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Protective Effect of Squalene on Certain Lysosomal Hydrolases and Free Amino Acids in Isoprenaline-Induced Myocardial Infarction in Rats

[1,3]K.H.S. Farvin, [2,3]A. Surendraraj and [3]R. Anandan
[1]Section of Aquatic Lipids and Oxidation, National Institute of Aquatic Resources (DTU-AQUA), Technical University of Denmark, B. 221, Søltofts Plads, DTU, DK-2800 Kgs., Lyngby, Denmark
[2]Institute of Food and Dairy Technology, TANUVAS, Alamathi P.O., Red Hills (via), Chennai-600 052, India
[3]Biochemistry and Nutrition Division, Central Institute of Fisheries Technology, Matsyapuri (PO), Cochin-682029, India

Abstract: This study was aimed to evaluate the preventive role of squalene on free amino acids and lysosomal alterations in experimentally induced myocardial infarction in rats. The levels of lysosomal enzymes (β-glucuronidase, β-galactosidase, β-glucosidase, acid phosphatase and cathepsin D) in plasma and lysosomal fractions, hydroxyproline content and free amino acids in heart tissue were determined. Isoprenaline administration to rats resulted in decreased stability of the membranes which was reflected by significantly ($p<0.001$) lowered activity of β-glucuronidase, β-galactosidase, β-glucosidase, acid phosphatase and cathepsin D in the lysosomal fraction with a concomitant increase in plasma. Significant ($p<0.001$) reduction was also observed in the levels of taurine and hydroxyproline with a concomitant increase in the levels of aspartate, glutamate and arginine in the heart tissue of isoprenaline administered rats compared to normal control rats. Pretreatment with squalene at 2% level along with feed for 45 days significantly ($p<0.001$) prevented these alterations in free amino acids, hydroxyproline and lysosomal hydrolases and maintained the rats at near normal. Thus, the results of this present study show that squalene protects the lysosomal membrane against isoprenaline induced oxidative stress. The overall cardioprotective effect of squalene is ascribable to its ability to strengthen myocardial membrane including lysosomal membrane by its membrane stabilizing action and consequent diminution in the liberation of hydrolytic enzymes or by counteraction of free radicals by its antioxidant nature.

Key words: Lysosomal hydrolases, taurine, hydroxyproline, aspartate, glutamate, arginine

INTRODUCTION

Cardiovascular diseases form a major health concern in recent years, causing severe illness and death throughout the world. In the developing world, demographic and lifestyle changes are resulting in an epidemiological transition from perinatal and infectious diseases to non-communicable diseases such as myocardial infarction. It is estimated that by the year 2020, up to three quarters of deaths in developing countries will result from non-communicable diseases and that myocardial infarction will top the list of killers (Gupta and Gupta, 1998). Over the past 40 years, the prevalence of myocardial infarction in urban India has increased by a factor of six to eight, to about 10% among persons 35-64 years of age (Reddy, 2002). Moreover, it is very much painful and of serious concern to realize that myocardial infarction in India occurs 10-15 years earlier as compared to that of West (Krishnaswami, 1998).

Considerable attention has been focused on lysosomal alterations that might accompany ischemic or hypoxyic myocellular damage, as these may exercise a pivotal role in the progressive modifications that lead from reversible myocardial ischemia to irreversible infarction (Okuda and Lefer, 1979; Wildenthal, 1978). A higher activity of lysosomal enzymes in inflammatory exudates serves as a good marker to assess the intensity of inflammation and severity of necrotic damage in condition like acute myocardial infarction. These hydrolytic enzymes are oozed out by the rupture of the lysosomal membrane, initiating the synthesis of inflammatory mediators such thromboxanes, prostaglandins and leukotrienes (Agha and Gad, 1995). Oxygen free radicals generated during ischemia in addition to the direct

Corresponding Author: Koduvayur Habeebullah Sabeena Farvin, Section of Aquatic Lipids and Oxidation, National Institute of Aquatic resources (DTU-AQUA), Technical University of Denmark, B. 221, Søltofts Plads, DTU, DK-2800 Kgs., Lyngby, Denmark Tel: + 45 45 25 25 42 Fax: +45 45 88 47 74

myocardial damaging effect may also be responsible for the cardiac damage through the release of lysosomal enzymes (Kalra *et al*., 1988). One approach to ameliorate the damage due to myocardial injury is to stabilize the membranes of ischemic myocytes, including lysosomal membrane and to protect the cells from autolytic and hydrolytic damage.

