Pyrethroid-induced biochemical changes and properties of human platelet membrane

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Abstract

The effect of allethrin and prallethrin based mosquito repellent pyrethroids on human platelet membrane, membrane cholesterol, phospholipids, lipid peroxidation in platelet membrane were investigated. Humans were chronically exposed to 7-10 years continuously for 8-10 hours per day by inhalation of these compounds exposure enter into circulation and changes in platelet membranes. Platelet membrane fluidity was determined by DPH and pyrene fluorescence anisotropy experiments show that allethrin and prallethrin exposed subjects, compared with controls, induced a significant decrease in platelet membrane fluidity measured by DPH and pyrene. A significant increase in the lipid peroxidation both exposed subjects. A significant decrease in the activity of platelet membrane-bound enzymes, the activity of total ATPase, Na^+/K^+-ATPase activity and Ca^{2+}-dependent ATPase activity and Mg^{2+}-ATPase are respectively.

Keywords: Pyrethroids; platelet membrane; ATPases; membrane fluidity; allethrin; prallethrin; lipid peroxidation

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Introduction

Pyrethroids are the widely used insecticides due to their potential insecticidal activity in India and other countries to get protection against mosquitoes and other insects for various domestic and agricultural purposes (Yoshio et al., 1999; Kakko et al., 2003; Moya-Quiles et al., 1995; Narendra et al., 2007; 2008a;b; Saim and Maeshwari 2013; Madhubabu and Yenugu 2014). Over the half of world population have been using pyrethroid insecticides which may account for more than 25% of the insecticide market of the industrial countries in 90's and their demand/use is increasing now in these countries (Casida and Quistab, 1998; Timothy et al., 2005), as prevalence of mosquitoes and other insects are more in many endemic parts

of the world. Initially, these pyrethroids were thought to be highly toxic to insects and less toxic to humans (Shaw and Chadwick, 1998). Now pyrethroid induced neurotoxicity and other toxic effects ranging from whole body tremors to convulsions and death are well documented (Timothy et al., 2005; Heredorf et al., 2004; He et al., 1989; Dorman and Beasley, 1991; Soderlund et al., 2002). There is few published data available so far on the effects of pyrethroids on humans, and now slowly the facts related to their toxicity are coming into light. The fatality with pyrethroids in India has been reported to be 12.5 to 25% (Pankaj and Prahlad, 2004). Allethrin and prallethrin are among the most widely used pyrethroid insecticides. Allethrin poisoning can be much frequent due to its easy accessibility as mosquito repellent and/or insecticidal sprays etc (Sinha et al., 1995) and often reports of pyrethroid poisoning in India are evident (Mishra and Singh, 2003; Ganga and Rajarajeswari, 2001). However, it is clear that no relevant data on chronic effects exist in open scientific literature related to pyrethroid toxicity in humans and animals (Pankaj and Prahlad, 2004; Kolaczinski and Curtis, 2004). Since these pyrethroids are used routinely and/or regularly as mosquito repellents and/or through agricultural/gardening sprays exposing people continuously to the inhalation of these compounds for longer durations, their inevitable chronic use aroused a concern among public now, which formed the basis for the design of the present study. The purpose of the present study is two fold; First, to detect the changes in membrane fluidity of human volunteers exposed to regular use of allethrin and prallethrin, and second to understand the role and status of Na^+/K^+-ATPase activity and Ca^{2+}-dependent ATPase activity and Mg^{2+}-ATPase in such users of allethrin and prallethrin.

Subjects for study

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 over night 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 & Isolation of platelets

Blood was drawn from human volunteers by venipuncture between 7 to 10 AM. The method of Aster and Jandl, (1964) was adopted for the isolation of platelets. Briefly, 10 ml of blood was collected with (ACD) acid citrate dextrose anticoagulant solution in the ratio of 9:1. The anticoagulated blood was centrifuged at 160g for 10 min to obtain PRP. The PRP obtained was again centrifuged at 160g to remove the red blood cells. The PRP was centrifuged at 300g for 5 min to pellet out platelets. The washing procedure was continued until the suspension was erythrocyte free and the purity was confirmed by microscopic examination. The platelet pellet was suspended in platelet storage buffer containing 0.109 M NaCl, 4.3 mM K_2HPO_4, 16 mM Na_2HPO_4, 8.3 mM NaH_2PO_4 and 5.5 mM glucose, pH 7.5, and stored bat 4^oC until further analysis.

