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Protective Effect of Squalene on Mitochondrial Alterations in Isoprenaline-Induced Myocardial Injury

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ABSTRACT

Reactive oxygen species (ROS) are reported to play an important role in producing lethal cell injury associated with cardiac ischemia during myocardial infarction. The effects of ROS should be greatest at the level of mitochondrial membrane constituents, including the complexes of the respiratory chain and phospholipids constituents particularly rich in unsaturated fatty acids, such as cardiolipin. In the present study we have investigated the protective effect of squalene supplementation on mitochondrial function in isoprenaline-induced myocardial infarction in rats. A dietary supplementation of 2% (w/w) squalene significantly minimized isoprenaline-induced alterations in mitochondrial energy status by maintaining the activities of TCA cycle enzymes (isocitrate dehydrogenase, *α*-ketoglutarate, succinate dehydrogenase and malate dehydrogenase) and respiratory marker enzymes (NADH dehydrognase and cytochrome-*c*-oxydase) at a higher level when compared to control rats that received isoprenaline. Squalene exerted an antioxidant effect by inhibiting mitochondrial lipid peroxidation. Supplementation with squalene also maintained the mitochondrial antioxidant defense system at a higher rate by increasing the level of reduced glutathione and the activities of glutathione-dependent antioxidant enzymes and antiperoxidative enzymes in the heart mitochondria. The results of this study provide evidence that dietary supplementation with squalene can improve heart mitochondrial function and prevent subsequent damage to the myocardium. The cardioprotective effect of squalene might be ascribable to its antioxidant property and membrane-stabilizing action.

Keywords: lipid peroxidation, membrane-bound ATPases, respiratory chain enzymes, TCA cycle enzymes
Abbreviations: **ATP**, adenosine triphosphate; **CAT**, catalase; **CDNB**, 1-chloro-2,4,dinitrobenzene; **GPx**, glutathione peroxidase; **GSH**, reduced glutathione; **GST**, glutathione-*S*-transferase; **ICDH**, isocitrate dehydrogenase; **KDH**, ketoglutarate dehydrogenase; **LPO**, lipid peroxides; **MDA**, malonaldehyde; **MDH**, malate dehydrogenase; **NADH**, reduced nicotinamide adenine dinucleotide; **SDH**, succinate dehydrogenase ; **SOD**, superoxide dismutase; **TCA**, trichloroacetic acid

INTRODUCTION

Mitochondria, "the power house of cells" are increasingly recognized as key players in cell survival, not only because of their traditional role as energy providers for vital cellular processes but also because of their critical involvement in programmed cell death via apoptosis (Matsuyama and Reed 2000). Acute myocardial infarction is associated with a reduced coronary blood flow and results in a decreased supply of oxygen and nutrients to the heart. This has an instant effect on myocardial energy production by mitochondria since the reduced availability of oxygen brings oxidative phosphorylation to a halt, thus inhibiting aerobic ATP synthesis (Ostadal *et al.* 1999). This fall in energy production by the mitochondria, which, in the absence of an adequate washout, causes abnormal accumulation of ions and metabolites, leads to a loss of mitochondrial function (Ingwall and Shen 1999). Loss of mitochondrial function is disastrous for the heart since ATP derived from oxidative phosphorylation is needed to maintain contractile activity (Lee and Allen 1991). It is critical that the mitochondrial inner membrane presents a permeability barrier to protons, since this is required to maintain the membrane potential and pH gradient that provide the driving force for ATP synthesis through oxidative phosphorylation (Matsuyama and Reed 2000). If the permeability barrier of the inner membrane is disrupted, mitochondria become uncoupled, and thus, can neither synthesise ATP by oxidative phosphorylation nor separate cytosolic and mitochondrial pools of metabolites (Hausenloy *et al.* 2002). Even glycolytically derived ATP is hydrolyzed by uncoupled mitochondria, as the ATP synthase reverses in the absence of a membrane potential or pH gradient. Thus the damage inflicted in mitochondria would ultimately result in irreversible injury and cell death (Crompton 1999; Paradies *et al.* 2004). However, if the mitochondria can be protected from damage, they may shift the balance away from irreversible injury towards cell recovery (Suleiman *et al.* 2001).