In recent years, accumulating evidence has indicated that the incidence and progression of cardiovascular disease may, to some extent, be modified by dietary means. Squalene is a remarkable bioactive substance present in deep sea shark liver oil in higher quantities (Hayashi and Takagi, 1981). It belongs to a class of antioxidants called isoprenoid, 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 (Qureshi *et al*., 1996; Ko *et al*., 2002; Ivashkevich *et al*., 1981). It has been found to be an efficient chemo preventive agent against variety of cancers, skin disorders and liver diseases (Xu *et al*., 2005). Its antiaging and detoxification have already been well studied (Passi *et al*., 2002; Richter and Schafer, 1982). Earlier, we have reported the protective effect of squalene on the tissue antioxidant defense system, mineral status, lipid and protein components in isoprenaline induced myocardial infarction in rats (Farvin *et al*., 2004-2007). But any *in vivo* study on the effect of squalene on lysosomal hydrolases, hydroxyproline and free amino acid profile has not been previously carried out. In view of the above facts, an attempt was made to assess the protective effects of squalene on isoprenaline-induced myocardial infarction in rats with respect to changes in the levels of lysosomal enzymes, hydroxyproline and free amino acids.

MATERIALS AND METHODS

Chemicals: Isoprenaline [isoproterenol; L-(3, 4-dihydroxyphenyl)-isopropylaminoethanol hydrochloride], tetraethoxy propane, p-nitro phenyl β-D-glucopyranoside, p-nitrophenol, p-nitro phenyl β-D-galactopyranoside, p-nitro phenyl β-D-glucuronide, haemoglobin and amino acid standards 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 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 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 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 [11mg (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, blood was collected using sodium citrate as anticoagulant and the plasma separated after centrifugation was used for biochemical analysis. 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.

Biochemical analysis

Assay of lysosomal enzymes: Lysosomal enzymes were determined both in plasma and lysosomal fractions. Lysosomal fraction was isolated by the method of Wattiaux (1977). The activities of the lysosomal enzymes β-D-glucuronidase by the method of Kawai and Anno (1971), β-D-galactosidase and β-D-glucosidase by the method of Conchie *et al.* (1967), acid phosphatase by the method of King (1965) and cathepsin-D by the method of Sapolsky *et al.* (1973) were determined.

Estimation of free amino acids and hydroxyproline content: The hydroxyproline content and free amino acids such as taurine, aspartate, glutamate and arginine in heart tissue were determined as per the procedure of Ishada *et al.* (1981). In brief: 300 mg of heart sample accurately weighed and extracted with 2.5 mL ethanol for three times. The homogenate was centrifuged at 3000 rpm for 10 min. Supernatant was transferred to another test tube and evaporated off the ethanol. Content made up to 1 mL with 0.05 M HCl. In the case of hydroxyproline,

200 mg heart tissue was digested overnight by using 10 mL of 6 N HCl in sealed test tubes. The digested sample was flash evaporated after adding water to remove HCl and the residue made up to 1 mL with 0.05 M HCl. Samples were filtered through a membrane filter of 0.45 μm and 20 μL of this is injected to an amino acid analyzer (HPLC- LC 10 AS) equipped with cation exchange column packed with a strongly acidic cation exchange resin, styrene di vinyl benzene copolymer with sulphonic group (ISC-07/S 1504 Na) having a length of 19 cm and diameter 5 mm.

