

Original Research

Pyrethroid Insecticide Effect on Platelet Biomembranes of Rats

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Abstract

The effect of Tetramethrin- and prallethrin-based mosquito repellent pyrethroids on rat platelet membranes of cholesterol, phospholipids, lipid peroxidation in platelet membrane was investigated. Rats were chronically exposed to these compounds by continuous inhalation for 8-10 hours a day for 2-3 months, which entered into circulation and counted changes in membranes of platelets. Platelet membrane fluidity was determined by DPH. Pyrene fluorescence anisotropy experiments revealed that tetramethrin and prallethrin exposure to subjects, compared with controls, induced a significant decrease in platelet membrane fluidity as measured by DPH and pyrene. A significant increase in lipid peroxidation was observed in the subjects exposed to both compounds. A significant decrease in the activities of platelet membrane-bound enzymes, viz., total ATPase, Na⁺/K⁺-ATPase and Ca²⁺-dependent ATPase and Mg²⁺-ATPase was also recorded.

Keywords: membrane fluidity, prallethrin, tetramethrin, pyrethroids, lipid peroxidation

Introduction

Pyrethroids are the most widely used insecticides due to their potential insecticidal activity in India and other countries for protection against mosquitoes and other insects for various domestic and agricultural purposes [1-10]. Pyrethroids are 2250 times more toxic to insects than mammals because insects have increased sodium channel sensitivity, smaller body size, and lower body temperature. Over half of the world's population have been using pyrethroid insecticides, which may account for more than 25% of the insecticide market of

the industrial countries in the 1990s, and their demand/use is increasing now in these countries [11-12] as the prevalence of mosquitoes and other insects has increased in many parts of the world. Initially, these pyrethroids were thought to be highly toxic to insects and less toxic to humans [13]. Now, pyrethroid-induced neurotoxicity and other toxic effects ranging from whole body tremors to convulsions and death are well documented [12, 14-17]. Very few reports are available so far on the effects of pyrethroids on rats and humans, and now slowly the facts related to their toxicity are coming to light. The fatality with pyrethroids in India has been reported to be 12.5 to 25% [18]. Prallethrin, 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

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mosquito repellents. Tetramethrin and prallethrin are among the most widely used pyrethroid insecticides. Tetramethrin poisoning can be much more frequent due to its easy accessibility as mosquito repellent and/or insecticidal sprays, [19] and often there are reports of pyrethroid poisoning in India [20-21]. However, it is clear that no relevant data on chronic effects exist in open scientific literature related to pyrethroid toxicity in humans and animals [18, 22]. 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 the public now, which formed the basis for the design of the present study. The purpose of the present study is twofold; first, to detect the changes in membrane fluidity of exposed rats to regular use of tetramethrin 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 tetramethrin and prallethrin.

Subjects for Study

The rats were exposed to either mosquito repellent coils or mats, both from markedly available. The coils are composed of (w/w) 0.1% *d-trans* tetramethrin, 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 (each group consisting of 8 rats). Release of the pyrethroid insecticide is either by burning the coil or placing the mat in the commercially available electric devices. The rats in the frequent were divided into 3 groups: Group I (control) did not use mosquito repellents; Group II was tetramethrin-exposed rats; Group III was prallethrin-exposed 2-month-old male albino rats of *Wistar* strain with body weight of 120-140 g, procured from the National Centre for Laboratory Animal Sciences (NCLAS), Hyderabad, A.P., India, and housed in individual cages in an air-conditioned room ($25 \pm 1^\circ\text{C}$) with light from 7 a.m. to 7 p.m. Hence the present work focused on the chronic effect of pyrethroid-based tetramethrin and prallethrin-induced platelet membrane damage/alterations with stress on its mechanism. This study was approved by the institutional ethical committee (JXC20171210) Qilu Hospital of Shandong University, China. Blood samples from overnight fasted subjects were used for the study.

Blood Collection and Plasma Biochemical Determinations

Plasma glucose was estimated by the method of Trinder [23]. Total cholesterol and triglycerides were measured with enzymatic Allian et al. [24]. HDL-cholesterol was determined after precipitation of VLDL and LDL with phosphotungstic acid and magnesium

chloride. VLDL- and LDL-cholesterol concentrations were calculated from the equation; $\text{LDL-Cholesterol} = \text{total cholesterol} - (\text{HDL cholesterol} + \text{VLDL-cholesterol})$, and $\text{VLDL cholesterol} = \text{Triglycerides}/5$. Urea, transaminases, total protein and plasma albumin [25] also were determined spectrophotometrically using commercial kits.

