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Role of nitric oxide in alcohol-induced changes in lipid profile of moderate and heavy alcoholics

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Abstract

Biochemical changes in plasma and red cell membrane in moderate and heavy alcoholics were investigated to compare them with teetotaters in the present study. Significant changes in lipid, lipoprotein profile, and lipid peroxidation were evident from the study suggesting the cardioprotective effect in moderate alcoholics, and adverse changes leading to cardiovascular risk in heavy alcoholics. Both nitrite and nitrate levels in plasma of moderate alcoholics increased significantly when compared with teetotaters and the increase is more pronounced in heavy alcoholics. The results of the present study showed no significant difference in osmotic hemolysis in red cells from moderate and heavy alcoholics incubated with NaCl at concentrations ranging from 0.1% to 0.9%. Further, the study showed a possible relationship of nitric oxide (NO) with changes in plasma lipid profile. To sum up, these changes in both moderate and heavy alcoholics clearly indicated the involvement of NO in rendering tolerance to alcohol-induced effects and also in modulation of alcohol effects. © 2008 Elsevier Inc. All rights reserved.

Keywords: Alcoholics; Hemolysis; Lipoproteins; Nitric oxide

Introduction

Alcohol is a widely consumed psychoactive drug throughout the world. Though considerable literature exists on adverse effects of heavy alcohol drinking (Durazzo et al., 2004; Klatsky et al., 2003) many studies clearly reveal that moderate alcohol consumption has several benefits that include protection against cardiovascular problems and various other diseases (Gaziano et al., 2000; Ogge et al., 2006). Most physicians are unable to recommend moderate alcohol consumption to people/patients in view of two reasons: (i) the exact beneficiary effects and their precise mechanisms are not fully understood (Albert et al., 2003; Pagel et al., 2004) and (ii) moderate alcohol consumption may further lead to its indiscriminate consumption causing health problems and social implications (Espinell et al., 2005; Lucas et al., 2005). Now it is evident that the levels of nitric oxide (NO), oxidation, and antioxidation systems in human body are in close relationship with the health (Huang et al., 2000; Zhou et al., 2000).

Recent reports, in general, clearly revealed the involvement of NO in modulation of physiological and pathophysiological processes, in maintenance of cardiovascular homeostasis, in regulation of vascular tone, and in neuronal transduction (Azizi et al., 2005; Blaise et al., 2005; Kleinbongard et al., 2006; Pacher et al., 2005). However, there is paucity of information concerning the precise role of NO in various events associated with alcoholism. Although, alcoholism is linked with cardiovascular disease and coronary heart disease by influencing atherogenesis for which lipoproteins and lipids are the excellent markers (Hines and Rimm, 2001), the precise effects of alcoholism on cardio-protection and erythrocyte hemolysis and the underlying protecive or lytic events are unclear. Reports recommended a need for such studies related to alcoholism (Lucas et al., 2005). Wallerath et al. (2003) reported an increased expression of endothelial NO synthase that leads to moderate, but sustained, elevation of vascular NO in humans consuming red wine. Also, evidences suggest that membranes are wholly, if not largely, responsible for various alcohol-induced effects (Glass et al., 1983; Rottenberg et al., 1992). The purpose of this study is therefore two fold: first, to understand the status of NO and the role of NO in the biochemical changes in plasma, red cell, and red cell
membrane, and second, to investigate the biochemical changes of lipids and lipoproteins of plasma and red cell membrane of moderate and heavy alcoholics.

Materials and methods

Subjects

Human male volunteers, aged 45–55 years, residing in Anantapur town, Andhra Pradesh, India, were the subjects for the present study. These volunteers were categorized into three groups viz., controls who were nonalcoholics (teetotalers), moderate alcoholics who consumed less than two drinks (below 70 g ethanol) of alcoholic beverage/day, and heavy alcoholics who took two to five drinks (more than 70 g ethanol) every day. The beverages consumed by the chosen alcoholics include 80 proof hard liquors such as whisky, rum, gin, and brandy of various brands containing up to 40% ethanol. All the volunteers were well explained about the experimentation, a written consent was obtained from them, and maintained on local diet. Enough care was taken to prevent the effects of diet, water, or sampling time, and daily activities of the subjects. The chosen subjects were not on medication for any known chronic disease or illness. The study was approved by our institutional review and ethical committees.

Blood collection and measurement of plasma parameters

Venous blood samples from volunteers were collected into heparinized test tubes after overnight fasting. Plasma and red cells were separated by centrifugation at 800 × g for 10 min and were used for analysis. Plasma cholesterol, high-density lipoprotein (HDL) (Alban et al., 1974), low-density lipoprotein (LDL), very low-density lipoprotein (VLDL) (Friedwald et al., 1972), and triglycerides (Fossati and Principi, 1982) were determined following kit methods.

Plasma and lysate samples were treated with 30% zinc sulfate to deproteinize samples followed by centrifugation at 4000 × g for 5 min. Nitrite was determined (Sastry et al., 2002) from 1-ml aliquots of plasma and erythrocyte lysate using Griess reagent (1% sulfanilamide, 2.5% phosphoric acid, and 0.1% 1-naphthylethylene diamine). One-milliliter aliquots of the supernatant were swirled for 90 min separately with activated cadmium granules for the conversion of nitrite to nitrate and then Griess reagent was added. Nitrite concentrations were estimated using a standard curve developed with sodium nitrite.

Lipid peroxidation

The extent of lipid peroxidation (LPO) was measured by the formation of malondialdehyde by treating the sample with 2 ml of thiobarbituric acid reagent, as described by Buege and Aust (1978).

