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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 phosphotidyl 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.,

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Abbreviations: DMPC: dimyristoylphosphatidylcholine; DPPC: dipalmitoylphosphatidylcholine;DSPC:distearoylphosphatidylcholine; DPH: 1,6-diphenyl-1,3-5-hexatriene; TMA-DPH: 1-[4-(trmethylammonium) phenyl]-6-phenyl-1,3,5-hexatriene.

2001; Zhou et al., 1994). 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 (0.1% 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 tranquillizers, 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). Nitrite 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 iso-propanol 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 50:20:10:10:5 (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., sphingomylin (SM), 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 produc-tion 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 incu-bated 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

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

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

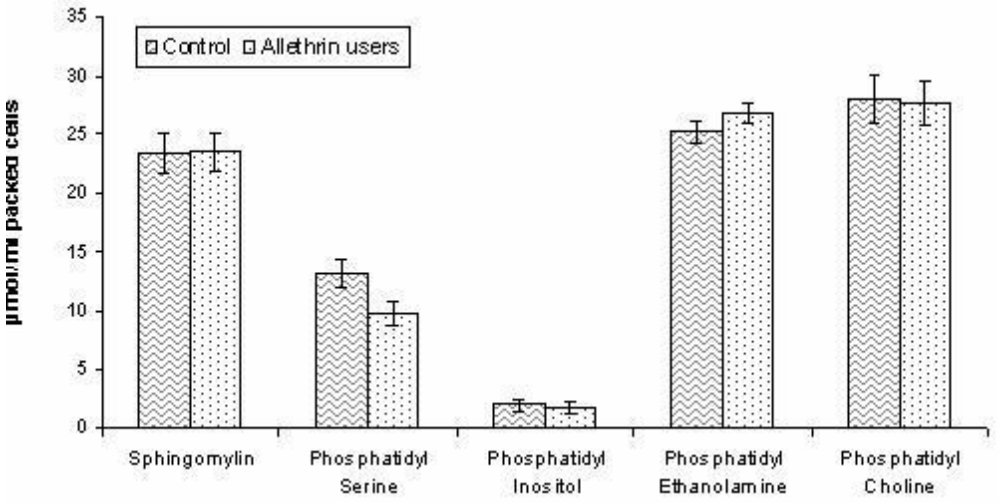

Figure 1. Effect of allethrin exposure on individual erythrocyte membrane phospholipids classes. Values are expressed as means ± SD. n = 12. PS= Significantly different from control ($P < 0.001$).

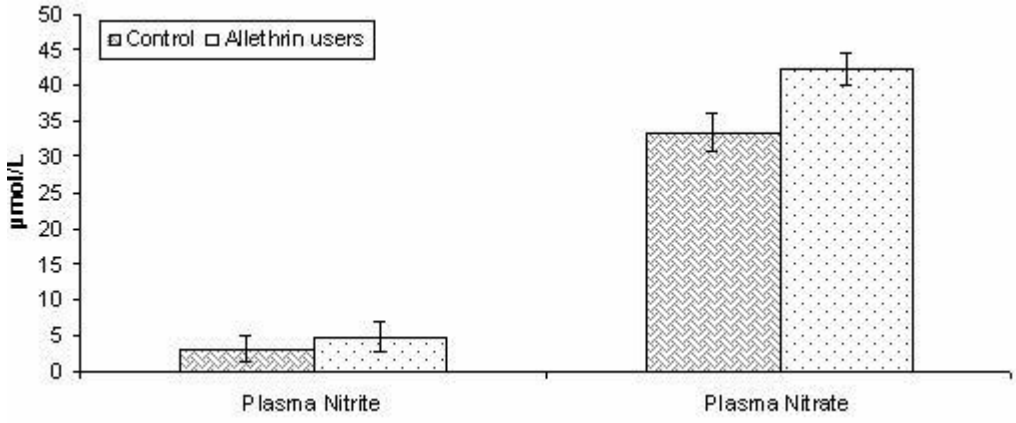

Figure 2A. Effect of allethrin exposure on NO_2 and NO_3 of plasma Values are expressed as means ± SD. n = 12. $P < 0.05$.

