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Biochemical Studies on the Cardioprotective Effect of Squalene against Isoprenaline-induced Myocardial Infarction in Rats

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This study was designed to examine the cardioprotective effect of squalene against isoprenaline-induced myocardial infarction in male albino rats. Levels of diagnostic markers [troponin T, homocysteine], lipoproteins [apolipoprotein A1, apolipoprotein B, lipoprotein (a)] (in plasma), total cholesterol, lipid peroxides (in plasma and heart tissue), and endogenous non-enzymatic antioxidants [vitamin C and vitamin E] (in heart tissue) were determined. Supplementation of squalene at 2% level along with feed for 45 days significantly prevented the isoprenaline-induced elevation in the diagnostic markers in plasma of experimental group of rats. It exerted an antilipidemic action against isoprenaline-induced hypercholesterolemia by maintaining the levels of cholesterol and lipoprotein components at near normalcy. Squalene supplementation exhibited an antioxidant effect against isoprenaline-induced myocardial infarction by blocking the induction of lipid peroxidation. A tendency to prevent the isoprenaline induced reduction in the non-enzymatic antioxidants such as vitamin E and C was also observed. The results of the present study indicate that the cardioprotective effect of squalene might be ascribable to its antilipidemic, antioxidant and membrane stabilizing properties.

Key words: Squalene, myocardial infarction, cholesterol, lipid peroxidation

Cardiovascular disease is a major public health concern and leading cause of death all over the world. According to WHO reports, about 16.7 million people around the globe die of myocardial infarction each year. This is about one-third of all deaths globally (WHO, 2004). It is predicted that heart disease and stroke will become the leading cause of both death and disability world-wide by the year 2020, with the number of fatalities projected to increase to more than 20 million a year and to more than 24 million a year by 2030. It is well evident that developing countries like India are strug-gling to manage the impact of infectious diseases simultaneously with the growing burden on society and health systems caused by non-communicable diseases such as myocardial infarction. Moreover, it is very much painful and serious concern to realize that myocardial infarction in India occurs 10 to 15 years earlier than in the west. It is also important to note that an increasing number of young Indians are falling prey to myocardial infarction and there are an estimated 45 million patients of coronary heart disease in India (Yusuf *et al.* 2001). As myocardial injury is irreversible in nature, most of the modern drugs available are effective in the prevention of spreading or dispersal of necrotic damage to the adjacent cells. So there is a need for search of new

cardioprotective agents, which could limit the myocardial injury by strengthening the heart muscle.

Squalene, an isoprenoid molecule present in deep-sea shark liver oil in higher quantities, has been reported to possess antioxidant (Ko *et al.* 2002) and membrane stabilizing properties (Ivashkevich *et al.* 1981). It is an intermediate metabolite in cholesterol metabolism and is secreted in human sebum, where it protects the skin from ultraviolet (UV) radiation (Kohno *et al.* 1995; Storm *et al.* 1993). Several experimental investigations demonstrated the detoxifying activities of the squalene against diverse chemicals such as hexachlorobiphenyl, hexachlorobenzene, arsenic, theophylline, phenobarbital and strychnine (Fan *et al.* 1996; Kamimura *et al.* 1992; Richter & Schafer, 1982). It has also been reported to possess anticarcinogenic activity against several carcinogens, including azoxymethane-induced colon cancer (Rao *et al.* 1998) and nicotine-derived nitrosaminoketone-(NMK) induced lung carcinogenisis (Smith *et al.* 1998). Its antiageing property has already been well established (Passi *et al.* 2002). Though the beneficial properties of squalene are promising and well studied, the cardioprotective effects of squalene have not yet been explored.