Erythrocyte membrane lipid analysis

Erythrocyte membranes were prepared following the method of Dodge et al. (1963). Erythrocyte suspension was washed with phosphate buffered saline (pH 7.2), and the cells were lysed with 5-mM phosphate buffer (pH 8.0) and spun at 15000 × g for 30 min. Hemoglobin-free ghosts obtained by another wash with 5-mM phosphate buffer were used for analysis. Lipid extraction from erythrocyte membranes was done by the method adopted by Peeterways and Hanahan (1964). To the lysed membrane preparations, 5 ml of methanol was added, followed by the addition of chloroform (10 ml). After 30 min, the filtrate was collected from the mixture, and the residue was used again for another extraction. The pooled filtrates were used for estimation of cholesterol (Zlatkis et al., 1953) and phospholipids (Connerty et al., 1961).

Osmotic hemolysis of red blood cells

Isolated red blood cells were incubated in different concentrations of NaCl ranging from 0.1% to 0.9% for 30 min with gentle stirring and hemoglobin released into supernatant from the red cells was determined after a spin at 2500 × g for 10 min and measuring absorbance at 540 nm (Kanai, 1988).

Statistical analysis

The data values are expressed as means ± S.E.M. The average value for each parameter was contrasted with the other values (P ≤ .05) using Duncan’s new multiple range test. Correlations between variables were assessed with Pearson’s correlation coefficients (r).

Results

As nitrite and nitrate concentrations in plasma and erythrocyte lysate are considered as reliable indices to assess the status of NO (Barbosa et al., 2006), they are measured in moderate and heavy alcoholics as well as in teetotalers and data are presented in Table 1. There was a modest increase in plasma nitrite and nitrate concentrations only in moderate alcoholics as compared to those of teetotalers. The increase was even more pronounced in heavy alcoholics. Thus, the increments of plasma nitrite and nitrate in moderate alcoholics over teetotalers were 31.4% and 39%, respectively. The corresponding figures in respect of plasma nitrite and nitrate in heavy alcoholics were 59.2% and 68.5%, respectively. Similarly, the values of nitrate and nitrate for erythrocyte lysate were 17.5% and 33.5% in moderate alcoholics and 9.7% and 25.1% in heavy alcoholics, respectively.

Table 2 presents the data on plasma lipid profiles such as total cholesterol, HDL, LDL, triglycerides, VLDL of teetotalers, moderate alcoholics, and heavy alcoholics. An
Table 1
Changes in plasma and erythrocyte lysate concentrations of nitrite and nitrate in alcoholics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Teetotalers</th>
<th>Moderate alcoholics</th>
<th>Heavy alcoholics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrite (pmol/l)</td>
<td>5.4 ± 0.30</td>
<td>7.1 ± 0.38</td>
<td>8.6 ± 0.33</td>
</tr>
<tr>
<td>Nitrate (pmol/l)</td>
<td>34.7 ± 0.37</td>
<td>48.3 ± 0.37</td>
<td>58.5 ± 0.43</td>
</tr>
</tbody>
</table>

Mean values (n = 12) in each row followed by the same superscript are not significant (P < .05) according to Duncan's multiple range test.

Table 2
Plasma lipid profiles of nonalcoholics, moderate alcoholics, and heavy alcoholics

<table>
<thead>
<tr>
<th>Lipid profile</th>
<th>Teetotalers</th>
<th>Moderate alcoholics</th>
<th>Heavy alcoholics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>190.0 ± 1.43</td>
<td>180.3 ± 1.24</td>
<td>220.1 ± 0.87</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>45.1 ± 1.41</td>
<td>50.6 ± 1.11</td>
<td>32.8 ± 1.26</td>
</tr>
<tr>
<td>LDL (mg/dl)</td>
<td>93.8 ± 1.01</td>
<td>80.7 ± 1.15</td>
<td>131.3 ± 1.38</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>149.3 ± 1.75</td>
<td>133.4 ± 1.59</td>
<td>180.1 ± 1.28</td>
</tr>
<tr>
<td>VLDL (mg/dl)</td>
<td>12.8 ± 1.18</td>
<td>9.5 ± 0.58</td>
<td>20.1 ± 1.10</td>
</tr>
</tbody>
</table>

Mean values (n = 12) in each row followed by the same superscript are not significant (P < .05) according to Duncan's multiple range test.

Table 3
Correlation values (r) between NOx and conventional cardiovascular risk factors

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Teetotalers</th>
<th>Moderate alcoholics</th>
<th>Heavy alcoholics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrite</td>
<td>-0.096</td>
<td>-0.40</td>
<td>-0.128</td>
</tr>
<tr>
<td>Nitrate</td>
<td>-0.096</td>
<td>-0.40</td>
<td>-0.128</td>
</tr>
<tr>
<td>Plasma LPO</td>
<td>-0.096</td>
<td>-0.40</td>
<td>-0.128</td>
</tr>
<tr>
<td>Erythrocyte NOx</td>
<td>-0.096</td>
<td>-0.40</td>
<td>-0.128</td>
</tr>
<tr>
<td>Membrane LPO</td>
<td>-0.096</td>
<td>-0.40</td>
<td>-0.128</td>
</tr>
</tbody>
</table>

Mean values (n = 12) in each row followed by the same superscript are not significant (P < .05) according to Duncan's multiple range test.

Discussion

The observation of modest hike in levels of NO2 and NO3 in moderate alcoholics, and overproduction of NO in heavy alcoholics when compared with controls followed by changes in lipoproteins and lipids in plasma in the present study suggested a possible involvement of NO in these changes. Earlier studies of acute and chronic alcohol consumption revealed such increases in plasma NO levels (Oekonomaki et al., 2004; Zima et al. 2001). Possible reasons for increased production of NO observed in the present study in alcoholic groups would be the upregulation of endothelial nitric oxide synthase (e-NOS), and/ or increased activity of nitric oxide synthase (NOS), and/or increased expression of isoforms of NOS contributing to the increase in HDL and decrease in total cholesterol. LDL, VLDL, and triglycerides were observed in moderate alcoholics when compared with teetotalers. On the contrary, there was decrease in HDL accompanied by increase in total cholesterol, LDL, VLDL, and triglycerides in heavy alcoholics. The release of hemoglobin on erythrocytic hemolysis from red cells of teetotalers, moderate alcoholics, and heavy alcoholics at all the selected concentrations of NaCl ranging from 0.1% to 0.9% remained the same and the data are furnished in Fig. 2.

