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CARDIOPROTECTIVE RESPONSE TO CHRONIC ADMINISTRATION OF VITAMIN E IN ISOPROTERENOL INDUCED MYOCARDIAL NECROSIS: HEMODYNAMIC, BIOCHEMICAL AND ULTRASTRUCTURAL STUDIES

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ABSTRACT

The present study evaluated the cardioprotective potential of vitamin-E by studying its effect on hemodynamic parameters, lipid peroxidation, myocyte injury marker and ultrastructural changes in model of isoproterenol-induced myocardial necrosis in rats. Wistar albino male rats (150-200 g) were randomly divided into saline, ISP control, and vit E groups. Vitamin E group was administered vitamin E at a dose of 100 mg/kg/day while saline and ISP control groups received saline orally for one month. On 29th and 30th day, ISP (85 mg/kg, sc) was administered at an interval of 24 h to vit E and ISP control rats. On 31st day, rats of all groups were anesthetized and hemodynamic parameters were recorded. At the end of experimentation, animals were sacrificed; hearts were excised and processed for biochemical and ultrastructural studies. ISP administration produced marked cardiac necrosis as evidenced by significant decrease in myocardial creatine kinase- MB as well as increase in malonaldialdehyde levels. ISP-induced myocardial necrosis resulted in myocardial dysfunction as evidenced by significant depression in heart rate and mean arterial pressure in the ISP control group as compared to saline control. Salient ultrastructural changes including extensive loss of myofibrils, muscle necrosis, loss of mitochondria, and formation of several intracytoplasmic vacuoles and lipid droplets further confirmed the ISP-induced myocardial damage. However, subsequent to ISP challenge, vit E treatment significantly preserved the myocardium by restoring myocardial CK-MB activity, inhibiting the ISP-induced lipid peroxidation and ultrastructural changes. Additionally, pre-and co-treatment of vit E prevented the deleterious ultrastructural changes caused by ISP. These beneficial effects of chronic vit E treatment also translated into significant restoration of the altered hemodynamic parameters. The present study clearly demonstrated the cardioprotective potential of vit E at dose of 100 mg/kg in ISP-induced model of myocardial necrosis in rats. The significant restoration of altered hemodynamic parameters, myocardial CK-MB activity, prevention of ISP-induced rise in lipid peroxidation and ultrastructural changes may confirm its cardioprotective effect.

KEY WORDS

Vitamin E, Isoproterenol, Myocardial necrosis, Rats

INTRODUCTION

Myocardial infarction (MI) is one of the most common manifestation of cardiovascular disease. The morbidity and mortality due to MI is now reaching epidemic proportion throughout the world. In recent years, accumulating evidence indicates that incidence and progression of cardiovascular disease may, to some extent, be modified by dietary means. In particular, attention has focused on the apparent beneficial effects of vitamin supplementation in reducing the incidence of cardiovascular disease (1).

Vitamin E (vit E) is most widely used vitamin in food supplements and cosmetic products. Owing to its wide array of biological actions, public and scientific interest has been

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directed towards the role of vit E in health promotion and disease prevention. It is the predominant lipophilic antioxidant in plasma membrane and tissues and is the most abundant antioxidant in low-density lipoprotein (LDL). Besides having antioxidant properties, vit E has been shown to slow or inhibit the oxidative modification of LDL that is responsible for development and progression of atherosclerosis (2). Moreover, high levels of vit E have been measured in the mitochondria, golgi apparatus, lysosomes, and endoplasmic reticulum (3), and recent studies have shown that vit E possesses a variety of cardiovascular effects including decreased platelet aggregation (4), arterial superoxide generation and increased eNOS mediated NO production (5).

Isoproterenol, a β-adrenergic agonist is a well-known inducer of myocardial necrosis and interstitial fibrosis at its supramaximal dosages. Mohanty et al. (6) have shown that isoproterenol leads to myocardial necrosis characterized by increased end-diastolic volume, end-diastolic pressure and left ventricular wall thickness. Some of the mechanisms proposed to explain the mechanisms of isoproterenol induced damage to cardiac myocytes include hypoxia due to myocardial hyperactivity and coronary hypotension, calcium overload, depletion of energy reserve and excessive production of free radicals resulting from oxidative metabolism of catecholamine. Free radical mediated peroxidation of membrane phospholipids and consequent changes in membrane permeability are the primary reasons for cardiotoxicity induced by isoproterenol (7-8). Oxidative stress also depresses the sarcolemmal Ca^{2+} transport and results in the development of intracellular Ca^{2+} overload and ventricular dysfunction (9). Oxidative stress has been implicated in the pathogenesis of myocardial ischemia. Therefore therapeutic interventions having antioxidant or free radical scavenging activity may exert beneficial effects against oxidative stress associated with various cardiovascular diseases, including ischemic heart disease (10-11).