Intraperitoneal administration of isoprenaline, a synthetic catecholamine and β-adrenergic agonist, into adult rats leads to biochemical and morphological alterations in the heart tissue of experimental animals similar to those observed in human myocardial infarction (Mohanty *et al.* 2004). Stimulation of β-adrenergic receptor through sympathetic neuronal activation by isoprenaline results in an increased heart rate (chronotropism), force of cardiac contraction (inotropism) and rate of cardiac relaxation (lusitropism) (Post *et al.* 1999). This results in elevated myocardial oxygen demands, which are not met by appropriate increases in oxygen supply i.e. coronary flow (Nikolaidis *et al.* 2002). This leads to an energy imbalance by Ca^{2+} overload which is accompanied by disruption of the mitochondria (Tavi *et al.* 2005) with inactivation of TCA cycle enzymes and an altered mitochondrial respiration (Prabhu *et al.* 2006). Isoprenaline is also well known to generate free radicals, which can initiate a wide range of toxic oxidative reactions,

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which include initiation of lipid peroxidation, direct inhibition of mitochondrial respiratory chain enzymes, initiation of glyceraldehydes-3-phosphate dehydrogenase and the inactivation of membrane sodium channels and other oxidative modification of proteins (Chagoya de Sánchez *et al.* 1997). All these adverse reactions play a significant role in irreversible damage to the myocardial membrane and pathogenesis of myocardial dysfunction. The extent of oxidative damage induced by isoprenaline can be exacerbated by decreased efficiency of antioxidant defense mechanism (Ganesan *et al.* 2009).

Squalene is a remarkable bioactive substance present in deep sea shark liver oil in higher quantities (Hayashi and Takagi 1981). Other natural sources of squalene include olive oil, cod liver oil, corn oil and a variety of other foods (Liu *et al.* 1976). Squalene is the principal hydrocarbon of human surface lipids amounting up to 11% of total surface fat (Passi *et al.* 2002). It belongs to a class of antioxidants called isoprenoids, which neutralize the harmful effects of excessive free radicals produced in the body. Squalene has been reported to possess antilipidemic, antioxidant and membrane-stabilizing properties (Ivashkevich *et al.* 1981; Ko *et al.* 2002; Qureshi *et al.* 1996). It has been found to be an efficient chemo-preventive agent against a variety of cancers, skin disorders, and liver diseases (Desai *et al.* 1996; Xu *et al.* 2005). Its antiaging, detoxification, cell invigoration and blood purifying properties have already been well studied (Richter and Schafer 1982; Passi *et al.* 2002; Buddhan *et al.* 2007). Earlier, we reported the protective effect of squalene on the tissue antioxidant defense system, mineral status, lipid, protein components, endogenous antioxidants, free amino acids and lysosomes in isoprenaline-induced myocardial infarction in rats (Farvin *et al.* 2004, 2005, 2006, 2007, 2009, 2010). In the present study an attempt has been made to assess the efficacy of squalene on mitochondrial energy metabolism in isoprenaline-induced myocardial injury, by virtue of its membrane-stabilizing and antioxidant properties.

MATERIALS AND METHODS

Chemicals

Epinephrine, isoprenaline, 1,1,3,3-tetra ethoxypropane malondialdehyde bis (diethyl acetal), 2,4 dinitrophenyl hydrazine (DNPH), α-ketoglutarate, sodium succinate, oxaloacetate, reduced glutathione (GSH), reduced nicotinamide adenine dinucleotide (NADH), adenosine triphosphate (ATP), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), ethylene diamine tetra acetate (EDTA), N-phenyl-*p*-phenylenediamine and 1-chloro-2,4,dinitrobenzene (CDNB) were purchased from M/s. Sigma Chemical Company, St. Louis. MO, USA. Squalene (specific gravity: 0.853; refractive index: 1.493; saponification value: 30; iodine value: 344; boiling point: 240-245°C) was prepared from the shark liver oil of *Centrophorus* sp. caught in the Andaman waters (Farvin *et al.* 2004). All other chemicals used were of analytical grade.

Animals

Male Wistar strain albino rats bred in our own animal house, weighing 100-120 g were selected for the study. The animals were housed individually in polyurethane cages under hygienic and standard environmental conditions (28 ± 2°C, humidity 60-70%, 12-h light/dark cycle). The animals were allowed food and water *ad libitum.* The experiment was carried out as per the guidelines of Committee for the Purpose of Control and Supervision of experiments on Animals (CPCSEA), New Delhi, India and approved by the Institutional Animal Ethics Committee (IAEC).

