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Protective Effect of Squalene on Endogenous Antioxidant Vitamins in Experimentally Induced Myocardial Infarction in Rats

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Abstract: In the present study an attempt has been made to assess the cardioprotective effect of squalene on isoprenaline-induced myocardial infarction in male albino rats with respect to changes in the levels of endogenous antioxidant vitamins in heart tissue. Levels of endogenous antioxidants such as ascorbic acid, α-tocopherol and endogenous squalene content in heart tissue were determined. Significant ($p<0.001$) reduction was observed in the levels of ascorbic acid, α-tocopherol and endogenous squalene content in the heart tissue of isoprenaline administered rats compared to normal control rats. It is worth noting that, the prior administration of squalene at 2% level along with feed for 45 days significantly ($p<0.001$) reduced the isoprenaline-induced decline in the levels of these vitamins and restored the membrane bound squalene content at near normal. The results of the present study indicates that the cardioprotective effect of squalene might be ascribable to its antioxidant property thereby sharing the responsibility of these antioxidant vitamins in counteraction of free radicals generated during isoprenaline-induced oxidative stress.

Key words: Squalene, α-tocopherol, ascorbic acid, myocardial infarction

INTRODUCTION

Myocardial infarction is the death of part of the heart muscle due to sudden loss of blood supply, which is typically caused by complete blockage of coronary arteries by blood clot (Qureshi *et al*., 2002). Myocardial injury is irreversible in nature and most of the drugs available are effective only for the prevention of spreading or dispersal of necrotic damage to the adjacent cells. Hence, it is important to find drugs capable of protecting myocardial cells from necrotic damage by strengthening the cardiac cell membrane. Since the major abnormalities noticed in myocardial infarction are lipidemia, peroxidation and loss of plasma membrane integrity (Rodriguez *et al*., 2005), the drug should possess antilipidemic, antiperoxidative and membrane stabilizing properties. Also, it should be devoid of any adverse side effects.

Squalene, an isoprenoid molecule present in shark liver oil in higher quantities, has been reported to possess antilipidemic, antioxidant and membrane stabilizing properties (Qureshi *et al*., 1996; Ko *et al*., 2002; Ivashkevich *et al*., 1981). A phase I trial in adult males given 869 mg of squalene daily for 20 weeks to study the cholesterol lowering effect of squalene showed that oral squalene is safe and tolerable (Chan *et al*., 1996). The preventive

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effect of squalene on mineral status (Farvin *et al*., 2005), lipid metabolism (Farvin *et al*., 2006), proteins and glycoprotein components in experimentally induced myocardial infarction in rats (Farvin *et al*., 2007), has already been explored. In the present study, an attempt has been made to assess the protective effect of squalene on endogenous non-enzymatic antioxidants in isoprenaline 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

Isoprenaline (isoproterenol; L-(3,4-dihydroxyphenyl)-isopropylaminoethanol hydrochloride), Ascorbic acid, α-tocopherol and squalene standards were purchased from M/s. Sigma Chemical Company, St. Louis. MO, USA in 2003 to 2006. Squalene (Specific gravity: 0.853; Refractive index: 1.493; Saponification Value: 30; Iodine value: 344; Boiling point: 240-245°C and 95% purity) 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.

Animals

Male Wistar strain 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 free access to food (M/s Sai Feeds, Bangalore, India) and water. 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 with added coconut oil at 2% level for 45 days and group II and IV animals were fed on commercial feed with added squalene at 2% level for a period of 45 days. After 45 days feeding, the group III and IV animals were intraperitoneally (i.p.) injected with isoprenaline (11 mg (dissolved in physiological saline) 100 g^{-1} b.wt. day^{-1} for 2 days) for the induction of myocardial infarction. Control animals (Group 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 excised immediately and washed with chilled physiological saline. The heart tissue homogenates prepared in ice cold 0.1 M Tris-HCl buffer, pH 7.2 was used for the biochemical analysis.

Determination of Ascorbic Acid (Vitamin C)

Ascorbic acid (Vitamin C) in the heart tissue was determined by the method of Roe and Kuether (1942). In brief : 1.0 mL of heart homogenate was taken into test tubes containing 1.0 mL 10% ice cold trichloro acetic acid (TCA) and centrifuged at 3000 rpm for 20 min. The 0.5 mL of supernatant was taken into another test tube, to this 2.0 mL 5% TCA and 0.1 mL DTC reagent (0.4 g thio urea, 0.05 g $CuSO_4$, 3 g 2,4 dinitrophenyl hydrazine (DNPH) dissolved in 100 mL of 9 N H_2SO_4) were added and incubated for 3 h at 37°C. Cooled to room temperature and 0.75 mL ice cold 65% H_2SO_4 was added. After 30 min absorbance was read

at 520 nm by using Shimadzu- UV spectrometer. A blank was carried out using 1.0 mL of distilled water. The standards of different concentrations were also treated similarly.