Based on these observations, vit E might have a protective effect on heart injury by ischemia. In the present study, isoproterenol-induced model of myocardial necrosis was used to investigate the cardio-protective effects of vit E. We also studied the effect of vit E treatment on hemodynamic parameters, lipid peroxidation, myocyte injury marker and ultrastructural changes to investigate mechanism of its cardioprotective effect.

MATERIALS AND METHODS

Drug and chemicals : Vitamin E and isoproterenol (ISP) were procured from Sigma Chemicals (St Louis, MO, USA) and all other chemicals were of analytical grade.

Animals : Wistar male albino rats, weighing 150 to 200g, 10 to 12 weeks old were used in the study. The study protocol was reviewed and approved by the Institutional Animal Ethics Committee and conforms with the Indian National Science Academy Guidelines for the Use and Care of Experimental Animals in research. Animals were obtained from the Central Animal House facility of All India Institute of Medical Sciences, New Delhi, India and were housed in polyacrylic cages (38x23x10 cm) with not more than four animals per cage. They were housed under standard laboratory conditions with natural light and dark cycles (approximately 12 h light/12 h dark) and maintained at humidity of 55±5% and an ambient temperature of 25±2°C. All experiments were performed between 9.00 and 16.00 h. The animals were allowed free excess to standard pellet diet (Ashirwad Industries Ltd.; India) and tap water *ad libitum*. The commercial pellet diet contained 24% protein, 5% fat, 4% fiber, 55% carbohydrate, 0.6% calcium, 0.3% phosphorous, 10% moisture and 9% ash w/w. The animals were allowed to acclimatize for one week before the experiments.

Treatment protocol : A total of 36 animals were randomly allocated into three main groups comprising of twelve animals each. Animals of group 1 assigned as control were orally administered normal saline (0.9 % NaCl) once daily for a month. Animals of group 2 assigned as ISP control were orally administered normal saline once daily for a month and in addition received ISP (85 mg/kg, *sc*) on 29th and 30th day, at an interval of 24 h. Animals of group 3 assigned as drug treated group were orally administered vit E (100 mg/kg) suspended in normal saline with the help of carboxy methyl cellulose (CMC) once daily for 1 month and in addition challenged with ISP (85 mg/kg, *sc*) on 29th and 30th day, at an interval of 24 h. Hemodynamic parameters were recorded on the 31st day, i.e. 24 h after last injection of ISP. At the end of experimentation, animals were sacrificed under overdose of anesthesia; hearts were excised and immediately processed for biochemical and ultrastructural studies. In each group, out of twelve rats, 8 rats were used for hemodynamic and biochemical studies and remaining 4 rats for ultrastructural studies. The standardized isoproterenol dose 85 mg/kg subcutaneously as selected according to the previous studies (12-13). The selection of dose 100 mg/kg of vit E was based on the previous studies (6, 14)

Hemodynamic studies : All animals were anesthetized intraperitoneally with pentobarbitone sodium (60 mg/kg). Atropine (4 mg/kg) was administered along with the anesthetic

to maintain heart rate especially during the surgery, and to reduce tracheo-bronchial secretions. The body temperature was monitored and maintained at 37°C during the experimental protocol. The neck was opened with a ventral midline incision to perform tracheostomy and rats were ventilated with room air from a positive pressure ventilator (Inco, India) using compressed air at a rate of 70 strokes/min and a tidal volume of 10ml/kg. Ventilator setting and PO_2 were adjusted as needed to maintain the arterial blood gas parameters within the physiological range. The left jugular vein was cannulated with polyethylene tube for continuous infusion of 0.9% saline solution. The right carotid artery was cannulated and the cannula was filled with heparinized saline and connected with CARDIOSYS CO-101 (Experimentria, Hungary) using a pressure transducer for the measurement of heart rate (HR), systolic arterial pressure (SAP), diastolic arterial pressure (DAP), and mean arterial pressure (MAP). Animals were allowed to stabilize for 15 minutes before recording the basal hemodynamic parameters.

