MOSQUITO REPELLENT PYRETHROID INDUCED BIOCHEMICAL AND BIOPHYSICAL CHANGES IN PLASMA AND ANTIOXIDANT STATUS IN HUMAN MALE VOLUNTEERS EXPOSED TO LONG TERM ALLETHRIN AND PRALETHRIN INHALATION

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ABSTRACT

Allethrin and prallethrin are type-I pyrethroids commonly used for domestic and agricultural purposes to get protection from mosquitoes and other insects. Use of these pyrethroids for longer durations (i.e. inhalation 8 hours per day not more than 10 hours per day) and for prolonged periods in the form of coils and mats, results in biochemical and biophysical changes in erythrocyte biomembrane has been established. The effect of allethrin and prallethrin on plasma nitrite and nitrate concentrations on the activities of red cell enzymes acetyl cholinesterase (AChE) and antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (Gpx) the content of reduced glutathione (GSH) and erythrocyte membrane lipid peroxidation (LPO) were determined in human male volunteers who has been exposed to allethrin and prallethrin for the past 7-10 years. The results were compared with controls (who did not use any mosquito repellents). It was observed that there was an increase in plasma nitrite and nitrate, increase in activities of defense enzymes and observed decrease levels of erythrocyte membrane lipid peroxidation (LPO), but a decreased activity of acetyl cholinesterase (AChE) was observed in allethrin and prallethrin exposed subjects. This study revealed a modest increase in nitric oxide production, increased antioxidant status followed by enhanced oxidative stress. A possible role of nitric oxide in the above changes cannot be ruled out, for which further experimentation is required.

KEYWORDS: Allethrin, Prallethrin, Antioxidant Enzymes, Acetyl Cholinesterase, Lipidperoxidation, Nitrite and Nitrate

Abbreviations: AChE: Acetyl Cholineesterase, SOD: Superoxide Dismutase, CAT: Catalase, Gpx: Glutathione Peroxidase, GSH: Reduced Glutathione, LPO: Lipid Peroxidation

INTRODUCTION

Pyrethroids are the most commonly used insecticides in India and other countries to get protection from mosquitoes, insects and pests for household and agricultural (Fradin, 1998; Moya-Quiles et al, 1995; Narendra et al, 2007; 2008a; 2008b; Sinha et al, 2004; Tsuji et al, 2002) purposes due to their high insecticidal activity and low toxicity in mammals (Elliot et al, 1967; Elliot, 1977; Miyamoto, 1976). Allethrin and prallethrin are among the most widely used pyrethroids in India and other countries including the United States (Liu et al, 2003; Ramesh and Vijayalakshmi, 2001). Pyrethroid-induced neurotoxicity, other toxic (acute and chronic) symptoms their deleterious effects on humans and experimental animals in recent reports aroused a concern among public regarding their chronic use (Kakko et al, 2003;
Inhalation is the route of exposure in humans inhaling pyrethroids continuously for 8 hours a day leads to entry of these compounds into circulation with maximal accumulation in all biomembranes and in specific tissues such as blood, nerve, adipose and other tissues due to their lipophilic nature (Sinha et al, 2004; Theeraphap et al, 2003). As inhaled pyrethroids directly enter the circulation and different tissues rapidly. Further distribution of these inhaled pyrethroids to different tissues cause effective damage. However, if these compounds reach the nervous system of mammals, including man in sufficient concentrations, they can cause adverse neurotoxic effects (Eriksson and Fradricksson, 1991; Malaviya et al, 1993; Narahashi, 1992). The pyrethroids are neurotoxins, based on their structure and their toxic effects. Observed in the rat and human beings, they are classified into two groups, namely type-I and type-II compounds. Type-I pyrethroids may cause ‘T syndrome’ with aggressive sparring, increased sensitivity to external stimuli and fine tremors progressing to whole-body tremors and prostration (Verschoyle and Barnes, 1972; Verschoyle and Aldridge, 1980). Type II pyrethroids produce ‘Cs syndrome’, characterized by burrowing, profuse salivation and coarse tremors progressing to choreoathetosis and chronic seizure (Ray and Cremer, 1979; Verschoyle and Aldridge, 1980).