Isolation of Platelets

Blood was drawn from rats by heart puncture between 7 and 10 a.m. The method of Aster and Jandl [26] was adopted for the isolation of platelets. Ten ml of blood was collected with acid citrate dextrose (ACD) anticoagulant solution in the ratio of 9:1. The anticoagulated blood was centrifuged at 160 g for 10 min to obtain platelet-rich plasma (PRP). This was again centrifuged at 160 g to remove the red blood cells. The PRP was centrifuged at 300 g 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 pellet of platelet was suspended in a 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 at 4°C until further analysis.

Isolation of Platelet Membrane

Platelet membrane was isolated based on the method adopted by Aneta et al. [27]. An 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°C for 2.5 h at 100000 g. Supernatant was discarded and the translucent platelet membrane pellet was carefully collected from the microfuge tube and used for estimating lipids and other parameters.

Estimating Lipids in Platelet Membrane

Lipids were extracted from the platelet membrane by the method of Lijiuan et al. [28]. 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. Platelet membrane cholesterol was estimated using ferric acetate/uranyl acetate reagent.

Lipid Peroxidation in Platelets

The extent of lipid peroxidation was measured by the formation of malondialdehyde (MDA) by the method of Negre-Salvayre et al. [29]. One ml of the 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 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 1.56×10^5 .

Assay of Platelet Membrane-Bound Enzymes

The activity of total ATPase was determined by the method of Evans [30] 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 [31] in the presence of Na^+ and K^+ ions. The level of Mg^{2+} -dependent ATPase was determined by the method of Ohnishi et al. [32], and Ca^{2+} -dependent ATPase activities were quantified by the method of Hjertson and Pan [33] using ATP as substrate in the presence of Mg^{2+} and Ca^{2+} ions, respectively. In all ATPases assays, the activity was expressed in terms of nanomoles of phosphorus liberated/minute per 1×10^5 platelets.