Fig. 1 compares alcohol-induced alterations in various cardiovascular risk factors and total nitrogen oxides (NOx) in three groups viz., teetotalers, moderate alcoholics, and heavy alcoholics. The release of hemoglobin on erythrocytic hemolysis from red cells of teetotalers, moderate alcoholics, and heavy alcoholics at all the selected concentrations of NaCl ranging from 0.1% to 0.9% remained the same and the data are furnished in Fig. 2.

Table 4
Changes in plasma LPO and membrane lipids in alcoholics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Teetotalers</th>
<th>Moderate alcoholics</th>
<th>Heavy alcoholics</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPO (umoles MDA/mg protein)</td>
<td>3.9 ± 0.36</td>
<td>2.5 ± 0.29</td>
<td>5.9 ± 0.26</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>98.8 ± 0.68</td>
<td>106.8 ± 0.84</td>
<td>130.8 ± 1.09</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>110.4 ± 0.59</td>
<td>114.8 ± 0.92</td>
<td>115.8 ± 1.63</td>
</tr>
</tbody>
</table>

Mean values (n = 12) in each row followed by the same superscript are not significant (P < .05) according to Duncan's multiple range test.

LPO = lipid peroxidation.
production of NO (Barua et al., 2003). Recent studies also revealed that high-density lipoproteins affect endothelial NO production (Liisanantti et al., 2004).

Increase in HDL followed by a decrease in LDL, VLDL, triglycerides, total cholesterol levels, together with a decreased LPO in moderate alcoholics in this study clearly suggested a possible reduction in risk of coronary heart disease. The mechanisms related to its protective effect on cardiovascular system are very complex and are not completely understood (Gu et al., 2001). Alcohol-induced modulation by increasing hepatic production or secretion of apolipoproteins or lipoprotein particles, increasing triglyceride lipases and decreasing the removal of circulating HDL with involvement of NO might have played a role in the observed effect (Dreon and Krauss, 1996). Potential action of cholesteryl ester transfer protein (CETP) provides an important mechanism for returning plasma cholesteryl esters to the liver and may therefore have a crucial atherogenic function (Hannuksela et al., 1992; Yang et al., 2002). NO production in moderate alcoholics might have interfered with the process either by nitrosation of the protein (CETP) affecting the function and/or the expression of the protein. Furthermore, decreased HDL, and increased LDL, VLDL, and increased LPO observed in heavy alcoholics point to the increased cardiovascular risk (Manninen et al., 1988; Seo et al., 2004; Soardo et al., 2005). Thus, NO may have biphasic role and a dose-dependent effect on alcoholic plasma lipoprotein profile and in cell metabolism as has been described earlier in macrophages, endothelial cells, and β-pancreatic cells (Bereta and Bereta, 1995; Mateo et al., 1995) in which glyceraldehyde-3-phosphate dehydrogenase and glycolytic flux were activated or inhibited, respectively, at low levels or high levels of NO (Galli et al., 1998). Many studies confirmed nitrosation of proteins and lipids affecting cellular signaling events and metabolic functions (Ischiropoulos and Gow, 2004; Kalyanaraman, 2004). The factors affecting e-NOS function and lipoproteins in alcoholics are well documented (Leighton et al., 2005). Earlier reports also revealed that production of NO for a brief period at low levels exerts a role in host defense causing beneficiary effects by modulating signal transduction (Beevi et al., 2004).

Fig. 1. A comparison of the changes in levels of lipids and NO in plasma of teetotalers, moderate alcoholics, and heavy alcoholics.

Fig. 2. Osmotic hemolysis as affected by alcoholism.
Antioxidant and free radical scavenging effects of NO might have helped in the observed decrease in LPO in moderate alcoholics. In heavy alcoholics, probably by reacting with O$_2$ NO forms superoxide nitroso free radical (ONOO$^-$), which possesses still more strong oxidative properties that can further attack and injure various cells in the body and deactivate the antioxidant machinery, resulting in the observed increase in LPO as noticed in the present study. NO behaves as an antioxidant or as a pro-oxidant depending on the conditions of oxidative stress (Andican et al., 2005; Chulz et al., 1999). Oxidative modification of LDL that plays an important role in pathogenesis and progression of atherosclerosis is prevented by NO (Zima et al., 2001). It is also pertinent and relevant to note another mechanism of the involvement of protein kinase C in alcohol-induced cardioprotection, which suggested the beneficial effects of chronic alcoholism by signaling elements and thereby reducing overall cardiovascular risk (Pagel et al., 2004). On the other hand, NO leads to adverse pathological consequences when generated at much higher concentrations for prolonged periods (Beevi et al., 2004). It is well known that the formation of peroxynitrite radical from NO interactions with superoxide leads to many perils such as cytotoxicity and other sequelae. In vivo peroxynitrite generation represents a crucial pathogenic mechanism in conditions such as stroke, myocardial infarction, chronic heart failure, diabetes, and other chronic diseases including cancer (Pacher et al., 2007).

No change in C/P ratio in moderate alcoholics noticed in the present study suggested no alterations in membrane fluidity. The observation of increased cholesterol content in heavy alcoholics with no change in phospholipid and the consequent increase in C/P ratio in present study indicative of decreased membrane fluidity is in agreement with the earlier studies (Parannaharma et al., 2004). Involvement of NO in fluidity changes and tolerance is evident from earlier studies (Muriel and Sandoval, 2000). This finding revealed the enrichment of membrane with cholesterol affecting lipid–lipid, lipid–protein, protein–protein interactions, and altered protein functions, which are complex (Borochov et al., 1979; Stibler et al., 1991).

