Antioxidant Defense of Dietary Squalene Supplementation on Sodium Arsenite-Induced Oxidative Stress in Rat Myocardium

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ABSTRACT

Arsenic is a naturally occurring element widely present in the environment, with drinking water accounting for the majority of chronic human arsenic exposure worldwide. Millions of people worldwide are at risk of cancer, heart disease and diabetes because of chronic arsenic exposure. In the present study, we have investigated the antioxidant defense of squalene on myocardial function in sodium arsenite-induced oxidative stress condition in rats. Oral administration of sodium arsenite [10 mg/kg body weight/day for a period of 30 days] caused a significant (p < 0.05) increase in the levels of diagnostic markers (troponin T, homocysteine and creatine phosphokinase-MB) in the plasma of rats, indicating necrotic damage to the myocardial membrane. Significant (p < 0.05) elevation in the level of lipid peroxidation with a parallel reduction in the level of reduced glutathione and the activities of glutathione-dependent antioxidant enzymes and antiperoxidative enzymes in the heart tissue was also noticed. The dietary supplementation of 2% squalene for a period of 30 days significantly (p < 0.05) attenuated the sodium arsenite-induced oxidative aberrations in the heart tissue and maintained the levels of diagnostic markers at near normal. The results of the present findings indicate that the cytoprotective potential of squalene is probably related to its ability to counteract free radical formation by its antioxidant nature.

Keywords: antioxidant status, arsenic poisoning, cardiotoxicity, diagnostic markers, isoprenoid, lipid peroxidation

INTRODUCTION

Arsenic is a metalloid element that is widespread in the aquatic environment as a result of both geogenic and anthropogenic processes (Mukherjee and Bhattacharya 2002). Millions of people worldwide are at risk of cancer, heart disease and diabetes because of chronic arsenic exposure (Soucy et al. 2004). Although several hypotheses have been proposed, the exact mechanism of arsenic toxicity has not yet been clearly defined. There are, however, studies which suggest that higher concentrations of arsenic cause oxidative stress, increased reactive nitrogen species, and inhibit enzyme and mitochondrial function (Hei et al. 1998; Lynn et al. 1998). Sodium arsenite induces myocardial dysfunction by a multiple step mechanism. A considerable body of clinical and experimental evidence now exists suggesting the involvement of a free radical-mediated oxidative process in the pathogenesis of arsenic poisoning (Mishra and Flora 2008). Alterations in tissue defense systems including chemical scavengers or antioxidant molecules and the enzymes catalase, superoxide dismutase, and glutathione peroxidase have been reported in sodium arsenite-induced myocardial toxicity (Maiti and Chatterjee 2001).

Until now the studies regarding treatment of arsenic toxicity are restricted mainly to some sulphydryl-containing chelating agents [meso 2,3-dimercaptosuccinic acid (DMSA), 2,3-dimercaptopropane-1-sulfonate (DMPS) or British Anti Lewisite (BAL; 2,3-dimercaprol), antioxidants [Vitamin C, Vitamin E and N-acetyl cysteine] and some micronutrients [zinc and selenium] (Aposhian and Aquashian 2006; Modi et al. 2006). Most of the conventional metal chelating agents and antioxidants have been reported to possess toxic side effects or disadvantages (Mehta et al. 2006). Thus, there has been increased interest in the therapeutic potential of natural products having antioxidant properties in reducing free radical-induced tissue injury (Gupta and Flora 2006).

Interestingly, squalene, an isoprenoid molecule (Fig. 1), which is present in large quantities in deep-sea shark liver oil and in smaller amounts (0.1-0.7%) in palm, wheat-germ, olive, and rice-bran oils, has been reported to possess antilipidemic, antioxidant and membrane-stabilizing properties (Qureshi et al. 1996; Farvin et al. 2006). It plays a role in enhancing health through its part in the building blocks of hormones and cholesterol and as an antioxidant. Squalene is secreted in human sebum, where it protects the skin from ultraviolet radiation (Kohno et al. 1995). Studies by Kamiura et al. (1992) demonstrated the detoxifying activities of squalene against diverse chemicals such as hexachlorobiphenyl, hexachlorobenzene, theophylline, phenobarbital and strychnine. Squalene has also been found to have a protective activity against several carcinogens, including azoxy-methane-induced colon caner and nicotine-derived nitrosaminokeetone-(NMK) induced lung carcinogenesis (Rao et al. 1998; Smith et al. 1998). Since squalene is being one of the most powerful antioxidant and antilipidemic agents, it has to be studied in detail as an important compound for better medicinal values.

