Pretreatment with a combination of quercetin and α-tocopherol ameliorates adenosine triphosphatases and lysosomal enzymes in myocardial infarcted rats

V.R. Punithavathi, P. Stanely Mainzen Prince *

Department of Biochemistry and Biotechnology, Annamalai University, Annamalai Nagar-608002, Tamil Nadu, India

A R T I C L E   I N F O
Article history:
Received 12 October 2009
Accepted 25 November 2009

Keywords:
Quercetin
α-Tocopherol
Isoproterenol
Myocardial infarction
Membrane bound ATPases
Lysosomal enzymes

A B S T R A C T

Aims: Membrane bound adenosine triphosphatases (ATPases) and lysosomal enzymes play an important role in the pathology of myocardial infarction. This study was aimed to evaluate the combined preventive effects of quercetin and α-tocopherol on membrane bound ATPases and lysosomal enzymes in isoproterenol induced myocardial infarcted rats.

Main methods: Male Wistar rats were pretreated with a combination of quercetin (10 mg/kg) and α-tocopherol (10 mg/kg) daily for 14 days. After the pretreatment period, isoproterenol (100 mg/kg) was injected to rats at an interval of 24 h for two days to induce myocardial infarction. The activities of ATPases and lysosomal enzymes were assayed.

Key findings: Isoproterenol treated rats showed decreased levels of heart creatine kinase and lactate dehydrogenase. The activity of sodium potassium adenosine triphosphatase was decreased and the activities of magnesium adenosine triphosphatase and calcium adenosine triphosphatase were increased in isoproterenol treated rats. Also, the activities of β-glucuronidase, N-acetylglucosaminidase, β-galactosidase, cathepsin-B and D were increased (serum and heart), but the activities of β-glucuronidase and cathepsin-D were decreased in lysosomal fraction and increased in cytosolic fraction of the heart in isoproterenol treated rats. Furthermore, the heart lipid peroxidation products were increased in isoproterenol treated rats. Combined pretreatment with quercetin and α-tocopherol to isoproterenol treated rats normalized all the biochemical parameters studied. The observed effects are due to their membrane stabilizing property and this property might be due to decreased lipid peroxidation.

Significance: Our study demonstrated that combined pretreatment was better than single pretreatment. This study may have significant impact on myocardial infarcted patients.

© 2009 Elsevier Inc. All rights reserved.

Introduction

Cardiovascular diseases (CVD) are the foremost cause of mortality worldwide. Reduction of mortality rate and prevention of myocardial infarction (MI) are of utmost importance. MI is the condition of necrosis of the myocardium that occurs as a result of imbalance between coronary blood supply and myocardial demand. ISO induced cardiac necrosis include increased oxygen consumption, insufficient oxygen utilization, increased calcium overload and accumulation, changes in myocardial cell metabolism, increased myocardial cAMP levels, deranged electrolyte milieu, alterations of membrane permeability, intracellular acidosis and increase in lipid peroxides (Bloom and Cancilla 1969).

Quercetin belongs to the family of flavonoids, found in many foods, including vegetables, tea, fruits and wine (Hertog et al. 1996). The antioxidant properties of quercetin might be due to its ability to chelate transition metal ions, such as Fe²⁺ and Cu²⁺ and scavenging free radicals (Sestili et al. 1998). It also prevents oxidation of low density lipoproteins in vitro (De Whalley et al. 1990). Varga et al. (1999) have reported that Ginko biloba extract (EGb 761) consisting of flavonoids (24% quercetin, kaempferol) and terpenoids (6% ginkgolides) inhibited nitric oxide production by concomitant inhibitory actions including the expression of iNOS mRNA and direct scavenging activity of nitric oxide in ischemic/reperfused myocardium. Also, Egb 761 improved cardiac function after ischemia in both preconditioned and preconditioned nondiabetic and streptozotocin induced diabetic rats (Tosaki et al. 1996).

Vitamin-E (α-tocopherol) is a lipid soluble antioxidant that protects poly unsaturated fatty acids and other components of the cell and organelle membranes from oxidation of reactive free radicals (Tappel 1972). Intake of vitamin-E is associated with decreased incidence of CVD (Gey et al. 1991). A study was conducted to compare the cardioprotective abilities of different isomers of tocotrienol against tocotrienol rich fraction in ischemia/reperfusion rat model. Tocotrienol fraction as well as all the isomers of tocotrienol (α, γ, and δ) used in this study provided...
cardioprotection as evidenced by their ability to improve post ischemic ventricular function and reduce myocardial infarct size in ischemia/ reperfusion rat model. Furthermore, the molecular mechanism of tocotrienol isoforms revealed that these isoforms reduced c-Src, but increased the phosphorylation of Akt, thus generating a survival signal (Das et al. 2008).