Myocardial infarction induced by isoprenaline [L- β- (3,4-dihydroxyphenyl)-a-isopropylaminoethanol hydrochloride], a β-adrenergic agonist has been reported to show many metabolic and morphologic aberrations in the heart tissue of the experimental animals similar to those observed in human myocardial infarction (Nirmala & Puvanakrishnan, 1996). It induces myocardial necrosis by a multiple step mechanism (Ravichandran *et al.* 1990). The primary disturbance of isoprenaline-induced myocardial infarction has been reported to enhance adenylate cyclase activity resulting in increased cAMP formation, which in turn would lead to increased lipid accumulation in the myocardium (Subhash *et al.* 1978). The administration of isoprenaline produces necrotic lesions and increased lipid peroxidation in the myocardium, which plays a crucial role in the pathogenesis of isoprenaline-induced myocardial infarction (Noronha-Dutra *et al.* 1984; Singal *et al.* 1982; Singal *et al.* 1983).

In the present study, an attempt has been made to assess the cardioprotective action of squalene in isoprenaline-induced myocardial infarction in rats by virtue of its hypolipidemic, antiperoxidative and membrane-stabilizing properties.

Materials & Methods

Isoprenaline, tetraethoxy propane and cholesterol were obtained 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: 240-245ºC) was prepared from the shark liver oil of *Centrophorus* sp. caught in the Andaman waters (Farvin *et al.* 2004). All the other chemicals used were of analytical grade.

Male Wistar strain albino rats, weighing 120-150g were selected for the study. The animals were housed individually in polypropylene cages under hygienic and standard environmental conditions (28±2ºC, 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*. 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 Institute Animal Ethics Committee (IAEC).

Seven days after acclimatization, the animals were divided into four groups of 6

rats each. Group I and Group III animals were fed on standard diet with added coconut oil at 2% level for 45 days and Group II and Group IV animals were fed on standard diet with added squalene at 2% level for the same period. After 45 days feeding, the Group III and Group IV animals were intraperitoneally (i.p.) injected with isoprenaline [11mg (dissolved in physiological saline)/ 100g body weight/ day for 2 days] for the induction of myocardial infarction. Control animals (Group I and Group 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, blood was collected using sodium citrate as anticoagulant and the plasma separated was used for assay of various biochemical parameters. The heart tissue was excised immediately and washed with chilled isotonic saline. Accurately weighed heart tissue was homogenized in ice-cold 0.1M Tris-HCl buffer, pH 7.2 and centrifuged. The supernatant was used for further biochemical analyses.

Troponin T was estimated in plasma samples by electrochemiluminescence immunoassay "ECLIA" on Roche Elecsys 1010/ 2010 and Modular Analytics E170 (Elecsys module) immunoassay analyzer. Homocysteine (Hcy) concentration in plasma was assayed by Microtiter plate assay package (Diazyme Laboratories). Apolipoprotein A1, apolipoprotein B, lipoprotein (a) were estimated in plasma samples using immunoturbidimetric test kit from DiaSys Diagnostic Systems GmbH, Germany. Total cholesterol content in plasma and heart tissue was estimated by the method of Parekh & Jung (1970) after extracting total lipids by the method of Folch *et al.* (1957). Lipid peroxide content in plasma and heart tissue was determined by the thiobarbituric acid (TBA) reaction as described by Okhawa *et al.* (1979). Endogenous antioxidants such as ascorbic acid (vitamin C) and α-tochopherol (vitamin E) were determined in the heart tissue by the methods of Roe & Kuether (1943) and Baker *et al.* (1980) respectively.

Results were expressed as mean ± SD. Multiple comparisons of the significant ANOVA were performed by Tukey's multiple comparison test. A p-value <0.05 was considered as statistically significant. All data were analyzed with the aid of statistical package program SPSS 10.0 for Windows.

Results & Discussion

Troponins are regulatory proteins essential for contraction and relaxation processes in myocardium. In the present study, a significant ($p<0.001$) increase was observed in the level of troponin T in plasma of Group III isoprenaline administered rats as compared to that of Group I control animals (Fig 1). This is in accordance with earlier reported studies (Acikel *et al.* 2004). Since cardiac troponins are not normally found in blood, an increased level of troponin T in serum indicates myocyte injury and necrosis.