Though the beneficial properties of squalene are pro-
mising and well studied, the protective effects of squalene against arsenic poisoning have not yet been explored. Hence, we thought it was important to study the effect of squalene on myocardial antioxidant system in experimental arsenic poisoning in rats by virtue of its hypolipidemic, antiperoxidative and membrane stabilizing properties.

**MATERIALS AND METHODS**

**Chemicals**

Epinephrine, tetraethoxy propane and reduced glutathione 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: 240–245°C) was prepared from shark liver oil of *Centrophorus sp.* caught in the Andaman waters. All the other chemicals used were of analytical grade.

**Isolation of squalene**

The fresh shark liver was chopped into pieces, kept in wire mesh baskets, and heated to 80°C in a 2% caustic soda solution for 30–40 min by dipping the liver in alkali, in an open kettle. The floating oil was skimmed off. Water content was removed by adding anhydrous sodium sulfate (25 g/100 ml), and the filtered oil was fractionally distilled under vacuum (2 mbar/760 mm Hg) for isolation of squalene. The low boiling fraction that distilled out at 125–140°C and the major high boiling fraction that distilled out at 240–245°C were separately collected, and the residue was discarded. The fractions were analyzed for purity using an Iatroscan MK-6s analyzer (Mitsubishi Kagaku Iatron Inc., Tokyo, Japan). The major high boiling fraction, which was 95% pure squalene, was stored in the presence of nitrogen in a dark brown bottle at -4°C and used for the experiment.

**Animals**

Wistar strain male albino rats, weighing 120–150 g were selected for the study. The animals were housed individually in polyurethane cages under hygienic conditions and maintained at room temperature. The animals were allowed food and water ad libitum. 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 (IAEC).

**Experimental protocol**

The animals were divided into four groups of 6 rats each. Group I and Group III animals were fed on commercial feed with added coconut oil at 2% (wt/wt) for 30 days and Group II and Group IV animals were fed on commercial feed with added squalene at 2% (wt/wt) for a period of 30 days. Group III and Group IV animals were orally administered with sodium arsenite [10 mg (dissolved in distilled water)/kg body weight/day] for a period of 30 days. Control animals (Group I and Group II) were orally administered with distilled water alone for 30 days.

At the end of the experimental period, the experimental animals were sacrificed by using chloroform anesthesia and blood was collected with ethylenediaminetetraacetate (EDTA) as an anti-coagulant for separation of plasma. The heart tissue was excised immediately and washed with chilled isotonic saline. Troponin-T content in plasma was determined by electrochemiluminescence immunoassay (ECLIA) using a Modular Analytics E170 (Elecys module) immunoassay analyzer. Homocysteine (Hcy) concentration in plasma was assayed by using the Microtiter Plate Assay package (Diazyme Laboratories). Creatine phosphokinase (CPK-MB) activity in plasma was determined by the method of Guzy (1977). The heart tissue homogenates prepared in ice-cold 0.1 M Tris-HCl buffer, pH 7.2 were used for the determination of lipid peroxides (LPO) (Ohkawa et al. 1979), reduced glutathione (GSH) (Ellman 1959), glutathione peroxidase [EC 1.11.1.9] (GPx) (Paglia and Valentine 1967), glutathione-S-transferase [EC 2.5.1.18] (GST) (Habig et al. 1974), catalase [EC 1.11.1.6] (CAT) (Takahara et al. 1960) and superoxide dismutase [EC 1.15.1.1] (Misra and Fridovich 1972).