ATPases of cardiac cells play a significant role in the contraction and relaxation cycles of cardiac muscle by maintaining normal ion levels (Ca$^{2+}$, Na$^+$, K$^+$, and Mg$^{2+}$) within the myocytes. Alterations in the properties of these ion pumps may affect cardiac function. ISO induced MI results in increased lysosomal hydrolases activity that may be responsible for tissue damage and infarcted heart (Ravichandran et al. 1990). ISO also alters the activities of ATPases (Yogee et al. 2006).

According to medical practitioners, a combination of drugs exhibits augmented protective efficacy than a single drug. Previously, we reported combined preventive effect of quercetin and α-tocopherol on lipids and glycoproteins in ISO treated rats (Punithavathi and Stanely Mainzen Prince 2009). In this study, we report the combined protective effects of quercetin and α-tocopherol on ATPases and lysosomal enzymes in ISO treated myocardial infarcted rats.

**Materials and methods**

**Experimental animals and diets**

All the experiments were carried out with male albino Wistar rats weighing 180–200 g, obtained from The Central Animal House, Rajah Muthiah Institute of Health Sciences, Annamalai University, Tamil Nadu, India. They were housed in polypropylene cages (47 cm × 34 cm × 20 cm) lined with husk, renewed every 24 h under a 12:12 h light/dark cycle at around 22 °C. The rats had free access to tap water and food. The rats were fed on a standard pellet diet (Pranav Agro Industries Ltd., Maharashtra, India). The experiment was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and approved by the Animal Ethical Committee of Annamalai University (Approval No.: 546: 20.3.2008).

**Drugs and chemicals**

Quercetin, ISO, N-phenyl-p-phenylene diamine, p-nitrophenyl-β-N-acetyl galactoside, p-nitrophenyl-N-acetyl-β-D-glucosaminide, p-nitrophenyl-β-D-glucuronide, α-N-benzoyl-DL-arginine-p-nitroanilide (BAPNA) hydrochloride and sodium dodecyl sulphate (SDS) were purchased from Sigma Chemical Co., St. Louis, MO, USA. α-Tocopherol, dimethyl sulfoxide (DMSO), adenosine triphosphate (ATP), ethylene diamine tetra acetic acid (EDTA) and trichloro acetic acid (TCA) were purchased from Himedia Laboratories, Mumbai, India. Thiobarbituric acid (TBA) and 1, 1’, 3, 3’ tetra methoxy propane were purchased from S.D Fine Chemicals, Mumbai, India. All other chemicals used were of analytical grade.

**Induction of experimental myocardial infarction and experimental design**

ISO (100 mg/kg) was dissolved in saline and subcutaneously injected to male albino Wistar rats at an interval of 24 h for 2 days. MI was confirmed by elevated activity of serum creatine kinase-MB (CK-MB) in rats. The rats were randomly divided into eight groups of six rats each. Group I: Normal control rats; Group II: Rats were orally treated with quercetin (10 mg/kg) alone daily for 14 days using an intragastric tube; Group III: Rats were orally treated with α-tocopherol (10 mg/kg) alone daily for 14 days using an intragastric tube; Group IV: Rats were orally treated with a combination of quercetin (10 mg/kg) and α-tocopherol (10 mg/kg) daily for 14 days using an intragastric tube; Group V: Rats were subcutaneously injected with ISO alone (100 mg/kg) at an interval of 24 h for 2 days (on 15th and 16th days); Group VI: Rats were pretreated with quercetin (10 mg/kg) alone daily for 14 days and then subcutaneously injected with ISO (100 mg/kg) for 2 days (on 15th and 16th days); Group VII: Rats were pretreated with α-tocopherol (10 mg/kg) alone daily for 14 days and then subcutaneously injected with ISO (100 mg/kg) for 2 days (on 15th and 16th days); Group VIII: Rats were pretreated with a combination of quercetin (10 mg/kg) and α-tocopherol (10 mg/kg) daily for 14 days and then subcutaneously injected with ISO (100 mg/kg) for 2 days (on 15th and 16th days). Normal control and ISO control rats received DMSO (0.5%) alone for 14 days of the experimental period. Quercetin and α-tocopherol were dissolved in DMSO (0.5%) and administered to rats 1 ml each orally using an intragastric tube daily for a period of 14 days. The dose and duration of pretreatment of quercetin and α-tocopherol were based on our earlier study (Punithavathi and Stanely Mainzen Prince 2009).