Biochemical studies : Hearts stored in liquid nitrogen were brought to room temperature and weighed. A 10% homogenate was prepared in ice-cold phosphate buffer (pH 7.4, 50 mM) and an aliquot of 0.2 ml was used for the assay of MDA.

Estimation of lipid peroxidation marker Malonaldialdehyde (MDA) : Malonaldialdehyde (MDA) was estimated by the method of Ohkawa and colleagues (15). To different aliquot volume of the standard 1,1,3,3-tetramethoxypropane (1-10 nM), 0.2 ml of 8.1% sodium dodecyl sulphate, 1.5 ml of 20% acetic acid and 1.5 ml of 0.81% thiobarbituric acid were added. The mixture was heated for 30 min at 95°C in a temperature controlled water bath. After cooling, 5 ml of n-butanol : pyridine (15:1) was added to it. The mixture was then centrifuged at 5000 rpm for 10 min. Absorbance of the organic layer was read spectrophotometrically at excitation wavelength 532 nm and emission wavelength 515 nm. The readings of absorbance were plotted against concentration of tetramethoxypropane to produce standard curve.

Estimation of myocyte injury marker: Creatine kinase-MB (CK-MB) : Creatine kinase-MB (CK-MB) was estimated spectrophotometrically using a standard enzyme kit supplied by Spinreact, S.A.-Spain (Cat.No.1001055), according to the method of Lamprecht and colleagues (16). The sample (50 µl) was added to cuvette containing 1.0 ml of prepared imidazole buffer consisting of 5.2 mM adenosine-mono-phosphate, 2.1 mM adenosine-di-phosphate, 2.1 mM nicotinamide adenine dinucleotide phosphate, 1.6 U/L glucose-6-phosphate dehydrogenase, 31.2 mM creatine phosphate and 21 mM N-acetyl cysteine. It was incubated for 120 sec at room temperature and the absorbance was recorded at 340 nm for 180 sec at 60 sec intervals. One unit of CK-MB isoenzyme is defined, as the amount of enzyme at 30°C will transfer 1µmol of phosphate from phosphocreatine to ADP per min at pH 7.4.

Ultrastructural studies (Transmission electron microscopy) : At the end of experiment, small pieces of myocardial tissue (approximately 1-2 mm in thickness) were immediately fixed in ice-cold Karnovsky's fixative. The tissues were then washed in phosphate buffer (0.1M, pH 7.4) and postfixed for 2 h in 1% osmium tetroxide in the same buffer at 4°C. The specimens were then washed in phosphate buffer, dehydrated with graded acetone and then embedded in araldite CY 212 to make tissue blocks. The semithin as well as ultrathin sections (80-100nm) were cut by an ultramicrotome (Ultracut E, Reichert, Austria). The sections were stained with uranyl acetate and lead acetate and examined under transmission electron microscope (Morgagni 268D, Fei Co., The Netherlands) operated at 60KV. At least four hearts from each group were examined for ultrastructural changes.

Statistical analysis : Descriptive statistics such as mean and standard deviation were calculated for all variables for each group. One-way Analysis of Variance (ANOVA) was applied for statistical analysis with post-hoc analysis (Bonferroni Multiple Range Test) for the evaluation of hemodynamic variables and Student's t test was used for biochemical analysis. The p value <0.05 has been considered as statistical significance level.

RESULTS

Effect of pre-and co-administration of vit E on hemodynamic parameter : In the ISP control group, a significant fall (24%, $P<0.05$, Table 1) in MAP was observed as compared to saline control group. However, vit E treatment significantly restored the MAP as compared to ISP control group ($P<0.05$). Similarly, ISP administration resulted significant decrease (36%, $P<0.05$) in HR as compared to saline control group. Chronic vit E treatment significantly prevented this ISP-induced fall in HR ($P<0.05$, Table 1).

Effect of pre-and co-administration of vit E on myocardial injury : Creatine kinase-MB (CK-MB) isoenzyme, a gold standard marker of myocyte injury, was significantly decreased (51%, $P<0.05$) in ISP control group as compared to saline control group (Table 2). However, chronic vit E treatment

Table 1 : Effect of vit E on hemodynamic parameters in different experimental groups

MAP (mm/Hg)	127.20±21.20	97.70±15.50#	101.60±12.36*
HR (Beats/min)	350.30±41.50	224.70±65.10#	291.80±30.31*

MAP: Mean arterial pressure; **HR:** Heart rate. #P<0.05 Vs Saline control, *P<0.05 Vs ISP control. Values are mean ± SD of eight experiments.

significantly attenuated the ISP-induced myocyte injury (P<0.05) in comparison to the ISP control group.