Experimental protocol

Seven days after acclimatization, the animals were divided into four groups of 6 rats each. Group I and III animals were fed on commercial feed (M/s Sai Feeds, Bangalore, India) with added coconut oil at 2% w/w for 45 days and Groups II and IV animals were fed on a commercial feed with squalene added at 2% (w/w) for a period of 45 days. After 45 days feeding, Groups III and IV animals were intraperitoneally (i.p.) injected with isoprenaline [11 mg (dissolved in physiological saline)/100 g body weight/day for 2 days] for the induction of myocardial infarction. Control animals (Groups I and II) were i.p. injected with physiological saline alone for 2 days.

At the end of the experimental period, i.e. 24 h after last injection of isoprenaline, the experimental animals were sacrificed. The heart tissue was dissected out immediately and washed with chilled physiological saline and a part of it was used for determining ATP content. Mitochondria were isolated from the heart tissue by the method of Johnson and Lardy (1967) and were used for the determination of TCA cycle enzymes, respiratory marker enzymes, lipid peroxides, reduced glutathione, glutathione-dependent antioxidant enzymes, antiperoxidative enzymes, membrane bound ATPase and mitochondrial calcium content.

Biochemical assays

1. TCA cycle enzymes

The activity of isocitrate dehydrogenase (ICDH) (EC 1.1.1.42) was assayed by the method of Bell and Baron (1960) and was expressed as nmol α-ketoglutarate liberated/min/mg protein. The activity of succinate dehydrogenase (SDH) (EC 1.3.99.1) was estimated according to the method of Slater and Bonner (1952). The rate of reduction of potassium ferricyanide was measured in the presence of sufficient potassium cyanide to inhibit cytochrome oxidase by following the rate of decrease in the optical density at 420 nm. The activity of SDH was expressed as µmol of succinate oxidized/min/mg protein. Malate dehydrogenase (MDH) (EC 1.1.1.37) activity was assayed by the method of Mehler *et al.* (1948). This was based on the measurement of the rate of oxidation of NADH in the presence of the enzyme and excess oxaloacetate. The activity of MDH was expressed as µmol of NADH oxidized/min/mg of protein. α-Ketoglutarate dehydrogenase (α-KDH)(EC 1.2.4.2) activity was estimated according to the method of Reed and Mukherjee (1969). It was based on the colorimetric determination of ferrocyanide produced by the decarboxylation of α-ketoglurate with ferricynide as electron acceptor and the colour intensity was measured at 540 nm in a Shimadzu UV1601 spectrophotometer. The activity of α-KDH was expressed as nmoles of potassium ferrocyaide liberated /min/mg protein.

2. Respiratory marker enzymes

The activity of NADH dehydrogenase (EC 1.6.99.3) was assayed according to the method of Minakami *et al.* (1962). The activity of NADH-dehydrogenase was expressed as µmol of NADH oxidized/min/mg of protein. Cytochrome-*c*-oxidase (EC 1.9.3.1) was assayed according to the method of Pearl *et al.* (1963). The enzyme activity was determined utilizing the accumulation of free radicals formed by the enzymatic univalent oxidation of a stable non-toxic substrate N-phenyl-*p*-phenylenediamine. The activity of the enzyme was expressed as change in optical density/min/mg protein.

3. Lipid peroxides

The heart mitochondrial lipid peroxide (LPO) content was determined by the thiobarbituric acid reaction as described by Ohkawa *et al.* (1979) in which the malonaldehyde (MDA) released served as the index of LPO. 1,1,3,3-tetra ethoxypropane malondialdehyde bis (diethyl acetal) was used as standard. The level of lipid peroxides was expressed as nmoles of MDA formed/mg protein.

4. Reduced glutathione and glutathione dependent antioxidant enzymes

Reduced glutathione **(**GSH) was determined by the method of Ellman (1959). The method is based on the reaction of reduced glutathione with 5,5-dithiobis(2-nitrobenzoic acid) to give a yellow-colored compound that has absorbance at 412 nm. The amount of glutathione was expressed as µmol/g wet tissue.