At the end of the experimental period, after 12 h of second ISO injection, (i.e. on 17th day) all the rats were anesthetized and then sacrificed by cervical decapitation. Blood was collected and plasma and serum were separated by centrifugation. Heart tissue was excised immediately and rinsed in ice-chilled saline.

**Separation of subcellular fractions**

The heart tissue samples were cut open and placed in isotonic saline to remove the blood. Then, the fresh heart tissues were homogenized in 0.25 M ice-cold sucrose solution at 4 °C. A portion of this homogenate was used to determine the total activity. Another portion of the homogenate was subjected to differential centrifugation and the different fractions were separated as follows: structural proteins, nucleus, and cell debris at 600× g, mitochondria at 1,20,000× g, and lysosomes at 15,000× g for 30 min and the supernatant, cytosol. Myocardial subfractions were treated with Triton X-100 (final concentration 0.2% v/v) in ice for 15 min prior to the determination of enzymic activities (Sathish et al. 2003).

**Assay of cardiac marker enzymes**

Activities of creatine kinase (CK) and lactate dehydrogenase (LDH) were measured in the heart tissue homogenate by standard commercial kits.

**Assay of membrane bound ATPases in the heart**

The activity of Na$^+$/K$^+$ ATPase in the heart tissue homogenate was assayed according to the method of Bonting (1970). The incubation mixture contained 1.0 ml of buffer, 0.2 ml of magnesium sulphate, 0.2 ml potassium chloride, 0.2 ml of sodium chloride, 0.2 ml of EDTA, 0.2 ml of ATP and 0.2 ml of heart tissue homogenate. The contents were incubated at 37 °C for 15 min. 1.0 ml of ice-cold 10% TCA was added at the end of 15 min to arrest the reaction. The amount of phosphorus liberated was estimated as described by Fiske and Subbarow (1925). 1.0 ml of the supernatant was made up to 4.0 ml with distilled water and 1.0 ml of 2.5% ammonium molybdate was added. This was incubated at room temperature for 10 min and 0.4 ml of amino naphthol sulfonic acid was added. The colour developed was read spectrophotometrically at 640 nm after 20 min.

The activity of Mg$^{2+}$/ATPase in the heart tissue homogenate was assayed according to the method of Ohnishi et al. (1982). The incubation mixture contained 0.1 ml of buffer, 0.1 ml of magnesium chloride, 0.1 ml of ATP, 0.1 ml of distilled water and 0.1 ml of heart tissue homogenate. The reaction mixture was incubated at 37 °C for 15 min. The reaction was then arrested by the addition of 0.5 ml of ice-cold 10% TCA. The amount of phosphorus liberated was estimated according to the method of Fiske and Subbarow (1925).
The activity of Ca\(^{2+}\) ATPase in the heart tissue homogenate was assayed according to the method of Hjerten and Pan (1983). The incubation mixture contained 0.1 ml of buffer, 0.1 ml of calcium chloride, 0.1 ml of ATP, 0.1 ml of distilled water and 0.1 ml of heart tissue homogenate. The contents were incubated at 37 °C for 15 min. The reaction was then arrested by the addition of 0.5 ml of ice-cold 10% TCA. The amount of phosphorus liberated was estimated according to the method of Fiske and Subbarow (1925).

**Assay of lysosomal enzymes in the serum, heart and subcellular fractions**

The activity of β-glucuronidase in the serum, heart tissue homogenate and subcellular fractions was assayed according to the method of Kawai and Anno (1971). A known amount of serum/aliquot of the heart tissue homogenate/subcellular fractions was added to 0.5 ml of incubation buffer containing 2 mM p-nitrophenyl-β-d-glucuronide (final concentration) and incubated at 37 °C for 2 h. The substrate, p-nitrophenyl-β-d-glucuronide was dissolved in 0.1 M acetic acid buffer. At the end of the incubation period, the reaction was arrested by the addition of 4.0 ml of 0.2 M glycine-NaOH buffer (pH 11.7) containing 2 M SDS and the contents were centrifuged at 1000×g for 15 min. To the aliquots of supernatants, 0.5 M NaOH was added and the absorbance was measured at 410 nm in a Systronics UV–visible spectrophotometer.