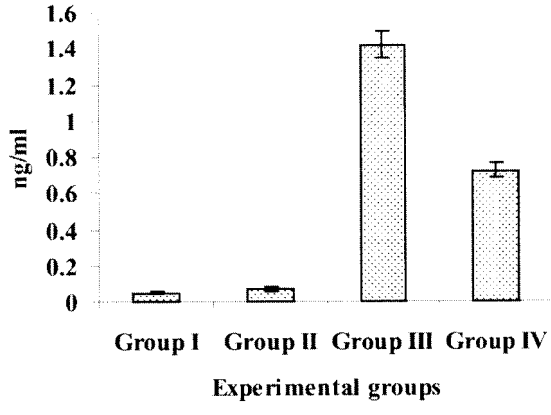

Fig 1. Level of Troponin T in plasma of normal and experimental groups of rats

Myocyte injury results in damage to contractile proteins and is a key mechanism responsible for the release of the structurally bound cardiac troponin T (Sarko & Pollack, 2002), and once outside the myocyte, these macromolecules are cleared from the interstitium by cardiac lymphatics. Eventually when the capacity of lymphatics to clear the macromolecules is exceeded, the markers become detectable in the peripheral circulation and are released with a stoichiometric relationship proportional to the amount of myocardial injury (Goldman *et al.* 2001). Recent years have witnessed an increased use of myocardial troponins as markers of myocardial injury. O" Brien *et al.* (1997) have shown that troponin T is a powerful biomarker in laboratory animals for sensitive and specific detection of cardiac injury.

Prior administration of squalene significantly ($p<0.001$) reduced the isoprenaline-induced release of troponin T from myocardium into the blood stream, thereby demonstrating its protective action on the cell membrane. It probably did so by maintaining the delicate balance of tonicity in cells in the myocardium. The presence of squalene in cell membrane is playing a major role in cell volume regulation by modulating the elasticity of plasma membrane. Cell volume affects the most basic processes of cell function, and as such it exerts an important role in the onset, severity, and outcome of myocardial infarction. Earlier reported studies indicate that squalene can overt severe osmolar changes associated with possible cell death (Ivashkevich *et al.* 1981).

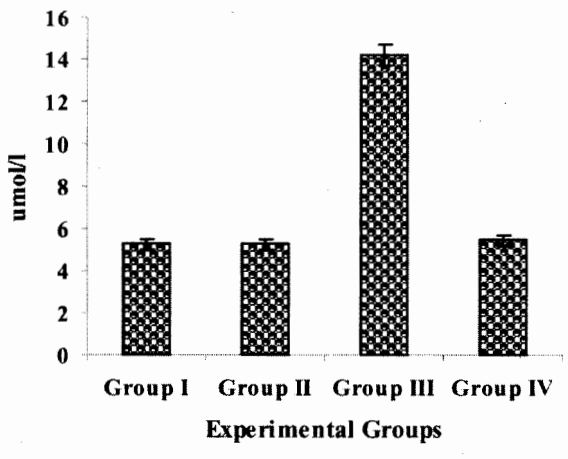

Fig 2. Level of homocysteine in plasma of normal and experimental groups of rats

In the present study, a significant ($p<0.001$) elevation in the level of homocysteine was noted in the plasma of Group III rats as compared to that of Group I control animals (Fig 2). This is in accordance with an earlier reported study (Hagar, 2002). Homocysteine is a thiol containing potentially cytotoxic 4-carbon α-amino acid formed during methionine metabolism. Recent prospective studies show that even mild hyperhomocysteinemia is associated with an increased risk of cardiovascular diseases independently of classical risk factors (Senaratne *et al.* 2000). Though the exact pathophysiological mechanism of homocysteine is still unclear, several experimental evidences are being reported in support of the involvement of homocysteine in the induction of myocardial infarction.

Homocysteine has been reported to induce atherosclerosis either by impairing coronary microvascular dilator function (Tawakol *et al.* 2002), or by stimulating smooth muscle proliferation (Tang *et al.* 1998), platelet activation, thrombogenesis (Rodgers and Kane, 1986), and endothelial dysfunction (Tsai *et al.* 1994). Both *in vivo* and *in vitro* studies suggest that homocysteine is a potent inducer of inflammatory processes in endothelial cells at the level of gene expression (Roth *et al.* 2001; Shai *et al.* 2004). Elevated level of homocysteine has also been reported to be associated with increased interleukin production in monocytes (Van Aken *et al.* 2000) and up regulation of vascular cell adhesion molecules (Silverman *et al.* 2002).

