Protective Effect of Squalene against Isoproterenol-Induced Myocardial Infarction in Rats

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Summary **The protective effect of squalene on membrane function and mineral status was examined in isoproterenol-induced myocardial infarction in male albino rats. The pretreatment with squalene at 2% level along with feed significantly reduced the isoproterenol-induced rise in the levels of plasma diagnostic marker enzymes (ALT, AST, LDH and CPK). It counteracted isoproterenol-induced lipid peroxidation in plasma and heart tissue, and maintained the level of reduced glutathione in the heart tissue at near normalcy. Supplementation of squalene also exerted membrane stabilizing action against isoproterenol-induced myocardial infarction by maintaining the activities of membrane-bound ATPases (Na^+, K^+ ATPase and Ca^{2+} ATPase) in heart tissue and the mineral status (sodium, potassium and calcium) in plasma and heart tissue at near normal levels. The cardioprotective effect of squalene might be ascribable to its antioxidant nature and membrane stabilizing property.**

Key Words: **squalene, isoproterenol, myocardial infarction, lipid peroxidation, mineral status, membrane-bound ATPases**

Introduction

Myocardial infarction, the most spectacular and lethal manifestation of cardiovascular disease, is the result of long process of subtle deterioration of the circulatory system and it is the common cause of mortality in all industrialized nations [*1*]. Till today, it remains a clinical challenge and a problem of great importance. There is an urgent need for the clinical development of safe and non-toxic cytoprotective agents for the adequate management of cardiovascular diseases. A better understanding of the processes involved in myocardial infarction has stimulated the search for new drugs, which could limit myocardial injury. The major abnormalities observed in myocardial infarction are lipidemia, peroxidation, and loss of membrane integrity [*2*, *3*].

Squalene is a remarkable bioactive substance present in shark liver oil in higher quantities [*4*]. It belongs to a class of antioxidants called isoprenoid, which neutralizes the harmful effects of excessive free radicals produced in the body. Squalene has been reported to possess antilipidemic, antioxidant and membrane stabilizing properties [*5–7*]. A phase I trial in adult males given 860 mg for squalene daily for 20 weeks to study the cholesterol-lowering effect of squalene showed that oral squalene is safe and tolerable [*8*]. Earlier, we have reported the preventive effect of squalene on tissue antioxidant defense system in experimentally induced myocardial infarction in rats [*9*]. In the present study, an attempt has been

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made to assess the protective effects of squalene on mineral status and membrane function in isoproterenol-induced myocardial infarction in rats, a well-established animal model for studying the effects of many drugs on the process of myocardial infarction.

Materials and Methods

Chemicals

Isoproterenol and tetraethoxypropane were obtained from M/s. Sigma Chemical Company (St. Louis, MO). 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 [*10*]. All the other chemicals used were of analytical grade.

Animals

Wistar strain male albino rats, 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 a standard diet [M/s Sai Feeds, Bangalore, India] and water *ad libitum*.

Induction of myocardial infarction

The myocardial infarction was induced in experimental rats by intraperitoneal (i.p.) injection of isoproterenol [11 mg (dissolved in physiological saline)/100 g body weight/day], for 2 days [*11*].

Experimental protocol

Seven days after acclimatization, the animals were divided into four groups of 6 rats each. Group I and Group III animals were fed standard diet with added coconut oil at 2% level for 45 days and Group II and Group IV were fed standard diet with added squalene at 2% level for the same period. After 45 days feeding, the Group III and Group IV animals were injected with isoproterenol as described above for the induction of myocardial infarction. Control animals (Group I and Group II) were injected with physiological saline alone for 2 days.

At the end of the experimental period, i.e. 24 h after last injection of isoproterenol, the experimental animals were sacrificed and blood was collected using heparin as anticoagulant for the separation of plasma. The heart tissue was excised immediately and thoroughly washed with cold physiological saline. The level of lipid peroxidation in plasma was determined by the method of Yagi [*12*] and in heart tissue by the method of Ohkawa *et al.* [*13*]. Reduced glutathione content was estimated by the method of Ellman [*14*]. The method described by Bonting [*15*] was followed for the determination of Na^+, K^+ ATPase activity in the heart tissue and the activity of Ca^{2+}ATPase activity was determined by the method of Hjerten and Pan [*16*]. The protein content was estimated by the method of Lowry *et al.* [*17*]. The sodium, potassium and calcium contents in plasma and heart tissue were determined by using Varian Atomic Spectrophotometer 220AA.