**Statistical analysis**

Results are expressed as mean ± SD. Multiple comparisons of the significant analysis of variance were performed by Duncan’s multiple comparison test. A P-value <0.05 was considered as statistically significant. All data were analyzed with the aid of a statistical package program, SPSS 10.0 for Windows.

**RESULTS AND DISCUSSION**

Arsenic is a naturally occurring element widely present in the environment, with drinking water accounting for the majority of chronic human arsenic exposure worldwide. Millions of people worldwide are at risk of cancer, heart disease and diabetes because of chronic arsenic exposure. Oxidative stress is one of the mechanisms with a central role involved in the pathogenesis of sodium arsenite-induced myocardial dysfunction. Natural products have been the starting point for the discovery of many important modern drugs. This fact has led to chemical and pharmacological investigations and general biological screening programs for natural products of all over the world. Squalene is a potent antioxidant molecule abundantly present in shark liver oil. In addition to shark liver oil, squalene is also found in variety of other foods, such as cod liver oil [520.3 µg/g], corn oil [278.9 µg/g], safflower oil [37.2 µg/g], cotton seed oil [27.8 µg/g], lard [22.5 µg/g], chicken meat [31.5 µg/g], duck meat [36.4 µg/g], halibut [96.9 µg/g], flounder [50 µg/g], tuna [149g/g], butter [61.3 µg/g], almond 13.2 [µg/g] and pistachio [15.1 µg/g] (Liu et al. 1976).

The focus of the current study was to evaluate the effects of dietary squalene supplementation for its antioxidant and membrane-stabilizing properties during experimentally-induced arsenic poisoning in rats.

Troponins are regulatory proteins essential for contraction and relaxation processes in the myocardium. Myocyte injury results in damage to contractile proteins and is a key mechanism responsible for the release of the structurally bound cardiac troponin T, and once outside the myocyte, these macromolecules are cleared from the interstitium by cardiac lymphatics (Sarko and Pollack 2002). In recent years an increase in the use of myocardial troponins as markers of myocardial injury has been witnessed. O’Brien et al. (1997) showed that troponin T is a powerful biomarker in laboratory animals for sensitive and specific detection of cardiac injury. Our findings confirm the same pattern, and show a significant (p < 0.05) increase in the level of troponin T in plasma of Group III sodium arsenite-administered rats as compared to that of Group I control animals (Table 1). Reports by Carnicer et al. (2006) have shown that its detection in systemic circulation could be considered not only a more sensitive but also a specific marker for assessing the severity of necrotic damage to myocardium. Investigations by Ganesan et al. (2007) have indicated that dietary intake attenuates the release of this regulatory protein from the ischemic myocardium by modulating the elasticity of the plasma membrane.

In the present study, the dietary supplementation of squalene significantly (p < 0.05) reduced the sodium arsinite-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 the cell membrane plays a major role in cell volume regulation by modulating the elasticity of the 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 dysfunction. Reports by Ivashkevich et al. (1981) indicate that squalene is capable of overtting severe osmolar changes associated with possible cell death.

Homocysteine is a thiol-containing potentially cytotoxic...
4-carbon α-amino acid formed during methionine metabolism. Recent studies have shown that mild hyperhomocysteinemia is associated with an increased risk of cardiovascular diseases independently of classical risk factors (Senaratne et al. 2000). In the present study, a significant (p < 0.05) elevation in the level of homocysteine was noted in the plasma of Group III rats as compared to that of Group I controls. This is in accordance with an earlier report (Gamble et al. 2007). Homocysteine has been reported to induce atherosclerosis either by impairing coronary microvascular dilator function (Tawakol et al. 2001; Shai et al. 2002). 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 (Rao et al. 2004; Shai et al. 2004). An elevated level of homocysteine is associated with increased intercellular lipid peroxidation in monocytes and up regulation of vascular cell adhesion molecules (Silverman et al. 2002).