Table 1 Activities of isocitrate dehydrogenase (ICDH), succinate dehydrogenase (SDH), α-ketoglutarate dehydrogenase (α-KDH), malate dehydrogenase (MDH), NADH-dehydrogenase and cytochrome-*c*-oxidase in the heart mitochondria of normal and experimental groups of rats.

Parameters	Group I	Group II	Group III	Group IV
TCA cycle enzymes				
ICDH	953.15 ± 80.23	987.32 ±76.45	652.72 ± 39.25 a,b	876.20 ± 76.25 c
SDH	370.8 ± 27.6	393.2 ± 28.3	197.4 ±13.5 a,b	285.3 ± 28.2 c
MDH	314.5 ± 26.2	318.33 ± 27.13	185.13 ± 14.81 a,b	296 ± 23 c
α-KDH	146.6 ± 10.2	150.35 ± 9.45	60.1 ± 22.9 a,b	125.1 ± 8.3 c
Respiratory marker enzymes				
NADH-dehydrogenase	30.61 ± 1.98	36.32 ± 2.14	20.35 ± 1.25 a,b	29.81 ± 1.76 c
Cytochrome-*c*-oxidase	$3.22 \pm 0.27 \times 10^{-2}$	$3.52 \pm 0.25 \times 10^{-2}$	$1.82 \pm 0.14 \times 10^{-2}$ a,b	$2.95 \pm 0.23 \times 10^{-2}$ c

Group I and Group II, normal control, rats received standard diet mixed with 2% coconut oil and 2% squalene, respectively, for a period of 45 days; Group III and Group IV, myocardial infarctions were induced by intraperitoneal (i.p) injection of isoprenaline [11 mg (dissolved in physiological saline)/100 g body weight/day for 2 days] after 45 days of feeding with standard diet mixed with 2% coconut oil and 2% squalene, respectively. Results are means ± SD for 6 animals. Values expressed: ICDH, nmol of α-ketoglutarate formed/min/mg protein; α-KDH, nmol of ferrocyanide formed/min/mg protein; SDH, µmol of succinate oxidised/min/mg protein; MDH, µmol of NADH oxidised/min/mg protein; NADH-dehydrogenase, µmol of NADH oxidised/min/mg protein; cytochrome-*c*-oxidase, change in optical density/min/mg protein. a: $P < 0.001$ significantly different compared with control animals; b: $P < 0.001$ significantly different compared with squalene administered normal rats; c: $P < 0.001$ significantly different compared with isoprenaline-induced myocardial infracted rats.

Glutathione peroxidase (GPx) [EC 1.11.1.9] activity was measured by the method of Pagila and Valentaine (1967). The enzyme activity was expressed as nmoles of GSH oxidized/min/mg protein. Glutathione-*S*-transferase (GST) (EC 2.5.1.18) activity was determined by the method of Habig *et al.* (1974). GST activity was expressed as µmol 1-chloro-2,4,dinitrobenzene (CDNB) conjugate formed/min/mg protein.

5. Antiperoxidative enzymes

Catalase (CAT) [EC 1.11.1.6] activity was assayed according to the method of Takahara *et al.* (1960). The enzyme activity was expressed as nmol of H_2O_2 decomposed/min/mg protein. Superoxide dismutase (SOD) (EC.1.15.1.1) activity was determined according to the method of Misra and Fridovich (1972) based on the oxidation of epinephrine-adrenochrome transition by the enzyme. One unit of SOD activity is calculated as the amount of protein required to give 50% inhibition of epinephrine autoxidation.

6. Mitochondrial membrane-bound ATPases

The method described by Bonting (1970) was followed for the determination of Na^+, K^+ ATPase activity in mitochondrial fraction. The activity of Ca^{2+} ATPase was determined by the method of Hjerten and Pan (1983). Mg^{2+} ATPase activity was determined by the method of Ohnishi *et al.* (1982). In all cases enzyme activity was expressed as µmol of inorganic phosphorous (Pi) liberated/ min/mg of protein.

7. Mitochondrial calcium content

Mitochondrial calcium content was determined using an atomic absorption spectrophotometer (Varian model 220 equipped with a deuterium back ground corrector) after digesting (Milestone ETHOS PLUS lab station Closed Vessel Microwave Digestion System) the sample with nitric acid and perchloric acid (9: 4) mixture as described by Ballentine and Burford (1957).

