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Research Article

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Cytoprotective Effect of Dietary Squalene Supplementation on Experimentally Induced Cardiomyopathy in Rats

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ABSTRACT

Background: Adriamycin is a broadspectrum, potent, older chemotherapy drug and antineoplastic agent used in the treatment of several cancers such as solid tumours, leukaemias, and lymphomas, playing a major role in cancer chemotherapy. Long-term use of this drug results in congestive heart failure and to overcome this effect dietary squalene intake reduces the adverse effects of adriamycin-mediated cardiotoxicity and cellular oxidative stress.

Methods: The current study aims to investigate the cytoprotective effects of dietary squalene supplementation on adriamycin-induced cardiomyopathy in rats in terms of alterations in Troponin T, homocysteine, diagnostic marker enzymes, and cardiac tissue histology.

Results: The findings show that a 1.5 percent dose of dietary squalene supplementation for 21 days reduced adriamycin-induced changes in homocysteine, troponin T, diagnostic marker enzymes, and lesions in cardiac tissues.

Conclusion: The outcomes of the study specified squalene's cytoprotective action which stabilizes membranes against adriamycin-induced oxidative membrane degradation, which is primarily responsible for heart cell irreversible necrosis.

Key-words: Adriamycin, Cardiomyopathy, Diagnostic marker enzymes, Homocysteine, Histopathology, Squalene, Troponin T

INTRODUCTION

Adriamycin is a potent and broad-spectrum anticancer drug, used in numerous cancer treatments such as solid tumours, leukaemias, and lymphomas. It plays the foremost role in cancer chemotherapy and remains to be the first-line antineoplastic drug. Adverse effects particularly dose-dependent cardiomyopathies leading to potentially fatal congestive heart failure have led to the limited clinical use of this drug [1].

After repetitive intake of Adriamycin for several weeks or months, the chronic side effects develop, which include chronic cardiomyopathy cardiovascular dysfunctions, congestive heart failure that are unchangeable, and also have a gloomy prognosis [2].

A delay in the onset of adriamycin-induced cardiac dysfunction has been associated with cardiomyopathy that becomes apparent 4-20 years later the completion of chemotherapy in some patients [3]. The clinical features associated with chronic cardiomyopathy are a striking decrease in blood pressure under 70/50 mmHg, tachycardia, dilatation of the heart, and ventricular failure. Diagnostic enzyme markers like creatinine phosphokinase, lactate dehydrogenase, and transaminases have also been stated to increase markedly in these conditions. Distortion in cardiomyofibrils, cytoplasmic vacuolization and increased number of lysosomes, and swelling of mitochondria are some of the anomalies in ultrastructure connected with adriamycin-induced cardiomyopathy [4,5].

Numerous metabolic and morphological anomalies were observed within the cardiac tissue from laboratory

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animals after the injection administration of Adriamycin, which is comparable to those detected in human cardiomyopathy. The common symptoms presented include impaired adrenergic stimulation, increased free radical formation, concealed mitochondrial function, altered calcium homeostasis, infiltration of inflammatory cells, and accumulation of fat [6-8]. Despite these opposing effects, Adriamycin is widely used as an anticancer drug, and thus there will be higher chances of patients getting cardiotoxic dosages of Adriamycin or even dying of this drug-related toxic effect. Thus, there is a need to ascertain major risk factors, to foretell those patients capable of bearing further doses of Adriamycin, and to perhaps diminish cardiomyopathic aberrations becoming apparent.

In modern medicines, many important drug discoveries have been started from the bioactive substances available in nature. All over the world, this fact has been channeled to biological screening programs for bioactive molecules which led to research in this area. Traditional Indian medicine has many bioactive molecules of interest and very little exploitation in this regard has happened. To cure many cardiac disorders, the isoprenoid chemical, squalene, found in deep-sea shark liver oil, was applied. Some amounts of squalene (0.1-0.7%) are also available in the palm, olive, wheat-germ, and rice-bran oils. About 11% of the total surface fat of human skin lipids is this hydrocarbon, which is also found in hair fat dermoid cysts, cerumen, and sebum [9].