Effect of pre-and co-administration of vit E on lipid peroxidation : Isoproterenol control group showed marked increase (49%, P<0.05) in lipid peroxidation in myocardium, measured as MDA content, as compared to saline control

Table 2 : Effect of vit E on lipid peroxidation and myocyte injury markers in different experimental groups

Parameters	Saline control	ISP control	ISP + vit E-100
MDA (nmol/g tissue)	66.5±13.9	99.9±9.5#	60.3±15.3*
CK-MB (IU/mg protein)	162.4±27.3	59.4±21.3#	112.3±23.4*

MDA: Malonaldialdehyde; **CK-MB:** Creatine phosphokinase-MB isoenzyme. #P<0.05 Vs Saline control, *P<0.05 Vs ISP control. Values are mean ± SD of eight experiments. One unit of CK-MB transfers 1mmol of phosphate from phosphocreatine to ADP per min at pH 7.4 at 30^0C.

group (Table 2). Administration of vit E at a dose of 100 mg/kg markedly reduced lipid peroxidation as evidenced by reduction (P<0.01) in myocardial MDA levels in comparison to the ISP control group.

Effect of pre-and co-administration of vit E on ultrastructural changes : The ultrastructure of the myocardium from saline control rats was normal in appearance showing sarcomere separated with rows of normal and abundant mitochondria (Plate 1). In ISP control group, the myocardial damage was marked by significant disruption of myofibrils (Plate 2A). Electron microscopic examination of the myocardial tissue revealed several intracytoplasmic vacuoles and lipid droplets (Plate 2B). Other ultrastructural changes include the appearance of small and irregular mitochondria with loss of cristae (Plate 2C). However, chronic pre-and co-treatment of animals with vit-E showed structural protection of the myocardium from these ISP-induced ultrastructural changes (Plate 3A and 3B).

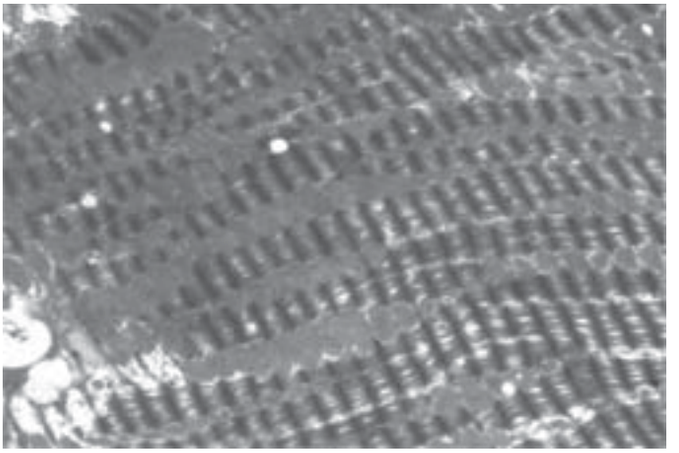

Plate 1: Electron micrograph showing normal ultrastructure of rat myocardium of sham group (1250 X).