The experiment was carried out according to 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.

Statistical analysis

Results are expressed as mean±SD. One-way analysis of variance (ANOVA) was carried out, and the statistical comparisons among the groups were performed with Tukey's test using a statistical package program (SPSS 10.0 for Windows).

Results

Significant rise observed in the levels of diagnostic marker enzymes (alanine amino transferase [ALT], aspartate amino transferase [AST], lactate dehydrogenase [LDH] and creatine phosphokinase [CPK]) in plasma of Group III myocardial infarction induced rats as compared to Group I control animals (Table 1). The pretreatment with squalene at 2% level along with feed significantly reduced the release of these diagnostic marker enzymes into the systemic circulation as compared with Group III rats. Intraperitoneal administration of isoproterenol caused a significant ($p<0.001$) increase in lipid peroxidation in plasma and heart tissue of Group III rats as compared with that of Group I rats (Table 2). This was paralleled by significant reduction in the level of reduced glutathione in the heart tissue as compared with that of normal control rats (Table 3). In Group IV rats, the prior administration of squalene significantly prevented the isoproterenol-induced lipid peroxidation in plasma and heart tissue and maintained the level of reduced glutathione at near normalcy in Group IV rats.

The activities of membrane-bound ATPases (Na^+,

Table 1. Levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and creatine phosphokinase (CPK) in plasma of normal and experimental groups of rats.

Groups	Group I	Group II	Group III	Group IV
ALT	102±9.1	110±9.4	386±36[a]	156±14[b,c]
AST	84.2±7.2	87.9±7.6	343±28[a]	126±11.5[b,c]
LDH	198±15.4	205±15.2	397±35[a]	231±19.3[b,c]
CPK	138±10.2	143±10.5	375±25[a]	168±12.7[b,c]

Group I and Group II, normal control, rats received standard diet mixed with coconut oil 2% and squalene 2% respectively for a period of 45 days; Group III and Group IV, myocardial infarction was induced by intraperitoneal (i.p.) injection of isoproterenol [11 mg (dissolved in physiological saline)/100 g body weight/day for 2 days] after 45 days of feeding with standard diet mixed with coconut oil 2% and squalene 2% respectively. Results are mean±SD for 6 animals. Values expressed: ALT, AST, and LDH, μmol pyruvate liberated/h/liter; CPK, μmol creatine liberated/h/liter. [a]$p<0.001$ significantly different compared with Group I control animals; [b]$p<0.001$ significantly different compared with Group III isoproterenol-induced myocardial infarcted rats; [c]$p<0.05$ significantly different compared with Group II animals.

Table 2. Level of lipid peroxides in plasma (nmol/ml) and heart tissue (nmol/mg protein) of normal and experimental groups of rats.

	Group I	Group II	Group III	Group IV
Plasma	1.78±0.14	1.52±0.12	4.21±0.34[a]	1.95±0.16[b,c]
Heart	0.94±0.08	0.86±0.08	1.99±0.11[a]	1.03±0.08[b,c]

Experimental conditions are the same as those in Table 1. Results are mean±SD for 6 animals. [a]$p<0.001$ significantly different compared with Group I control animals; [b]$p<0.001$ significantly different compared with Group III isoproterenol-induced myocardial infarcted rats; [c]$p<0.05$ significantly different compared with Group II animals.

Table 3. Level of reduced glutathione (GSH) and the activities of Ca^{2+} ATPase and Na^+, K^+ ATPase in heart tissue of normal and experimental groups of rats.