In this study, it is observed that the prior administration of squalene significantly ($p<0.001$) reduced the level of homocysteine in plasma of Group IV rats as compared to that of Group III myocardial infarction induced rats (Fig 2). It probably did so by inhibiting the production of monocyte/macrophage-derived interleukins, which triggers firm adhesion of rolling monocytes to vascular endothelium, a necessary prelude to the initiation of atherosclerosis (Gerszten *et al.* 1999). The HMG-CoA reductase inhibitors like lipophilic cerivastatin and fluvastatin have been reported to reduce the cardiovascular risk and vulnerability of atherosclerotic plaque through non-lipid mechanisms such as inhibition of interleukin expression (Ito *et al.* 2002). Since squalene is more lipophilic than statins, it is more permeable to vascular smooth muscle cells. Hence, it is possible that similar to prime HMG-CoA reductase inhibitor, squalene may also inhibit both homocysteine and interleukin production.

The present study reveals that animals treated with isoprenaline showed a significant ($p<0.001$) increase in the levels of total cholesterol in plasma and heart tissue as compared to that of normal control animals (Fig. 3 & 4), indicating the isoprenaline-induced hypercholesterolemic condition. Hypercholesterolemia has long been recognized as one of the major reversible risk factors for coronary heart disease. High level of circulating cholesterol and its accumulation in the heart tissue are well associated with cardiovascular damage (Joan *et al.* 1984; Buring *et al.* 1992). Our finding is in agreement with an earlier reported study (Ithayarasi & Devi, 1997b), which indicates that high levels of cholesterol in serum and heart of isoprenaline-treated rats is mainly responsible for altered cardiovascular functions. Also the levels of apolipoprotein B and lipoprotein (a) was found to be increased drastically with a concomitant decline in the apolipoprotein A1 in Group III isoprenaline-administered rats compared to group I control rats (Fig. 5 & 6). According to earlier reports apolipoprotein B and lipoprotein (a) were thought to be better predictors of acute myocardial infarction than total cholesterol and LDL-cholesterol (Sharrett *et al.* 2001; Graziani, *et al.* 1998).

Apolipoprotein A1 is primarily found in high-density lipoprotein (HDL) particle and serves the function of preventing the

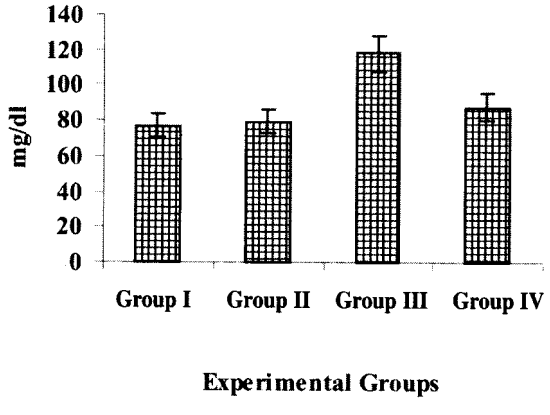

Fig 3. Level of cholesterol in plasma of control and experimental groups of rats

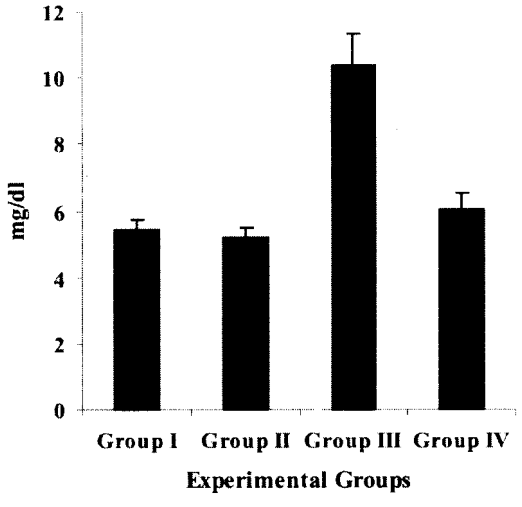

Fig 4. Level of cholesterol in heart tissue of control and experimental groups of rats