The instrument was equipped with Shimadzu FL 6A fluorescence detector and Shimadzu CR 6A Chrompac recorder. The mobile phase consists of two buffers, Buffer A (trisodium citrate 16.35 g L^{-1}, absolute ethanol 70 m L^{-1}, perchloric acid 8.3 m L^{-1}, pH 3.2) and buffer B (trisodiumcitrate 58.8 g L^{-1}, boric acid 12.4 g L^{-1}, 4 N NaOH 22.5 m L^{-1}, pH 10). Gradient system was followed for the effective separation of amino acids. The oven temperature was maintained at 600°C. The total run was programmed for 62 min. The amino acid analysis was done with non-switching flow method and fluorescence detection after post-column derivatization with o-phthaldehyde. In the case of hydroxyproline, imino group is converted to amino group with hypochlorite. Amino acid standard was also run to calculate the concentration of amino acids in the sample. The amount of each amino acid is expressed as μmol amino acid/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 multiple comparison test. A p-value <0.05 was considered as statistically significant. All data were analyzed with the aid of statistical package program Graphpad prism 4 (Graphpad Software Inc., San Diego, USA).

RESULTS

Table 1 and 2 show the activities of lysosomal hydrolases (β-glucuronidase, β-galactosidase, β-glucosidase, acid phosphatase and cathepsin D) in plasma and heart tissue of control and experimental groups of rats respectively. There was a significant ($p<0.001$) increase noted in the levels of lysosomal hydrolases in plasma of Group III myocardial infarction induced rats as compared to that of Group I control rats, indicating the severity of isoprenaline-induced necrotic damage to the myocardium (Table 1). A parallel decline in the activities of these hydrolytic enzymes in lysosomal fraction of the heart tissue was also observed (Table 2). In the present study, supplementation of squalene along with feed resulted in significant ($p<0.001$) reduction in the isoprenaline-induced release of these hydrolytic enzymes from the lysosomes and maintained the rats in near normal.

Table 1: Activities of β-glucuronidase, β-galactosidase, β-glucosidase, cathepsin D and acid phosphatase in plasma of normal and experimental groups of rats

	Group			
Parameters	I	II	III	IV
β-glucuronidase	9.53±0.69	9.36±0.65	15.8±0.75^{a,b}	10.8±0.71^{c}
β-galactosidase	9.56±0.42	8.18±0.44	15.4±0.58^{a,b}	8.23±0.44^{c}
β-glucosidase	7.12±0.62	7.57±0.68	14.7±0.82^{a,b}	7.74±0.68^{c}
Cathepsin D	8.71±0.69	9.11±0.72	14.9±0.89^{a,b}	9.58±0.74^{c}
Acid phosphatase	0.12±0.009	0.09±0.004	0.89±0.032^{a,b}	0.27±0.013^{c}

Group I and 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 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 45days of feeding with standard diet mixed with 2% coconut oil and 2% squalene, respectively. Results are Mean±SD for 6 animals. Values expressed: β-glucuronidase, β-galactosidase and β-glucosidase, μmol of p-nitrophenol liberated/h/mg protein; Cathepsin D, μmol of tyrosine liberated/h/100 mg protein; Acid phosphatase, IU L^{-1}. $^{a}p<0.001$ 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 2: Activities of β-glucuronidase, β-galactosidase, β-glucosidase, cathepsin D and acid phosphatase in lysosomal fractions of normal and experimental groups of rats

	Group			
Parameters	I	II	III	IV
β-glucuronidase	31.99±2.20	30.76±2.10	18.4±0.92^{a,b}	27.57±1.8^{c}
β-galactosidase	23.20±1.74	23.40±1.73	16.3±1.28^{a,b}	21.4±1.42^{c}
β-glucosidase	21.10±1.42	21.30±1.41	13.1±0.98^{a,b}	19.4±1.14^{c}
Cathepsin D	64.10±5.42	65.30±5.63	46.6±3.54^{a,b}	60.3±5.21^{c}
Acid phosphatase	87.80±4.82	94.30±5.31	64.5±2.81^{a,b}	90.4±5.12^{c}