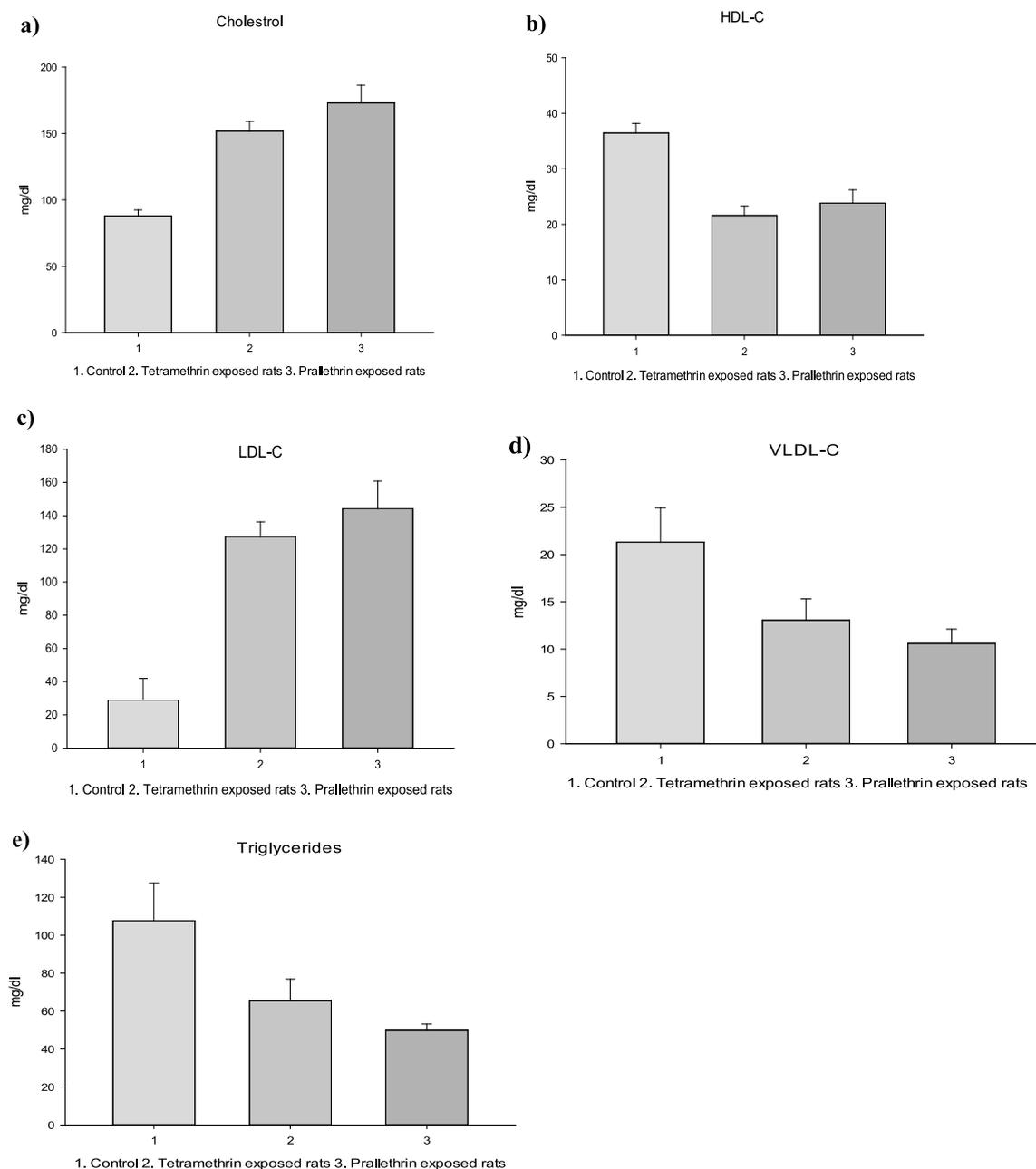


Fig. 1(a-e). Pyrethroid-induced lipid profile alterations of control and exposed rats. Values are shown as Mean \pm Standard Deviation (n = 8). Different superscript letters in the same line indicate statistically significant difference for $P < 0.05$. NS = Not Significant.

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 [34] from the equation:

$$r = (I_{||} - I_{\perp}) / (I_{||} + 2I_{\perp}G)$$

$$(I_{||} + 2I_{\perp}G)$$

...where G is an instrumental correction factor, and are, respectively, and the intensities were 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 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°C.