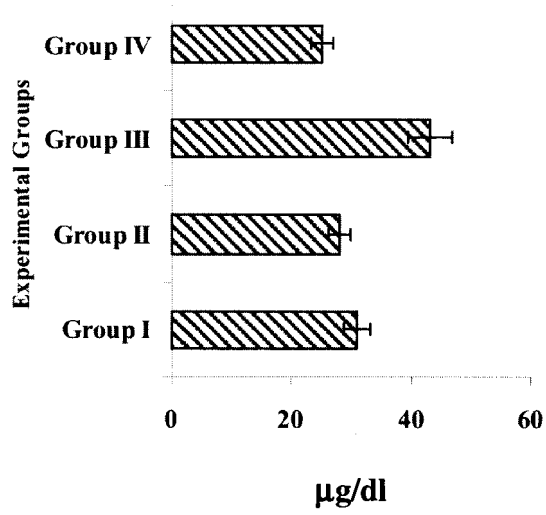

Fig 5. Level of lipoprotein (a) in plasma of control and experimental groups of rats

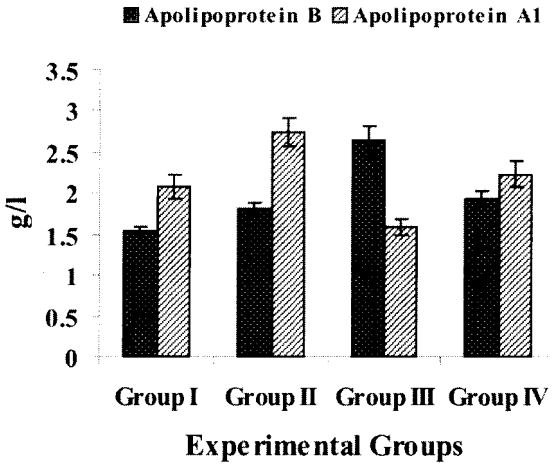

Fig 6. Level of apolipoprotein A1 and apolipoprotein B in plasma of control and experimental groups of rats

deposition of cholesterol-loaded macrophages on the arterial wall as foam cells, which is the prominent early feature of atherosclerotic lesion formation ultimately resulting in atherosclerosis. Over 90% of low density lipoprotein (LDL) particle is composed of apolipoprotein B. It can be used for assessing the cholesterol depositing capacity of the blood (Sehayek *et al.* 1994). Lipoprotein (a) is predominantly a genetic trait bound to both HDL and LDL and it is considered as a marker of atherosclerosisis (Simon *et al.* 1993) and has many properties in common with low-density lipoprotein (LDL). Isoprenaline mainly increases the low-density lipoproteins (LDL) cholesterol level in the blood, which in turn and leads to the build up of harmful deposits in the arteries, and thus favors induction of myocardial infarction (Goldstein and Brown, 1984). Increased cholesterol levels in the heart during myocardial infarction could be due to increased uptake of LDL from the blood by the tissues (Mathew *et al.* 1981). The abnormal cholesterol deposition is favored by the dangerous tendency of cholesterol to passive exchange between the plasma lipoproteins and the cell membranes (Brown and Goldstein, 1986).

In the present study, supplementation with squalene significantly ($p<0.001$) prevented the isoprenaline-induced elevation in the levels of total cholesterol, apolipoprotein B and lipoprotein (a) in Group IV rats as compared to that of Group III rats (Fig 5 & 6). Also the level of apolipoprotein A1 maintained at near normalcy. It probably did so by its hypocholesterolemic property.