8. Determination of myocardial ATP content

The level of ATP in the heart was determined by a modified method of Ryder (1985) using a Shimadzu LC 10 ATvp HPLC-PDA system with a Hypersil C18 RP column (4.6 mm × 250 mm). Nucleotide separation was achieved by isocratic elution with phosphate buffer solution prepared by mixing 0.04 M KH_2PO_4 and 0.06 M K_2HPO_4 in a 1: 1 proportion. The flow rate in HPLC was maintained at 1.5 ml/min throughout the chromatographic separation. Quantification of each nucleotide breakdown product was done by comparing the peak area of the samples with peak area of the standards corresponding to each sample. The ATP content was expressed as µmol/g wet tissue.

Statistical analysis

Results were expressed as mean ± SD. One way analysis of variance (ANOVA) was carried out, and the statistical comparisons among the groups were performed with Bonferroni's multiple comparison test. $P < 0.05$ was considered as statistically significant. All data were analyzed with the aid of statistical package program Graphpad prism 4 (Graphpad Softwarer Inc., San Diego, USA).

RESULTS AND DISCUSSION

The major source of energy for contraction comes from the oxidative metabolism of mitochondria in the myocardial cell. Thus the myocardial cell with high energy requirement is more likely to be affected when this organelle is dysfunctional (O' Rourke *et al.* 2005). Mitochondria consume 90% of the oxygen used by the cell, and the mitochondrial respiratory chain generates a continuous flux of oxygen radicals (Paradies *et al.* 2004). Mitochondrial-mediated ROS generation leads to primary reaction and damage in the immediate area surrounding where these ROS are produced, as they are highly reactive and short-lived species. Therefore, as major sources of ROS production, mitochondria could also be major targets of free radical attack (Petrosillo *et al.* 2005). For this reason the function of mitochondria in heart disease is of particular interest.

A significant ($P < 0.001$) decline was noticed in the activities of TCA cycle enzymes (ICDH, SDH, α-KDH and MDH) and respiratory marker enzymes (NADH dehydrogenase and Cytochrome-*c*-oxidase) (**Table 1**) in the heart mitochondria of Group III isoprenaline-administered rats as compared to control rats. This suggests that the mitochondrial oxidative phosphorylation was operating at a lower level despite the higher energy demand in the ischemic myocardium. This observation is in accordance with an earlier reported study (Ebenezar *et al.* 2003) wherein they also found a marked decrease of these enzymes after the intraperitoneal administration of isoprenaline. These enzymes are located in the outer membrane of the mitochondria, which could have been affected by the excessive production of free radicals induced by isoprenaline (Ebenezar *et al.* 2003). The NADH/NAD ratio has been reported to rise in isoprenaline-induced ischemic heart mitochondria, when the metabolic overload on cells was prolonged (Ramasamy *et al.* 1998). This in turn may result in diminution in the activities of TCA cycle enzymes by the mechanism of mass action as observed in the present study. In hypoxic condition, decreased NADPH and NADH oxidation accelerates the inactivation of cyt-P450 to cyt-p420 and is associated with destruction of the nucleus, mitochondria and endoplasmic reticulum (Martin *et al.* 1998). Changes in the concentration of respiratory components, phosphorylative activity, cytochrome-c-oxidase activity and adenylate charge level have also been reported in isoprenaline-induced cardiac damage in rats (Sampath and Kannan 2009). Our observation also confirmed the same pattern and showed a significant ($P < 0.001$) reduction in the level of ATP content in the heart tissue of Group III isoprenaline-induced rats when compared with Group I controls (**Fig. 1**).

