction ischemia/reperfusion that increases infarct size [10]. The cardiac dysfunction and remodeling are reduced by suppression of iNOS enzyme [11]. A systematic review showed that the genetic polymorphism of apolipoprotein E affects the risk of myocardial infarction [12]. *ApoE* is a key protein in lipoprotein metabolism and can attach to the ligand for the low-density lipoprotein receptors. Shiwei Lv et al. showed betaine supplementation reduces atherosclerosis in *ApoE* -deficient rats [13]. Transcriptional regulation of *ApoE* is associated with DNA methylation and carbon metabolism [14]. Some articles reported a relationship between betaine and lipid metabolism in the *ApoE* -deficient rats [15]. Lipid peroxidation is associated with cardiovascular disease [16]. Body pro-antioxidant imbalance leads to lipid peroxidation. It could be considered as a biomarker for body antioxidant power meanwhile it can serve as a pathologic agent. Superoxide dismutase and other enzymatic antioxidant systems prevent the peroxidation of lipids and further myocardial infraction incidences [17]. We explored the protective efficacy of betaine and possible underlying mechanisms at cellular and molecular levels.

MATERIALS AND METHODS

Animal and Treatment

We declare that the animal care and use were approved by Biomedical Research Ethics Committee, Urmia University of Medical Sciences. The code of ethics was ir.umsu.rec.1395.270 (https://umsu.ac.ir/uploads/tarh95_68681.pdf). rat cardiac biomarker confirmed myocardial infraction. As we described in the previous article, 48 rats were divided into four groups receiving betaine at 50, 150, 250 mg/kg of body weight and deionized water [1]. After a 60-day treatment, myocardial infraction was induced by isoproterenol 100 mg/kg. The heart tissue and blood were collected for further analysis.

PBMCs Isolation and Real-time PCR

PBMCs were isolated by Ficoll® and centrifugation based on the standard protocol

[18]. By lysis buffer and solid phase extraction method, the total RNA was obtained. After cDNA synthesis, the samples were stored at -20 Celsius. The gene expressions were analyzed using RT-PCR and *Bcl-2* and *apoE* gene primers (Table 1). The replicated products were undergone agarose gel electrophoresis and quantified by image j software.

Superoxide Dismutase and Lipid Peroxidation Assays

The superoxide dismutase levels were measured using Cayman kit (cat number 706002). Using xanthine oxidation and Formosan dye color changing, the enzyme activity was determined. We also used Cayman kit (cat number 705002) for the measurement of malondialdehyde by thiocyanate as a chromogen.

TUNEL assay

By the TUNEL assay, apoptosis was assessed in the dissected tissues. This method is based on the detection of the single- and double-stranded DNA breaks with enzyme linked antibody. Using Situ Cell Death Detection Kit (roche) was used according to the company protocols. After dewaxing and hydration, a proteinase k treatment was performed. Terminal nucleotidyl transferase containing buffer was applied to each sample. The labeled DNA breaks were detected by monoclonal antibody and HRP-DAP stain.

Immunohistochemistry

Using 10% formalin solution, the tissues were fixed. Ethanol replacement and xylene treatment used to proper tissue blocks. The time and concentration of solution were papered and administered according to the standard protocols. The anti-iNOS antibody (cat number: ab15323, (1:200, Abcam, MA) used for the detection of rat nitric oxide synthase in the heart tissues.

Statistical analysis

Using SPSS software version 23.0, we tested one-way analysis of variance (ANOVA) to compare the mean of the groups. We also considered $p < 0.05$ statistically significant.

RESULTS

Bcl-2 and ApoE Gene Expression

The Bcl-2 gene is shown in Figure 1. Betaine induced the *Bcl-2* gene and the treatment group has higher PBMCs *Bcl-2* than the controlled group. Betaine at 150 mg/kg has the maximum effects. We observed a similar gene expression pattern for *apoE* gene, where betaine increases the gene expression more than the controlled group and the most effective dosage was 150mg/kg (Figure 2).