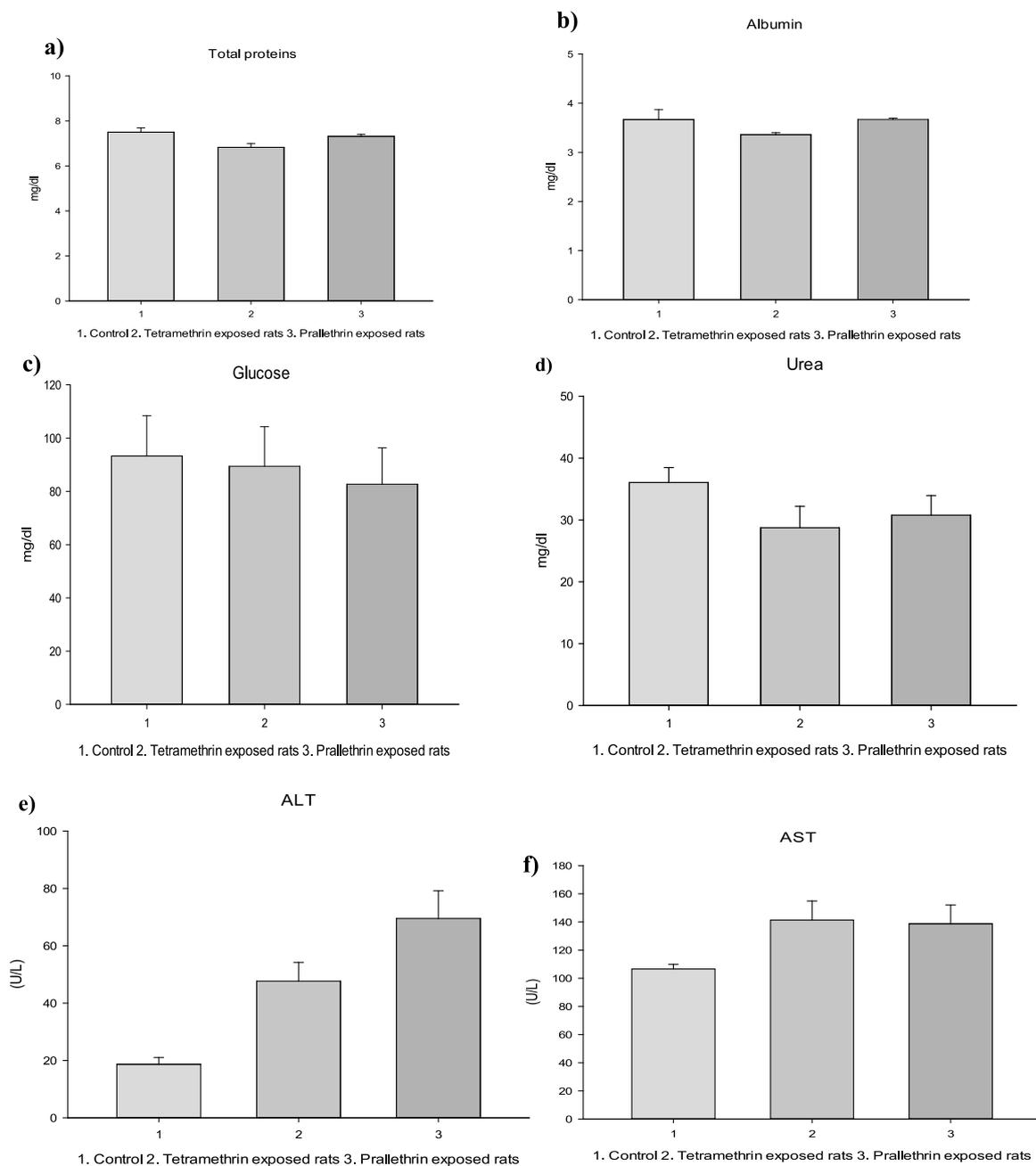


Fig. 2(a-f). Pyrethroid-induced alterations of control and exposed rat plasma profile.

Values are shown as Mean \pm Standard Deviation ($n = 8$). Different superscript letters in the same line indicate statistically significant difference for $P < 0.05$. NS = Not Significant.

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 tested at ($P\leq 0.05$).

Results

The results obtained in this study are clear by revealing a significant increase in platelet membrane cholesterol (C) and a decrease in phospholipid (P) content with no significant change in the protein contents in platelet membranes of tetramethrin and prallethrin-exposed subjects group II and group III when compared to controls group I. The increase in consequent C:P ratio in tetramethrin and prallethrin-exposed subjects was observed when compared with control. Furthermore, a significant increase in platelet membrane lipid peroxidation in the experimental subjects is obvious from this study (Table 1). Data furnished in Table 2 indicate decreased levels of platelet membrane-bound enzymes total ATPase, Na^+ , K^+ -ATPase, Mg^{2+} -ATPase, and Ca^{2+} -ATPase in platelets of tetramethrin- and prallethrin-exposed subjects group II and group III when compared to controls group I. Plasma cholesterol and LDL-C levels increased significantly

with no change triglycerides, HDL-C, VLDL-C (Fig. 1a-e). Glucose levels decreased in rats exposed to both while total proteins and albumin levels increased slightly when compared to control, and no change in ALT, AST, or urea levels were observed (Fig. 2a-f). Hemoglobin levels in pyrethroid-inhaled rats decreased significantly, whereas RBC, WBC, and platelet counts were altered in tetramethrin- and prallethrin-exposed rats (Fig. 3a-d). Modifications in platelet fatty acid chain length (fatty acid composition 16.0, 20.4 significantly) also was observed in (Fig. 4a-c). Data presented (Fig. 5a, b) showed that use of pyrethroids led to a significant decrease in platelet membrane fluidity in rats exposed to both.

Discussion

In the present study, the platelet membrane of tetramethrin- and prallethrin-exposed subjects showed an increase in cholesterol and decrease in phospholipid contents when compared to control, with no change in platelet membrane protein moiety, which suggested alterations in membrane organization affecting lipid-lipid, lipid-protein, and protein-protein interactions in the membrane, and with an increase in C/P ratio and a significant increase in lipid peroxidation (Table 1). Liver is the central organ involved in metabolism of