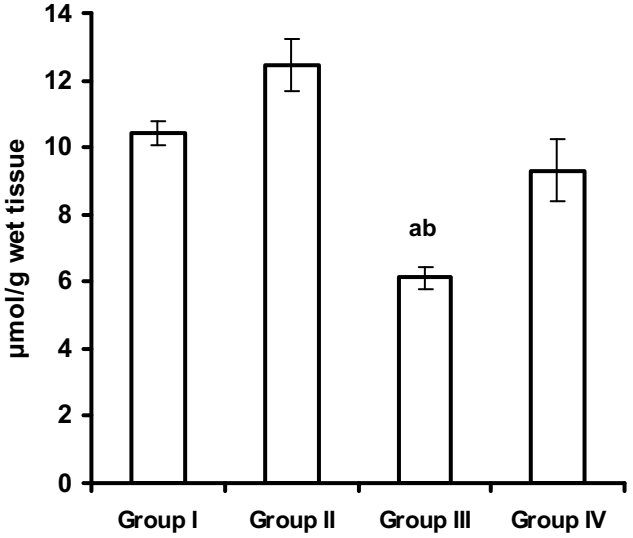

Fig. 1 The levels of ATP in the heart tissue of normal and experimental groups of rats. Group I and Group II, normal control, rats received standard diet mixed with 2% coconut oil and 2% squalene, respectively, for a period of 45 days; Group III and Group IV, myocardial infarctions were induced by intraperitoneal (i.p) injection of isoprenaline [11 mg (dissolved in physiological saline)/100 g body weight/day for 2 days] after 45 days of feeding with standard diet mixed with 2% coconut oil and 2% squalene, respectively. Results are means ± SD for 6 animals. The statistical comparisons among the groups were performed with Bonferroni's multiple comparison test. $P < 0.05$ was considered as statistically significant. a: $P < 0.001$ significantly different compared with control animals; b: $P < 0.001$ significantly different compared with squalene-administered normal rats; c: $P < 0.001$ significantly different compared with isoprenaline-induced myocardial infracted rats.

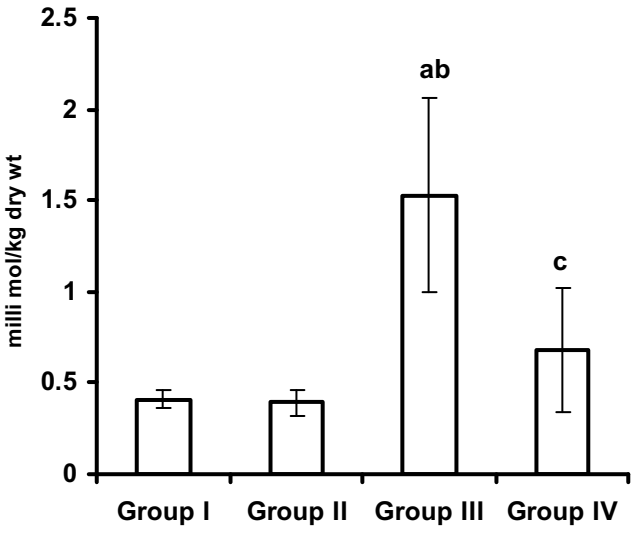

Fig. 2 The levels of mitochondrial calcium content in normal and experimental groups of rats. Description of the groups is same as in **Fig. 1**. Results are means ± SD for 6 animals. The statistical comparisons among the groups were performed with Bonferroni's multiple comparison test. $P < 0.05$ was considered as statistically significant. a: $P < 0.001$ significantly different compared with control animals; b: $P < 0.001$ significantly different compared with squalene-administered normal rats; c: $P < 0.001$ significantly different compared with isoprenaline-induced myocardial infracted rats.

In the present study, the prior administration of squalene at 2% (w/w) maintained the level of ATP and the activities of TCA cycle enzymes and respiratory marker enzymes significantly ($P < 0.001$) at near normalcy in Group IV rats compared to Group III myocardial infarction-induced rats, reflecting its ability to maintain the function of the heart mitochondria at near normal status. The protective action of squalene on mitochondrial energy status is probably related to its ability to modulate the physiochemical properties of the mitochondrial membrane lipid bilayer. Like other non-bilayer forming lipids such as ubiquinone, squalene has been reported to lie in the centre of the lipid bilayer and inhibit proton leaks (Haub *et al.* 2002). Since cellular membranes maintain a proton electrochemical gradient as a principal energy transducer, proton leakages unproductively consumes a cell's ATP. Hence it is possible that squalene might have rendered the protection by maintaining a proper membrane structure and fluidity for the transport of materials across the membrane as well as the transmission of signals across the membrane. Squalene has also been reported to protect mitochondrial function in the liver of aged rats (Buddhan *et al.* 2007).