Plate 2A

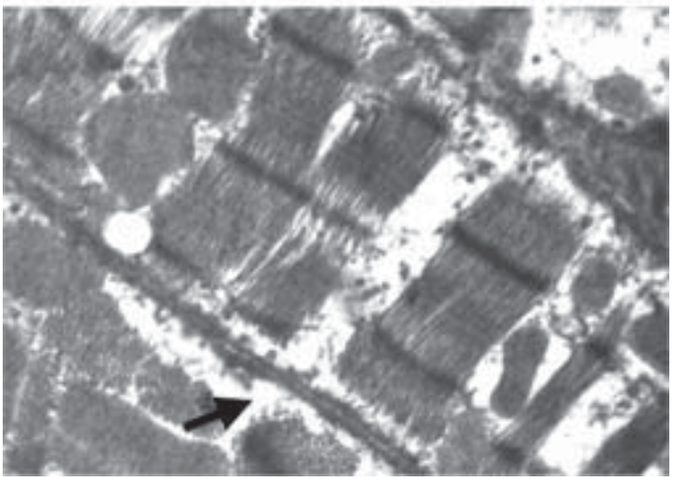

Plate 2B

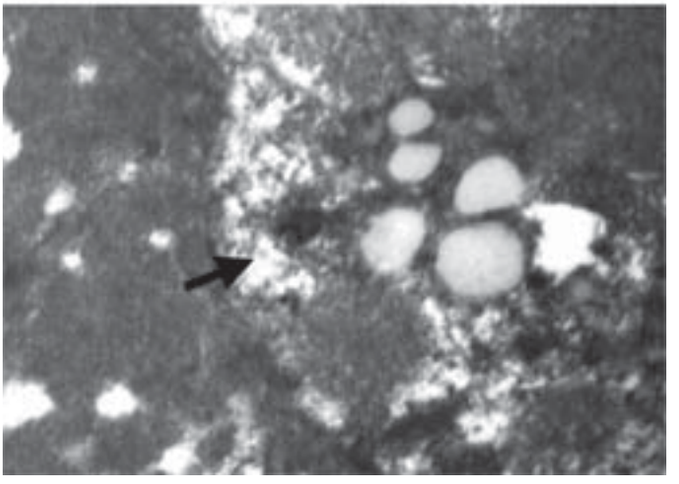

Plate 2C

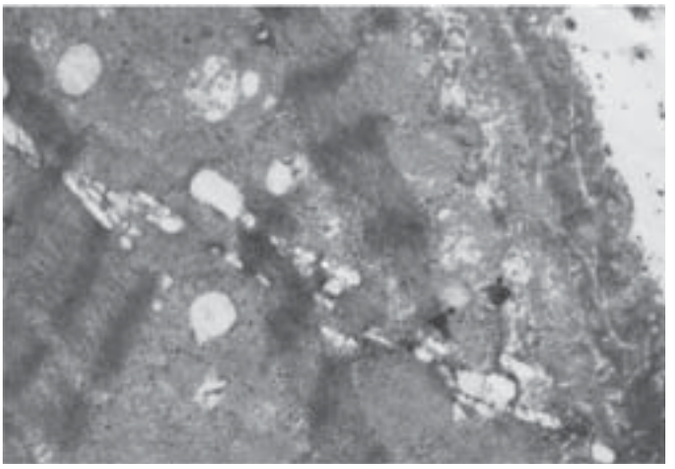

Plate 2: Electron micrographs of rat myocardium of isoproterenol control group showing A) extensive muscle necrosis with significant disruption of myofibrils (2800 X) B) several intracytoplasmic vacuoles and lipid droplets (4440 X) C) small and irregular mitochondria with loss of cristae (2800 X).

Plate 3A

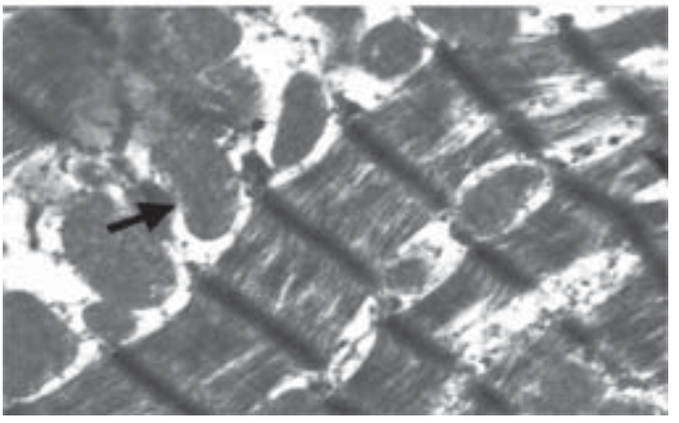

Plate 3B

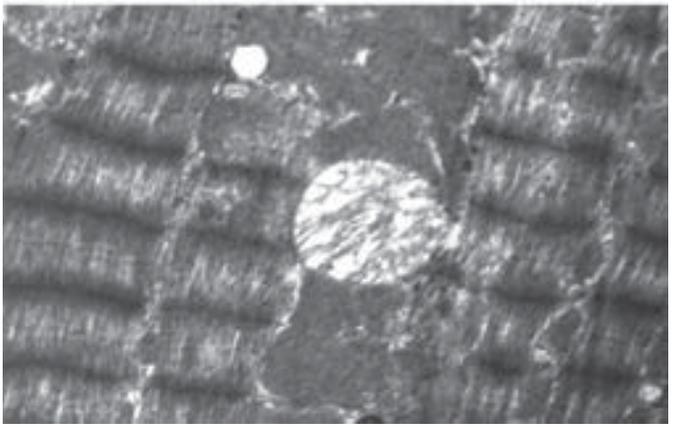

Plate 3: Electron micrographs of rat myocardium treated with vit E 100 mg/kg for 30 days in addition to challenged with isoproterenol showing A) very less muscle necrosis with slight disruption of myofibrils (2800X) B) high power of mitochondria showing normal architecture (4400 X).