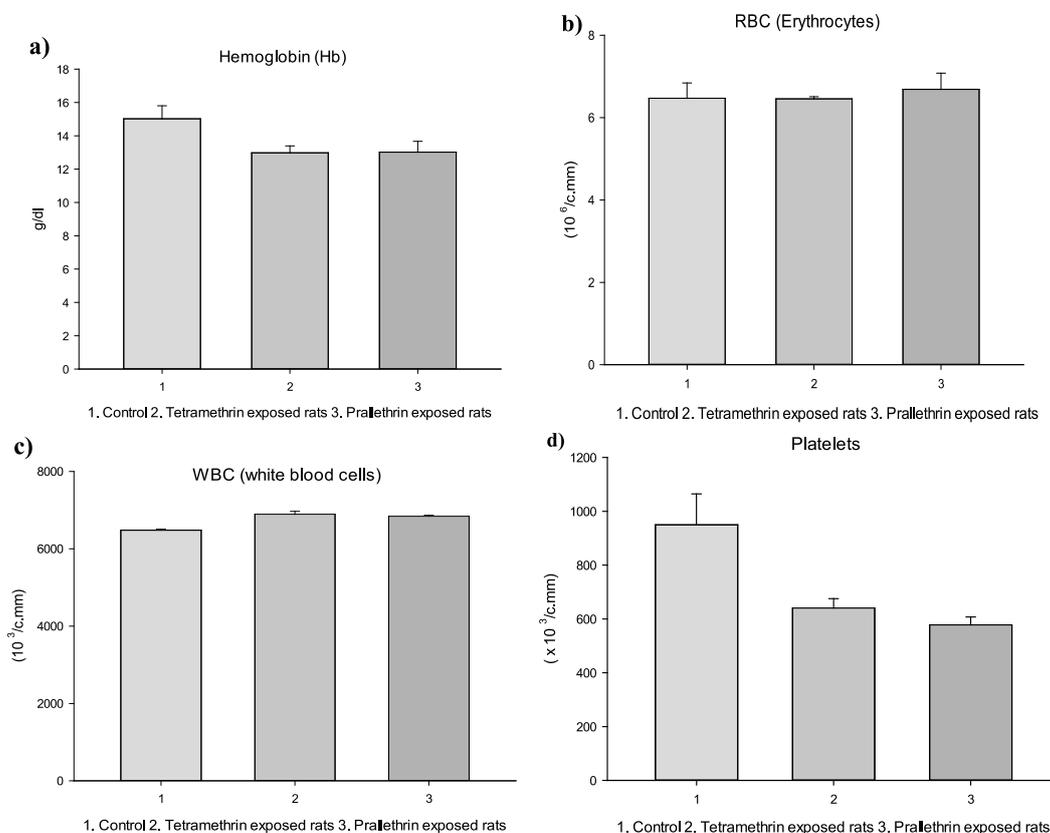


Fig. 3(a-d). Pyrethroid-induced changes of Hb, RBC, WBC, and platelets of exposed rats

Values are shown as Mean±Standard Deviation (n = 8). Different superscript letters in the same line indicate statistically significant difference for $P < 0.05$. NS = Not Significant.

cholesterol, phospholipids, triglyceride and lipoproteins. The functional impairments of liver would result in the reduced capacity to synthesize many important biomolecules, including lipids. Results from the 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 [35]. Our study showed an increase in the cholesterol/phospholipids ratio, leading to a decrease in membrane fluidity. Increased c/p ratio in tetramethrin- and prallethrin-based pyrethroid-induced platelet membrane indicates decreased fluidity, thereby influencing viscoelastic properties of the membrane, which is in agreement with other reports [36-38]. This result was further confirmed 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 indicative of the decreased platelet membrane fluidity. This finding contradicts the results reported by Hrelia et al. [39]. This decrease in membrane fluidity might affect receptor functions by changing the lateral mobility or clustering of receptors in their vertical orientation [40]. Phospholipids have been reported to influence membrane fluidity. Our study suggests that the increase in the cholesterol/phospholipid ratio might have affected membrane fluidity. If membrane fluidity decreases, the secretory function of the platelet will be severely affected. The procoagulants will not be secreted at a normal level, thereby affecting aggregation. Fig. (1a-e) shows a significant increase in serum cholesterol in pyrethroid groups and a significant reduction of its HDL fraction compared to the control group. LDL-cholesterol no difference was found in relation to triglycerides and VLDL-cholesterol, as compared to control animals. This apparently unexpected result could be explained by the activity of lipoprotein