The activity of β-N-acetyl glucosaminidase in the serum and heart tissue homogenate was assayed by the method of Moore and Morris (1982). A known amount of serum/aliquot of the heart tissue homogenate was added to 0.5 ml of incubation buffer containing 2 mM p-nitrophenyl-N-acetylβ-d-glucosaminidase (final concentration) and incubated at 37 °C for 2 h. The substrate, p-nitrophenyl-N-acetylβ-d-glucosaminidase was dissolved in 0.1 M citrate buffer. At the end of the incubation period, the reaction was arrested by the addition of 4.0 ml of 0.2 M glycine-NaOH buffer (pH 11.7) containing 2 M SDS and the contents were centrifuged at 1000×g for 15 min. To the aliquots of supernatants, 0.5 M NaOH was added and the absorbance was measured at 410 nm in a Systronics UV–visible spectrophotometer.

The activity of β-galactosidase in the serum and heart tissue homogenate was assayed by the method of Conchie et al. (1967). The incubation mixture contained 2.0 ml of 0.2 M disodium hydrogen phosphate, 0.1 M citrate buffer, 0.5 ml of 5 mM p-nitrophenyl-β-d-galactoside and known amount of serum/heart tissue homogenate. Incubation was carried out for 1 h at 37 °C. The reaction was terminated by the addition of 0.2 M glycine-NaOH buffer. The reaction mixture was centrifuged at 1000×g for 15 min and the absorbance of the released p-nitrophenol in the supernatant was measured at 410 nm in a Systronics UV–visible spectrophotometer.

The activity of cathepsin-B in the serum and heart tissue homogenate was assayed by the method of Barrett (1972). A known amount of serum/aliquot of the heart tissue homogenate was activated by 2 mM cysteine and 1 mM EDTA in a buffer of 0.14 M sodium dihydrogen phosphate (pH 6.0) and after the addition of 50 μl of BAPNA the solution was incubated for 1 h. The enzyme activity was arrested by the addition of 2.0 ml of 2 M Tris–HCl buffer (pH 9.0) and the yellow colour developed was read at 410 nm in a Systronics UV–visible spectrophotometer.

Cathepsin-D activity in the serum, heart tissue homogenate and subcellular fractions was assayed by the method of Sapolsky et al. (1973). A known amount of serum/aliquot of the heart tissue homogenate/subcellular fractions was incubated with 1.5% of hemogoblin in 1.0 M acetate buffer (pH 3.0) for 2 h. The enzyme activity was arrested by the addition of 10% TCA and the liberated TCA soluble products were filtered and neutralized with 1 N NaOH. The tyrosine content of the filtrate was determined by Folin’s phenol reagent according to the procedure of Lowry et al. (1951). The blue colour developed was read at 620 nm.

**Estimation of lipid peroxidation products and protein in the heart**

Thiobarbituric acid reactive substances (TBARS) in the heart tissue homogenate were estimated by the method of Fraga et al. (1988). To 1.0 ml of heart tissue homogenate, 2.0 ml of TBA–TCA–HCl reagent was added, mixed thoroughly and kept in a boiling water bath for 15 min. The reaction mixture was cooled and centrifuged at 1000×g for 10 min. The supernatant was taken and the absorbance was measured at 535 nm.

Lipid hydroperoxides (LOOH) in the heart tissue homogenate were estimated by the method of Jiang et al. (1992). A volume of 1.8 ml of the Fox reagent was mixed with 0.5 ml of heart tissue homogenate and incubated for 30 min at room temperature. The colour developed was read at 560 nm.

The content of the protein in the heart tissue homogenate and subcellular fractions was determined by the method of Lowry et al. (1951). About 0.5 ml of heart tissue homogenate/subcellular fractions was precipitated with 0.5 ml of 10% TCA and centrifuged for 10 min and the precipitate was dissolved in 1.0 ml of 0.1 N NaOH. About 0.1 ml of aliquot was taken and made up to 1.0 ml with distilled water. Then, 4.5 ml of alkaline copper reagent was added and allowed to stand at room temperature for 10 min. After incubation, 0.5 ml of Folin’s–Ciocalteau reagent was added and the blue colour developed was read at 620 nm after 20 min.

