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Cardioprotective Effects of Co-administration of Pomegranate Extract and Vitamin E on Electrocardiographic, Biochemical and Apoptotic Changes in Isoproterenol Induced Myocardial Infarction in Rats

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ABSTRACT

Background: The present study was planned to assess the cardioprotective effects of Pomegranate fruit extract alone and along with vitamin E by evaluating electrocardiographic changes and biochemical changes in isoproterenol (ISO) induced myocardial infarction in rats. **Materials and Methods:** Myocardial infarction was induced by subcutaneous injection of isoproterenol (200 mg kg^{-1}) for two consecutive days at an interval of 24 h. Rats were treated with pomegranate (100 mg kg^{-1} day^{-1}, p.o.) and vitamin E (50 mg kg^{-1} day^{-1}, p.o.) for a period of 30 days and isoproterenol was injected on 29th and 30th day. On day 31st electrocardiographic changes and biochemical changes were monitored from all the experimental groups. **Results:** Isoproterenol injected rats showed a significant alteration in ECG pattern. It also showed significant increase in C-reactive protein, myeloperoxidase levels, lipid peroxidation and Caspase-3 protease activity. In addition, it also exhibited alteration in the levels of electrolytes (Na^+, K^+ and Ca^{++}). Significant alteration of ATPases was also observed. Treatment with Pomegranate fruit extract alone and along with vitamin E significantly prevented the isoproterenol induced alteration in electrographic changes and other biochemical alterations. **Conclusion:** The present result showed that treatment of pomegranate fruit extract alone and along with vitamin E in ISO injected rats significantly attenuates myocardial infarction.

Key words: Pomegranate, myocardial infarction, vitamin E, antioxidant, apoptosis

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INTRODUCTION

Cardiovascular disease (CVDs), is one of the leading causes of death and is expected to be the most significant cause of mortality in India by 2020. These are primarily caused by the chronic deficiencies of essential nutrients such as coenzymes, cellular energy carriers, antioxidants and vitamins. Many dietary antioxidants and some non-nutrient based antioxidants from plants are increasingly being recognized as possible health promoters in reducing the risk of CVDs[1,2].

Isoproterenol (ISO), a β-adrenergic agonist, induced myocardial infarction in rats which is confirmed by hyperglycemia, hyperlipidemia, alteration in enzymes activity, ECG changes, biochemical alterations and formation of reactive oxygen species and thereby oxidative stress. MI induced by ISO in animals, shows many metabolic and morphologic aberrations similar to those observed in man. In previous studies, the protective effects of green tea, lycopene and *Lagenaria sicessaria* in ISO induced myocardial infarction in rats has been reported[3-7].

Punica granatum belongs to the family of Punicaceae, is commonly known as pomegranate, grenade, grants and punica apple. Extracts of all parts of the fruit appear to have therapeutic properties. The most therapeutically beneficial pomegranate constituents are ellagic acid, ellagitannins (including punicalagins), punicic acid, flavonoids, anthocyanidins, anthocyanins and estrogenic flavonols and flavones[8]. Epidemiological data shows an

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inverse association between cardiovascular risk and consumption of vitamin E from supplements or dietary consumption[9,10]. Antioxidants are uniquely different from each other and study synergistically when used in combination[11,12]. So, from the above observation the present study was designed to evaluate the effect of Pomegranate fruit extract alone and in combination with vitamin E on ECG changes and biochemical alterations in ISO induced myocardial infarction in rats.

MATERIALS AND METHODS

Drugs and chemicals: (±)-Isoproterenol hydrochloride (ISO) and vitamin E (DL-α-Tocopherol acetate) were purchased from Sigma Aldrich Co. St. Louis. MO. USA. Alcoholic extract of Pomegranate fruit (PGFE) was gifted by Cherain Chemicals, Baroda, India. The entire chemical used in this study was of analytical grade.