	Group I	Group II	Group III	Group IV
GSH	5.21±3.16	5.61±3.43	2.88±0.21[a]	4.76±0.25[b,c]
Ca^{2+} ATPase	0.78±0.04	0.84±0.04	0.54±0.01[a]	0.69±0.04[b,c]
Na^+, K^+ ATPase	1.36±0.07	1.28±0.08	0.75±0.04[a]	1.09±0.07[b,c]

Experimental conditions are the same as those in Table 1. Results are mean±SD for 6 animals. Values expressed: GSH, μg/g wet tissue; Ca^{2+} ATPase and Na^+, K^+ ATPase, μmol inorganic phosphorous (Pi) liberated/min/mg protein. [a]$p<0.001$ significantly different compared with Group I control animals; [b]$p<0.001$ significantly different compared with Group III isoproterenol-induced myocardial infarcted rats; [c]$p<0.05$ significantly different compared with Group II animals.

Table 4. Levels of potassium, sodium and calcium in plasma (mmol/liter) and heart tissue (mg/g wet tissue) of normal and experimental groups of rats.

	Group I	Group II	Group III	Group IV
Plasma:				
Potassium	7.18±0.61	7.04±0.64	10.3±0.89[a]	7.92±0.68[b,c]
Sodium	108±8.72	112±10.8	65.4±4.11[a]	94.3±7.2[b,c]
Calcium	64.5±5.24	69.6±5.15	118±10.7[a]	78.4±7.07[b,c]
Heart:				
Potassium	123±9.51	128±9.07	194±15.8[a]	143±12[b,c]
Sodium	321±26.4	316±27.4	189±14.2[a]	278±21.3[b,c]
Calcium	81.5±5.84	85.9±5.65	132±9.41[a]	95.8±7.24[b,c]

Experimental conditions are the same as those in Table 1. Results are mean±SD for 6 animals. [a]$p<0.001$ significantly different compared with Group I control animals; [b]$p<0.001$ significantly different compared with Group III isoproterenol-induced myocardial infarcted rats; [c]$p<0.05$ significantly different compared with Group II animals.

K^+ ATPase and Ca^{2+} ATPase) were significantly decreased in the heart tissue of Group III myocardial infarction induced animals as compared with that of Group I control rats (Table 3). Also significant alteration in the levels of sodium, potassium and calcium in serum and heart tissue mineral status was observed in isoproterenol-administered rats (Table 4). In the present study, the prior administration of squalene at 2% level along with feed significantly ($p<0.001$) prevented all these isoproterenol-induced adverse effects and maintained the levels of evaluated parameters nearly at normal values. The Group II normal rats receiving squalene alone did not show any significant change when compared with normal rats, indicating that it does not *per se* have any adverse effects.

Discussion

Significant ($p<0.001$) increase noticed in the levels of AST, ALT, LDH and CPK in plasma of Group III isoproterenol-administered rats (Table 1), which is in line with earlier reports [*18*], might be due to enhanced susceptibility of myocardial cell membrane to the isoproterenol-mediated peroxidative damage, resulting in increased release of these diagnostic marker enzymes into the systemic circulation. In the present study, the prior administration of squalene at 2% level along with feed was significantly ($p<0.001$) prevented the isoproterenol-induced elevation in the levels of diagnostic marker enzymes in plasma of Group IV animals compared with those Group III isoproterenol-injected rats, indicating the cytoprotective activity of squalene. Squalene, which is lipophilic in nature, could be compared to any other lipophilic agents, such as vitamin E, antipyrine and nifidine. The lipophilic β-blocking drugs intercalate into the lipid matrix and impart stabilization to myocardial cell membranes in relation to the degree of their lipophilicity [*19*]. Hence, it is possible that likewise squalene may also prolong the viability of myocardial cell membrane stabilizing action [*7*].