**Statistical analysis**

Statistical analysis was performed by One-way Analysis of Variance (ANOVA) followed by Duncan’s multiple range test (DMRT) using Software Package for the Social Science (SPSS) software package version 12.00. Results were expressed as mean ± S.D. for six rats in each group. P values < 0.05 were considered significant.

**Results**

We have done a pilot study to find out the dose dependent effect and duration of pretreatment of quercetin and α-tocopherol at two different doses, quercetin (5 and 10 mg/kg) and α-tocopherol (5 and 10 mg/kg) in ISO treated rats. Every week, we have examined the activity of cardiac marker enzyme, serum creatine kinase activity in rats pretreated with a combination of quercetin and α-tocopherol and then induced with ISO. On the 7th day, we could find a partial reduction in serum creatine kinase activity. On the 14th day of pretreatment with a combination of quercetin and α-tocopherol, we could observe normalized activity of serum creatine kinase compared to normal control rats. The effect exerted by 10 mg/kg of quercetin and 10 mg/kg of α-tocopherol showed highest significant effect than the lower dose, 5 mg/kg of quercetin and 5 mg/kg of α-tocopherol. Thus, we have chosen the time period of pretreatment and dose of the study. Since the 14th day of pretreatment with this combination gave us normalized activity of serum creatine kinase, we did not see the effect of this combination beyond the 14th day.

Figs. 1 and 2 show the activity of CK, LDH and lipid peroxidation products (TBARS and LOOH) in the heart of normal and experimental rats. ISO treated rats showed a significant (P < 0.05) decrease in the activity of CK and LDH in the heart tissue homogenate and a significant (P < 0.05) increase in the levels of TBARS and LOOH in the heart tissue homogenate compared to normal control rats. Combined pretreatment with quercetin (10 mg/kg) and α-tocopherol (10 mg/kg) daily for a period of 14 days normalized (P < 0.05) the activity of these enzymes (Fig. 1) and lipid peroxidation products in the heart tissue homogenate (Fig. 2) compared to ISO alone treated rats.

Rats treated with ISO showed a significant (P < 0.05) decrease in the activity of Na\(^+\)/K\(^+\) ATPase in the heart tissue homogenate and a significant (P < 0.05) increase in the activities of Mg\(^{2+}\) ATPase and Ca\(^{2+}\) ATPase in the heart tissue homogenate compared to normal control rats.
Combined pretreatment with quercetin (10 mg/kg) and α-tocopherol (10 mg/kg) normalized (P<0.05) the activities of Na+/K+ ATPase, Mg²⁺ ATPase and Ca²⁺ ATPase in the heart tissue homogenate compared with ISO alone treated rats (Fig. 3).

ISO treated rats showed a significant (P<0.05) increase in the activities of β-glucuronidase, β-N-acetyl glucosaminidase, β-galactosidase, cathepsin-B and D in the serum compared to normal control rats. Combined pretreatment with quercetin (10 mg/kg) and α-tocopherol (10 mg/kg) normalized (P<0.05) the activities of these enzymes in the serum compared to ISO control rats (Table 1).

ISO treated rats showed a significant (P<0.05) increase in the activities of β-N-acetyl glucosaminidase, β-galactosidase and cathepsin-B in the heart compared to normal control rats. Combined pretreatment with quercetin (10 mg/kg) and α-tocopherol (10 mg/kg) normalized (P<0.05) the activities of these enzymes in the heart compared to ISO alone treated rats (Table 2).

Table 3 shows the total activity of β-glucuronidase in the heart and subcellular fractions in normal and experimental rats. ISO treated rats showed a significant (P<0.05) increase in the activity of this enzyme in the heart and cytosolic fraction and a significant (P<0.05) decrease in the activity of this enzyme in lysosomal fraction compared with normal control rats. Combined pretreatment with quercetin (10 mg/kg) and α-tocopherol (10 mg/kg) normalized (P<0.05) the activity of this enzyme in the heart, cytosolic fraction and lysosomal fraction compared to ISO alone treated rats.

ISO treated rats showed a significant (P<0.05) increase in the activity of cathepsin-D in the heart and cytosolic fraction and significant (P<0.05) decrease in lysosomal fraction compared to normal control rats. Combined pretreatment with quercetin (10 mg/kg) and α-tocopherol (10 mg/kg) normalized (P<0.05) the activity of cathepsin-D in the heart, cytosolic fraction and lysosomal fraction compared to ISO alone treated rats (Table 4).