Though ethanol-induced hemolysis has been very well documented, results of the present study showed no change in hemolysis of red cells from moderate and heavy alcoholics when incubated with different concentrations of NaCl, suggesting increased resistance/tolerance in red cells from chronic alcoholics against hemolysis. Increased bioavailability of NO might have protected the cells from hemolysis by its free radical scavenging and antioxidant effects (McCuskey et al., 1995; Nanji et al., 1995; Ockonomaki et al., 2004). Studies of Knanna et al. (1993) suggested the development of rapid functional tolerance to ethanol, which was attributed to NO. Decreased NO bioavailability was reported to be associated with hemolysis (Barvitenko et al., 2005; Minneci et al., 2005). The present study demonstrates the role of NO in vasoprotective and cardioprotective effects in moderate alcoholics, and increased cardiac risk and atherogenesis in heavy alcoholics when compared with teetotalkers. Furthermore, nitrosative stress appears to play a key role in the observed adverse effects in heavy alcoholics.

Acknowledgments

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References


Chronic exposure to pyrethroid-based allethrin and prallethrin mosquito repellents alters plasma biochemical profile

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Glucose
Nitric oxide
Lipo proteins
Pyrethroids

A B S T R A C T
Continuous exposure of humans to pyrethroid-based mosquito repellents for longer durations may lead to adverse health effects. No information is available on long-term use of these mosquito repellents pertaining to the biochemical changes in human subjects. Therefore, the present study is an attempt to evaluate the status of health in human volunteers exposed to two commercially available mosquito repellent pyrethroids, allethrin and prallethrin, in terms of changes in plasma biochemical profile. Results of this study showed less but significant increase in the levels of plasma glucose, phospholipids, nitrite and nitrate, lipid peroxides with a decrease in plasma cholesterol. No significant changes were observed in the contents of total protein, albumin, globulin, HDL-C and LDL-C. However, SGPT activity increased significantly in persons exposed to only allethrin. Though the present investigation involving a limited number of human subjects indicates the onset of both protective changes as well as derangement in metabolism, a detailed and rigorous study is greatly warranted to arrive at a definite conclusion about the effects of pyrethroid mosquito repellents.

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I. Introduction

Pyrethroids are widely used insecticides in agriculture, household and for industrial purpose in India and other countries to get protection from mosquitoes, cockroaches and other insects (Tsuji et al, 2002; Bryant and Bite, 2003; Das et al, 2003; Kakko et al, 2003; Sinha et al, 2004; WHO, 2004; Narendra et al, 2007). Pyrethroids have been subdivided into two classes according to their structural, toxicological, and pharmacological differences. Structurally, type I pyrethroids (allethrin and prallethrin) do not contain a cyano group, while type II pyrethroids (deltamethrin, cypermethrin, and fenvalerate) contain the alpha-cyano group. Both non-cyano-substituted and cyano-substituted pyrethroids show insecticidal action and low toxicity to mammals. Allethrin and prallethrin (Figs. 1 and 2) are the chief constituents of various mosquito repellent-insecticides in India (Ramesh and Vijayalakshmi, 2001; Liu et al, 2003). Pyrethroid-induced neurotoxicity and other toxic (acute and chronic) symptoms, and their deleterious effects in humans and experimental animals caused a concern on their chronic use. Inhalation and the consequent entry of these compounds into circulation by their prolonged exposure leads to accumulation in tissues such as blood, nerve, adipose and other tissues causing effective damage (Kulkarni and Hodgson, 1980; El-Dessoky et al, 1986; Sein et al, 1987) mainly to plasma RBCs, other blood cells and vascular system (El-Elimay, 1986; Moya-Quiles et al, 1995). Bio-membranes are largely, if not totally, responsible for characteristic actions of many pyrethroids and are known targets for pyrethroid action and toxicity because of the lipophilic nature of pyrethroids (Moya-Quiles et al, 1994, 1996a,b; Narahashi et al, 1995; Narahashi, 1996; Kakko et al, 2003; Narendra et al, 2007). Earlier studies have revealed their acute toxic effects in experimental animals, but limited literature is available on prolonged use of these pyrethroid mosquito repellents (Ganga and Rajarajeshwari, 2001; Mishra and Singh, 2003; Kolaczynski and Curtis, 2004; Pankaj and Prahad, 2004; Narendra et al, 2007). The present preliminary study is, therefore aimed to evaluate systematically the effect of chronic exposure of pyrethroid-based mosquito repellents (allethrin and prallethrin) on human plasma profile which reflects the physiological status of the exposed individuals.

2. Materials and methods

2.1. Subjects

The volunteers were using either Jet® mosquito repellent coils or mats, both from Godrej Sara Lee Ltd., Mumbai, India. The coils are

Abbreviations: A/G, albumin/globulin; HDL-C, high density lipoprotein cholesterol; IU, international unit; LDL-C, low density lipoprotein cholesterol; LPO, lipid peroxidation; MR, mosquito repellent; NO, nitric oxide; NO2, nitrite; NO3, nitrate; RBC, red blood cells; SGOT, serum glutamate oxaloacetate transaminase; SGPT, serum glutamate pyruvate transaminase; VLDL-C, very low density lipoprotein cholesterol; w/w, weight/weight.

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Fig. 1. Chemical structure of aliethrin.

Fig. 2. Chemical structure of prallethrin.

Blood samples, drawn from human volunteers by venipuncture between 7 and 10 AM into heparinized test tubes, were used immediately for plasma analysis. Plasma glucose was estimated by Trinder (1969), amino acids by Moore and Stein (1948), triglycerides by Fossati and Principe (1982), cholesterol and HDL-C by Allain et al. (1974), LDL-C and VLDL-C by Friedwed et al. (1972), phospholipids by Connerty et al. (1961), glycolipids by Roughan and Batt (1968), nitric oxide (NOx and NOy) by Sastry et al. (2002), lipid peroxidation by Buege and Aust (1978), iron by Ramsay (1958), proteins by Reinhold (1953), albumin and globulin by Wootton (1974), SGOT and SGPT by Henry (1974) methods.

Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Controls I</th>
<th>Aliethrin users II</th>
<th>Prallethrin users III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose (mg/dl)</td>
<td>87.75±2.60a</td>
<td>98.49±1.80b</td>
<td>101.58±2.38c</td>
</tr>
<tr>
<td>Glycolipids (mg/dl)</td>
<td>333.12±4.27a</td>
<td>395.64±4.51b</td>
<td>395.40±1.37c</td>
</tr>
<tr>
<td>Total proteins (g/ml)</td>
<td>6.90±0.17a</td>
<td>6.32±0.03a</td>
<td>6.44±0.14a</td>
</tr>
<tr>
<td>Albumin (g/ml)</td>
<td>4.73±0.19a</td>
<td>4.39±0.17a</td>
<td>4.67±0.46a</td>
</tr>
<tr>
<td>Globulin (g/ml)</td>
<td>2.46±0.06a</td>
<td>2.40±0.11a</td>
<td>2.30±0.07a</td>
</tr>
<tr>
<td>A/G ratio</td>
<td>1.86±0.03a</td>
<td>1.96±0.04a</td>
<td>1.95±0.02a</td>
</tr>
<tr>
<td>Plasma amino acids (mg/dl)</td>
<td>5.50±0.10a</td>
<td>6.14±0.04a</td>
<td>6.08±0.03a</td>
</tr>
<tr>
<td>Plasma iron (µg/dl)</td>
<td>118.12±6.85a</td>
<td>82.91±16.30b</td>
<td>90.41±8.21c</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM, in each column followed by the same letter are not significantly different (P<0.05) from each other according to Duncan’s Multiple Range (DMR) test, n=12.
Table 2
Influence of chronic pyrethroid inhalation on plasma lipoprotein patterns

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Controls I</th>
<th>Allethrin users II</th>
<th>Prallethrin users III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>211.00 ± 5.08</td>
<td>184.46 ± 6.64</td>
<td>189.90 ± 4.35</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dl)</td>
<td>45.89 ± 4.41</td>
<td>49.83 ± 2.98</td>
<td>48.31 ± 7.29</td>
</tr>
<tr>
<td>LDL-cholesterol (mg/dl)</td>
<td>71.00 ± 6.68</td>
<td>56.41 ± 5.96</td>
<td>55.21 ± 7.29</td>
</tr>
<tr>
<td>VLDL-cholesterol (mg/dl)</td>
<td>10.57 ± 1.81</td>
<td>35.35 ± 2.49</td>
<td>36.81 ± 2.70</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>153.04 ± 9.17</td>
<td>18156 ± 16.17</td>
<td>175.93 ± 1.24</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM, in each column followed by the same letter
are not significantly different (P < 0.05) from each other according to Duncan's
Multiple Range (DMR) test. n = 12.

Fig. 3. Influence of allethrin and prallethrin inhalation in plasma nitrite and nitrate.

Table 3
Pyrethroid inhalation induced changes in plasma

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Controls I</th>
<th>Allethrin users II</th>
<th>Prallethrin users III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma lipid peroxidation</td>
<td>5.25 ± 0.06</td>
<td>5.84 ± 0.02</td>
<td>5.50 ± 0.50</td>
</tr>
<tr>
<td>Serum glutamate oxalo acetate</td>
<td>38.67 ± 3.40</td>
<td>27.54 ± 1.16</td>
<td>30.40 ± 1.95</td>
</tr>
<tr>
<td>transamine (SOCT) (U/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum glutamate pyruvate</td>
<td>33.83 ± 2.58</td>
<td>48.31 ± 5.18</td>
<td>42.57 ± 5.55</td>
</tr>
<tr>
<td>transamine (SGPT) (U/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospholipids (mg/dl)</td>
<td>246.46 ± 3.33</td>
<td>266.50 ± 1.78</td>
<td>262.61 ± 2.47</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM, in each column followed by the same letter
are not significantly different (P < 0.05) from each other according to Duncan's
Multiple Range (DMR) test. n = 12. IU = international unit.