Pyrethroids are highly hydrophobic compounds and this suggests that their action in biological membranes might be related to association with integral proteins and with phospholipids (Michelangeli et al, 1990). Biomembranes are largely, if not totally, responsible for various pyrethroid induced toxicity. Since biomembranes are the known targets because of the lipophilic nature of the pyrethroids (Kakko et al, 2003; Moya-Quiles et al, 1994; 1996a; 1996b; Narahashi et al, 1995; 1996; Narendra et al, 2007; 2008a; 2008b). Current literature also reveals that reactive oxygen species have been implicated in the toxicology of pyrethroids (Kalle et al, 1999). Hence the present study is aimed at investigating and evaluating the changes in antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and GPx and reduced concentration of glutathione (GSH) in erythrocytes of exposed to inhalation of allethrin and prallethrin subjects and the activities of red cell enzyme acetyl cholinesterase (AChE) and erythrocyte membrane lipid peroxidation (LPO) and plasma nitrite (NO$_2$) and nitrate (NO$_3$) in human subjects.

**MATERIALS AND METHODS**

**Subjects**

The volunteers were using either Jet® mosquito repellent coils or mats, both from Godrej Sara Lee Ltd, Mumbai, India. The coils are composed of (w/w) 0.1% d-trans allethrin, 52.9% wood flour, 35% coconut shell powder, 12% starch, and the mats contained (w/w) 1.6% d-trans prallethrin and 98.4% relevant ingredients as indicated by the manufacturers. Release of the pyrethroid insecticide is either by burning the coil or placing the mat in the commercially available electric devices. All the subjects were known to get exposed to allethrin or prallethrin for at least 8h/day but not 10h/day, and the subjects had no known history of exposure to any other similar pyrethroids. Three groups, each group consisting of 24 male volunteers aged between 35-45 years, included in the present study were: Group I, controls who did not use mosquito repellents; Group II, allethrin exposed subjects; Group III prallethrin exposed subjects. All the volunteers were well explained about the experimentation and their written consent was obtained. This study was approved by the institutional ethical committee. Blood samples from overnight fasted subjects were used for the study. All the volunteers in the present study were free from any other chronic disease or illness, and, were teetotalers with no smoking habit and free from use of any tranquillizers, drugs and anaesthetics.
Blood Collection and Sample Preparation

A 10 ml sample of blood from study subjects was obtained from the brachial vein and each 5 ml collected in vials containing heparin. In one blood sample, erythrocytes were separated by centrifugation at 1273 g (3000 rpm) for 15 min. The buffy coat was removed and erythrocytes were washed three times with physiological saline. Aliquots of erythrocytes were kept at -20°C except the samples for catalase assay. The other 5 ml of the blood sample was used for the study of plasma nitrite and nitrate, erythrocyte membrane lipid peroxidation. The samples were transported on ice to the laboratory and were processed within two hours.

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.

Lipid Peroxidation in Erythrocytes

The extent of lipid peroxidation was measured by the formation of malondialdehyde (MDA) by using the method of (Buege and Aust, 1978). One ml of erythrocyte membrane was taken in a test tube to which 2 ml of reagent (15% w/v TCA, 0.375% w/v TBA and 0.25N HCl) was added and kept in boiling water bath for 15 minutes and the contents were allowed to cool and then centrifuged at 1000 g for 10 minutes. The supernatant was transferred into a separate test tube and the absorbance of the sample was read at 535 nm by a UV/Visible spectrophotometer against the reagent blank assuming the molar extinction coefficient to be 1.56x10^5.

Measurement of Plasma Nitrite and Nitrate

Plasma samples were treated with 30% zinc sulfate to deproteinize samples followed by centrifugation at 4000 x g for 5 min. Nitrite was determined by the method of Sastry et al, (2002) from 1.0 ml aliquots of plasma using Griess reagent (1% sulfanilamide, 2.5% phosphoric acid, and 0.1% 1-naphthylethylene diamine). One ml 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.