Isolation of platelet membrane

Platelet membrane was isolated based on the method adopted by Lind et al. (1982). Briefly, equal volume of platelet suspension and Triton X-100 lysis buffer was taken in microfuge tubes and mixed by inversion. The clarified suspension of platelets was immediately centrifuged at 4^{o}C for 2.5 h at 100000g. Supernatant was discarded and the translucent platelet membrane pellet was carefully collected from the microfuge tube and used for the estimation of lipids and other parameters.

Estimation of lipids in platelet membrane

Lipids were extracted from the platelet membrane by the method of Schick et al. (1983). The membrane preparation containing 2 mg of protein/ml was mixed with chloroform/methanol mixture (2:1, v/v) in the ratio of 1:9 (v/v). The solution was homogenized at low speed and the organic lipid layer was carefully separated and evaporated to dryness in a conical flask. The lipid was dissolved in a known amount of chloroform/ methanol mixture. Total phospholipids were estimated in terms of inorganic phosphorus by the method of Fiske and Subbarow (1925) after Barlett (1959) perchloric acid digestion. Platelet membrane cholesterol was estimated by using ferric acetate/uranyl acetate reagent (Jung and Parekh, 1971).

Lipid peroxidation in platelets

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 platelet 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 1000g 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 $1.56x10^{5}$.

Assay of platelet membrane-bound enzymes

The activity of total ATPase was determined by the method of Evans, (1969) by using ATP as the substrate in the presence of Na^{+}, K^{+} , Mg^{2+}, and Ca^{2+}ions.

Na^{+}, K^{+}-ATPase activity was measured according to the method of Bonting (1970) in the presence of Na^{+} and K^{+} ions. The level of Mg^{2+}-dependent ATPase was determined by the method of Ohnishi et al. (1982) and Ca^{2+}-dependent ATPase activities were quantified by the method of Hjerton and Pan (1983) using ATP as substrate in the presence of Mg^{2+} and Ca^{2+} ions, respectively.

In all the ATPases assays, the activity was expressed in terms of nanomoles of phosphorus liberated/minute per 1 X 10^{5} platelets.

Fluorescence measurement

Fluorescence measurements on platelet membranes and lipid extracts were performed on a spectrofluorometer. Steady-state fluorescence anisotropy (*r*) measurements for DPH and pyrene were obtained using the excitation and emission wavelengths at 360 and 430 nm, respectively. The degree of fluorescence anisotropy (*r*) was calculated according to Shinitzky and Barenholz (1978) from the equation:

$$r = \frac{(I\| - I\_g)}{(I\| + 2I\_G)}$$

where G is an instrumental correction factor, and are, respectively, the intensities measured with the polarization plane parallel and perpendicular to that of the exciting beam. The final protein concentration in the assay was 0.4 mg/ml, while the probe concentration was 10^{-6} M. Fluorescence measurements performed

on lipid extracts according to Folch et al., (1957) were normalized to the same content of proteins (0.4 mg/ml). Samples were suspended in 10 mM Tris pH 7.4 and the measurements were performed at 25^0C.

Statistical analysis

The results of the study are expressed as mean ± SD. Statistical analysis was performed using Duncan's Multiple Range (DMR) test. The Significance was set at ($P \leq 0.05$).

Results

The results obtained in this study it is clear that there was a significant increase in membrane cholesterol (C) and decrease in phospholipid (P) contents with no significant change in the protein contents in platelet membranes of allethrin and prallethrin exposed subjects group II and group III when compared to controls group I. Increase in consequent C:P ratio in allethrin and prallethrin-exposed subjects when compared with controls (Table I). Data presented in Fig-1 showed that pyrethroids use a significant decrease in platelet membrane fluidity. Further, a significant increase in platelet membrane lipid peroxidation in the experimental subjects is obvious from this study (Fig. 2). Data furnished in Table. 2 indicated decreased levels of platelet membrane bound enzymes total ATPase, Na^+, K^+-ATPase, Mg^{2+}-ATPase, Ca^{2+}-ATPase in platelets of allethrin and prallethrin exposed subjects group II and group III when compared to controls group I.