Vitamin E ("-Tochopherol)

α-Tochopherol (Vitamin E) was determined in the heart tissue by the method of Baker *et al.* (1980). In brief: 200 mg heart sample was homogenized with 2.0 mL of ethyl alcohol and centrifuged at 3000 rpm for 10 min. One milliliter of the supernatant was taken into test tubes containing 1 mL of 2% epinephrine and incubate at 70°C for 2 min. To this 0.3 mL of saturated KOH was added and kept in a water bath at 70°C for 2 min. After cooling in ice bath, 1 mL distilled water and 4.0 mL hexane was added and centrifuged at 2800 rpm for 10 min. Three milliliter of the upper hexane layer was taken into a test tube and evaporated to dry. To the residues and standards (0.1-0.5 mL), 3.0 mL ethyl alcohol, 0.2 mL bathophenanthroline, 0.2 mL $FeCl_3$ and 0.2 mL H_3PO_4 was added. Mixed well and absorbance was read at 550 nm by using Shimadzu-UV spectrometer.

Determination Endogenous Squalene Content

Endogenous squalene content of the heart tissue was estimated by a modified method of Liu *et al.* (1976) using GC-MS (Thermo trace GC ultra) equipped with Elite 220 capillary column (30 m×0.54 mm dia) and a flame ionization detector. The carrier gas used was nitrogen with a flow rate of 0.8 mL min^{-1}. Initial temperature was set at 220°C and was increased 3°C min^{-1} until the temperature of 260°C was reached. Injector and detector temperature was kept at 260 and 275°C, respectively. Squalene separated was identified by comparison of retention time with those obtained by standard. Measurement of peak areas and data processing were carried out by Thermo chrom cord software.

Statistical Analysis

Mean values of data obtained were calculated and the results were expressed as Mean±SD of 6 animals. One way Analysis of Variance (ANOVA) was carried out and the statistical comparisons among the groups were performed with *Bonferroni* multiple comparison test by using a statistical package program Graphpad prism 4 (Graphpad Software Inc., San Diego, USA). A p-value <0.05 was considered as statistically significant.

RESULTS AND DISCUSSION

In the present study, a significant ($p<0.001$) decline was observed in the content of non-enzymic antioxidants such as ascorbic acid and α-tocopherol in the heart tissue of Group III isoprenaline-administered rats as compared to Group I control rats (Fig. 1a, b). Which concurs with an earlier reported study (Pinelli *et al.*, 2004). Severe oxidative challenge results in the reduction in the levels of hydrophilic antioxidants like vitamin C, which in turn leads to significant depletion in lipophilic antioxidants like ubiquinol and vitamin E (Molyneux *et al.*, 2002), as observed in the present study. A significant ($p<0.001$) reduction was also observed in the levels of endogeneous squalene content in the heart tissue of Group III isoprenaline-administered rats as compared to Group I rats (Fig. 2). The decline noted in the levels of these antioxidant vitamins and endogenous squalene content in the heart tissue indicates the severity of oxidative stress in isoprenaline-induced myocardial infarction condition. The generation of free radicals in isoprenaline-induced myocardial infarction is probably exceeded the free radical scavenging capability of these non-enzymic antioxidants, resulting in deterioration of membrane integrity.