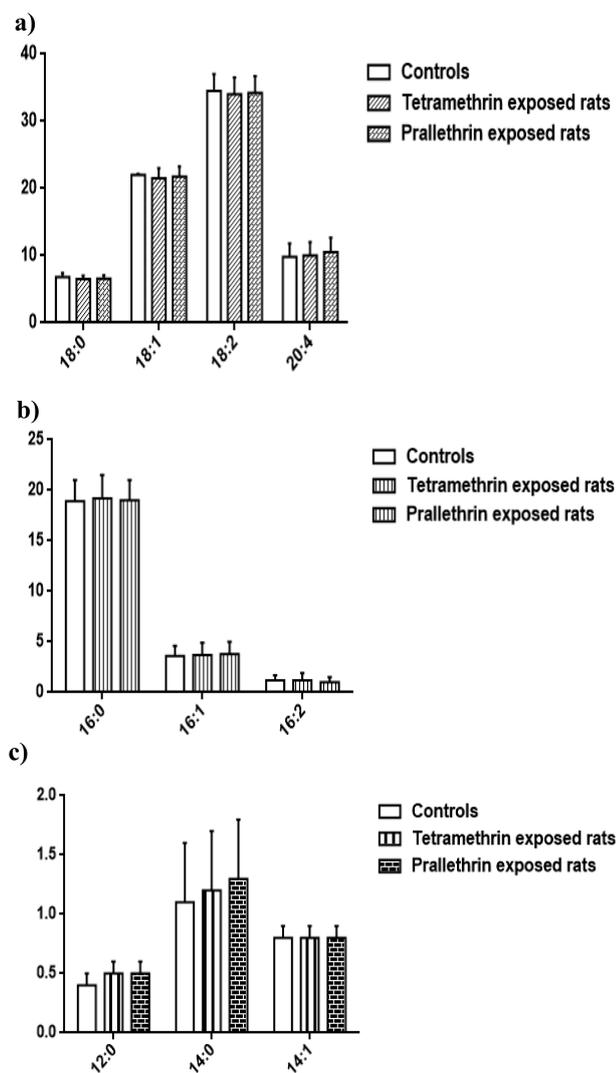


Fig. 4(a-c). Effect of mosquito repellent pyrethroid exposure on fatty acid composition of platelet membrane. Values are shown as Mean±Standard Deviation (n = 8). Different superscript letters in the same line indicate statistically significant difference for $P < 0.05$. NS = Not Significant.

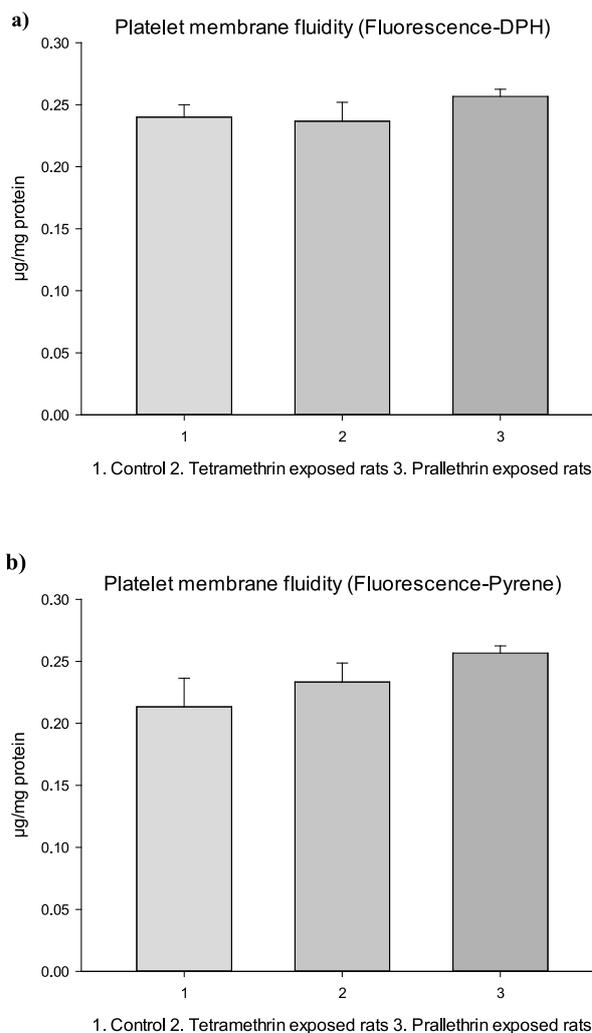


Fig. 5(a-b). Effect of mosquito repellent pyrethroid exposure on platelet membrane fluidity. Values are shown as Mean±Standard Deviation (n = 8). Different superscript letters in the same line indicate statistically significant difference for $P < 0.05$. NS = Not Significant.