Squalene plays a foremost role in the upkeep of good health and holds antioxidant, antilipidemic, and membrane-stabilizing properties [10]. It partakes well in the synthesis of cholesterol, hormones, and vitamins and acts as a powerful endogenous antioxidant. To protect the skin from ultraviolet radiation, it is secreted in human sebum [11] and has been ascribed to possess cytoprotective [12] and anti-ageing properties [13]. Squalene has been found to inhibit the growth of cancer cells and neutralize carcinogens [14]. Squalene may be a good antidote, which can minimize the toxicity of unintentional drugs. Research revealed that squalene can act synergistically with marine polyunsaturated fatty acids to enhance myocardial dysfunction and a study has shown that the content of squalene, coenzyme Q10 (Co Q10), and vitamin E in skin surface lipids increases from childhood to adulthood and decreases again in old age, indicating that antioxidants can protect the body against exogenous oxidative damage and age-related diseases. The report shows that squalene is not toxic as a dietary supplement in food and capsules, and there is no problem after using squalene. In the current study, attempts have been made to examine the cytoprotective, antioxidant, hypolipidemic, and membrane stability properties of squalene in adriamycin-induced cardiomyopathy in rats.

MATERIALS AND METHODS

Place of Study- The study was conducted in July 2013, Department of Biochemistry, Research and Development, Bharathiar University, Coimbatore, Tamil Nadu, India

Drugs and chemicals- Squalene (Refractive Index: 1.493; Specific Gravity: 0.853; Iodine Number: 344; Boiling Point: 240–245°C; Saponification Value: 30) is a gift carefully prepared by Dr. T.K. Thankkappan, Chief Scientist, ICAR- Central Institute of Fisheries Technology, Cochin 682029, India. Get Adriamycin, lactate, and aspartate from Sigma Chemical Company, St. Louis, Missouri, United States. Other chemicals purchased are of analytical quality.

Animals- Male Wistar rats weighing 120-150 g are kept under standard environmental conditions. The animals receive standard pellet feed and free drinking water from M/s Sai Foods in Bangalore, India.

Experimental protocol- Four groups of six rats in each group were used for the study, and experiments were conducted by the rules of the Committee for Control and Supervision of Animal Experiments in New Delhi, India (CPCSEA). Group I and Group III animals were fed commercial feed supplemented with 1.5% coconut oil for 21 days. For Group II and Group IV, animals were fed commercial feed supplemented with a 1.5% level of squalene for 21 days. Intraperitoneal (IP) injection of Adriamycin [15 mg/kg (IP) in 6 equal injections within 2 weeks] was injected into group III and group IV animals to induce cardiomyopathy. Physiological saline was i.p. injected in control animals (Group I and Group II).

After the completion of the experiment, the experimental rats were killed and for collecting the blood and the separation of plasma, an anticoagulant was added to the blood. Heart tissue was removed and immediately washed with frozen isotonic saline.

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A part of the tissue was fixed in 10% buffered formalin for histopathological interpretation. Troponin T is measured using an electro-chemiluminescence immunoassay. A microtiter plate assay kit (Diazyme Laboratories) was used to determine the plasma homocysteine concentration (tHcy). Aspartate aminotransferase (ALT), alanine aminotransferase (AST), creatine phosphokinase (CPK), and lactate dehydrogenase (LDH) it is measured in plasma.

Statistical Analysis- Results are expressed as mean±SD. Significant ANOVA was used for multiple comparisons using Duncan's multiple comparison tests. The $p<0.05$ is considered statistically significant. All data is analyzed with the help of the statistical software package SPSS 10.0 for Windows.