The descriptions of the groups are same as Table 1. Results are Mean±SD for 6 animals. Values expressed: β-glucuronidase, β-galactosidase and β-glucosidase, μmol of p-nitrophenol liberated/h/mg protein; Cathepsin D, μmol of tyrosine liberated/h/100 mg protein; Acid phosphatase, μmol of phenol released/h/100 mg of 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

Figure 1 and 2 show the levels of hydroxyproline and free amino acids in the heart tissue of control and experimental groups of rats. A significant ($p<0.001$) decrease was observed in the levels of myocardial taurine and hydroxyproline content in Group III myocardial infarction induced rats as compared to Group I normal rats (Fig. 1a, b). In contrast, the levels of aspartate, glutamate and arginine showed a significant ($p< 0.001$) increase in the heart tissue of Group III isoprenaline-administered rats as compared to Group I control rats (Fig 2a, b). The rats pretreated with squalene significantly ($p<0.001$) prevented

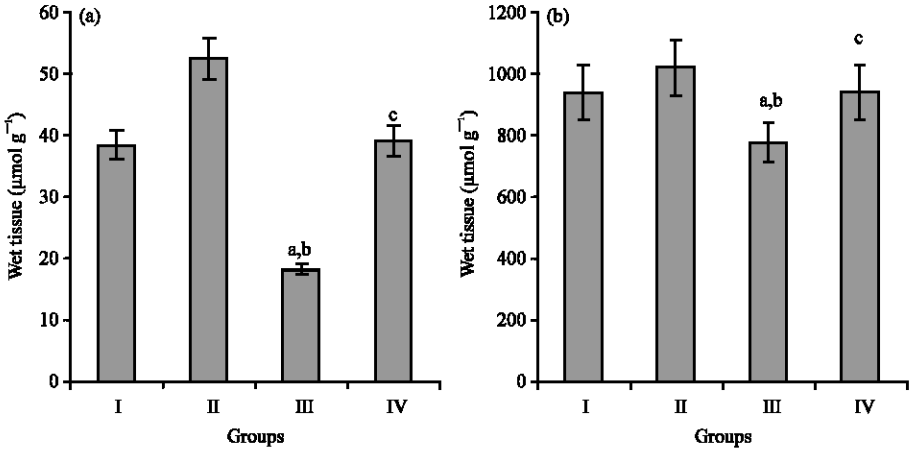

Fig. 1: Levels of (a) taurine, (b) hydroxyproline in heart tissue of control and experimental groups of rats. Group I and II, normal control, rats received standard diet mixed with 2% coconut oil and 2% squalene, respectively, for a period of 45 days; Group III and Group IV, myocardial infarctions were 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 Mean±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

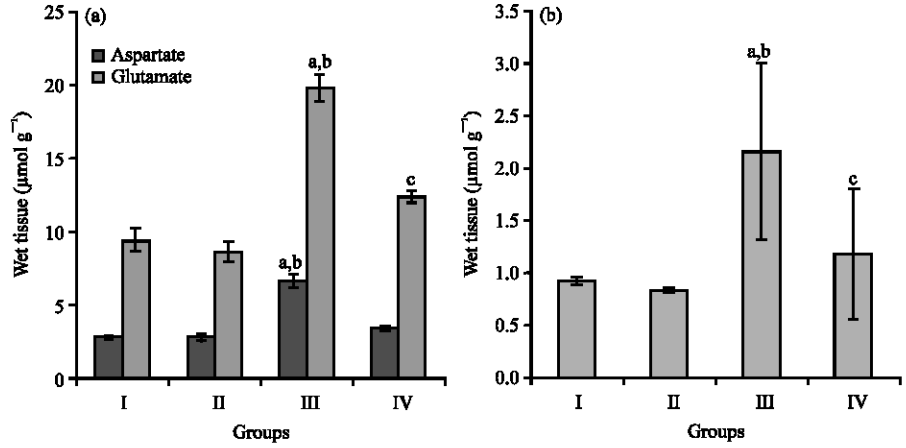

Fig. 2: Levels of (a) aspartate and glutamate, (b) arginine in heart tissue of control and experimental groups of rats. The descriptions of the groups are same as Fig. 1. Results are Mean±SD for 6 animals. [a]$p<0.001$ significantly different compared with control animals; [b]$p<0.001$ significantly different compared with squalene administered normal rats; [c]$p<0.001$ significantly different compared with isoprenaline-induced myocardial infracted rats