Experimental animals: Male adult albino rats (200-250 g) were used in the present study. The animals were housed in an air conditioned room and were kept in standard laboratory conditions under normal light and dark cycle (12 h light/dark) maintained at an ambient temperature 25±2°C. The animals were fed standard pellet diet and water *ad libitum*. The experimental protocol was approved by the Institutional Animal Ethics Committee (404/02/a/CPCSEA) of Pharmacy Department, The Maharaja Sayajirao University, Vadodara.

Experimental design and protocol: Animals were divided into different groups each group consists of six animals. The experimental protocol was for 30 days:

Group 1: Control rats received distilled water for 30 days and normal saline (1 mL kg^{-1}, s.c) on 29th and 30th day

Group 2: Received distilled water for 30 days and ISO (200 mg kg^{-1}, s.c) in normal saline on 29th and 30th day at an interval of 24 h

Group 3: Received PGFE (100 mg kg^{-1} day^{-1}, p.o.)+vit. E (50 mg kg^{-1} day^{-1}, p.o.)

Group 4: Received vit. E (50 mg kg^{-1} day^{-1}, p.o.) for 30 days and ISO on 29th and 30th day

Group 5: Received PGFE (100 mg kg^{-1} day^{-1}, p.o.) for 30 days and ISO on 29th and 30th day

Group 6: Received vit. E (50 mg kg^{-1} day^{-1}, p.o.)+PGFE (100 mg kg^{-1} day^{-1}, p.o.) for 30 days and ISO on 29th and 30th day

Measurements of electrographic changes: At the end of treatment period i.e., on day 31st, ECG of all the groups were recorded under light ether anaesthesia through needle electrodes (Lead II) using Biopac MP30 data acquisition system (Biopac Systems, Santa Barbara, CA). The changes in ST interval, QT interval and RR interval were recorded.

Biochemical estimation

Assessment of serum markers enzyme: The level of AST, ALT, lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) were estimated using standard diagnostic kit. Serum protein and uric acid levels were determine using reagent kit (Span Diagnostic Ltd, Gujarat, India).

Separation of LDH isoenzymes by electrophoresis: LDH-isoenzymes were separated by agarose gel electrophoresis[13]. Agarose gel (1% w/v) was prepared and poured immediately on the glass slide. Ten microliter of serum sample was loaded into the wells and the gel was run. After that, the gel was stained using the reported regents. The photographs were taken using Alpha Ease Fc Imaging system, USA.

Estimation of electrolytes and membrane bound phosphates: The sediment after centrifugation of tissue homogenate was resuspended in ice-cold tris buffer (10 mM, pH 7.4) to get a final concentration of 10% w/v and were used for the estimation of Na^+/K^+ATPase[14] Ca^{2+}ATPase[15] and Mg^{2+}ATPase[16]. The levels of Na^+ and K^+ were estimated using commercial kits (Monozyme India Ltd, Secunderabad). Level of Ca^{+2} was measured by the O-cresophthalein complex one method using a reagent kit (Span Diagnostic Ltd, Gujarat, India).

Estimation of C-reactive protein, myeloperoxidation and lipid peroxidation: Quantitative estimation of CRP in serum was performed as per instructions provided by SPINREACT kit (Latex turbidimetry method) S.A. Ctra. Santa Coloma, Spain. Tissue myeloperoxidation activity was measured using a modified spectroscopic method described by Bradley *et al.*[17]. Tissue was homogenized and the supernatant was used for estimation of lipid peroxidation level[18].

Estimation of caspase-3 protease level: The levels of caspase-3 Protease were estimated as per the instruction provided by BioVision (Caspase-3/CPP32 Colorimetric Assay Kit, USA). Micro-titer plate was read at 405 nm in a micro plat reader (BIO RAD, Model 680XR).

Statistical analysis: Results are presented as Mean±SEM. One-way analysis of variance (ANOVA) followed by Bonferroni multiple comparisons using a computer based fitting program (Prism, Graph Pad) was used to analyse results. Differences were considered to be statistically significant when $p<0.05$.