Acetylcholinesterase Activity in Erythrocytes

Acetylcholinesterase activity in erythrocytes was measured by (George and Abernethy, 1983). The rate of hydrolysis of acetylthiocholine iodide (substrate) in erythrocyte suspension at pH 7.6 was measured at 440 nm by the reaction of thiocholine iodide with DTNB to give the yellow 5-thio-2-nitrobenzoate anion with UV spectrophotometer. The enzyme activity was expressed as KU/L. Haemoglobin in blood was estimated using Drabkin’s reagent by the method of (Dacie and Lewis, 1984). The protein content was determined by the method of Lowry et al, (1951).
Determination of Plasma Biochemical Profile

The activities of alkaline phosphatase, lactate dehydrogenase, carbonyl groups and –SH groups were determined.

Erythrocyte Antioxidant Enzyme Activities

Erythrocytes were washed thrice with 0.9% Nacl and suspend in 1 volume of 0.9% Nacl. The packed cell volume was adjusted to 5% with PBS-pH 7.5 (10 mM phosphate buffer saline). Hemoglobin content in erythrocytes was determined. Reduced glutathione (GSH) content was estimated and expressed as µmol/g Hb. The superoxide dismutase (SOD) activity was measured based on the ability of the enzyme to inhibit the autoxidation of adrenaline and activity was expressed as units/mg Hb/min. The catalase (CAT) activity in hemolysate was estimated and the activity of the enzyme was calculated using the extinction coefficient of H₂O₂ as 0.071 cm⁻¹ mol⁻¹ and expressed as IU x 10⁴/g Hb at 37°C. The glutathione peroxidase (GPx) activity was measured and the activity was expressed as µmol of glutathione oxidized min/mg Hb.

Statistical Analysis

The results of the study are expressed as mean ± SEM. Statistical analysis was performed using Duncan’s Multiple Range (DMR) test. The significance was set at (P ≤ 0.05).

RESULTS

Data presented in table 1 suggests that a significant increase in the activity of antioxidant enzymes such as superoxide dismutase (+9%, +10%), catalase (+12%, +10%) GSH (+20%, +21%) and GPx (-13%, -21%) is reduction in concentration of glutathione peroxidase (GPx) in erythrocytes of experimental subjects to pyrethroids, group II and III when compared to controls group I. The data presented in Table 2 indicated that exposure of two different pyrethroids allethrin and prallethrin by inhalation but significant increase in enzyme activities of plasma ALP and LDH of mosquito repellent pyrethroids and control groups. Experimental subjects (allethrin users and prallethrin users) significantly increased (p≤0.05) plasma enzyme activities when compared to control group. Plasma protein carbonyl content levels increased significantly in exposed subjects (group II and III) when compared to controls, and total sulphydryl groups were significantly decreased in group II and III human exposed subjects, respectively when compared to controls (group I) who do not use any mosquito repellents.

Plasma lipid peroxidation was significantly increased in allethrin and prallethrin subjects when compared to controls our previous report (Narendra et al, 2007, 2008a, 2008b). Data presented in Figure 1 suggests that a significant decrease in erythrocyte membrane lipid peroxidation (-16%, -16%) in experimental subjects (group II and III) when compared to controls, significant decrease (p≤0.008) our previous report (Narendra et al, 2007, 2008a, 2008b). Figure 2 shows that there was significant inhibition of acetyl cholinesterase activity (-19%, -16%) in red cell membrane in experimental subjects (group II and III) when compared to controls. Increased concentration of nitrite (+13%, +15%) and nitrate (+20%, +19%) in plasma suggest an increased production of nitric oxide in human volunteers exposed to mosquito repellent pyrethroids (allethrin and prallethrin) when compared to controls Figure 3. Nitric oxide levels were increased in plasma and red cell lysate of controls and mosquito repellent pyrethroid users. A significant increase (p≤0.05) in protein carbonyl content, significant decrease (p≤0.05) in total sulphhydril groups were followed by a significant increase (p≤0.05) in nitrites/nitrates of plasma and erythrocyte lysate of allethrin and prallethrin experimental subjects when compared to
controls Figure 3.