all these isoprenaline induced changes and maintained the levels of free amino acids in the myocardium near normal.

DISCUSSION

The present observation of reduction in the activity of lysosomal hydrolases in lysosomes with a concomitant increase in activity in plasma shows the severity of myocardial necrosis. The present observation concurs with an earlier report by Ganesan and Anandan (2009), that the cytosolic acid hydrolases liberated from lysosomes induce dysfunction and destruction of mitochondria, sarcolemma and other organelle. Measurement of these hydrolytic enzymes in systemic circulation is frequently used as an index of lysosomal

membrane integrity (Ravichandran *et al*., 1990). Prior oral administration of squalene significantly prevented these isoprenaline induced changes in lysosomal hydrolases and maintained the enzyme activities in near normalcy in group IV rats as compared with group III rats. This favorable protective effect might be related to its ability to exert stabilization on lysosomal membranes. Since the release of lysosomal enzymes is crucial in the pathogenesis of ischemic myocardial injury and related inflammation process, it is likely that the reduction of such enzyme release would prove beneficial, indirectly confirming the salubrious effect of the squalene. Earlier report by Upasani and Balaraman (2001) indicates that lipophilic antioxidant molecules are capable of stabilizing the fragile lysosomal membranes by reducing inflammation process and inhibiting lipid peroxidation. Squalene has been reported to exert membrane stabilization through forcing structural interactions with lipid membranes and the isoprenyl group of squalene plays a major role in this interaction process (Kohno *et al*., 1995). Earlier studies have shown that squalene is capable of inhibiting oxidative deterioration of phospholipids associated with cellular membranes and lipoproteins particles (Dessi *et al*., 2002). Reports by Haines (2001) indicated that squalene plays a major role in the maintenance of the balance between the hydrophilic and hydrophobic clusters present inside the cell membrane and also capable of suppressing the effect of hydrolyzed products, which affect the membrane stability.

The α-amino acids glutamate, aspartate and alamine, together with the β-amino acid taurine constitute a very significant proportion (67%) of the free amino acid pool in the rat heart (Chapman *et al*., 1993). Earlier studies (Warskulat *et al.,* 2004; Keith *et al.,* 2001) demonstrated that pathology develops in the myocardium if the animal is depleted of taurine stores either through a taurine deficient diet or use of taurine transport antagonists. The present findings confirm the same pattern and showed a significant ($p<0.001$) decrease in myocardial taurine content in group III myocardial infarction induced rats as compared to group I normal rats (Fig. 1a). The rats pretreated with squalene showed a significant ($p<0.001$) increase in cardiac taurine content as compared to Group III rats. Taurine is primarily utilized in biological system for the elimination of cholesterol from the body through the bile acid conjugation reactions. Earlier report by Tilvis and Miettinen (1983), indicate that supplementation of squalene increases the fecal excretion of cholesterol without the involvement of taurine-mediated bile acid conjugation reactions, which may be the possible reason for the increase noticed in the cardiac taurine content in squalene-supplemented rats. Hence, it is possible that the supplementation of squalene is capable of preserving the cellular taurine content for other biological processes such as cell membrane stabilization, antioxidation, detoxification and osmoregulation in the myocardium.