Nitric oxide synthesis -2 protein levels

Table 1. *apoE* and *Bcl-2* gene primers for RT-PCR analysis

apoE	Forward	5TGAACCGCTTCTGGGATTAC3
	Revers	5TGTGTGACTTGGGAGCTCTG3
Bcl-2	Forward	5CTTCAGGGATGGGGTGAACT3
	Revers	5CAGCCTCCGTTATCCTGGAT3

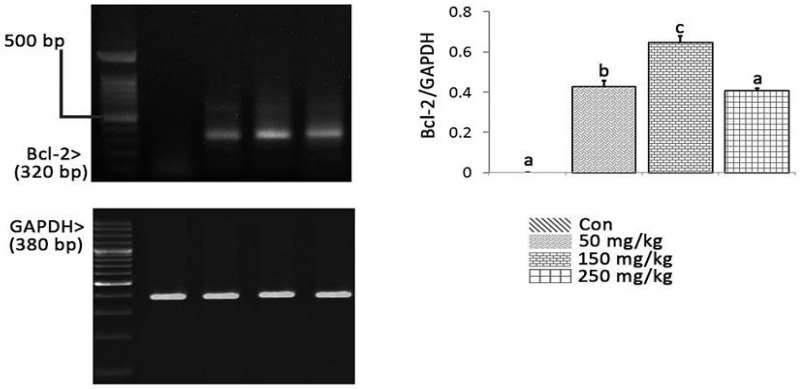

Figure 1. The *Bcl-2* gene expression in the PBMC of rats receiving betaine and water .GAPDH was used as a housekeeping gene. The quantified amount was depicted in charts.

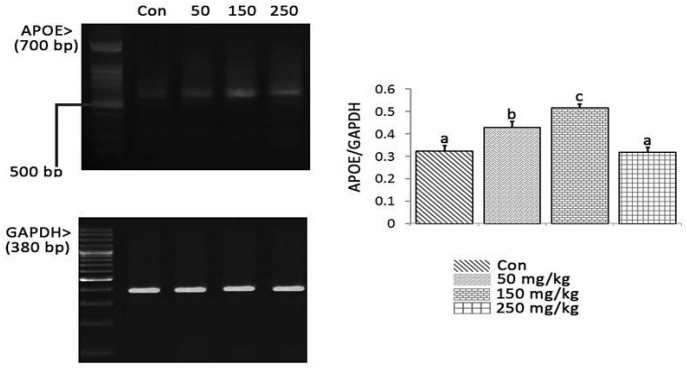

Figure 2. The *apoE* gene expression in the PBMC of rats receiving betaine and water .GAPDH was used as an internal control gene. The quantified amount was depicted in charts.

Table 2. Statistical analysis of iNOS gene in the heart tissues.

(I)	(II) Treatment	Sig.
control	G1	0.64
	G2	0.892
	G3	0.919

The immunohistochemistry staining of rat heart samples including apex and caudal and cranial parts showed betaine supplement has no significant effect on NOS-2 expression in the heart tissues. Figures 3 and 4 depict the tissues staining with specific antibodies and the statistical analysis of the images. Table 2 shows the variance analysis between groups.

Apoptosis TUNEL assay results

Figures 5 and 6 show the post myocardial infraction apoptosis in the heart and the statistical finding respectively. Betaine