Reactive oxygen species (ROS) are reported to play an important role in producing lethal cell injury associated with cardiac ischemia during myocardial infarction (Ferrari *et al.* 2004). The effects of ROS should be greatest at the level of mitochondrial membrane constituents, including the complexes of the respiratory chain and phospholipids constituents particularly rich in unsaturated fatty acids, such as cardiolipin (Paradies *et al.* 2004). In the present study, the level of lipid peroxidation was significantly ($P < 0.001$) higher in the heart mitochondria of Group III isoprenaline-administrated rats compared to Group I controls (**Table 2**). This was paralleled by a significant ($P < 0.001$) decline in the level of reduced glutathione and the activities of glutathione-dependent antioxidant enzymes (GPx and GST) and antiperoxidative enzymes (SOD and CAT) in the heart mitochondria of myocardial infarction-induced Group III rats when compared to Group I controls (**Table 2**). The reduction in the level of GSH was either due to increased degradation or decreased synthesis of glutathione and the decreased availability of GSH might have lead to the lowered activities of GPx and GST. Increased generation of reactive oxygen radicals such as superoxide and hydrogen peroxide is associated with the inhibition of SOD and CAT (Farvin *et al.* 2004). The intracellular calcium concentration in mitochondria has been reported to rise in isoprenaline-induced myocardial stress (Tavi *et al.* 2005). Our results also confirm the same (**Fig. 2**). Intracellular Ca^{2+} is an inducer of phospholipase A2, which degrades membrane phospholipids

Table 2 levels of lipid peroxides (LPO), reduced glutathione (GSH) and activities of glutathione peroxidase (GPx), glutathione-*S*-transferase (GST), catalase (CAT) and superoxide dismutase (SOD) in heart mitochondria of normal and experimental groups of rats.

Parameters	Group I	Group II	Group III	Group IV
LPO	0.84 ± 0.04	0.74 ± 0.03	1.32 ± 0.07 a,b	0.89 ± 0.03 c
GSH	3.14 ± 0.28	3.58 ± 0.31	1.12 ± 0.08 a,b	2.91 ± 0.17 c
GPx	2.79 ± 0.24	3.12 ± 0.29	1.31 ± 0.18 a,b	2.26 ± 0.26 c
GST	1175 ± 109	1201 ± 115	491 ± 32 a,b	820 ± 76 c
CAT	11.6 ± 0.75	13.69 ± 0.82	6.13 ± 0.37 a,b	9.45 ± 0.72 c
SOD	5.97 ± 0.28	6.25 ± 0.31	2.51 ± 0.17 a,b	5.01 ± 0.24 c

Descriptions of the groups are same as in **Table 1**. Results are means ± SD for 6 animals. Values expressed: LPO, nmol malondialdehyde released/mg protein; GSH, µmol/g wet tissue; GPx, nmol GSH oxidised/min/mg protein; GST, µmol 1-chloro-2,4-dinitrobenzene conjugate formed/min/mg protein; CAT, nmol H_2O_2 decomposed/min/mg protein; SOD, one unit of SOD activity is the amount of protein required to give 50% inhibition of epinephrine autoxidation. a: $P < 0.001$ significantly different compared with control animals; b: $P < 0.001$ significantly different compared with squalene-administered normal rats; c: $P < 0.001$ significantly different compared with isoprenaline-induced myocardial infracted rats.

Table 3 The activities of Ca^{2+}-ATPase, Na^{+}-, K^{+}-ATPase and Mg^{2+}-ATPase in heart mitochondria of normal and experimental groups of rats.

Parameters	Group I	Group II	Group III	Group IV
Ca^{2+}-ATPase	1.16 ± 0.11	1.05 ± 0.07	0.74 ± 0.05 a,b	0.98 ± 0.09 c
Na^{+}-, K^{+}-ATPase	1.61 ± 0.07	1.93 ± 0.06	1.08 ± 0.05 a,b	1.39 ± 0.08 c
Mg^{2+}-ATPase	0.81 ± 0.04	0.83 ± 0.07	0.63 ± 0.02 a,b	0.78 ± 0.04 c

Descriptions of the groups are same as in **Table 1**. Results are means ± SD for 6 animals. Values expressed as μ moles of inorganic phosphorous (Pi) liberated/min/mg protein. a: $P < 0.001$ significantly different compared with control animals; b: $P < 0.001$ significantly different compared with squalene administered normal rats; c: $P < 0.001$ significantly different compared with isoprenaline-induced myocardial infracted rats.