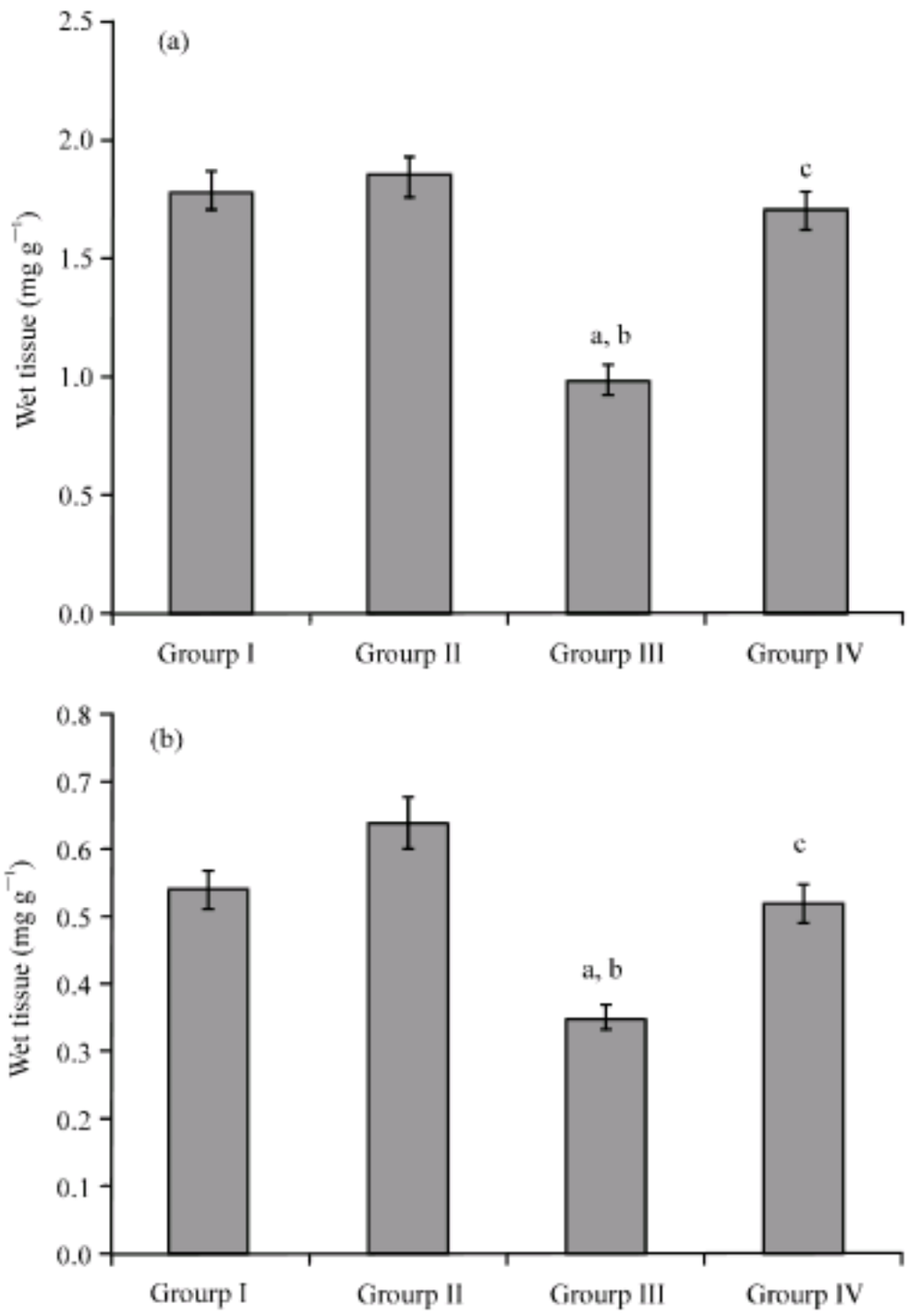

Fig. 1: Levels of (a) vitamin C, (b) vitamin E content in heart tissue of control and experimental groups of rats. Group I and Group II, normal control, rats received standard diet mixed with 2% coconut oil and 2% squalene, respectively, for a period of 45 days; Group III and Group IV, myocardial infarctions were induced by intraperitoneal (i.p) injection of isoprenaline (11 mg (dissolved in physiological saline) 100 g^{-1} b.wt. day^{-1} 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. [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

Vitamin E has a strong antioxidant capacity and has been used in several ischemia-reperfusion studies (Singh *et al*., 1996; Kushi, 1999), which have demonstrated that vitamin E attenuates membrane related morphological and biochemical alterations resulting from myocardial infarction. Not only can the vitamin C directly quench superoxide radicals in aqueous milieu before they can attack lipids but it can also reduce tocopheroxyl radicals and so removes free radicals from the lipid phase (Frei *et al*., 1989). 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 decrease in vitamin E