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 bilayerorder in the temperature range of phase transition when incorporated into liposomes made with DMPC, DPPC and DSPC. Studies using DPH and TMA-DPH fluores-cence polarization technique revealed no change in membrane fluidity when membranes from normal human

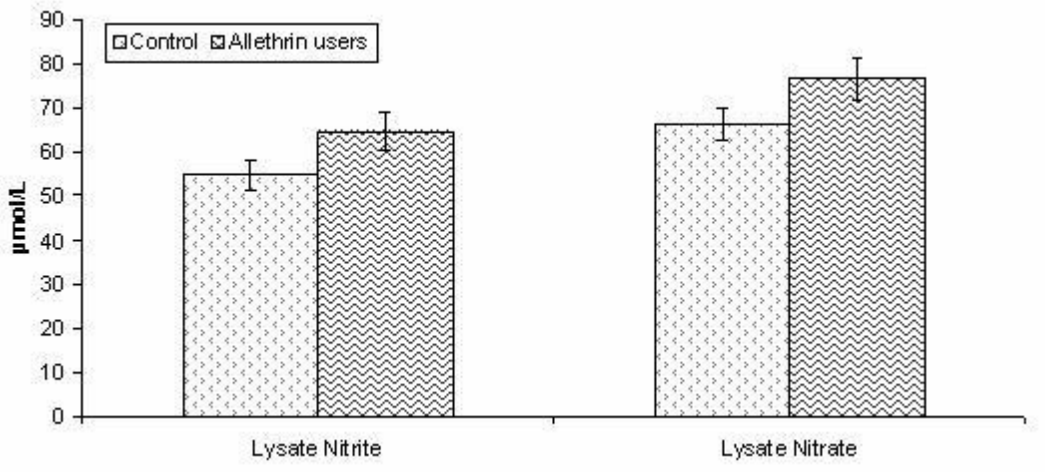

Figure 2B. Effect of allethrin exposure on NO_2 and NO_3 of lysate. Values are expressed as means± SD. n = 12. $P < 0.05$

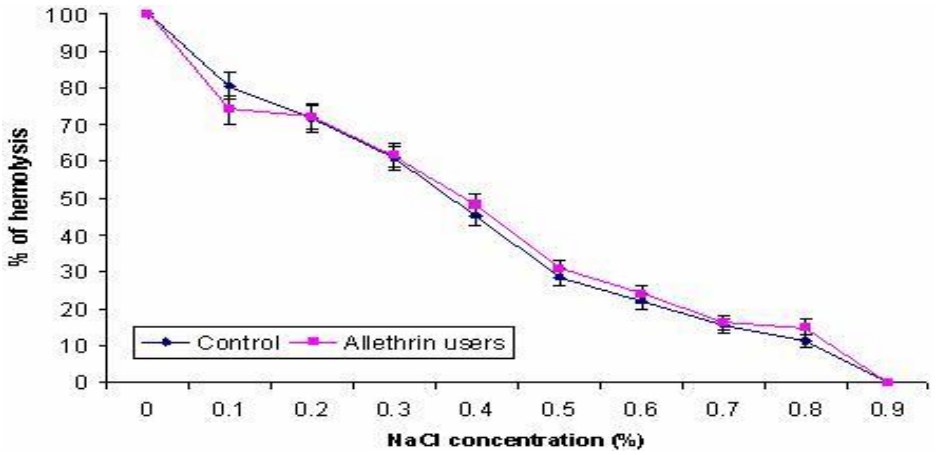

Figure 3. Changes in human red cell osmotic fragility as induced by allethrin exposure. Values are expressed as means ± SD. n = 12.

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 pyrethriod 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. liphophilic nature of the pyrethroid facilitates its miscibility with the hydrophobic moiety and probably by forming pyrethriod- phospholipid mixture patches (aggregates/domains) and thereby substituting the depleted lipid content and restoring the basic structure and physic-cal state of the bilayer without affecting fluidity of the biomembranes (Moya Quile 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 interacttion 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 develop-ment of resistance *in vivo* against osmotic haemolysis. However Moya-Quiles et al. (1994, 1995) reported alle-thrin-induced haemolysis when normal red cells were incubated for a period of 3 h. Increments in nitrite and nitrate 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. De-creased 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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