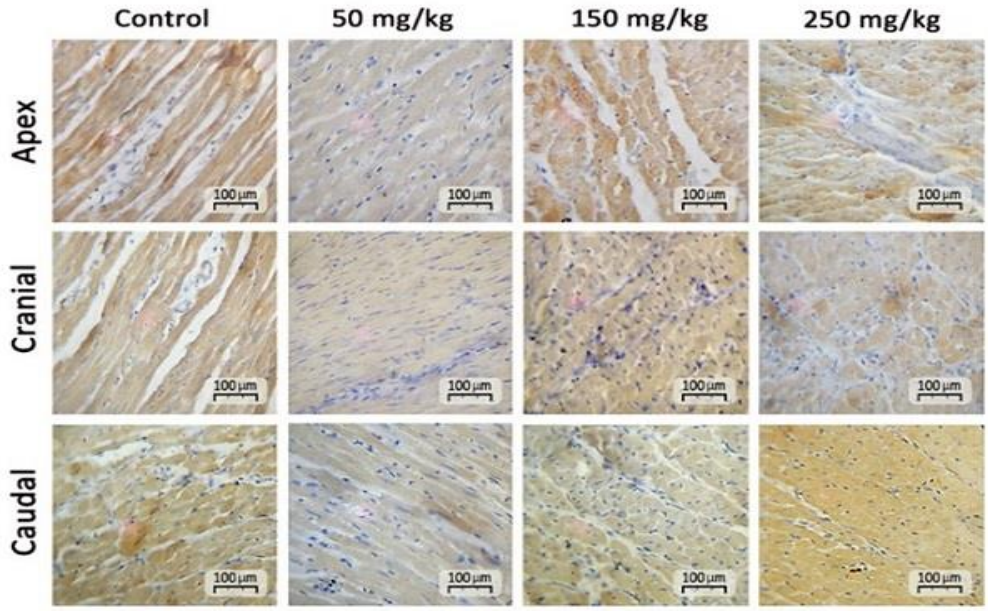

Figure 3. The protein levels of NOS-2 in the heart tissues. The brown diaminobenzidine staining shows the expression of NOS-2 protein in the tissues.

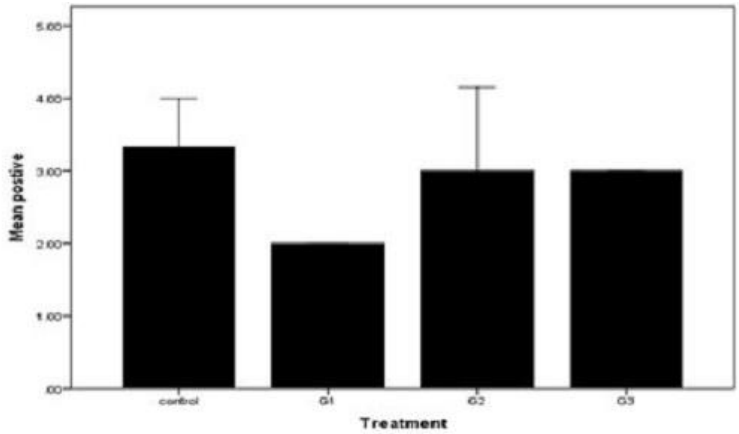

Figure 4. The statistical analysis of iNOS protein in the heart tissues . The data of the stained sections was quantified and expressed as graphs. The error bars show the SD of means treated by betaine (G1=50mg/kg, G2=150mg/kg, G3=250mg/kg).

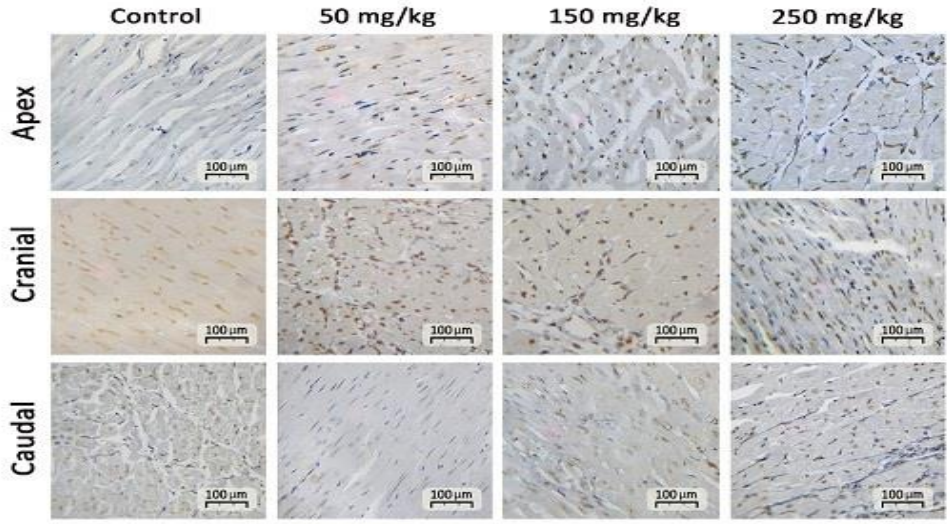

Figure 5. The apoptotic cells with DNA breaks labeled by brown dye. The brown diaminobenzidine staining shows the apoptotic cells in the tissues (TUNEL assay).

manner and the 250 mg/kg dosage has a significant effect (P= 0.038). The P value for the mean difference between group 2 (150mg/kg) and group 1 (50mg/kg) with the control groups were 0.116 and 0.359, respectively.