RESULTS

Cytoprotective effect of squalene against adriamycin-induced cardiomyopathy in rats- The animals of groups I and III were fed with commercial feed supplemented with 1.5% coconut oil for 21 days, and the animals of groups II and IV were fed with commercial feed supplemented with 1.5% squalene for 21 days. Group III and group IV animals were injected intraperitoneally (i.p.) with Adriamycin [15 mg/kg (i.p.) In 6 equal injections over 2 weeks] to induce cardiomyopathy. Value expressed: troponin T-ng/ml; homocysteine µmol/l. The results are the Mean±SD of 6 animals; one-way analysis of variance; Duncan's multiple comparison tests. Values with different letters (a, b, c) are significantly different from each other ($p<0.05$) (Table 1).

Table 1: The level of troponin T and homocysteine in the plasma of the rat aberration control group and experimental group

Parameters	Group I	Group II	Group III	Group IV
Troponin T	0.05±0.01a	0.05±0.01a	1.85±0.09b	0.11±0.01c
Homocysteine	4.82±0.28a	4.96±0.33a	14.32±1.27b	5.48±0.46a

Diagnostic marker enzymes- Animals in groups I and III were fed commercial feed supplemented with 1.5% coconut oil for 21 days, and animals in groups II and IV were fed commercial feed supplemented with 1.5% squalene for 21 days. Group III and Group IV animals were injected intraperitoneally (i.p.) with doxorubicin [15 mg/kg (i.p.) in 6 equal injections over 2 weeks] to induce cardiomyopathy. The numerical value indicates the number of micromoles of ALT, AST, and LDH of pyruvate released/h/l; the creatine formed by CPK µmol/h/l. The results are the mean±SD of 6 animals; one-way analysis of variance; Duncan's multiple comparison tests. Values with different letters (a, b, c) are significantly different from each other ($p<0.05$) (Table 2).

Table 2: Plasma levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), creatine phosphokinase (CPK), and AST / ALT ratio in the groups of normal and experimental rats

Parameters	Group I	Group II	Group III	Group IV
ALT	$115.3±8.24^{a}$	$109.8±7.45^{a}$	$256.3±16.8^{b}$	$134.1±11.2^{c}$
AST	$132.2±9.12^{a}$	$126.2±8.93^{a}$	$294.6±18.5^{b}$	$161.7±12.4^{c}$
LDH	$155.6±11.4^{a}$	$143.1±10.2^{a}$	$318.7±21.2^{b}$	$179.2±15.6^{c}$
CPK	$137.3±10.5^{a}$	$127.9±9.74^{a}$	$278.3±17.6^{b}$	$164.2±10.9^{c}$
AST/ALT Ratio	$1.18±0.02^{a}$	$1.16±0.02^{a}$	$1.12±0.01^{b}$	$1.20±0.02^{c}$

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Histopathological Study- Histological annotations were made to the myocardial tissues of normal and experimental groups of animals to confirm the cytoprotective activity of squalene against adriamycin-induced cardiomyopathy. Fig. 1 shows disclosed regular myofibrillar architecture with striations, bifurcated appearance, and permanency with contiguous myofibrils by microscopic examination of heart tissue slices of Group I normal rats. Squalene received histological inspections on the heart tissue of normal rats of Group II in Fig. 2 did not display any significant variations when compared with control rats, showing that it does not per se have any adversative effects. Fig. 3 showed architectural abnormalities in cardiac tissue sections of Group III rats after administration of Adriamycin as compared to the normal Group I control rats such as mild to diffused cloudy swelling, focal vacuolar disintegration, and occasional pericentral infiltration of round cells. The light microscopical analysis of Group IV squalene supplemented rats on the heart tissue sections was shown in Fig. 4, which exhibited regular architecture of myofibrillar striations, branched appearance, and continuity with adjacent myofibrils as compared to the normal Group I control rats.