Collagen fibers of the myocardium is a network connecting myocytes, thus maintaining the architecture of the ventricles and transmitting contractile force from myocytes to the ventricular lumen. Significant decline observed in the hydroxyproline content in the heart tissue of group III isoprenaline induced rats compared to group I control rats (Fig. 1b) is an indication of enhanced degradation of collagen network, which plays a major role in maintaining the structural and functional integrity of the myocardium. This is in accordance with an earlier reported study (Nirmala and Puvanakrishnan, 1996), which have shown that degradation of collagen occurs in the ischemic myocardium as a result of increase in the activities of lysosomal enzymes, enhanced lysosomal fragility and infiltration of inflammatory cells. Prior administration of squalene at 2% level along with feed significantly ($p<0.001$) prevented the isoprenaline-induced reduction in hydroxyproline content in the heart tissue of Group IV animals as compared to that of Group III rats. It probably did so by strengthening the structural integrity of the lysosomal membrane, thereby reducing the release of proteolytic enzymes from lysosomes.

Aspartate and glutamate play an important metabolic role in ischemic myocardium. A significant increase was observed in the levels of aspartate and glutamate in the heart tissue of group III isoprenaline-administered rats as compared to group I control rats (Fig. 2a). This might be due to increased degradation of both structural and functional proteins in the infarcted myocardium. Cardiac swelling subsequent to ischemia (Steenbergen *et al*., 1985) may also contribute to increasing extra cellular concentrations of amino acids such as aspartate and glutamate by a process known as regulatory volume decrease which leads to activation of anion channels in the cell membrane leading to diffusional efflux (Song *et al*., 1996). Loss of the plasma membrane integrity as a result of enhanced phospholipase A2 activity and reversal of Na^+ dependant transporters by isoprenaline may also contributes to amino acid efflux (Backstrom *et al*., 2003). In the present study, the prior administration of squalene at 2% level along with feed significantly ($p<0.001$) prevented the isoprenaline-induced increase in aspartate and glutamate levels in heart tissue of group IV animals as compared to those group III isoprenaline-injected rats (Fig. 2a). It probably did so by strengthening heart tissue by its membrane stabilizing action which might have prevented the amino acid efflux. Its membrane stabilizing property is well established (Ivashkevich *et al*., 1981).

The results of the present study showed a significant elevation in the level of arginine in heart tissue of group III isoprenaline-administered rats as compared to that of group I normal rats (Fig. 2b). This is in accordance with an earlier study (Gustafsson and Brunton, 2000), which indicates that iNOS (inducible nitric oxide synthase) mediated nitrative stress plays a pivotal role in isoprenaline-induced myocardial apoptosis and cellular injury. Isoprenaline-mediated β-adrenergic receptor stimulation results in a phenotypic up regulation of iNOS in the heart and increase release of pro-inflammatory mediators, which in turn trigger increased NO production from arginine. Though NO is short-lived and a relatively unreactive radical, it can combine with superoxide to form the potent oxidant-ONOO-(peroxynitrite), which plays a significant role in iNOS-mediated postischemic cells damage (Arstall *et al*., 1999). Prior administration of squalene significantly maintained the cardiac arginine content at near normalcy as compared to that of group III rats (Fig. 2b). It probably did so by blocking the availability of excess superoxide radicals required for the formation of peroxynitrite radicals from arginine-derived NO through its superoxide scavenging capability. Earlier reports by Aioi *et al.* (1995) showed that squalene, an alleviator of skin irritation, suppressed the lauroylsarcosine-mediated production of superoxide radicals from cultured keratinocytes and peritoneal exudates leukocytes by a mechanism similar to that of vitamin E, which attenuates O^{2-} production by stimulated macrophages through interaction with the cell membrane.

In conclusion, the results of the present investigation reveals that squalene proved to be more effective in mitigating the deleterious effect of isoprenaline induced aberrations in free amino acids and lysosomal integrity in experimental rats. The overall cardioprotective effect of squalene is probably related to its ability to strengthen myocardial membrane including lysosomal membrane by its membrane stabilizing action and consequent diminution in the liberation of hydrolytic enzymes or by counteraction of free radicals by its antioxidant nature.

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