Serum SOD and LPO Levels

Serum SOD and LPO levels were measured and the statistical findings represented in the Table 3. Betaine supplementation increases the SOD levels dose dependently. The difference was statistically significant. LPO, the lipid peroxidation biomarker, was reduced by betaine. The 250mg/kg was the most effective dosage in terms of lipid peroxidation.

DISCUSSION

The Isopernaline administration induced the myocardial infraction, and the troponin levels and blood pressure measurement confirmed the reported results in our previous articles [1]. Betaine reduces the troponin levels that was in concordance with Zheng et al study, where they reported a protective effect for betaine against isopernaline-induced myocardial infraction [19]. It is a well-documented fact that apoptosis and necrosis are the cellular events after myocardial infraction that mediates the cardiac injury. Betaine reduces the apoptosis rates in the heart tissue at 150mg/kg dosage. This protective effect may be due to the osmomodualtoy properties of betaine. Galvez et al. found hyperosmolar

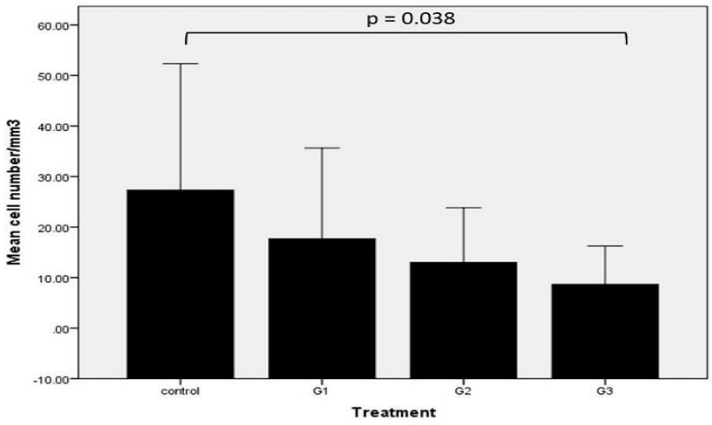

Figure 6. The statistical analysis of apoptosis in heart tissues .The data of the apoptotic cells of the sections was quantified and expressed as graphs .The error bars show the SD of means. Treatment includes (G1=50mg/kg, G2=150mg/kg, G3=250mg/kg).

Table 3. The statistical Findings related to Serum SOD and LPO

Treatments	SOD (U/L)		LPO (nmol/ml)	
	mean	P value	Mean	P value
Control	15.554± 4.471	None	3.188± 0.504	None
G1(50mg/kg)	17.300± 3.573	0.584	3.567±0.437	0.0807
G2(150mg/kg)	24.101±8.730	0.001	2.962±0.401	0.056
G3(250mg/kg)	24.427± 7.343	0.001	2.116±0.249	0.001

condition could regulate the signaling pathway leading to betaine transporter expression [20]. Betaine impedes the myosin denaturation and controls the IGF-1 dependent cardimyocte differentiation. Then, we can conclude, the

effects of betaine are mediated by osmomudulation at least in part [21]. However, the role of betaine as an antioxidant was confirmed by our results that showed the SOD and LPO levels were increased and reduced respectively. The third angel of betaine effect is the involvement of betaine in the carbon metabolism that we disused elsewhere [1]. We showed that the *apoE* expression was increased by betaine supplements. Lever et al. found that patients with acute coronary syndrome and lower betaine levels have disturbed lipid profile. Martins et al. reported that betaine reduces serum cholesterol levels. Wang et al. found the effect of betaine on lipid profile is mediated with SOD and glutathione levels [22]. The role of SOD on cardiovascular disease and myocardial infraction is undeniable and our results showed the SOD levels changing in the rat models. SOD is regulated by environmental and epigenetic factors. This regulation may be due to the regulatory effects of betaine on gene expressions. Wilcken et al. reported that homocysteine, the methyl deficiency biomarker has correlations with superoxide dismutase [23]. LPO biomarker was reduced in the serum of betaine treated rats. Therefore, we conclude that the antioxidant property of betaine is the important factor in the protective effect of betaine against myocardial infarction. Some studies confirmed this finding.

CONCLUSION

Our results show that betaine is an effective supplement to prevent the myocardial infraction and its severity in dose dependent manner. The mechanism has a multi-facial nature and needs to be studied in more scrutiny.

ETHICS

All ethical standards have been respected in this study.

CONFLICT OF INTEREST

None declared.

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