DISCUSSION

Isoproterenol (ISP), a synthetic β-adrenergic agonist, by its positive inotropic and chronotropic actions increases the myocardial oxygen demand that leads to ischemic necrosis of myocardium in rats similar to that seen in human myocardial infarction. A number of patho-physiologic mechanisms have been outlined to explain the ISP-induced myocardial damage, viz. altered membrane permeability, increased turn over of nor-epinephrine, and generation of cytotoxic free radicals. In addition, ISP administration reduces blood pressure that triggers reflex tachycardia, thereby increases myocardial oxygen demand (17-19).

In the present study, ISP administration in rats resulted in marked depression in HR and MAP similar to that reported in earlier studies (6). Increased generation of cytotoxic free radicals is one among the several mechanisms proposed to explain the ISP-induced myocardial necrosis (20). Large number of studies have demonstrated that free radicals initiate lipid peroxidation resulting in alteration of membrane integrity, fluidity and permeability (21). In addition, ISP administration resulted in marked rise in lipid peroxidation, expressed as MDA content, as compared to saline control group, which is in line with earlier reported studies (22). In the present study, rats pre- & co-treated with vit E showed significant decrease in the level of myocardial lipid peroxides as compared to ISP control group, indicating antioxidant activity of vit E against ISP-induced lipid peroxidation.

We also observed a marked decline in myocardial CK-MB activity after ISP administration as mentioned earlier. CK-MB, a golden standard marker of myocyte injury or death, leaks out from myocardium due to disintegration of the contractile apparatus and increased sarcoplasmic permeability (23). The marked rise in lipid peroxidation with concomitant fall in myocardial CK-MB activity indicates the ISP-induced myocardial damage. Additionally, ultrastructural perturbations in ISP administered rat hearts further confirmed the injured state of myocardium. The rats chronically fed with vit E showed cytoprotective activity of it as evidenced by significant restoration of myocardial CK-MB activity, preservation of myofibrils and mitochondrial morphology.

Hemodynamic changes observed in the present study following ISP administration are suggestive of its myocardial deleterious effects. In the present study myocardial dysfunction was clearly evident by a significant fall in HR and MAP in the ISP control group, which might be due to ISP induced myocardial necrosis. Marked decline in myocardial CK-MB

activity also reconfirms the injured state of myocardium. In addition, absence of positive chronotropic effect in the presence of reduced MAP suggests impairment of conduction system of the heart following ISP-induced ischemic myocardial necrosis. Usually fall in MAP due to ISP administration is expected to increase HR and myocardial contractility through baroreceptor response. But these effects were not observed in the present study, which is an indication of ISP-induced injury to cardiac reflexes. However, pre-and co-treatment of animals with vit E restored the altered hemodynamic parameters such as MAP and HR. Thus, present study demonstrated the cardioprotective activity of vit E as shown by its beneficial effects against ISP-induced biochemical, hemodynamic and ultrastructural perturbations.

Studies have demonstrated that ISP-induced oxidative stress results in hyperstimulation of beta-adrenoreceptor, which ultimately leads to cardiotoxicity (20). Oxidative stress may also depress the sarcolemmal Ca^{2+} transport and result in the development of calcium overload and cardiac dysfunction (9). Several studies have demonstrated the therapeutic potential of antioxidants against these deleterious changes (8). Hence, it is possible that vit E may also provide cardioprotection against ISP-induced cardiac dysfunction by its strong antioxidant property. Thus, preservation of cardiac reflexes, attenuation of ISP-induced myocardial oxidative stress and cytoprotective activity produced by vit E may be responsible for improved cardiac performance.

In conclusion, the present study results indicate that the pre and co-treatment with vitamin E at dose of 100 mg/kg for one month prevents the ISP-induced myocardial infarction in rats. Improved hemodynamics, antioxidant, anti-peroxidative and myocardial preservative effects contribute to the overall cardioprotective action of vitamin E.

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