Discussion

In the present study increased in cholesterol and decreased in phospholipid contents in platelet membrane of allethrin and prallethrin exposed subjects when compared to controls, with no change in platelet membrane protein moiety suggested alterations in membrane organization affecting lipid-lipid, lipid-protein, and protein-protein interactions in the membrane, and with increase in C/P ratio in data presented in table 1. The liver is the central organ for cholesterol, phospholipids, triglyceride and lipoprotein metabolism. The functional impairments of liver would result in the reduced capacity to synthesize many important biomolecules, including lipids. Present study observed increased levels of cholesterol and decreased levels of phospholipids. Platelet dysfunction in liver diseases is associated with an abnormality in lipid composition. Present study show an increase in the cholesterol/phospholipids ratio leads to a decrease in membrane fluidity. Increased c/p ratio in allethrin and prallethrin based pyrethroid induced platelet membrane indicates the decreased fluidity, influencing viscoelastic properties of the membrane whish is in agreement with other reports (Beauge et al., 1985; Stibler, 1991; Paramahamsa et al., 2004). This result was confirmed further by the fluorescence anisotropic studies using pyrene which showed a decrease in translational mobility of pyrene due to increased intra membrane micro viscosity, which is indicate of the decreased platelet membrane fluidity (Bryszewska, 1986). This finding contradicts the results reported by Hrelia et al (1986). This decrease in membrane fluidity might affect receptor functions by changing the lateral mobility or clustering of receptors in their vertical orientation (Brulet and McConnell, 1976). Phospholipids have been reported to influence membrane fluidity. Our study suggests that the increase in the cholesterol/phospholipid ratio might have affected the membrane fluidity. If the membrane fluidity decreases, the secretary function of the platelet will be severely affected, the procoagulants will not be secreted at a normal level, and there by aggregation will be affected. Fig. 1 shows the influence anisotropy (r) values on controls and allethrin and prallethrin exposed subjects from platelet membranes observed decrease in membrane fluidity in experimental subjects group II and III when compared to controls group I in both DPH and pyrene using probes. Pyrethroids are fat-soluble pesticides, and there fore they accumulate in fat deposits in the body (pers. com. 2002). The highest concentration of fat in the body is in the brain due to the lipid-based myelin sheaths surrounding every nerve cell (pers. Com. 2002). Generally, the fluidity of

biomembranes is responsible for their functional integrity, which is largely determined by the levels of protein and cholesterol/phospholipid ratio. A significant alteration in the level of lipids in platelet membrane would contribute to the seretory functions and there by to the defective adhesive and aggregation properties. The myelin sheath serves as an insulator and conductor, protecting the nerve and giving the neural impulses speed and direction. All types of pesticides can degrade the myelin sheath (pers. Com. 2002). If demyelinization occurs, nerve impulses are either slowed (sometimes to the point of halting), or they misfire because there is no conductor to give them direction. The observed increase of lipid peroxidation in allethrin and prallethrin exposed subjects when compared to controls and corroborates the reports of others (Uysal et al., 1986; Meagher et al., 1999). Data presented in table 2 inhibitions of ATPases, the enzymes associated with the platelet membrane, like ATPases and nucleotidases, are essential for the membrane-related functions like adhesion, aggregation and secretion of granules. Na^+, K^+-ATPase belongs to a family of ATPases which are present in virtually all mammalian cell membranes. Other members of this family include enzyme dependent upon magnesium (Mg^{2+}-ATPase) and calcium (Ca^{2+}-ATPase). Because Na^+, K^+-ATPase transports Na^+ ions extracellulerly and K^+-ions intracellularly, it plays an important role in maintaining the level of membrane polarization (Powell and Cantley, 1980). Consequently, modulation of Na^+, K^+-ATPase activity may lead to alterations in the function of various cell types, including peripheral blood mononuclear cells. In our study, it is observed that there is a significant reduction in the activity of Na^+, K^+-ATPase which might result in reduced cation exchange through the membrane and thereby it's reduced energy-dependent secretory fuctions. Biochemical characterization of the calcium ATPases isolated from human platelet intracellular and plasma membranes has been reported (Enouf et al., 1989). This enzyme catalyses Ca^{2+} dependent exchange of hydride ions to calcium ions which influence the rate of signal transduction which is highly essential for the timely release of secretory granules and formation of actomyosin complex in the platelet membrane during adhesion. So, the observed low level of calcium ATPase activity would have also influenced the defective platelet activities such as adhesion, aggregation and secretion.