In the present study, we observed that the dietary intake of squalene significantly (p < 0.05) reduced the level of homocysteine in plasma of Group IV rats as compared to that of Group III sodium arsenite administered rats. It probably did so by inhibiting the formation of homocysteinerich macrophage-derived intercellulars, 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 fluoravastatin 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 likewise the prime HMG-CoA reductase inhibitor squalene may also inhibit both homocysteine and interleukin production.

The plasma concentration of CPK-MB, a cardiac diagnostic marker enzyme was significantly higher (p < 0.05) in sodium arsenite administered rats (Group III) compared to the controls (Group I) (Table 3). This is in line with an earlier reported study (Saad et al. 2006), which showed that the level of CPK-MB released from the damaged myocardium into the blood stream was directly proportional to the number of necrotic cells present in the heart tissue. The release of CPK-MB reflects non-specific alterations in the plasma membrane integrity and permeability as a response to sodium arsenite intoxication. Supplementation of squalene significantly (p < 0.05) prevented the sodium arsenite-induced concentration of CPK-MB in plasma of Group IV animals compared to Group III rats, indicating the cytoprotective activity of squalene. The membrane stabilizing action of squalene is comparable to any other membrane-stabilizing agents like antipyrine and nifedine, which can intercalate into the lipid matrix and impart stabilization to myocardial cell membranes (Farvin et al. 2005). It is possible that likewise squalene may also prolong the viability of myocardial cell membranes from necrotic damage by its membrane stabilizing property.

Biological membranes are sensitive to lipid peroxidation induced by reactive oxygen species. The oxidation of unsaturated fatty acids in biological membranes may cause impairment of membrane function, decrease in membrane fluidity, inactivation of membrane receptors and enzymes, increase of non-specific permeability to ions and disruption of membrane structure. Oxygen-free radicals are implicated as mediators of tissue injury in sodium arsenite-induced cardiovascular pathology (Kekreja and Hess 1992). In the present investigation, a significant (p < 0.05) rise in the level of lipid peroxidation was observed in the heart tissue of Group III sodium arsenite-administered rats as compared to controls. Lipid peroxidation has been identified as one of the basic deteriorative reaction in cellular mechanisms of the sodium arsenite-induced myocardial dysfunction (Yáñez et al. 1991). A parallel decline (p < 0.05) in the level of GSH (Table 3) in the heart issue of Group III animals was also observed. Peroxidation of endogenous lipid might be a major factor involved in the cytotoxic nature of sodium arsenite. Our results also suggested that sodium arsenite-administered rats might be less resistant and more susceptible to lipid peroxidation in the presence of promoters like ascorbate, FeSO₄ and t-BH (Table 2).

Lipid peroxidation of membranes is regulated by the availability of substrate in the form of polysaturated fatty acids (PUFA), the availability of inducers such as free radicals and the exited state molecules to initiate propagation, the antioxidant defense status of environment and the physical status of membrane lipids (Dhandapani et al. 2007). The unpaired electron present in the hydroxyl free radical reacts with polysaturated fatty acids to form reactive lipid radicals harmful to the structural and functional integrity of the myocardial membrane. The results of the present study suggested that the high vulnerability myocardium to peroxidative damage may be due to a decline in the level of free radicals for scavengers.