Discussion

Increase in plasma glucose levels in the experimental subjects (group II allethrin-exposed subjects, group III prallethrin-exposed subjects) when compared to controls who do not use any pyrethroids to repellents suggests an interference of allethrin and prallethrin in glucose metabolism. In general, maintenance of stable levels of blood glucose is a complex process and is one of the finely regulated homeostatic mechanisms in which various tissues, hormones, enzymes and other factors take part (Eton et al., 1970; Kahn, 1985; Anderson and Gell, 1994). Besides, the intracellular metabolic adjustments and modulation of the sensitivity of insulin receptor action are responsible for glucose homeostasis. Blood glucose concentration is regulated by the net result of two processes glycogen synthesis and glycogenolysis in liver (Blavatnaya et al., 2001). In addition to this, the blood glucose levels are regulated by hepatic and renal gluconeogenic production of the glucose on one hand and degradation of peripheral glucose on the other (Randall et al., 1984). Although the observed increase in plasma glucose in this preliminary study is significant, the glucose level still falls within the normal range. To know the significance of increase in blood glucose concentration and its effects, further studies are needed. The observed increase in plasma glucose may be due to change in one or many of the above mentioned factors (Cremer and Seville, 1982; Manna et al., 2004). Further, the involvement of catecholamines and/or other hormones in this process cannot be ruled out. Increase in nitric oxide levels in plasma and increased lipid peroxidation suggest a role played by NO in regulation of blood glucose to keep glucose concentration in normal range, as NO signaling has been reported to play a new role in regulating glucose metabolism in insulin intensive tissues where it could function in parallel to insulin (Cremer and Seville, 1982; Cremer et al., 1983). Nitric oxide (NO) appears to protect membrane integration and function by blocking lipid-derived radicles and thereby antagonizing oxidative and photooxidative stress which affect glucose homeostasis (Burg, 1995; Hotta, 1997; Ramana et al., 2003). Hence an indirect role of nitric oxide may contribute to the observed change in blood glucose in the present study. Though very few data on blood glucose concentration and tissue glucose degradation (El-Dermederash et al., 2003; El-Dermederash et al., 2004; Eraslan et al., 2007) are available from humans or animals exposed to allethrin and prallethrin, previous studies revealed that an acute administration of clesmethrin type I and deltamethrin type II cause an increased rate of blood flow and glucose transport into tissues and also increased glucose utilization by brain and other tissues resulting in hypoglycemia in rats followed by some toxic symptoms such as tremors and convulsions, etc. (Cremer and Seville, 1982; Cremer et al., 1983). On the contrary, the data obtained from the present study showed an elevated plasma glucose concentration in humans exposed to chronic inhalation of allethrin or prallethrin. The results suggest there are mechanisms to keep that elevated glucose concentrations to meet the demand of increased utilization of glucose by tissues and also to prevent hypoglycemia which may arise as a result of increased glucose uptake by tissues. Decrease in plasma proteins and albuims followed by marked increase in plasma free amino acid levels in the present study point to increased degradation of proteins in allethrin/prallethrin-exposed subjects when compared to controls. Probably, enhanced activities of one or many proteases/peptidases might have contributed for the same. The decrease in iron and glycolipids in plasma of allethrin or prallethrin-exposed persons (Table 1) may be due to an enhanced transport of iron and glycolipids to blood cells and other cells. It should be noted that the toxicity towards various biochemical parameters observed in the present study may not be due to the impact of inert chemical constituents present in the formulation. Marked increase in concentration of VLDL-C followed by increase in triglyceride levels with significant change in cholesterol and HDL-C suggests some cardiovascular risk in group II and group III when compared to group I. However, a decrease in LDL-C and increased NO levels in plasma, suggest some protective measure (Doumas et al., 1971; Yousef et al., 2003). The magic role played by NO is suspected here in lowering plasma LDL-C levels and in increasing lipid peroxidation. Observed changes in plasma cholesterol, other lipids (triglycerides), lipoprotein patterns (VLDL-C and HDL-C), plasma SGOT and SGPT and lipid peroxidation (Tables 2 and 3) may reflect liver damage and be indicative of coronary and cardiovascular risk. Observed increase in the activity of plasma SGPT in allethrin using volunteers indicated liver damage,
probably by the direct effects of allethrin on membranes of hepatocytes, via interdigitation of pyrethroid between phospholipids, and/or indirect effects caused by products derived from pyrethroid metabolism. The operation of certain rapid protective mechanisms to counteract the above mentioned risk and damage is evident from enhanced generation of nitric oxide (NO). Though direct NO production was not determined, plasma nitrite (NO2) and nitrate (NO3) were determined which asserted an increased production indirectly by other possible protective mechanisms of nitric oxide (McCuskey et al., 1995; Nanji et al., 1995; Oekonomaki et al., 2004; Narendra et al., 2007). Although the significant changes observed in plasma constituents appear to be smaller and fall within normal range, clinical alterations can be ignored totally. In view of the disturbances in metabolism in experiments, a regular monitoring of health in humans exposed to pyrethroids is necessary since long-term occurrence of small disturbances would lead to any clinical manifestation and/or aggravate any disease condition and further use may predispose the subjects to clinical manifestations.

5. Conclusions

Increase in plasma glucose levels in exposed subjects appears to be an adaptive biochemical mechanism to prevent hypoglycemia. Protein degradation in exposed subjects results in an elevation of plasma free amino acids. There is some tissue damage (probably liver) and cardiovascular risk in exposed subjects and the operation of some counter acting mechanism(s) is also obvious. Increased production of plasma nitric oxide (NO), nitrate (NO3) and nitrite (NO2) levels were observed. Further studies are needed to correlate the toxic effects of prolonged use of allethrin and pyrethrin on health status of individuals.

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Supplementary data

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References


Full Length Research Paper

Allethrin-induced biochemical changes and properties of human erythrocyte membrane

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Erythrocyte membranes from twelve human volunteers exposed regularly to allethrin, a mosquito repellent of type-I pyrethroid, were analyzed for cholesterol (C), phospholipids (P), and individual phospholipid classes to assess changes induced by this toxicant. A decrease in C and P moieties with no change in C: P ratio was observed with allethrin exposure. A significant reduction in the amount of phosphatidyl serine (PS) was noticed indicating that PS is an allethrin sensitive phospholipid species. Furthermore, decreased red cell membrane lipid peroxidation (LPO) and with no change in osmotic haemolysis of erythrocyte was observed. Increased plasma and red cell nitrate and nitrite were evident suggesting that the bioavailability of nitric oxide may have rendered tolerance to erythrocyte membrane by protecting the cell from haemolysis and oxidative damage due to its free radical scavenging and antioxidant effects.

Keywords: Allethrin, nitric oxide, osmotic haemolysis, phosphatidyl serine, rbc biochemical changes.