Earlier Qureshi *et al.* (1996) have shown that squalene intake is effective in lowering the plasma total cholesterol, VLDL cholesterol and LDL cholesterol levels in experimental animals. In the present study also a slight reduction in the level of total cholesterol was observed in Group II squalene fed normal animals, establishing the anticholesterolemic property of squalene. The increase noticed in the apolipoprotein A1 also exhibits the beneficial action of squalene in preventing the cardiovascular complications. Dietary squalene was shown to inhibit the activity of 3-hydroxy methylglutaryl coenzyme A (HMG CoA) reductase, an endoplasmic reticulum (ER) protein, which catalyzes the rate-determining reductive deacylation of HMG CoA to mevlonate in cholesterol biosynthesis (Standberg *et al.* 1989). The

hypocholesterolemic effect of squalene observed in the present study is probably related to its ability to modulate cholesterol metabolism either by regulating HMG CoA reductase activity through a feed back inhibition mechanism or by increasing cholesterol esterification process in the liver, as reported earlier by Khor & Chieng (1997). Further more oral supplementation of squalene has been previously reported to upregulate the fecal excretion of cholesterol and its non-polar derivatives as bile acid conjugates in experimental animals (Standberg *et al.* 1990; Nakamura *et al.* 1997).

Lipid peroxidation *in vivo* has been identified as one of the basic deteriorative reaction in cellular mechanisms of the myocardial ischemia (Singal *et al.* 1983). Injection of isoprenaline induced a significant ($p<0.001$) increase in the level of lipid peroxidation (Fig. 7) in the heart tissue of Group III isoprenaline-administered rats as compared to Group I rats. This result agrees with the findings of Nirmala & Puvanakrishnan, (1996b), which indicates that lipid peroxidation is also a key factor involved in the pathogenesis of isoprenaline-induced myocardial infarction. The enhanced lipid peroxidation in heart tissue of isoprenaline-administered rats might have been due to the reduction in tissue antioxidant defense status, as reported earlier (Sushmakumari *et al.* 1989; Padma & Devi, 2002).

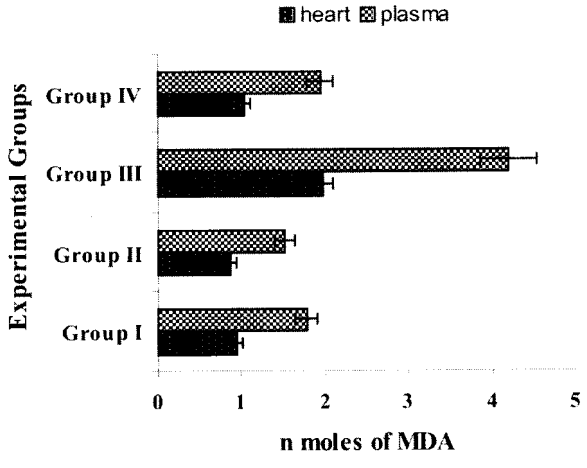

Fig 7. Levels of lipid peroxides in heart tissue and plasma of control and experimental groups of rats

Lipid peroxidation of membranes is regulated by the availability of substrate in the form of PUFA, the availability of inducers such as free radicals and excited state molecules to initiate propagation, the antioxidant defense status of environment and the physical status of the membrane lipids (Anandan *et al.* 1998). Isoprenaline has also been proposed to be acting as a cardiotoxic agent due to its ability to destruct myocardial cells, possibly by a free radical mechanism (Singal *et al.* 1982). The reaction of isoprenaline-induced hydroxyl radicals (OH) with polyunsaturated lipids present in the myocardial cell membranes ultimately leads to the formation of lipid radicals and eventually to short-chain aldehydes and hydroxy alkenals. These events can be followed by the formation of conjugated dienes, malondialdehyde, and alkanes.

In the present study, the animals supplemented with squalene showed a significant ($p<0.001$) decrease in the level of lipid peroxidation in the plasma and heart tissue, thus indicating the antioxidant nature of squalene in experimentally induced oxidative stress condition. The antioxidant effect is probably due to the presence of isoprenoid unit in the structure of squalene. The unpaired electron present in the hydroxyl radical ($OH^{\cdot}$) generated during isoprenaline-induced myocardial infarction might have been trapped for dismutation by its free radical scavenging isoprenoid unit.

