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Comparative cytotoxic and genotoxic effects of permethrin and its nanometric form on human erythrocytes and lymphocytes *in vitro*

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ABSTRACT

The research on the novel pesticides such as nanopesticides has become inevitable to control the mosquito population. Nanopermethrin (NP), one of such kind was formulated in pesticide loaded oil-in-water (o/w) microemulsion by rapid evaporation. Even though NP possess improved efficacy against the target pests, the toxicological investigation on the human or mammalian system remains unexplored. So, the present study focused on a comparative investigation of the cytotoxic and genotoxic effects of NP *in vitro* and its commercial parental bulk form of permethrin (BP) on human peripheral erythrocyte/lymphocyte by erythrocyte morphology analysis, cell viability assay, and cytokinesis-block micronucleus (CBMN) assay. The NP and BP concentrations (10, 25, 50 and 100 μg/ml) interacted with human blood cells, and the morphological changes were observed using a phase contrast microscope. The drastic increase of echinocyte was observed at 24, 48 and 72 h treatment as compared with the control. The cell viability studies have shown the significant decrease with increase in NP and BP concentration. CBMN study showed a series correlation in the number of micronuclei, bridge, bud, trinucleated and tetranucleated when interacted with different levels of NP and BP, as comparative to control $^{*}p < 0.05$, $^{**}p < 0.001$, $^{***}p < 0.0001$.

1. Introduction

Permethrin belongs to type I synthetic group of pyrethroid pesticide, is a chrysanthemic acid which is naturally occurring insecticidal ester [1]. It has found reliable application to kill and resist the dreadful mosquitoes and has become an integral part of pest control. These advantages of permethrin found use in mosquito nets and insect repellent clothing to prevent the bite of several dreadful disease-causing mosquitoes [2,3]. This has saved many human lives against the dreadful mosquito-borne diseases such as malaria, lymphatic filariasis, dengue and so on [4].

In spite of these advantages, the accidental and occupational exposure and uptake of permethrin by oral, inhalation or dermal absorption were determined to be applied a dose of 2% [5–7]. The permethrin was found to cause toxicity which is leading to gulf war syndrome for the military soldiers [8]. The biomonitoring studies on soldiers have also shown the increased internal exposure and uptake for the frequent use in battle dress uniforms of permethrin treated [9,10]. Moreover, the repetitive applications of permethrin will contaminate the groundwater, sediments, soil, animals and plants have caused different environmental issues [11]. Also, the presence of toxic solvents for solubilizing the hydro immiscible pesticides has also raised several environmental concerns [12]. So, the need of an alternative form of permethrin with improved specificity and reduced environmental toxicity is much anticipated.

In accordance, the nano-particular form of permethrin (NP) was formulated in pesticide loaded o/w microemulsion by rapid evaporation [13]. The previous study reported that the NP was found to be possessing better efficacy against the mosquito populations even at lower exposure concentrations [14]. According to [15] the non-target toxicity of NP against *Escherichia coli*, *Bacillus subtilis* *Lycopersicum esculentum*, *Cucumis sativus*, *Zea may,* and *Allium cepa* has shown no significant toxicity. In this context, present work was focused on exploring further the toxicological profile of NP and BP in the mammalian system. The cytotoxic and genotoxic *in vitro* studies on the human peripheral erythrocytes/lymphocytes were investigated on BP and NP by erythrocyte morphology analysis, cell viability assay, and CBMN assays respectively.

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2. Materials and methods

2.1. Nanopermethrin formulation

Permethrin (BP) is a poorly-water soluble parental form was formulated into nanometric powder (NP) in pesticide loaded o/w microemulsion by rapid evaporation [13]. In brief 4 wt% of BP dissolved in 14 wt% of *n*-butyl-acetate (nBuAc). Along with 9 wt% ammonium-glycyrrhizinate (AG), 9 wt% soybean-lecithin (SbPC), and 9 wt% secondary butyl-alcohol (secBuOH) were added to be a part of the organic phase. The organic phase was added to the aqueous phase (water) constituting 18 wt% of sucrose and equilibrated at 25 °C up to the formation of an isotropic solution. This leads to the formation of permethrin loaded oil-in-water microemulsion, which was lyophilized (Heto dry-winner lyophilizer-model DW3, Denmark) at −47 ± 3 °C. The resultant solid nanopowder consists of permethrin which was easily hydrodispersive in nature. The analytical quantification of the permethrin concentration was determined in the NP by using Reverse phase-HPLC (LAChromElite, Hitachi, Japan) and was found to be 16.18 wt% [14].

2.2. Preparation of test concentrations

The test concentrations of 10–100 μg/ml of BP and NP were prepared based on the active ingredient (AI) composition in distilled water (DW) (cascada bio-water purification system, USA). Initial dilution of hydro immiscibility BP was prepared with dimethylsulphoxide (DMSO) and further dilution with DW. The effect of AI employed for NP preparation on erythrocytes/lymphocytes was also tested.