RESULTS

Effect of PGFE and vitamin E on ECG pattern: Effects of PGFE and Vit. E on ECG changes in normal and ISO injected rats were recorded (Table 1). ISO injected rats showed a significant ($p<0.01$, $p<0.001$) elevation of ST-interval, QT interval and a significant ($p<0.001$) decrease in RR interval as compared to control group. Co-administration of PGFE and Vit. E in ISO injected rats (Vit. E+PGFE+ISO) showed a significant ($p<0.001$, $p<0.01$, $p<0.05$) decrease in ST interval, QT interval and a significant increase in RR interval as compared to PGFE and vit. E alone treated group.

Effect of PGFE and vitamin E on serum markers of cardiotoxicity: The activities of serum marker enzymes such as AST, ALT, ALP, uric acid and LDH were found to be significantly ($p<0.001$) increase and total protein was significantly ($p<0.001$) decreased in ISO injected rats compared to control rats. Co-administration of vit. E and PGFE in ISO injected rats showed a significant ($p<0.01$) decrease in the level of serum AST, ALT, ALP, uric acid and LDH and a significant ($p<0.001$, $p<0.05$) increase in the level of total protein as compared to ISO injected rats (Fig. 1, 2 and 3) was observed.

Effect of PGFE and Vitamin E on LDH isoenzyme pattern: ISO injected rats showed an increase intensity of LDH-1 and LDH-2 isoenzyme bands. PGFE+ISO treatment decreased the intensity of LDH1 and LDH2 isoenzyme compared to ISO injected rats. Co-administration of vit. E and PGFE in ISO injected rats (vit. E+PGFE+ISO) showed further reduction in the intensity of LDH-1 and LDH-2 isoenzyme bands (Fig. 4).

Effect of PGFE and vitamin E on membrane bound ATPase and electrolytes levels: ISO injected rats showed a significant ($p<0.001$) decrease in Na^+/K^+-ATPase, Mg^{+2}-ATPase activities and a significantly ($p<0.001$) increase in Ca^{+2}-ATPase activity was also observed. Co-administration of vit. E and

Table 1: Effect of PGFE alone and in combination with Vitamin E on ECG changes in normal and ISO injected rats

Groups	ST elevation	QT interval	R-R interval
Control	0.184±0.0022	0.071±0.0006	0.170±0.0007
ISO	0.302±0.0040***	0.081±0.0012**	0.158±0.0017**
Vit. E+PGFE	0.182±0.0032	0.070±0.0021	0.170±0.0005
Vit. E+ISO	0.267±0.0054**	0.078±0.0076	0.162±0.0010*
PGFE+ISO	0.230±0.0032**	0.077±0.0038	0.164±0.0024**
Vit. E+PGFE+ISO	0.222±0.0056^{b***}	0.074±0.0017^{ab*}	0.166±0.0011^{a**}

Values are expressed as Mean±SEM. Level of significance is considered as $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$. ISO injected group compared with control, treatment groups were compared with ISO injected group. $^{a}p<0.05$ compared with vit. E+ISO group, $^{b}p<0.05$ compared with PGFE+ISO group

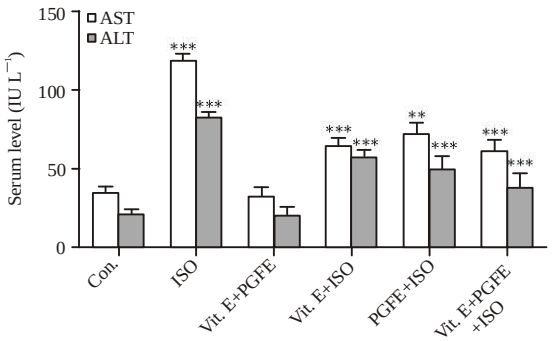

Fig. 1: Effect of PGFE alone and in combination with vitamin E on the levels serum AST and ALT level in the heart of normal and ISO injected rats