A significant ($p<0.001$) decline was observed in the content of non-enzymic antioxidants, ascorbic acid and α-tocopherol,

in the heart tissue of Group III isoprenaline-administered rats as compared to Group I control rats (Fig 8). This is in line with an earlier reported study (Senthil *et al.* 2004). Vitamin C is the primary antioxidant in plasma and cells and is the first antioxidant to be depleted under conditions of oxidative stress. It prevents oxidative modification of both the cytosolic and membrane components of the cells. Not only can the vitamin C directly quench superoxide radicals in aqueous milieu before they can attack lipids, but it can also reduce formation of tocopheroxyl radicals and so inhibits free radicals formation in the lipid phase (Frei *et al.* 1989). Vitamin E is a prominent membrane constituent of cardiac muscle, which halts lipid peroxidation by acting as a peroxyl radical trapping, chain-breaking antioxidant (Hensley *et al.* 2004). It is the only endogenous lipid soluble vitamin stabilizing the lipid bilayer of cell membranes, where it interacts with phospholipases to reduce membrane rearrangements (Schafer *et al.* 2002). It plays a major role in maintaining the structural integrity of cell membranes by limiting lipid peroxidation by reative oxygen species (Azzi *et al.* 2003). As long as the concentrations of redox cycling antioxidants, ascorbate and glutathione are maintained in the myocardium, distal antioxidant system would not be consumed. The reduction noted in the levels of these antioxidant vitamins in isoprenaline-induced myocardial infarction condition might be due to increased utilization of these vitamins for the removal of free radicals produced during isoprenaline induced oxidative stress. The generation of free radicals in isoprenaline-induced myocardial infarction might have exceeded the ability of these free radical scavenging non-enzymic antioxidants to dismute the radicals, resulting in membrane damage and reduction of scavengers.

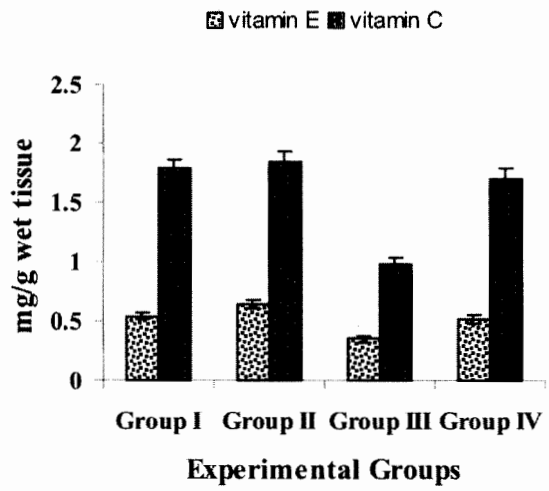

Fig 8. Levels of ascorbate and α-tocopherol in heart tissue of control and experimental groups of rats

It is worth noting that, the prior administration of squalene at 2% level along with feed significantly ($p<0.001$) reduced the isoprenaline-induced decline in the levels of these vitamins in Group IV animals as compared to those of Group III isoprenaline-injected rats. It probably did so by sharing the responsibility of these antioxidant vitamins in counteraction of free radicals generated during isoprenaline-induced oxidative stress.

$$LOO^{\cdot} + SQ \longrightarrow LOOH + SQ^{\cdot}$$

Where $LOO^{\cdot}$ denotes a lipid peroxy radical, SQ denotes squalene.

SQ consists of six 2-methyl-2-pentene units and the electron donating property of the methyl group at the 2-position is likely to play an important role in the quenching activity. In fact, the replacement of hydrogen by methyl group in ethylene results in the reduction of the ionization potential. In SQ, the electron donating property of methyl groups bonded to quaternary carbons will be essential to the small ionization potential. Further more, the methyl groups also supply hydrogen for the ene reaction (Gilbert &

Baggott, 1991), which may facilitate the quenching of singlet oxygen. Molecules containing a structure such as C-C (CH3) =CH-C are expected to have large quenching activity.

In conclusion, the results of the present study indicate that the cardioprotective effect of squalene against isoprenaline-induced myocardial infarction may probably be related to its hypercholesterolemic property, to the counteraction of free radicals by its antioxidant nature, to the strengthening of myocardial membrane by its membrane stabilizing action, or to its ability to maintain near to the normal level of endogenous antioxidants like vitamin E, and vitamin C, which protects myocardial membrane against oxidative damage by decreasing lipid peroxidation.

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