For all the biochemical parameters studied, pretreatment with quercetin (10 mg/kg) alone and α-tocopherol (10 mg/kg) alone showed significant (P<0.05) effects in all the biochemical parameters studied in ISO treated rats. But, combined oral pretreatment with quercetin (10 mg/kg) and α-tocopherol (10 mg/kg) normalized all the biochemical parameters studied and the effect was better than single pretreatment alone (quercetin alone or α-tocopherol alone) in ISO treated rats. Treatment with quercetin (10 mg/kg) alone, α-tocopherol (10 mg/kg) alone and combined treatment with quercetin (10 mg/kg) and α-tocopherol (10 mg/kg) to normal control rats did not show any significant effect in all the biochemical parameters studied.

Discussion

In this study, combined pretreatment with quercetin (10 mg/kg) and α-tocopherol (10 mg/kg) maintains the membrane integrity and restores the activities of ATPases and lysosomal enzymes in ISO treated myocardial infarcted rats. We observed decreased activities of cardiac marker enzymes such as CK and LDH in the myocardium. The myocardial cells containing these enzymes are damaged or destroyed because of deficient oxygen and glucose supply and the cell membrane becomes permeable or may rupture, which results in the
leakage of these enzymes. This accounts for the lowered activities of these enzymes in the heart of ISO treated MI in rats. This may be due to the damage caused to the sarcolemma by ISO, rendering it leaky (Mathew et al. 1985). Combined pretreatment with quercetin and α-tocopherol normalized the activities of these enzymes by their antioxidant activity, which may explain their ability to protect the myocardium from damage by preventing the leakage of these enzymes from the heart.

Lipid peroxidation is a well established mechanism of cellular injury and has been used as an indicator of oxidative stress. Measurements of TBARS and LOOH, both products of lipid peroxidation were also used in the present study as that kind of markers. Increased levels of TBARS and LOOH implicate an increased production of free radicals in ISO treated MI. The increased free radicals react with the lipid bilayer of intracellular organelles including lysosomes, which destabilizes lysosomal membranes and results in the rupture of lysosomes. It was reported that exposure of mammalian cells to oxidant stress causes early lysosomal organelles including lysosomes, which destabilizes lysosomal membranes and results in the rupture of lysosomes. This accounts for the lowered activities of these enzymes in the heart of ISO treated MI in rats. This may be due to enhanced lipid peroxidation by free radicals on ISO induction, since Na+/K+ ATPase is a SH group containing enzyme and is lipid-dependent (Ithayarasi and Devi 1997). Decreased activity of Na+/K+ ATPase can lead to a decrease in sodium efflux, thereby altering membrane permeability (Finotti and Palatini 1986). Ca2+-ATPase regulates the calcium pump activity (Levy et al. 1994). Enhanced Ca2+-ATPase activity observed in ISO treated rats is due to the activation of adenylate cyclase by ISO. Calcium overload in the myocardial cells during ischemia activates the Ca2+-dependent ATPase of the membrane depleting high energy phosphate stores, thereby indirectly inhibiting Na+ and K+ transport and inactivation of Na+/K+ ATPase (Ithayarasi and Devi 1997). Mg2+-ATPase activity is involved in other energy requiring process in the cell and its activity is sensitive to lipid peroxidation. Combined pretreatment with quercetin and α-tocopherol normalized the levels of lipid peroxidation products in ISO treated rats. This effect clearly revealed the antilipid peroxidation property of quercetin and α-tocopherol.