2.3. Erythrocyte morphology analysis

Human blood collected from healthy individuals (n = 3), between 25 and 30 years was chosen for this study. The exclusion criteria for the donors were considered to follow as non-smokers and non-medication therapy. The 1 ml of blood cells was incubated with BP and NP (10, 25, 50 and 100 μg/ml) for 24, 48 and 72 h at 37 °C. A thin blood smear was prepared from heparinized blood using wedge slide technique [16]. The erythrocyte morphology images were captured using a microscope (Leica microsystems DM2500, Germany). Abnormal erythrocyte (echinocyte) was enumerated from the total 1000 scored cells.

2.4. Blood collection and isolation of human lymphocytes

The blood samples collected from healthy volunteers aged about 25–30 years (n = 3) and the samples were drawn according to an approval of Institutional Ethical Committee, VIT University. The written informed consent was obtained from all the donors. From human peripheral blood the lymphocytes were isolated using HiSepTM (HiMedia) separation media, and 1×10^6 cells was cultured using HiKaryoXL RPMI Medium (HiMedia) contains fetal bovine serum (FBS), phytohemagglutinin (PHA-M), L-glutamine, penicillin, streptomycin, sodium bicarbonate and incubated for 24 h at 37 °C (5% CO_2). The trypan blue exclusion method used for cell count and measured the viable cells using hemocytometer [17].

2.5. Cytotoxicity by MTT assay

MTT assay has been used as the standard method for determining the cytotoxicity of nanoparticles using cell culture techniques [18]. After the incubation for 24 h, human lymphocyte cells were calculated and 1×10^4 cells loaded into the 96-well plates with various concentrations of NP and BP (10, 25, 50 and 100 μg/ml) incubated for 24 h at 37 °C. The cells were treated with 5 mg/ml solution of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) for 4 h in 5% of CO_2 at 37 °C and to solubilize the formed formazan the 50 μl of DMSO added. The viable cells were read by ELISA multi-well plate reader (BioTek power wave XS2, USA) at 590 nm. The untreated human lymphocyte cells were taken as a control [19].

2.6. Genotoxicity by cytokinesis-block micronucleus assay (CBMN)

For the CBMN assay, the 5 ml of HiKaryoXL RPMI Medium (HiMedia) added with 0.5 ml of the blood sample. The cells were incubated at 37 °C for 72 h and added 6 μl/ml of cytochalasin-B at 44th h. Human lymphocytes exposed to 10, 25, 50 and 100 μg/ml concentrations of NP and BP for micronucleus (MN) study. After 24th h of culture initiation, the cells were exposed to the samples. The harvesting initiated at 72nd hr and the cells were resuspended using hypotonic solution (0.075 M KCl) and fixative (cold methanol: acetic acid (3:1)) solution. The slides prepared after fixation and air dried. The slides were stained with Giemsa (5%) for 10 min and washed with DW. For MN analysis, 1000 binucleates cells were scored for each concentration of BP and NP [20].

2.7. Statistical analyses

The erythrocyte morphology analysis, cell viability, and MN assay data were analyzed statistically with the unpaired *t*-test for ANOVA (analysis of variance), and the significance differences were determined between control and treated groups. Prism graph pad version (6.0) used for the analyses.

3. Results

3.1. Nanopermethrin formulation

The rapid evaporation of permethrin loaded o/w emulsion by lyophilisation led to the formation of permethrin nanopowder (NP). The NP composition was found to consist of permethrin, 29.5 wt% of AG, 28 wt% of sucrose and 29.5 wt% of SbPC [13]. Permethrin content in NP was found to be 16.18 wt%, and the mean hydrodynamic diameter of NP was found to be 199.01 ± 1.4 nm [14]. The NP has exhibited good kinetic stability, low viscosity, amorphicity and hydrodispersive stability [13,15].

3.2. Erythrocyte morphology analysis

The blood sample-interacted with BP and NP influences the erythrocyte morphology and induced echinocyte (Figs. 1 and 2). The blood sample-interacted with BP has displayed a many numbers of crenated cells (echinocyte) than the cells treated with an NP in dose-dependent manner. In 50 and 100 μg/ml of treated BP showed significantly ($p < 0.05$, $p < 0.001$) increase in echinocyte compared with NP. The significant difference ($p < 0.05$, $p < 0.001$, $p < 0.0001$) found in different concentrations treated BP as compared with control. The similar toxicological influence was found in the NP interacted with blood, except 10 and 25 μg/ml at 72 h (Table 1A–1B).

3.3. Cytotoxicity by MTT assay

The cell viability was calculated after 24 h of BP and NP interacted with MTT. The present study proved that there is a decrease in cell viability compared to BP and NP in an increasing dosage of samples. The BP and NP showed a significant ($p < 0.001$) reduction in the percentage of cell viability at 50 and 100 μg/ml of