Conclusion

Increase in platelet membrane cholesterol and decreased in phospholipids, with no change in proteins were observed in exposed subjects appears to be an adaptive biochemical changes in platelet membrane. Decreased membrane fluidity of platelet membrane and increase in lipid peroxidation, a significant decrease in platelet membrane bound enzymes were observed. Results of our study reveal that pyrethroid based mosquito repellents allethrin and prallethrin toxicity of platelet membrane in the human male volunteers may blood cell apoptosis. Further studies are needed to correlate the toxic effects of prolonged use of allethrin and prallethrin on health status of individuals.

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Table 1. Levels of phospholipid and cholesterol in the platelets and cholesterol /phospholipid ratio in the platelets of control, allethrin and prallethrin subjects.

Parameter	Groups		
	Controls	Allethrin users	Prallethrin users
Cholesterol (ng/1 10^5platelets)	0.182±0.009a	0.233±0.009b	0.251±0.006b
Phospholipid (ng/1 10^5platelets)	0.216±0.009a	0.153±0.006b	0.142±0.005b
Platelets Cholesterol/Phospholipid ratio	0.842a	1.522b	1.767b

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.

Table 2. Activity of total Na^+, K^+ , Mg^{2+} and Ca^{2+} ATPases in the platelet membrane of allethrin and prallethrin users and in normal healthy volunteers (controls).

Parameter	Groups		
	Controls	Allethrin users	Prallethrin users
Total ATP ase	96.31±2.15a	75.79±3.68b	75.13±3.60b
Na^+, K^+-ATP ase	28.57±1.04a	23.11±1.16b	24.17±0.57b
Mg^{2+}-ATP ase	47.94±1.00a	38.15±0.74b	38.91±0.82b
Ca^{2+} -ATP ase	41.98±1.30a	29.98±0.97b	29.19±1.59b

Activities are expressed as nmoles of phosphorus liberated/min per 1 X 10^5 platelets. 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.

Table 3. Clinical characteristics of pyrethroid based mosquito repellents allethrin and prallethrin users and controls

Parameter	Groups		
	Control	Allethrin users	Prallethrin users
Total Number	24	24	24
Height (cm)	160.93±5.04	161.98±4.99	161.27±6.55
Weight (kg)	51.91±4.35	53.00±5.11	54.75±6.15
BMI (kg/m^2)	32.44	32.44	34.00
Platelet Count (Mean ± SD) 10^5 cells/µl	258±59	230±61	234±64
Below 1×10^5 cells/µl	None	18	20
Above 2×10^5 cells/µl	None	6	4
Percentage platelet aggregation when compared to age and sex matched normal volunteers 80%	None	5	4
Percentage platelet aggregation when compared to age and sex matched normal volunteers 50%	None	19	20