A significant decreased activity of Na+/K+ ATPase and significant increased activities of Mg2+-ATPase and Ca2+-ATPase in the heart were observed in ISO treated rats. Decreased activity of Na+/K+ ATPase could be due to enhanced lipid peroxidation by free radicals on ISO induction, since Na+/K+ ATPase is a SH group containing enzyme and is lipid-dependent (Ithayarasi and Devi 1997). Decreased activity of Na+/K+ ATPase can lead to a decrease in sodium efflux, thereby altering membrane permeability (Finotti and Palatini 1986). Ca2+-ATPase regulates the calcium pump activity (Levy et al. 1994). Enhanced Ca2+-ATPase activity observed in ISO treated rats is due to the activation of adenylate cyclase by ISO. Calcium overload in the myocardial cells during ischemia activates the Ca2+- dependent ATPase of the membrane depleting high energy phosphate stores, thereby indirectly inhibiting Na+ and K+ transport and inactivation of Na+/K+ ATPase (Ithayarasi and Devi 1997). Mg2+-ATPase activity is involved in other energy requiring process in the cell and its activity is sensitive to lipid peroxidation. Combined pretreatment with quercetin and α-tocopherol normalized the activities of Na+/K+ ATPase, Mg2+-ATPase and Ca2+-ATPase in ISO treated rats. Restoration of Na+/K+ ATPase activity due to quercetin and α-tocopherol combined pretreatment in ISO treated rats could regulate the intracellular Ca2+ levels, thereby protecting the myocardium from excess damage by maintaining the membrane integrity. Elevated levels of Na+ concentration resulted in depressed effects of Ca2+ and augment Ca2+ influx (Gudbjorson et al. 1983). This could be due to the ability of...
quercetin and α-tocopherol to protect the ‘SH’ groups from the oxidative damage through the inhibition of peroxidation of membrane lipids. This effect is due to the membrane stabilizing properties of quercetin and α-tocopherol. The membrane stabilizing property might be due to the blocking of lipid peroxidation in cell membranes.

Lysosomes are a distinct group of cell organelles that contain a variety of acid hydrolases (Saflig 2005). The stability and integrity of lysosomal membrane is vital to maintain normal levels of lysosomal enzymes in tissues and body fluids. Lysosomal enzymes are important mediators of acute MI and their release into the cytoplasm stimulates the formation of inflammatory mediators such as oxygen radicals and prostaglandins. Since lysosomal hydrolases have been implicated in the propagation of the cellular injury during the early stages of acute MI and this action is crucial in protecting the heart against ischemic damage. Lysosomal destabilization may be prevented either by inhibition of cellular peroxidation or by prevention of iron catalyzed oxidative reactions, which involve peroxidation of cellular membranes, energy depletion and leakage of lysosomal content (Karthiskeyan et al. 2007). It is possible that stabilization of myoccardial cell membranes, particularly the lysosomal membranes, may prolong the viability of ischemic cardiac muscle.

Lysosomal membrane plays a vital role in the regulation of lysosomal enzyme secretion in pathophysiology (Pillay et al. 2002) and in various inflammatory conditions (Ignarro 1974). Increased activities of lysosomal enzymes (β-glucuronidase, β-N-acetyl glucosaminidase, β-galactosidase, cathepsin-B and D) were observed in the serum and heart of ISO treated rats. Increased lipid peroxidation observed in ISO treated rats could have resulted in the leakage of serum and myocardial acid hydrolases from the enclosed sacs due to lysosomal membrane damage. Macikova et al. (1999) also reported an increase in the activities of serum and heart lysosomal enzymes in ISO treated rats. It has been shown that the cytosolic acid hydrolases released from lysosomes and from the sarcoplasmic reticulum induce the dysfunction and destruction of mitochondria, sarclemma and other organelles (Kennett and Weglicki 1978). Combined pretreatment with quercetin and α-tocopherol normalized the activities of lysosomal enzymes both in the serum and myocardium by its inhibitory effect on lipid peroxidation, thereby preventing lysosomal damage induced by ISO treated rats.

Conflict of interest statement
The authors declare that there are no conflicts of interest.

Acknowledgement
We thank Annamalai University for the financial assistance in the form of “University Research Fellowship” to Mrs. V.R. Punithavathi.

**Table 4**

Activity of cathepsin-D in the total heart and subcellular fractions of the heart in normal and isoproterenol (ISO) induced myocardial infarcted rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cathepsin-D (μM of tyrosine liberated/h/100 mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heart</td>
</tr>
<tr>
<td>I</td>
<td>31.2±2.4 a</td>
</tr>
<tr>
<td>II</td>
<td>31.3±2.9 a</td>
</tr>
<tr>
<td>III</td>
<td>31.3±2.7 a</td>
</tr>
<tr>
<td>IV</td>
<td>31.4±2.5 a</td>
</tr>
<tr>
<td>V</td>
<td>51.3±4.7 a</td>
</tr>
<tr>
<td>VI</td>
<td>37.9±3.9 a</td>
</tr>
<tr>
<td>VII</td>
<td>43.8±3.8 a</td>
</tr>
<tr>
<td>VIII</td>
<td>32.2±2.5 a</td>
</tr>
</tbody>
</table>