Table 1. Pyrethroid effect on platelet parameters.

Parameter	Groups		
	Controls	Tetramethrin exposed Rats	Prallethrin exposed Rats
Cholesterol (ng/1 10 ⁵ platelets)	0.174±0.009a	0.212±0.009b	0.244±0.006b
Phospholipid (ng/1 10 ⁵ platelets)	0.206±0.009a	0.164±0.006b	0.158±0.005b
Platelets Cholesterol/Phospholipid ratio	0.84a	1.29b	1.54b
Lipid Peroxidation (MDA formed (µmoles x 10 ⁵ platelets)	0.62±0.04	1.20±0.05	1.18±0.05

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 = 8.

lipase. Lipid metabolism has also been assessed besides other biochemical parameters thus, aiming at a picture of the hepatic function of the animals since the liver is the major organ responsible for maintenance of homeostasis related to lipid metabolism. Fig. 2(a-f) shows a significant decrease in serum albumin, glucose and total proteins in exposed subjects, but no change in ALT, AST and urea was observed in the two groups when compared to control. This finding corroborated how the high caloric value of the diet might have impaired the absorption of proteins and other nutrients. Decreased platelet counts and haemoglobin concentration in experimental rats exposed to (tetramethrin and prallethrin) significantly exposed slight increases in WBC counts. In RBC count significant increases in prallethrin rats saw no change in tetramethrin rats compared to control (Fig. 3a-d). Fatty acid composition was altered significantly in subjects exposed to both (fatty acid chain 16.0, 20.4 significantly) Fig. 4(a-c). Data presented in Fig. 5(a-5b) shows the on-influence anisotropy (r) values in control and tetramethrin- and prallethrin-exposed subjects platelet membranes showed a decrease in membrane fluidity in group II and group III experimental subjects when compared to control group I in both DPH and pyrene-using probes. Pyrethroids are fat-soluble pesticides, and therefore they accumulate in fat deposits in the body [41]. The highest concentration of fat in the body is in the brain due to the lipid-based

myelin sheaths surrounding every nerve cell [41]. 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 secretory functions and thereby on 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 [41]. If demyelination 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 in lipid peroxidation in tetramethrin- and prallethrin-exposed subjects when compared to controls corroborates the reports of others [42-43]. Data presented in Table 2 reveal the inhibition 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 enzymes dependent upon magnesium (Mg²⁺-ATPase) and calcium (Ca²⁺-ATPase). Because Na⁺, K⁺-ATPase transports Na⁺ ions extracellularly and K⁺-ions intracellularly,

Table 2. Effect of mosquito repellent use on platelet ATPases.

Parameter	Groups		
	Controls	Tetramethrin exposed Rats	Prallethrin exposed Rats
Total ATP ase	24.31±1.10a	15.40±1.80b	16.10±1.60b
Na ⁺ , K ⁺ -ATP ase	18.22±1.08a	13.50±1.28b	14.60±0.78b
Mg ²⁺ -ATP ase	18.40±1.20a	14.15±0.74b	15.64±1.02b
Ca ²⁺ -ATP ase	14.68±1.28a	10.28±0.97b	11.44±1.34b

Activities are expressed as nmoles of phosphorus liberated/min per 1 X 10⁵ 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 = 8.

it plays an important role in maintaining the level of membrane polarization [44]. 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 its reduced energy-dependent secretory functions. Biochemical characterization of the calcium ATPases isolated from human platelet intracellular and plasma membranes has been reported [45]. This enzyme catalyses Ca²⁺-dependent exchange of hydride ions to calcium ions which influence the rate of signal transduction is essential for the timely release of secretory granules and the 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.

Conclusions

An increase in platelet membrane cholesterol and decrease in phospholipids, with no change in proteins, were observed in exposed subjects, which appears to be an adaptive biochemical change in platelet membrane. Decreased membrane fluidity of platelet membrane, increased lipid peroxidation and a significant decrease in platelet membrane-bound enzymes were recorded. Further studies are needed to correlate the toxic effects of prolonged use of tetramethrin and prallethrin on rat platelet membrane.

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Conflict of Interest

The authors declare no conflict of interest.

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