INTRODUCTION

Pyrethroid insecticides have been used for more than 40 years in view of their wide availability, and consequently accounting for 25% of the world insecticide market (Kakko et al., 2003, Shafer et al., 2005). As such, their use has risen dramatically over the past 10 years in India (Ramesh and Vijayalakshmi, 2001). Available literature suggests that indoor pyrethroid exposure is of considerable magnitude in India and other countries including the United States (Bateman, 2000; Pankaj and Prahlad, 2004; Narahashi, 2000) as a result of the widespread use of pyrethroid-based repellents to control a variety of pests such as mosquitoes and cockroaches (Narahashi, 2000; WHO, 2000) due to their high insecticidal and low mammalian acute toxic effects (Kakko et al., 2003). Though severe toxicity of pyrethroids has been uncommon in developed countries, it appears to be common in developing countries because of their extensive and intensive use for agricultural and domestic purposes (Kakko et al., 2003, Shafer et al., 2005; Bateman, 2000). No relevant data or considerable literatures are available on the chronic toxic effects of these compounds in humans (Pankaj and Prahlad, 2004; Kolaczinski and Curtis, 2004; Mishra and Singh, 2003). Allethrin, a type-I pyrethroid, is among the top few commonly used insecticides having maximal human exposure for prolonged periods as it is used as a chief component of mosquito repellents (Anvita et al., 2006; Tsuji et al., 2002). In addition to inhalation, slow but significant absorption and accumulation in epidermis, (Ray and Forshaw, 2000) when these pyrethroids are used in closed and poorly ventilated areas expectedly expose humans to the risk of severe toxicity (Chen et al., 1991). There has been a growing concern among the public regarding the routine and prolonged use of mosquito repellents such as allethrin (Anvita et al., 2006; Tsuji et al., 2002). Biomembranes are wholly, if not largely, responsible for various pyrethroid induced toxicity. Since biomembranes are the known targets because of the lipophilic nature of the pyrethroids (Narahashi, 1996). Also evidences from the available literature suggests that metabolic status of nitric oxide (NO) and functional status between oxidative and antioxidant system are in close relationship with health (Tang et al., 2000; Huang et al., 2000). Recent reports revealed the involvement of nitric oxide in various physiological and pathological processes (Jun et al., 2000; Worthington et al., 1997; Zema et al.)
There is paucity of information concerning the effects on humans due to prolonged and long-term use of allethrin (Pankaj and Prahlad, 2004; Kolaczinski and Curtis, 2004; Mishra and Singh, 2003). The purpose of the present study is two fold: first, to detect the changes in red cell membranes of human volunteers exposed to regular use of allethrin, and second, to understand the role and status of nitric oxide in such users of allethrin.

MATERIALS AND METHODS

Subjects

Twelve human male volunteers, aged between 35 - 45 (mean age 41 ± 2 years) residing in Anantapur town in Andhra Pradesh taking local diet and using allethrin-containing mosquito repellent coil (C1: 1% w/w) for protection from mosquitoes during nights, were chosen as experimental subjects. All the subjects were exposed to allethrin for at least 8h/day and not more than 10h/day, and the subjects were not using other pyrethroids or any other insecticide for the purpose. Commercially available allethrin containing mosquito repellent coils designed for the release of the pyrethroid have been regularly used by the volunteers for the past 7 - 10 years. The protocol of the study was explained to the volunteers and their written consent was obtained. In the present study all volunteers were free from any chronic disease or illness and teetotalers with no smoking habit and free from use of any tranquilizers, drugs and anaesthetics. Controls (age, sex and diet matched) who did not use any mosquito repellent were selected for the study. The experimentation were explained to all the volunteers about and their written consent was obtained. This study was approved by institutional ethical committee (No.25/1/99-AWD). Blood samples from over night fasted subjects were used for the study.

Collection of blood and analysis

Blood samples drawn from human volunteers by venipuncture between 7 to 10 am into heparinized test tubes, were used immediately for plasma and red cell analysis. Erythrocyte membrane proteins were estimated by the method of Lowry et al. (1951). Membrane cholesterol was estimated as outlined by Zlatkis et al. (1953). Membrane phospholipids were estimated by the method of Connerty et al. (1961): Individual phospholipid classes in the red cell membrane were determined (Skipski et al., 1964), and membrane lipid peroxidation extent was measured by thiobarbituric acid (TBA) reaction with the formation of malondialdehyde (MDA) following the method of Buege and Aust (1978). Nitric oxide and nitrate in plasma and erythrocyte lysate were estimated by Griess reaction (Sastry et al., 2002).

Osmotic haemolysis of red blood cells

Isolated red blood cells (RBC) were incubated in different concentrations of NaCl ranging from 0.1 to 0.9% for 30 min with gentle stirring. Then RBC suspensions were centrifuged at 700 X g for 5 min and the optical density of the supernatant was determined at 540 nm (Nicak and Mojzis, 1992).

Isolation of erythrocytes

Erythrocytes were isolated by using the method of Beutler (1975). Anticoagulated blood were passed through the cellulose column and the filtrate was collected to remove lymphocytes, platelets etc. The filtrate was diluted with saline and erythrocytes were collected by centrifugation at 1000 rpm for 10 min. This washing step was repeated until the erythrocytes for study were obtained.

Erythrocyte membrane preparation

Erythrocyte membranes were prepared using the method adopted by Dodge et al. (1963). Erythrocyte suspension was washed with phosphate buffered saline (pH 7.2), and then cells were lysed with 5 mM phosphate buffer (pH 8.0) and spun at 15000 X g for 30 min. The supernatant was removed carefully and by using the same buffer the latter step was repeated to obtain haemoglobin-free ghosts for further analysis.

Erythrocyte membrane lipid analysis

Lipid extraction was done by the method adopted by Peeterways and Hanahan, (1964). Lipids extracted from a portion of the erythrocyte membrane suspension with isopropanol and chloroform and aliquots were taken for estimation of cholesterol and phospholipids. Erythrocyte membrane phospholipids separated on silica gel H (Merck) using two dimensional thin layer chromatography with chloroform-methanol-aqueous ammonia 65:35:5 (v/v) as the first solvent and chloroform-acetone-methanol-acetic acid-water 50:20:10:5 (v/v) as the second solvent were measured as inorganic phosphorus after digestion with sulphuric acid (Fiske and Subbarow, 1925).

Statistical data analysis

The results of the study are expressed as mean ± SD. Statistical analysis was performed using student t- test. The significance was set at 0.05.