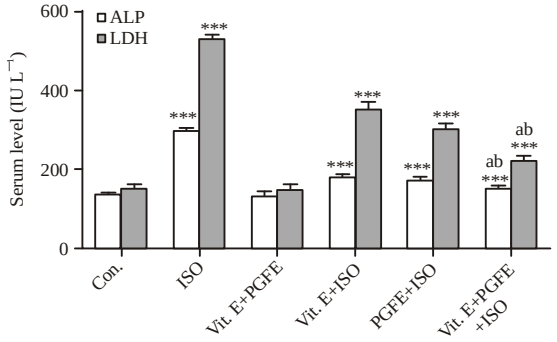

Fig. 2: Effect of PGFE alone and in combination with vitamin E on serum ALP and LDH levels in treatment and ISO injected rats

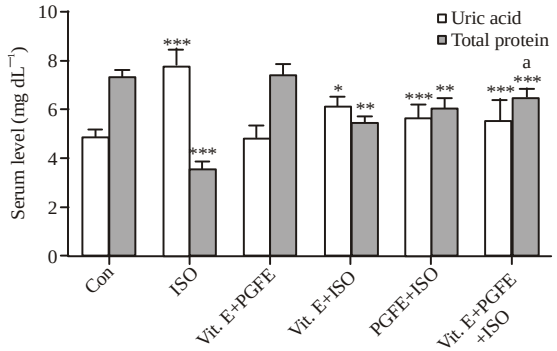

Fig. 3: Effect of PGFE alone and in combination with vitamin E on serum protein and uric acid levels in normal and ISO injected rats

PGFE in ISO injected rats showed significant ($p<0.001$) increase in the activities of Na^+/K^+ and Mg^{+2}-ATPase with a significant ($p<0.001$) decrease in Ca^{+2}-ATPase compared to ISO injected rats (Fig. 5).

The levels of electrolyte in control and ISO injected rats are shown in Table 2. ISO injected rats showed a significant ($p<0.001$) increase in sodium, calcium levels and a significant ($p<0.001$) decrease in potassium level. Co-administration of vit. E and PGFE in ISO injected rats significantly ($p<0.001$) decreased the levels of sodium, calcium and significantly ($p<0.001$, $p<0.05$) increased the level of potassium as compared to ISO injected rats (Table 2). This combination did not produce further significant improvement in ATPase activities and electrolyte levels as compared to vit. E+ISO and PGFE+ISO treated groups.

Effect of PGFE and vitamin E on inflammatory markers and lipid peroxidation: Rats injected with

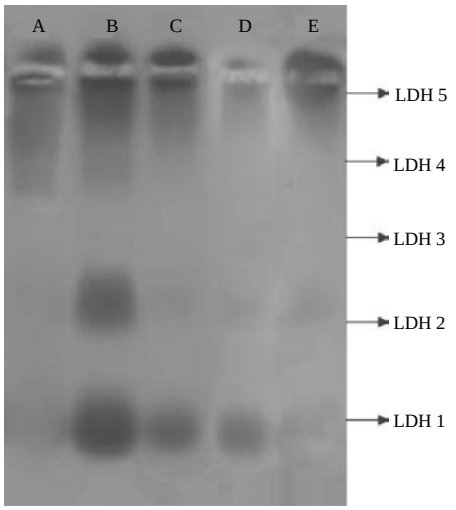

Fig. 4: Effect of PGFE alone and in combination with vitamin E on LDH isoenzyme pattern in normal and ISO injected rats. A: Control, B: ISO, C: Vit. E+ISO, D: PGFE+ISO, E: Vit. E+PGFE+ISO

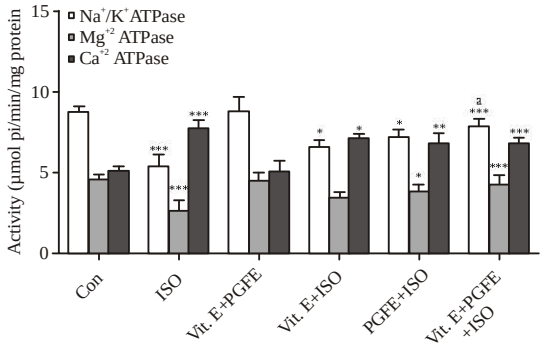

Fig. 5: Effect of PGFE alone and in combination with vitamin E on the activities of sodium, potassium and calcium ATPases in the heart of normal and ISO injected rats