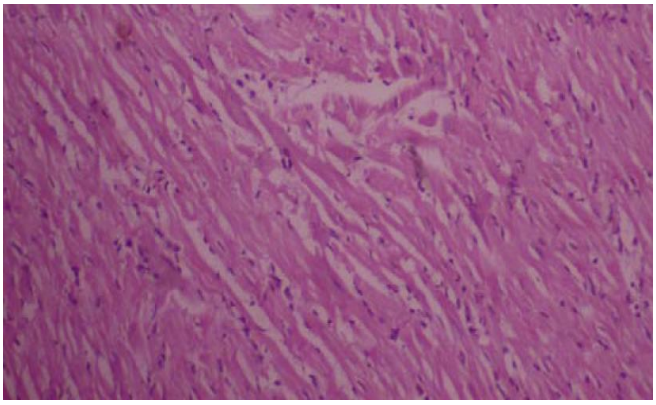

Fig. 1: The structure of normal heart tissue in group I rats

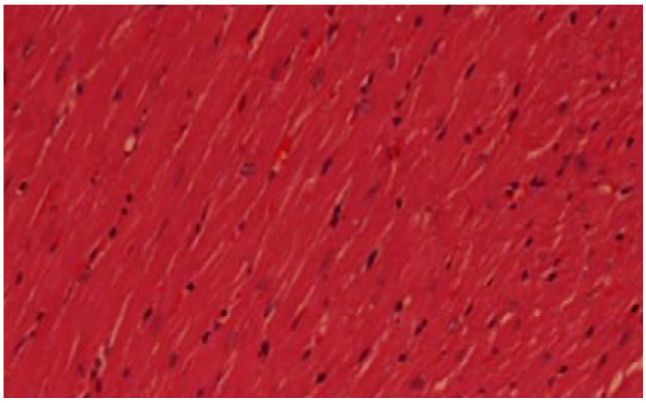

Fig. 2: The structure of the heart tissue of the squalene supplemented animals did not change significantly compared to normal conditions

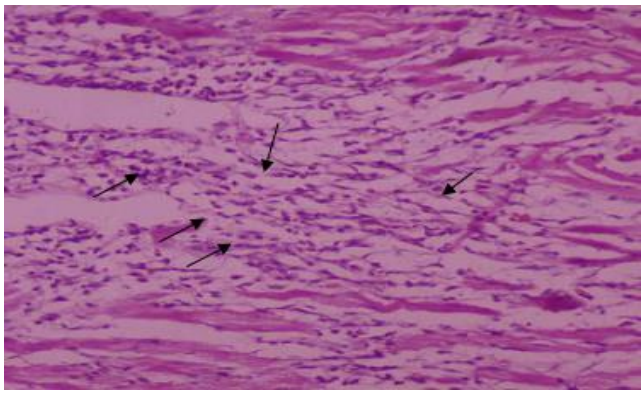

Fig. 3: The structure of the cardiac tissue of rats intoxicated by adriamycin in group III showed rupture of the fibers of the muscle cells and accumulation of inflammatory cells

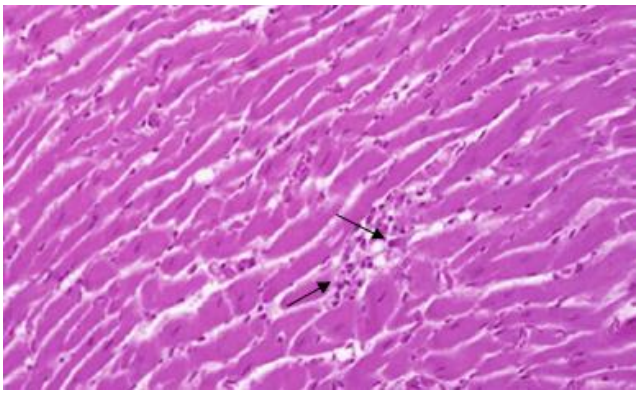

Fig. 4: The structure of the heart tissue of animals poisoned by squalene and adriamycin, showing a slight and precise breakdown of muscle fibers and the presence of less inflammatory cells.