A compromise of lysosomal membrane integrity may lead to an undesirable elevation of enzymes in both intra and extracellular space, which could pave the way for cellular and tissue disorders, including apoptosis (George 2008). The integrity of the lysosomal membrane is crucial to the maintenance of normal levels of lysosomal glycohydrolases and cathepsins in tissues and body fluids. The release of β-glucuronidase is an index of lysosomal membrane integrity (Michihara et al. 2005). The decrease in the activities of β-glucuronidase and cathepsin-D in the lysosomal fraction indicates decreased stability of membranes (Satish et al. 2003). The elevated activities of these enzymes in the cytosolic fraction are due to release of these enzymes from lysosome to the cytosol in ISO treated rats. Combined pretreatment with quercetin and α-tocopherol inhibited the release of these enzymes from the lysosomal fraction in ISO treated rats. The distribution of these enzyme activities between the cytosol to lysosomal and cytosol to total activities reveals decreased lysosomal stability in ISO treated rats. Combined pretreatment with quercetin and α-tocopherol inhibited the release of lysosomal enzymes as well as decreased the activity of total lysosomal hydrolases, thereby enhancing the stability of lysosomes. This effect might be due to the membrane stabilizing properties of quercetin and α-tocopherol.

Kalra and Prasad (1994) have suggested that oxygen free radicals produced during ischemia may be responsible for the cardiac damage through the release of lysosomal enzymes. The protective effects of quercetin and α-tocopherol may also be mediated by the scavenging of oxygen free radicals with the resultant preservation of cellular viability serving secondarily to preserve lysosomes as well. Quercetin and α-tocopherol maintained the membrane integrity and protected lysosomal enzymes against lipid peroxide mediated radicals generated in the lysosomal membrane by ISO. This effect is due to membrane stabilizing effects of quercetin and α-tocopherol.

Our study showed combined pretreatment with quercetin and α-tocopherol normalized the activities of membrane bound ATPases and lysosomal enzymes by inhibiting lipid peroxidation in ISO treated rats. The observed effects are due to membrane stabilizing properties of quercetin and α-tocopherol. The membrane stabilizing property of quercetin and α-tocopherol might be due to inhibiting lipid peroxidation. According to our findings, a 70 kg person requires 700 mg of quercetin and 700 mg of α-tocopherol daily. Thus, a combination of quercetin (10 mg/kg) and α-tocopherol (10 mg/kg) may be useful for the treatment of myocardial infarcted patients.

**Conclusion**

Our study revealed that combined pretreatment with quercetin and α-tocopherol protected the lysosomal enzymes and membrane bound ATPases in ISO treated myocardial infarcted rats. Combined administration of quercetin (10 mg/kg) and α-tocopherol (10 mg/kg) to normal control rats had no effect on the measured biochemical parameters and no adverse effects were observed. Our study demonstrated that combined pretreatment was better than single pretreatment. This study further strengthens the cardioprotective effects of quercetin and α-tocopherol. Also, quercetin and α-tocopherol were safe and highly effective in preventing cardiac dysfunction in rats, possibly due to their membrane stabilizing property. This study may have significant impact on myocardial infarcted patients. Further in vitro studies are underway in our laboratory to find out the exact mechanism of action of quercetin and α-tocopherol.

Group I: Normal control rats, Group II: Rats were treated with quercetin (10 mg/kg) alone, Group III: Rats were treated with a combination of quercetin (10 mg/kg)+α-tocopherol (10 mg/kg), Group IV: Rats were treated with quercetin (10 mg/kg) alone+ISO (100 mg/kg), Group V: ISO treated rats (100 mg/kg), Group VI: Rats were pretreated with quercetin (10 mg/kg)+ISO (100 mg/kg), Group VII: Rats were pretreated with α-tocopherol (10 mg/kg) alone+ISO (100 mg/kg), Group VIII: Rats were pretreated with a combination of quercetin (10 mg/kg)+α-tocopherol (10 mg/kg)+ISO (100 mg/kg). Values are mean±S.D. for six rats in each group; values not sharing a common superscript (a, b, c, and d) differ significantly with each other (P<0.05, DMRT).

**A**, ratio of cytosol (free) to lysosomal (bound) activity; **B**, ratio of cytosol to total activity.

**Table 4**

Activity of cathepsin-D in the total heart and subcellular fractions of the heart in normal and isoproterenol (ISO) induced myocardial infarcted rats.
References