DISCUSSIONS

Pyrethroid based allethrin and prallethrin are enter easily into humans who are exposed to them for continuously 8-10 hours a day; these compounds enter into circulation with maximal accumulation in all biomembranes and in specific tissues such as blood, nerve, adipose and other tissues with an increased uptake due to their lipophilic nature (Sinha et al, 2004; Theeraphap et al, 2003). In the present study there was a significant decrease in erythrocyte membrane lipid peroxidation previous report (Narendra et al, 2007; 2008) and acetyl cholinesterase activity was inhibited in red cell membrane of allethrin and prallethrin exposed subjects when compared to controls (who did not use any mosquito repellents).

There was an increase in activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), decreased glutathione (GSH) in erythrocytes and decreased concentration of glutathione peroxidase (GPx) in erythrocytes of exposed subjects (group II and III) when compared to controls. Superoxide anion radicals (O$_2^-$) hydrogen peroxide (H$_2$O$_2$) and hydroxyl radicals (OH) are palls of oxidative metabolism. Damage at the cell level by oxidants is attenuated by antioxidant enzymes such as SOD, CAT and GPx. Oxidative stress, generated by xenobiotics, induces disturbances in antioxidant enzyme systems. Recent reports show the generation of free radicals in fenvalerate and cypermethrin-induced pyrethroid treated mice and rats an in increased activity of superoxide dismutase and catalase (Gabbinelli et al, 2002; Maiti et al, 1995). Pyrethroids can be expected to have two modes of action: it may induce oxidative stress and, it is a hydrophobic compound it may accumulate in cell membrane and disturb membrane structure (Gabbinelli et al, 2002).

The activities of two cytosol antioxidant enzymes, SOD, and CAT, were altered in table 1. Pyrethroids and their esters, from cyanohydrins, which decompose to cyanides and aldehydes. Cyanide ions are mainly converted to thiocyanate and CO$_2$. The aldehydes and other lipophilic conjugates may produce oxidative stress in pyrethroid toxicity. Although only few studies have investigated the effects of pyrethroids on AChE in vivo, the results of these studies are contradictory (Bandyopadhyay, 1982; Gazula and Kosagi, 1995; He et al, 2002; Hossain et al, 2005; Reddy et al, 1991). In the present study inhibition of AChE activity has been inhibited in pyrethroid exposed subjects (group II and III) when compared to controls. Inhibition of AChE is believed to be the principal mode of action of pyrethroid compounds.

The inhibition in AChE activity in target tissues is often followed as a measure of pesticide intoxication (Jayatatnam and Maroni, 1994; Kale et al, 1999). In many of the earlier studies, correlation between blood AChE inhibition and inhibition in target tissues has been shown (Kale et al, 1999; Koshakji et al, 1973; Su et al, 1971; Yang and Dettbarn, 1996). Allethrin and prallethrin of AChE in RBC inhibited the activity, AChE is synthesized in liver and RBC (Kale et al, 1999). The present results also show significant correlation between decrease in erythrocyte membrane lipid peroxidation and inhibition of AChE activity in RBC. Nitric oxide (NO) a labile molecule with half life of only a few seconds, is synthesized mainly in the endothelium (Moncada et al, 1991). It is rapidly oxidized by tissue oxygen to the stable end products, nitrate (NO$_3^-$) and nitrite (NO$_2^-$).

In the circulation, nitrite is almost converted to nitrate by hemoglobin (Stamler et al, 1992). The best index of overall NO production, therefore, is the total concentration of both nitrate and nitrite. An increased nitric oxide levels in plasma compartments of experimental are evident from measurements of nitrite and nitrate concentrations in plasma. Nitric oxide is endogenously produced chiefly by endothelial cell nitric oxide synthase (NOS) and is released into blood
stream where it is quickly scavenged by Hb in erythrocyte or oxidized to nitrite and further oxidized to nitrate (Ignarro et al, 1993). In addition a significant increase of nitric oxide (NO) production occurs in other tissues as different isoforms of NOS exist.