DISCUSSION

In the myocardium, troponin T is a key regulator of the contraction and relaxation process observed with actin filaments. One of the main indicators of myocardial dysfunction in the systemic circulation is elevated troponin T levels. A study showed that troponin T is an effective biomarker for sensitive detection in experimental animals, and it has specific damage to the heart [15]. In this study, compared with the control animals in group I, the level of troponin T in the plasma of rats in group III given adriamycin was significantly increased ($p<0.05$). This is consistent with previously established studies, which showed that the detection of troponin in the systemic circulation is more sensitive and

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specific for assessing the severity of cardiomyopathy-induced adriamycin Markers [16]. Dietary supplementation of squalene significantly reduced ($p<0.05$) the release of adriamycin-induced troponin T from the heart tissue into the systemic circulation, indicating its protective effect on the myocardial membrane system. This may be done by maintaining a delicate balance of cardiomyocyte tension.

Squalene present in the cell and subcellular membranes has the function of regulating cell volume and regulating the elasticity of the plasma membrane. Since regulation of cell volume affects cell function, the presence of squalene in the membrane plays an important role in protecting the myocardium from necrotizing lesions. As in previous studies, isoprenoid squalene molecules can avoid the severe osmotic pressure changes associated with apoptosis. In the process of methionine metabolism, thiols and homocysteine containing cytotoxic 4-carbon alpha-amino acids are produced, which can impair the function of coronary microvascular dilators or promote smooth muscle proliferation [17], thrombosis, platelet activation [18] and endothelial abnormalities [19]. Homocysteine is a gene in endothelial cells [20]. A powerful mediator of inflammation progression and elevated homocysteine levels are associated with interleukins in monocytes [21]. The increase in production is related to the positive regulation of vascular cell adhesion molecules [22].

The increased risk of cardiovascular disease independent of classic risk factors is even associated with mild hyperhomocysteinemia [23]. In the current study, compared with group I control rats, the level of homocysteine in the plasma of group III animals taking adriamycin significantly increased, which is different from the previous A published study is consistent [24]. The exact pathophysiological role of homocysteine-induced cardiomyopathy is unclear, and there is a large amount of research evidence supporting the role of homocysteine in the development of myopathy aberrations.

Compared with group III cardiomyopathy-induced animals, dietary squalene supplementation greatly reduced the plasma homocysteine content of group IV rats. This may be due to the inhibition of monocyte/macrophage-derived interleukin production, which triggers the firm adhesion of rolling monocytes to the vascular endothelium, which is the cause of atherosclerosis [25]. A study conducted in 2002 showed that the lipophilic inhibitors cerivastatin, fluvastatin, and HMGCoA reductase reduce cardiovascular risk and atherosclerosis through non-lipid mechanisms (such as inhibition of interleukin expression) plaque fragility [26]. The lipophilic nature of squalene is more than statins thereby increasing their permeability to vascular smooth muscle cells and thus inhibiting the production of both interleukin and homocysteine like HMG-CoA reductase inhibitor.

The damaged myocardium released diagnostic marker enzymes (CPK, CPK-MB, LDH, AST, ALT, and alkaline phosphatase) into the systemic circulation after adriamycin-induced myocardial injury. The number of damaged myocytes present in the myocardium is directly related to circulatory levels of marker enzymes in plasma. These enzymes are considered to be the best markers of myocardial damage because they have specific and catalytic effects on all other biomolecules leaking from damaged heart tissue. The levels of diagnostic marker enzymes concentration in plasma showed a significant ($p<0.05$) rise in Group III adriamycin-administered animals as compared to Group I control rats. This is consistent with the results of a previous study [27], which clarified the intensity of necrotic damage to the myocardial cell membrane caused by adriamycin. Release of the labelled enzyme reveals nonspecific abnormalities in plasma membrane integrity and permeability in response to adrenergic stimulation. The cytoprotective activity of squalene was confirmed, because compared with group III, consumption of squalene by oral route significantly offset ($p<0.05$) the increase in the intensity of diagnostic marker enzymes in the plasma of group IV animals caused by adriamycin mouse. Squalene is lipophilic and can be combined with any other lipophilic drugs, such as antipyrine, vitamin E, and nifedipine [28].