Significant ($p<0.001$) rise observed in the level of lipid peroxidation in the plasma and heart tissue of isoproterenol-administered rats (Table 2), which is in line with an earlier reported study [*20*], indicated the extent of necrotic damage to the myocardial membrane. The reduction noticed in the level of GSH in isoproterenol-induced myocardial infarction was either due to increased degradation or decreased synthesis of glutathione. Depletion of GSH results in enhanced lipid peroxidation, and excessive lipid peroxidation can cause increased GSH consumption [*21*], as observed in the present study. In Group IV rats, supplementation of squalene at 2% level along with feed significantly ($p<0.001$) prevented the isoproterenol-induced lipid peroxidation and maintained the level of reduced glutathione at near normal level as compared with Group III animals. This antioxidant effect of squalene is probably due to the presence of isoprenoid unit in the structure of squalene [*22*]. The unpaired electron present in the hydroxyl radical ($OH^{\cdot}$) generated during isoproterenol-induced myocardial infarction might have been trapped by its free radical scavenging isoprenoid unit.

Significant ($p<0.001$) reduction noticed in activities of the membrane-bound ATPases (Na^+, K^+ ATPase and Ca^{2+} ATPase) in the heart tissue of Group III isoproterenol-induced myocardial infracted rats as compared with Group I normal rats (Table 3), which is in line with previous reported study, reflected a severe derangement of subcellular metabolism and structural alterations in myocardial membrane [*23*]. Oxidative stress, which is usually associated with increased generation of reactive oxygen species (ROS), modifies membrane phospholipids and proteins leading to lipid peroxidation and oxidation of thiol groups [*24*]. These changes are considered to alter myocardial membrane permeability and configuration in addition to producing functional modification of lipid dependent as well as –SH dependent membrane-bound ATPases [*25*]. Elevated levels of ROS in the myocardium may result in cellular defects including a depression in sarcolemmal Ca^{2+} ATPase and Na^+, K^+ ATPase activities, which in turn may lead to decreased calcium efflux and increased calcium influx respectively [*26*, *27*]. The depression in Ca^{2+} regulatory mechanism by ROS ultimately results in intracellular calcium overload and cell death [*28*]. In the present study, prior administration of squalene significantly ($p<0.01$) maintained the activities of membrane-bound ATPases at near normal as compared with that of Group III animals. It probably did so by preventing the oxidation of membrane phospholipids and membrane-bound protein –SH groups from ROS by its free radical quenching capability [*6*, *22*].

The significant ($p<0.001$) rise observed in the level of calcium in the plasma and heart tissue of isoproterenol-administered Group III rats as compared with that Group I control animals (Table 4) is in

accordance with an earlier reported study [*29*, *30*], which indicated that the chronological changes of calcium, magnesium and water contents were well correlated to the morphological early changes of myocardial fibers. Isoproterenol administration could maximally increase Ca^{2+} influx in whole heart tissue [*31*, *32*]. Since plasma membrane Ca^{2+} ATPase extrudes Ca^{2+} from the cytoplasm of all the cells [*33*] blocking of intracellular Ca^{2+} ATPase under the condition of isoproterenol-induced myocardial infarction results in fast cellular Ca^{2+} accumulation. Thus influx of calcium into the cell is uncontrolled under isoproterenol-induced myocardial infarction, leading to the disturbance in equilibrium between intracellular and extracellular calcium concentrations [*34*].

Active calcium transport and resultant low calcium concentration are the essential requirement for active Na^+/K^+ transport. Since sodium and calcium are thought to be competitive at a number of membrane sites, it seems likely that a high concentration of Ca^{2+} in myocardial cells of ischemic rats would compete with sodium specific sites at the inner surface of the membrane [*35*], and this may lead to decrease in sodium content. Also, failure of sodium pump results in a depletion of plasma sodium and rise in plasma potassium concentration as observed in the present study (Table 4). In the present study, supplementation of squalene at 2% level along with feed significantly ($p<0.001$) prevented all these isoproterenol-induced adverse effects on the levels of these minerals and maintained the rats at near normal status. It probably did so by protecting the plasma membrane-bound ATPases from the free radical attack by its free radical scavenging capability.

The results of the present study indicate that the cardioprotective effect of squalene against isoproterenol-induced myocardial infarction may probably related to a counteraction of free radicals by its antioxidant nature, to a strengthening of myocardial membrane by its membrane stabilizing action, or to its ability to maintain near to the normal level of GSH, which protects myocardial membrane against oxidative damage by decreasing lipid peroxidation.

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