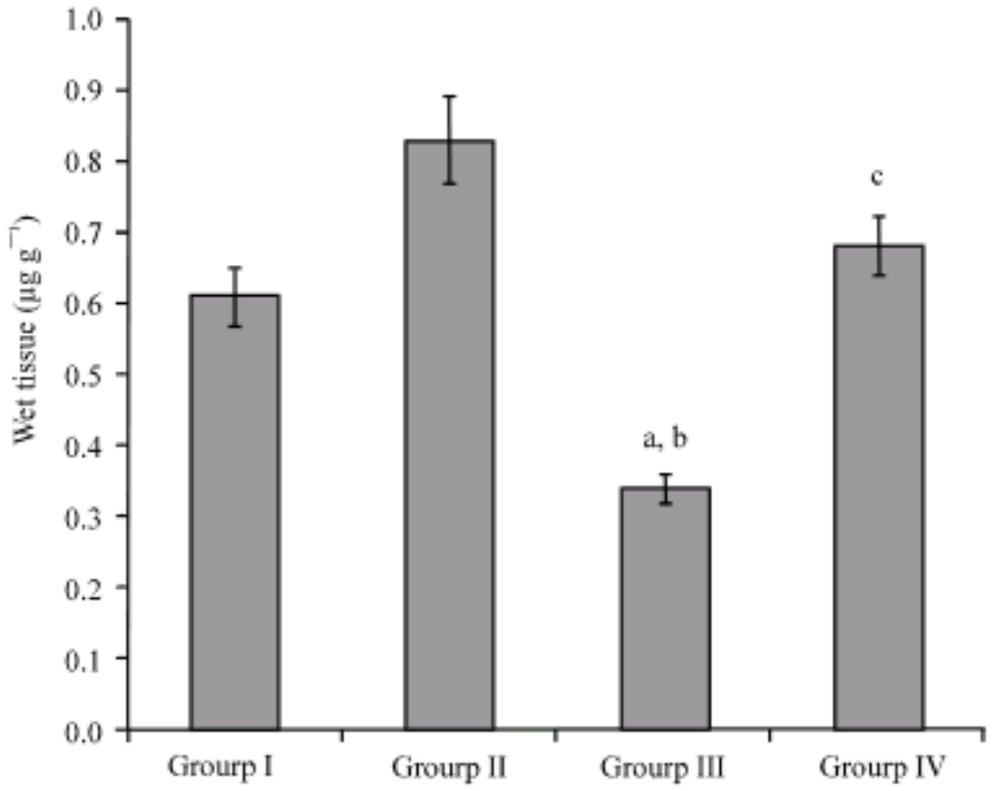

Fig. 2: Levels of Endogenous squalene content (µg g^{-1} wet tissue) in heart tissue of normal and experimental groups of rats. Description of the different groups are same as in Fig. 1. Results are Means±SD for 6 animals. [a]p<0.001 significantly different compared with control animals, [b]p<0.001 significantly different compared with squalene administered normal rats, [c]p<0.001 significantly different compared with isoprenaline-induced myocardial infracted rats

in our study could be due to the increased utilization in scavenging the oxyradicals generated or could be due to decreased vitamin C concentration because vitamin C can regenerate α-tocopherol by reducing α-tocopheryl radicals present on the surface of the membranes (Freiglben and Packer, 1993).

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 (Fig. 1a, b). It probably did so by sharing the responsibility of these antioxidant vitamins in counteraction of free radicals generated during isoprenaline-induced oxidative stress. Moreover, Administration of squalene restored the membrane bound squalene content in the heart tissue (Fig. 2). The unpaired electron present in the hydroxyl radical (OH) generated during isoprenaline-induced myocardial infarction might have been trapped for dismutation by its free radical scavenging isoprenoid unit. Miyachi *et al.* (1983) reported that squalene functions as an efficient quencher of singlet oxygen and prevents the corresponding lipid peroxidation in human skin. Of all the other organs and tissues, heart tissue was reported to be richest in squalene, after skin and adipose tissues (Liu *et al.*, 1976). Squalene occurs in the midplane of the lipid bilayer and stabilizes the layers of cellular and subcellular membranes through the formation of complexes with the fatty acids in the phospholipid bilayer membranes (Hauβ *et al.*, 2002). The presence of squalene in the membrane phospholipid bilayer might have rendered the heart muscle more stable against isoprenaline-induced oxidative injury. The antioxidative property of squalene has been already well established (Ko *et al.*, 2002). Squalene 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. Further more, the methyl groups also supply hydrogen for the ene reaction (Gilbert and Baggott, 1991), which may facilitate the quenching of singlet oxygen.

In conclusion, the results of the present study reveal that, squalene is very effective in mitigating the deleterious effect of isoprenaline-induced aberration in endogenous antioxidant vitamins in experimental rats. The overall cardio protective effect of squalene is probably related to the counteraction of free radicals by its antioxidant nature or by its membrane stabilizing action.

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