Regarding the degree of lipophilicity, lipophilic β-blocking molecules insert into the lipid bilayer and stabilize the muscle cell membrane**.** Since then, it is conceivable that squalene may also extend the sustainability of myocardial cell membrane necrosis damage through membrane stabilization. In the myocardial tissues of normal and experimental animals, histological annotations were made to verify the cytoprotective activity of squalene against adriamycin-induced cardiomyopathy.

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Microscopic study of cardiac tissue sections from normal Group I rats revealed the structure of regular myofibrils with stripes, the appearance of bifurcations, and the permanence of continuous myofibrils. However, compared to normal control group I rats, adriamycin administration can cause structural abnormalities, such as mild to diffuse cloudy swelling, focal vacuolization, and group III rats heart tissue sections Occasionally infiltrates around the centre of round cells. Congestion, dilation of the hepatic sinusoids, occasional cell proliferation, central necrosis, and fibrous hyperplasia in the portal area were also observed. These structural irregularities may be due to the drop in oxygen supply and increased parallel wall stress. Current observations corroborate previously reported research [29,30] indicating that adriamycin-induced histological changes are noted in the left ventricular sub-endocardium.

Therefore, in the current study, light microscope analysis of the fourth group of rat heart tissue sections supplemented with squalene showed the regular structure of the myofibril stripes, the appearance of branches, and the continuity with the adjacent myofibrils. The myocardial fibers are well protected and are similar to the myocardial fibers of control rats, representing the cytoprotective effect of squalene. Previous research is pointed out that oral squalene improves the morphological changes of the heart caused by isoproterenol through its membrane-stabilizing properties [31]. A histopathological study demonstrated that squalene intake can protect experimental animals from cyclophosphamide-induced tissue damage [32]. Compared to control rats, histological examination of heart tissue from normal rats that received squalene alone (group II) did not show any significant change, indicating that it did not have any adverse effects on its own.

Squalene dietary supplementation has a protective effect on adriamycin-induced cardiomyopathy in rats. The overall cardioprotective effect of squalene may be related to its ability to stabilize the membrane against the deterioration of the oxide film induced by Adriamycin, which is the main cause of irreversible necrosis of cardiomyocytes.

CONCLUSIONS

Adriamycin is a powerful extensively used anticancer drug that plays an important role in cancer chemotherapy. The clinical efficacy of this drug is very limited as it causes continuous cardiomyopathy or congestive heart failure in cancer patients. The heart tissue has particularly become toxic which is a major cause of morbidity and mortality due to its complex etiology. Despite its side effects, Adriamycin is widely used and Adriamycin is the best-selling anticancer drug in the world. A better understanding of the underlying mechanisms of adriamycin-induced cardiomyopathy has led to the development of new cardioprotective therapies. Correcting possible myocardial dysfunction can be a useful and practical principle in the treatment of patients with cardiomyopathy. Squalene is an isoprenoid molecule used in Indian folk remedies, present in large quantities in cod liver oil and extracted from deep-sea sharks to treat cardiovascular disease. It has been reported to have important membrane stabilizing properties.

The target of the present study is to examine the cytoprotective effects of dietary supplementation of squalene against adriamycin-induced cardiomyopathy in rats, an animal model for cardiomyopathy of human beings, and the results correlate the statement by maintaining the levels of diagnostic markers at near normalcy and through inhibiting the formation of lesions in the cardiac tissue in the experimental rats.

CONTRIBUTION OF AUTHORS

Research concept- Dr. Pallavi Srivastava
Research design- Dr. Pallavi Srivastava and Mr. Agnivesh
Supervision- Dr. Pallavi Srivastava
Materials- Dr. Pallavi Srivastava and Mr. Agnivesh
Data collection- Dr. Pallavi Srivastava and Mr. Agnivesh
Data analysis and Interpretation- Dr. Pallavi Srivastava
Literature search- Dr. Pallavi Srivastava
Writing article- Dr. Pallavi Srivastava and Mr. Agnivesh
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Article editing- Dr. Pallavi Srivastava and Mr. Agnivesh
Final approval- Dr. Pallavi Srivastava and Mr. Agnivesh

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