Nitric oxide plays a principle role in basal blood flow regulation and vascular homeostasis. Besides many other physiological processes are mediated and regulated by direct or indirect actions of NO and/or NO derived species. NO is capable of diffusion to great distances at physiological oxygen tension in tissue maintaining a teleological balance. This has been observed in erythrocytic red cell mass and plasma mass (Wang et al, 2004). Though direct NO production was not determined, plasma nitrite (NO₂) and nitrate (NO₃) were determined and observed in the present study asserted an increased production and bioavailability of nitric oxide that might have rendered tolerance against haemolysis by the free radical scavenging effect and indirectly by other possible protective mechanisms of nitric oxide (McCuskey et al, 1995; Nanji et al, 1995; Narendra et al, 2007; 2008; Oekonomaki et al, 2004).

CONCLUSIONS

Increase in erythrocyte membrane antioxidant enzymes such as SOD, Catalase, and decreased GSH, decreased activity of GPx exposed subjects appears to be an adaptive biochemical changes in erythrocyte membrane. Decreased levels of erythrocyte membrane lipid peroxidation and acetyl cholinesterase activity. Increased production of plasma nitric oxide (NO), nitrite (NO₂) and nitrate (NO₃) levels were observed. Further studies are needed to correlate the toxic effects of prolonged use of allethrin and prallethrin on health status of individuals.

Table 1: Activities of Antioxidant Enzymes and Glutathione Levels in Control and Allethrin and Prallethrin Subjects

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Controls</th>
<th>Allethrin Users</th>
<th>Prallethrin Users</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide Dismutase (SOD) (Units / mg Hb/min)</td>
<td>6.24±2.82</td>
<td>9.83±2.76</td>
<td>10.12±2.86</td>
</tr>
<tr>
<td>Catalase (CAT) (IU/10⁴/gm Hb)</td>
<td>12.08±1.74</td>
<td>17.06±1.82</td>
<td>17.81±1.86</td>
</tr>
<tr>
<td>Red Cell Reduced glutathione (GSH)(µmol/gm Hb)</td>
<td>8.82±0.08</td>
<td>10.60±0.08</td>
<td>10.65±0.05</td>
</tr>
<tr>
<td>Glutathione Peroxidase (GPx) (µmol of GSH oxidized/min/mg Hb)</td>
<td>17.86±1.62</td>
<td>11.58±1.55</td>
<td>12.75±1.48</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 = 24.

Table 2: Effect of Chronic Pyrethroid Inhalation on Plasma ALP and LDH, Carbonyl Content, SH Groups in Human Male Volunteers

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Controls</th>
<th>Allethrin Users</th>
<th>Prallethrin Users</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline Phosphatase (ALP) (IU/L)</td>
<td>60.24±2.82</td>
<td>86.12±2.06</td>
<td>89.15±2.64</td>
</tr>
<tr>
<td>Lactate Dehydrogenase (LDH) (IU/L)</td>
<td>312.04±10.54</td>
<td>417.06±31.82</td>
<td>470.81±34.86</td>
</tr>
<tr>
<td>Carbonyl groups (nmols/mg protein)</td>
<td>0.82±0.08</td>
<td>1.65±0.08</td>
<td>1.54±0.05</td>
</tr>
<tr>
<td>SH groups groups (nmols/mg protein)</td>
<td>4.34±0.62</td>
<td>2.58±0.55</td>
<td>2.75±0.48</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 = 24.
Mosquito Repellent Pyrethroid Induced Biochemical and Biophysical Changes in Plasma and Antioxidant Status in Human Male Volunteers Exposed to Long Term Allethrin and Prallethrin Inhalation

Figure 1: Effect of Pyrethroids on Erythrocyte Membrane Lipid Peroxidation (LPO)

Values are expressed as Mean ± SEM, in each column followed by the same letter are not significantly different ($P \leq 0.05$) from each other according to Duncan’s Multiple Range (DMR) test, n = 24.

Figure 2: Effect of Pyrethroids on RBC Acetyl Cholinesterase (AChE)

Values are expressed as Mean ± SEM, in each column followed by the same letter are not significantly different ($P \leq 0.05$) from each other according to Duncan’s Multiple Range (DMR) test, n = 24.

Figure 3: Influence of Allethrin and Prallethrin Inhalation in Plasma Nitrite and Nitrate

Values are expressed as Mean ± SEM, in each column followed by the same letter are not significantly different ($P \leq 0.05$) from each other according to Duncan’s Multiple Range (DMR) test, n = 24.

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