ISO showed a significant ($p<0.001$) increase in serum CRP (Fig. 6a) level tissue MPO activity (Fig. 6b) and tissue lipid peroxidation (Fig. 6c) contents as compared to control group. However, co-administration of vit. E and PGFE in ISO injected rats showed better improvement in maintaining the levels of CRP, MPO and lipid peroxidation contents as compared to vit. E+ISO or PGFE+ISO treated groups (Fig. 6a-c).

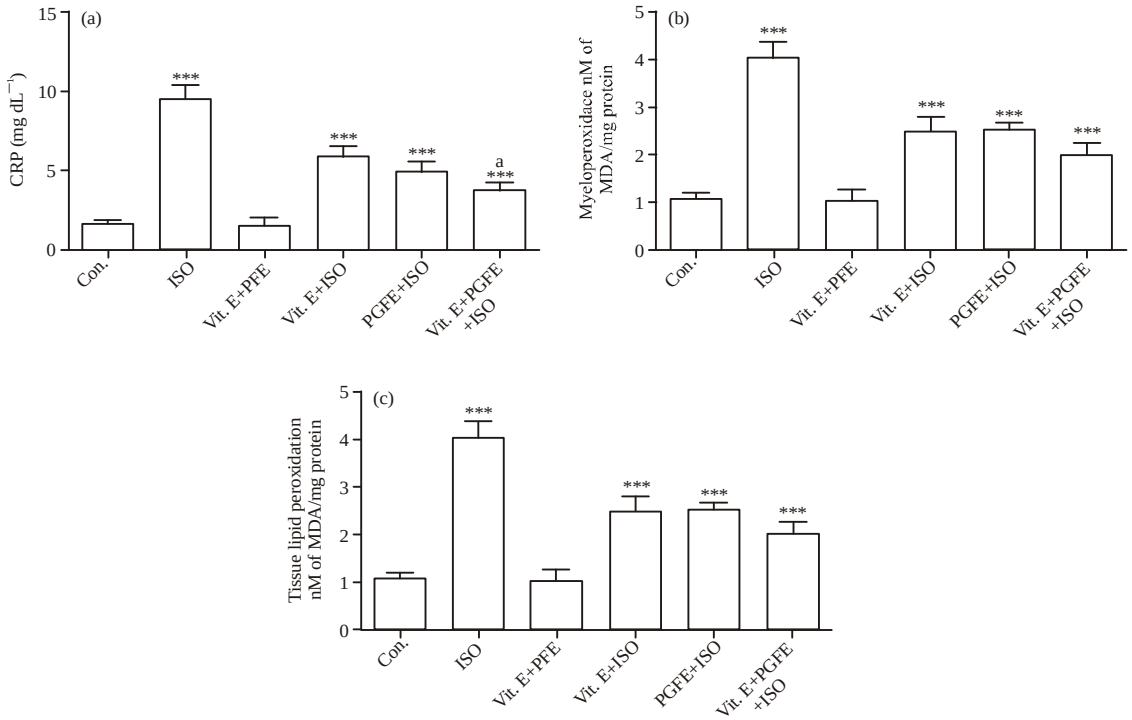

Fig. 6(a-c): Effect of PGFE alone and in combination with vitamin E on (a) CRP, (b) Myeloperoxidase and (c) lipid peroxidation levels normal and ISO injected rats

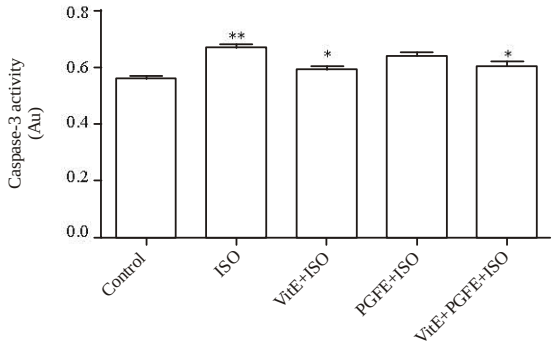

Fig. 7: Effect of PGFE alone and in combination with vitamin E on caspase-3 protease activity in normal and ISO injected rats