RESULTS

Data presented in Table 1 suggested a significant decrease in erythrocyte membrane cholesterol (25%) and phospholipid (21%) moieties as well as membrane lipid peroxidation (17%) with no significant change in membrane protein content in allethrin users. However, there was no change in the membrane C: P ratio. Pyrethroid use did not alter the contents of erythrocyte membrane individual phospholipid classes viz., phosphatidyl ethanolamine (PE), phosphatidyl inositol (PI), phosphatidyl choline (PC), but the concentration of erythrocyte phosphatidyl serine (PS) decreased significantly (Figure 1). Increased concentrations of nitrite and nitrate in plasma and lysate suggest an increased production of nitric oxide in human volunteers exposed to mosquito repellents when compared to controls (Figures 2A and Figures 2B). There was no significant change in osmotic haemolysis of erythrocyte when red cells from allethrin users were incubated in different concentrations of NaCl (0.1 to 0.9%) when compared with controls (Figure 3).

DISCUSSION

The only in vitro experiments of Moya-Quiles et al. (1994,
Table 1. Alterations in biochemical composition of erythrocyte membrane induced by allethrin exposure

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Controls</th>
<th>Allethrin users</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane proteins (mg/dl)</td>
<td>241.98±5.29</td>
<td>248.68±8.73</td>
<td>NS</td>
</tr>
<tr>
<td>Membrane cholesterol (µg/mg protein)</td>
<td>100.35±2.58</td>
<td>75.48±7.53</td>
<td>0.001</td>
</tr>
<tr>
<td>Membrane phospholipids (µg/mg protein)</td>
<td>112.98±4.08</td>
<td>88.04±8.03</td>
<td>0.001</td>
</tr>
<tr>
<td>Membrane lipid peroxidation (µmol/mg protein)</td>
<td>0.59±0.11</td>
<td>0.49±0.03</td>
<td>0.008</td>
</tr>
<tr>
<td>C/P ratio</td>
<td>0.88</td>
<td>0.89</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD. n = 12. NS = Not Significant

Figure 1. Effect of allethrin exposure on individual erythrocyte membrane phospholipids classes. Values are expressed as means ± SD. n = 12. PS = Significantly different from control (P < 0.001).

Figure 2A. Effect of allethrin exposure on NO₂ and NO₃ of plasma. Values are expressed as means ± SD. n = 12. P < 0.05.

1995) suggested a possible insertion and aggregation of allethrin in the lipid bilayer of model membranes creating special domains with a consequent increase in membrane instability and also allethrin induced fluidizing effect. They also noticed that allethrin modified bilayer order in the temperature range of phase transition when incorporated into liposomes made with DMPC, DPPC and DSPC. Studies using DPH and TMA-DPH fluorescence polarization technique revealed no change in membrane fluidity when membranes from normal human
human erythrocytes were incubated in presence of allethrin (Moya Quiles et al., 1995). The present study thus compared the biochemical composition of erythrocyte membranes of allethrin users and humans that were not exposed to this repellent. The present study observation of decrease in membrane cholesterol, total phospholipid concentrations and membrane lipid peroxidation with no change in membrane protein moiety and C: P ratio (an index of fluidity) in volunteers using allethrin suggested no change in membrane fluidity. However, it led to a significant change in lipid packing in membrane, facilitating the easy entry of the pyrethroid into the bilayer which may lead to increased interaction among membrane constituents and also with the pyrethroid. The fluidity of the membrane has been shown to depend mainly on cholesterol content and the orientation of lipid molecule and their composition of fatty acyl chains in the membrane. lipophilic nature of the pyrethroid facilitates its miscibility with the hydrophobic moiety and probably by forming pyrethroid-phospholipid mixture patches (aggregates/domains) and thereby substituting the depleted lipid content and restoring the basic structure and physical state of the bilayer without affecting fluidity of the biomembranes (Moya Quiles et al., 1995).

No change in the contents of PE, PC, PI and SM, but a change in the content of PS suggested the specific interaction of allethrin with phospholipid PS which is an important and sensitive phospholipid species that is influenced by allethrin. Phosphatidyl serine is an important membrane phospholipid that is associated with several regulatory, structural and other proteins, and membrane skeletal proteins such as spectrin localized within the membrane through their interaction with phosphatidyl serine. On the other hand, disruption of lipid asymmetry leading to exposure of PS on the outer surface of the plasma membrane creates a procoagulate surface on
platelets that may serve as a trigger for macrophage recognition of apoptic cells (Manno et al., 2000). The structure and physico-chemical properties of allethrin may help in the formation of aggregates without affecting the hydrocarbon or polar head group domains of the bilayer. Furthermore, allethrin may lie in an extended orientation in the bilayer with the carbonyl group of cyclopentane ring at the lipid-water interface protecting membrane from disordering effect (Moya Quiles et al., 1994). All these likely changes would affect the lipid packing order in erythrocyte membrane and possibly in other cellular membranes. No significant change in haemolysis of erythrocyte collected from allethrin exposed humans when treated at different concentrations of NaCl observed in the present study suggested the development of resistance in vivo against osmotic haemolysis. However Moya-Quiles et al. (1994, 1995) reported allethrin induced haemolysis when normal red cells were incubated for a period of 3 h. Increments in nitrate and nitrite of plasma and lysate in allethrin users would indicate the possible role of nitric oxide (NO) in rendering tolerance against haemolysis (McCuskey et al., 1995; Nanji et al., 1995; Oekonomaki et al., 2004). The present observation of decreased membrane LPO in suggested reduced susceptibility of membrane for damage. Decreased membrane phospholipid moiety and increased NO scavenging effect on free radicals might have contributed for the observed decrease in LPO in erythrocyte membrane of humans using allethrin. The essential role of NO as an endothelial vasodilator in the maintenance of cardiovascular homeostasis as well as intact erythrocytic functions has been demonstrated (Barbosa et al., 2006; Minneci et al., 2005). It is also evident from earlier studies that non availability of NO may lead to haemolysis and other pathological consequences (Minneci et al., 2005; Barvitenko et al., 2005; Peters et al., 2003).

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