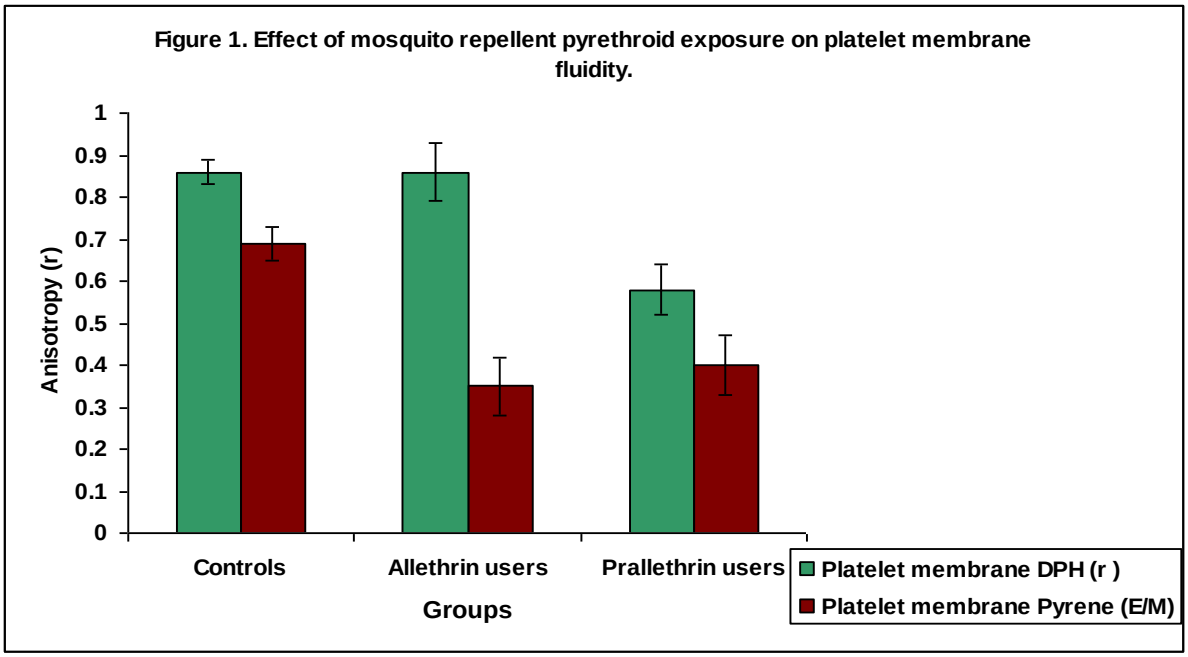

Figure 1. Effect of mosquito repellent pyrethroid exposure on platelet membrane fluidity.

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.

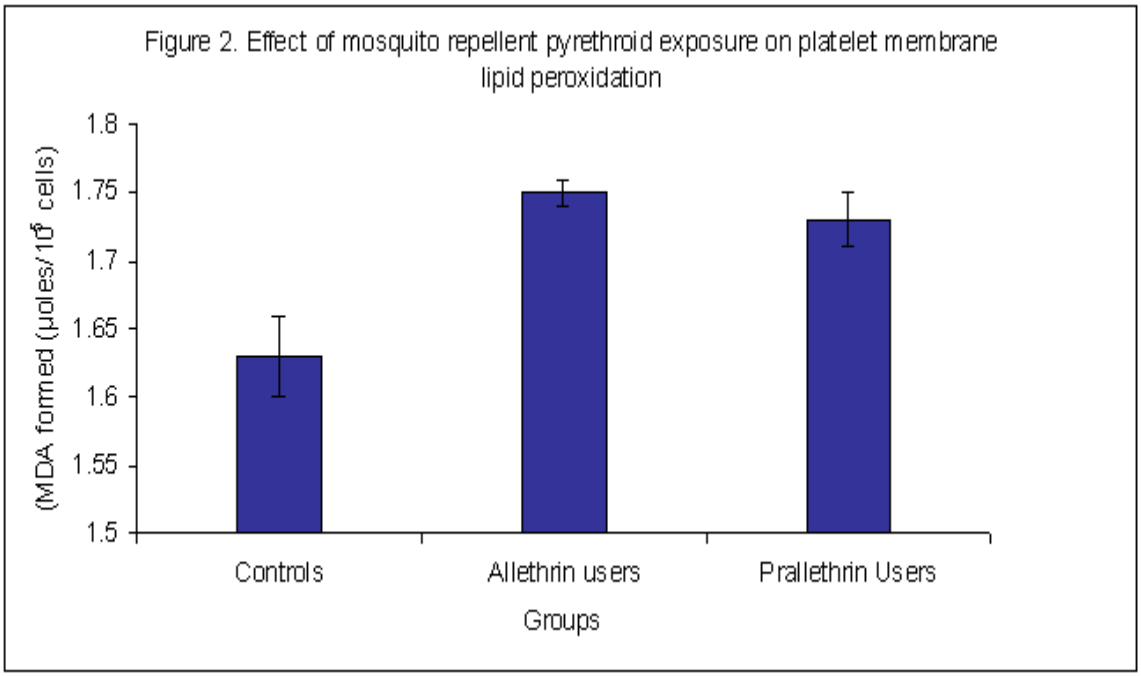

Figure 2. Effect of mosquito repellent pyrethroid exposure on platelet membrane lipid peroxidation.

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.