Table 2: Effect of PGFE alone and in combination with vitamin E on the levels of sodium, potassium and calcium in the heart of normal and ISO injected rats

Groups	Sodium (Na^+)	Potassium (K^+)	Calcium (Ca^{++})
Control	6.088±0.125	8.978±0.164	10.81±0.107
ISO	7.728±0.312****	6.162±0.216****	13.83±0.344****
Vit. E+PGFE	6.111±0.103	8.832±0.108	10.93±0.132
Vit. E+ISO	7.444±0.175	7.462±0.189****	12.63±0.291**
PGFE+ISO	6.832±0.211**	7.232±0.223**	12.88±0.303*
Vit. E+PGFE +ISO	6.599±0.203****	8.126±0.108****	12.04±0.210**

Values are expressed as Mean±SEM. Level of significance is considered as *p<0.05, **p<0.01 and ****p<0.001, ISO injected group compared with control and treatment groups were compared with ISO injected group

Effect of PGFE on Caspase-3 protease activity: ISO injected rats showed a significant ($p<0.01$) increase in caspase-3 activity as compared to control group. Treatment with PGFE alone (PGFE+ISO) did not produce significant effects on caspase-3 activity. Co-administration of vit. E+PGFE+ISO showed similar effects as shown by Vitamin E alone (Fig. 7).

DISCUSSION

CVDs are primarily caused by chronic deficiencies of vitamins and other essential nutrients and consumption of such nutrition are reported to protect the myocardium from damage/infarction. Electrocardiographic changes are generally considered as the main criteria for the diagnosis of myocardial infarction. In the present study, ISO injected rats showed a significant elevation of ST segment, QT interval and reduction in R-R interval. These alterations in ECG could be due to the consecutive loss of cell membrane potential in injured myocardium. Patient with myocardial ischemia also shows ST segment elevation[19]. Similarly, ST elevation is also reported in animal models of myocardial infarction[20]. Treatment with PGFE and vit. E in ISO injected rats significantly prevented the altered ECG pattern towards normal suggesting the cell membrane stabilizing potential of this combination.

In the present study, ISO injected rats showed a significant elevation in the levels of AST, ALT, ALP and LDH, indicated the severity of membrane damage and loss of functional integrity of cell membrane integrity[4,20]. ISO undergo auto-oxidation and generates free radicals which further produced oxidative stress. Increase production of free radicals and oxidative stress initiate lipid peroxidation of the membrane, leading to loss of structural and functional integrity of the membrane. A significant increase was observed in the activities of LDH and ALP, which might be due to the leakage of enzymes from the heart as a result of oxidative stress and necrosis induced by ISO[21]. The increase in the expression of cardiac specific LDH isoenzymes confirms the cardiac injury induced by ISO.

Studies have shown that uric acid is an important independent risk factor for cardiovascular mortality and in the development of MI[22]. It has been reported that during hypoxic condition xanthine dehydrogenase is converted to xanthine oxidase by the oxidation of -SH-groups. Xanthine oxidase then catalyses the conversion of hypoxanthine to xanthine, uric acid and superoxide[23]. This could be one of the reasons for the elevated levels of serum uric acid in the present study. Administration of PGFE alone and along with vit. E significantly decreased the elevated levels of serum marker enzymes towards normal. This reduction in marker enzyme levels could be due to the strong protective effects of these combinations which prevent membrane integrity and/or permeability thereby restricting the leakage of these enzymes from the myocardium[24]. Further the combination significantly decrease the level of uric acid and increase the level of serum protein, this might be the antioxidant property of this combination which prevents the -SH- group of enzyme from oxidation and thereby reduced the elevated uric acid level.