In the present study, the dietary supplementation of squalene significantly (p < 0.05) inhibited the sodium arsenite-mediated lipid peroxidation in the heart tissue of Group IV rats, establishing its antioxidant nature. Squalene is highly lipophilic and, when administered exogenously, it can readily pass across the membrane lipid bilayer. The ability of squalene to diffuse into intracellular compartments...
helps in the capability of this isoprenoid as a potent antioxidant (Buddhan et al., 2007). The unpaired electron present in the hydroxyl radical (OH) generated during sodium arsenite-induced myocardial toxicity might have been trapped for dismutation by its free radical scavenging isoprenoid unit. Studies by Miyachi et al. (1983) have shown that subsequent to oxidative stress, such as sunlight exposure, squalene functions as an efficient quencher of singlet oxygen and it prevents the corresponding lipid peroxidation in human skin surface. An earlier report (Dhandapani et al. 2003) indicated that dietary supplementation of squalene modulated PUFAs-mediated peroxidative damage to the myocardial membrane by its antioxidant and membrane stabilizing properties. The rate constant of quenching of singlet oxygen by squalene is much larger than those of other lipids, and to be comparable to 3,5-di-r-butyl-4-hydroxytolene (BHT). Reports by Kohno et al. (1995) have shown that squalene is not particularly susceptible to peroxidation in the presence of membrane lipid bilayer. Squalene has already been reported to exert antioxidant action through forcing structural interactions with membrane lipids (Buddhan et al. 2007). The lipid-soluble squalene has been reported to have a greater free radical scavenging activity than the known potent non-enzymatic antioxidants GSH, and vitamins C and E. Earlier reports by Das et al. (2003) have shown that squalene exerts a significant protective action against cisplatin-induced toxicity in neuroblastoma cells similar to that of GSH, which is well known to detoxify platinum compounds by enhancing the GSH-GST detoxification system. It also suggests that squalene has a selective in vitro cytoprotective effect on bone marrow-derived hemopoietic stem cells that is equipotent to GSH.

In conclusion, the results of the present study indicate that the dietary supplementation of squalene prevents sodium arsenite-induced myocardial toxicity in rats. The overall protective effect of squalene is probably due to its membrane stabilizing action, or to a counteraction of free radicals by its antioxidant nature, or to its ability to maintain the normal status the activities of the free radical scavenging enzymes and the level of reduced glutathione, which protect myocardial membrane against peroxidative damage by decreasing lipid peroxidation and strengthening the myocardial membrane.

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Table 3

Table 3 Level of reduced glutathione and the activities of glutathione peroxidase (GPX), glutathione-S-transferase (GST), catalase (CAT) and superoxide dismutase (SOD) in the heart tissue of normal and experimental groups of rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH</td>
<td>5.68 ± 0.38 a</td>
<td>6.23 ± 0.45 b</td>
<td>2.89 ± 0.18 c</td>
<td>4.75 ± 0.29 d</td>
</tr>
<tr>
<td>GPX</td>
<td>2.92 ± 0.21 a</td>
<td>3.18 ± 0.28 a</td>
<td>1.38 ± 0.11 c</td>
<td>2.81 ± 0.19 a</td>
</tr>
<tr>
<td>GST</td>
<td>1287.14 ± 97.45 a</td>
<td>1315.34 ± 108.07 a</td>
<td>763.49 ± 62.12 b</td>
<td>1118.26 ± 83.09 c</td>
</tr>
<tr>
<td>CAT</td>
<td>9.18 ± 0.88 a</td>
<td>9.63 ± 0.92 a</td>
<td>4.32 ± 0.25 b</td>
<td>7.85 ± 0.63 c</td>
</tr>
<tr>
<td>SOD</td>
<td>4.32 ± 0.21 a</td>
<td>4.09 ± 0.18 a</td>
<td>1.28 ± 0.69 b</td>
<td>3.87 ± 0.15 c</td>
</tr>
</tbody>
</table>

Group I and Group II: Normal control rats were received standard diet mixed with 2% coconut oil (wt/wt) and 2% squalene (wt/wt) respectively for a period of 30 days; Group III and Group IV: Experimental rats were received standard diet mixed with 2% coconut oil (wt/wt) and 2% squalene (wt/wt) respectively for a period of 30 days and orally administered with sodium arsenite [10 mg/kg body weight/day] for a period 30 days. Results are mean ± SD for 6 animals. Values expressed: GSH, nmol g^-1 wet tissue; CAT, nmol H2O2 decomposed min^-1 g^-1 protein; GPx, nmol GSH oxidized min^-1 g^-1; SOD, one unit of the SOD activity is the amount of protein required to give 50% inhibition of epinephrine autooxidation. One way ANOVA, Duncan’s multiple comparison test. Values that have a different letter differ significantly (p < 0.05) with each other.

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