(Caro and Cederbaum 2007). The free radicals produced as a result of phospholipase A2-stimulated lipid peroxidation may attack RNA polymerase corresponding to these antioxidant enzymes (Meng *et al.* 2006).

In the present study, the prior administration of squalene significantly ($P < 0.001$) counteracted the isoprenaline-induced lipid peroxidation and maintained the level of reduced glutathione and the activities of antioxidant enzymes at near normalcy in the heart mitochondria of Group IV rats (**Table 2**). This might be attributed to the free radical scavenging potential of squalene by its isoprenoid unit (Kohno *et al.* 1995). Highly lipophilic antioxidant molecules such as vitamin E have been reported to trap free radicals by electron donating and radical resonating mechanisms, thereby blocking the lipid peroxidation chain reaction (Stahl and Sies 1997). Since squalene is more potent than vitamin E as a free radical scavenger (Kohno *et al.* 1995) and as a stabilizer of cellular and subcellular membranes (Ivashkevich *et al.* 1981; Buddhan *et al.* 2007). It is possible that stabilization of myocardial membranes by squalene, particularly the mitochondrial membranes, may prolong the viability of ischemic cardiac muscle from isoprenaline-induced peroxidative damage.

Mitochondrial ATPases are lipid dependent as well as -SH dependent membrane-bound enzymes, and any alterations in the membrane lipid leads to change in the membrane fluidity which in turn affect the activities of these enzymes. In the present study, a significant ($P<0.001$) reduction was noticed in the activities of mitochondrial membrane-bound ATPases (Ca^{2+}-ATPases, Na^{+}/K^{+} ATPases, Mg^{2+}-ATPase) in Group III isoprenaline-administered rats compared with Group I normal rats (**Table 3**). This is in line with previous reported studies (Uyemura and Curti 1991; Haraguchi *et al.* 2000), which showed a severe derangement of subcellular metabolism and structural alterations in the mitochondrial membrane. Oxidative stress, which is usually associated with an increased generation of ROS, modifies membrane phospholipids and proteins leading to lipid peroxidation and oxidation of thiol groups (Suzuki *et al.* 1991). Hence, the marked loss noticed in the activities of mitochondrial ATPases in isoprenaline administered rats might be due to loss of protein -SH, which resulted due to increased lipid peroxidative damage to myocardial membrane (Acosta *et al.* 1984; Ganesan *et al.* 2009).

Pretreatment with squalene at 2% (w/w) along with feed significantly ($P < 0.001$) prevented the isoprenaline-induced alterations in mitochondrial membrane-bound ATPases in the heart tissue of Group IV rats compared to Group I rats (**Table 3**). It probably did so by the restoration of enzymic and non-enzymic mitochondrial antioxidants (Aioi *et al.* 1995), which protect the membrane-bound enzymes from free radicals-mediated inactivation. Feeding -SH generating substances or free radical scavengers in the diet has been reported to restore the cellular thiol content and the membrane functions (Levine *et al.* 1993; Senthilkumar *et al.* 2006). Squalene has been reported to act as a chain-breaking antioxidant by donating its labile hydrogen atom from isoprenyl groups to lipid peroxyl and alkoxyl radical intermediates of lipid peroxidation, thus terminating chain reaction (Koizumi 1994). In biomembranes, squalene has been found to have potent antioxidant activity due to its ability to penetrate to a precise site into the membrane, which may be the important feature of protection against highly reactive radicals (Ivashkevich *et al.* 1981).

CONCLUDING REMARKS

The results of the present study indicate that dietary supplementation of squalene at 2% (w/w) ameliorates isoprenaline-induced aberrations in the myocardial mitochondrial energy status, antioxidant defense system and membrane bound ATPases in experimental rats. The overall cardioprotective effect of squalene is probably related to its ability to strengthen the myocardial membrane by its membrane stabilizing action, or to its ability to maintain the myocardial energy status (ATP) at higher level by maintaining the activity of TCA cycle enzymes and respiratory marker enzymes at near normalcy, and/or to its free radical-scavenging ability against isoprenaline-induced lipid peroxidation, which is primarily responsible for the irreversible necrosis of the myocardial membrane.

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