In the cell, membrane bound phosphatase (ATPases) are involved in the energy dependent transport of sodium, potassium, magnesium and calcium translocation. An increase in sodium and calcium along with decrease in potassium was observed in ISO injected rats which might be due to altered ATPases activity in membrane as a result of lipid peroxidation produced by ISO.

Increased concentration of sodium might be due to decrease in Na^+/K^+ATPase[25]. In the present study, treatment of PGFE alone and its co-administration with Vit. E significantly increased the activities of Na^+/K^+ ATPase and Mg^{2+} ATPase and decreased the activity of Ca^{2+} ATPase. These combinations also prevent the alteration in Na^+, K^+ and Ca^{2+} levels. The protective effects of these combinations could be due to the prevention of -SH- group from oxidative damage. The present study shows the beneficial effect of PGFE along with vit. E in reducing lipid peroxidation. This might be the reason for better effects of PGFE along with vit. E in attenuating membrane bound ATPases and electrolyte levels.

The CRP has been used as a responsive marker of acute cardiovascular events. It has been reported that serum CRP concentrations are inversely associated with dietary intake of fruits, vegetables and tea, which are rich in polyphenolic antioxidants[26]. Increased MPO activity indicates an acute inflammation and leukocyte accumulation in heart tissues of the ISO-injected rats[27]. Treatment of PGFE in combination with vit. E significantly reduced the elevated CRP levels and MPO activity suggesting potent anti-inflammatory activity.

Increased levels of lipid peroxidation products can also injure blood vessels, increased adherence and aggregation of platelets to the injured sites[28]. The main target for reactive oxygen species appears to be polyunsaturated fatty acids (PUFA), a precursor for lipid peroxidation. Malondialdehyde is a major lipid peroxidation end product; increased malondialdehyde content may contribute to increased generation of free radicals, altered membrane structure and decreased activities of antioxidant enzymes[29]. In the present study a significant increase in the lipid peroxidation in ISO injected rats could be attributed to the accumulation of lipids in the heart. Oxidative stress in tissues resulted in increased production of ROS and depletion of the antioxidants in the defense system, which is in close relationship with induction of LPO[11,12]. It has been reported that, auto-oxidation of ISO produces quinones, which react with oxygen to produce superoxide anions and hydrogen peroxide, leading to oxidative stress and depletion of the endogenous antioxidant system[30]. Treatment of PGFE and vit. E in ISO injected rats significantly decreased lipid peroxidation and thereby reduced oxidative stress. Several reports are available in this context that antioxidants in combination shows better effects rather than alone in attenuating oxidative stress[3,4,6,11,12].

Increased caspase-3 protease activity indicates cardiac apoptosis. β-adrenergic receptor (AR) stimulation by catecholamine, induces cardiac apoptosis or/and necrosis. It has been reported that oxidative stress provoke DNA damage and antioxidants supplement inhibit DNA fragmentation and apoptosis[31]. PGFE alone

and along with vit. E treatment showed significant reduction in caspase-3 protease activity indicating the anti-apoptotic effects of the combination.

Pomegranates are predominantly rich in polyphenols, including primarily hydrolysable ellagitannins, anthocyanins and other polyphenols. Ellagitannins found in the outer part of the fruit are largely responsible for the antioxidant activity of the pomegranate[8]. The presence of different polyphenolic compounds in pomegranate could be responsible for its exhibited potent antioxidant activity in the present study. It has been reported that polyphenols donate hydrogen atoms to tocopheryl radicals and enhance the antioxidant efficiency of alpha tocopherol and eliminates the so called tocopherol mediated peroxidation[31]. This may possibly be the reason for the beneficial effect of pomegranate and vitamin E in attenuating myocardial infarction in the present study.

CONCLUSION

In conclusion, the present study showed that acute ISO injection leads to induces myocardial infarction in animals which is confirmed by alteration in ECG pattern, biochemical changes, inflammatory markers, lipid peroxidation and apoptotic markers. Co-administration of PGFE and vit. E protects ISO induced myocardial infarction than alone antioxidant treatment and this effect of the combination might be due to potent antioxidant activity.

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