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Permethrin induced cytotoxicity of rat splenocytes: Protective effect of N-acetylcysteine

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Permethrin is a synthetic insecticide, extensively used in pest control. Exposures to permethrin have been attributed to increased cell death. The mechanism for its toxicity is still not clear. Hence, in the present study we determined the molecular mechanism associated with permethrin induce cytotoxicity. Rat splenocytes were incubated with increasing concentration of permethrin (0-39 ug/Ml) for 6 to 24 h. Cytotoxic effect of permethrin was evaluated by MTT assay. To assess the mechanism of cytotoxicity, different biochemical indices of cell death, namely annexin V binding assay, DNA fragmentation assay, and levels of caspase 3 were analyzed. To evaluate the oxidative stress, glutathione depletion and melondialdehyde levels were analyzed. MTT assay revealed that permethrin induces cytotoxicity in dose-dependent way. In annexin-V binding assay, above 7.8 µg/mL concentration, significant necrosis of cells was noticed and consistent with DNA fragmentation assay. A significant dose and time dependent depletion of cellular glutathione (GSH) and increased MDA levels were observed and consistent with the percentage of cells undergoing apoptosis. Co administration of N-acetycysteine mitigates permethrin- induced apoptosis, showing the role of oxidative stress in apoptosis induction. The present study demonstrated the role of oxidative stress in permethrin-induced cytotoxicity in rat splenocytes *in vitro*.

Keywords: Apoptosis, Cytotoxicity, Insecticide, Necrosis, Oxidative stress, Pesticide

Permethrin is an extensively used synthetic pyrethroid for agriculture and public health purposes in India. Due to the widespread application of permethrin and increased persistence embodied risk to non target organisms. A significant amount of permethrin residues were detected in fruits and vegetables that may create a dietary risk to non-target animals including human[1]. Studies have reported quantifiable levels of permethrin residues in human samples[2,3]. To assess the environmental risk to non target organisms, the valuation of pesticide residues should be correlated with the monitoring of biomarkers that act as early warning signals. Several studies suggest that permethrin causes neurotoxic effects. It interacts with voltage-dependent sodium channels in excitable membranes that cause the channels to remain open much longer than normal resulting in enhanced neurotransmitter release[4,5].

The spleen is the primary site of blood filtration in the body and hosts a wide range of immunologic functions alongside its roles in hematopoiesis and red blood cell clearance. Cells specific to spleen are responsible for monitoring the blood and initiating adoptive immunity[6]. Unintended exposure to human beings during handling, storage, and disposal causes occupational hazards increasing issue on health risk assessment towards the toxicity, including cytotoxic, and genotoxic effects[7]. A significant target of oxidative damage is DNA that promotes apoptosis[7-9]. An alteration in DNA indicates early damage in affected organisms that indentify the genotoxic potential of pesticide for effective risk assessment. DNA is oxidized as a result of attack by free radicals being release exogenously and endogenously[8]. Pesticide induced oxidative stress has been observed by the increased level of malondialdehyde (MDA) i.e. index of lipid peroxidation and by differentially modified endogenous antioxidants causing moderate to severe pathophysiological consequences[8,9].

N-acetylcysteine (NAC), an aminothiol, and a synthetic precursor of intracellular cysteine used therapeutically in various disorders related to oxidative stress[10]. It has been reported that NAC is a scavenger of hydrogen peroxide (H_2O_2), hypochloric

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acid (HOCl), hydroxyl radical (•OH) as well as it is a powerful antioxidant[9]. Several therapeutic effects of NAC has been reported such as oral administration of NAC can prevent the radiocontrast-induced nephropathy, schizophrenia, bipolar disorder, pancreatic β-cell dysfunction, chronic obstructive pulmonary disease (COPD), exacerbations, etc[11-13]. It also reduces the toxic effects of some pesticide and chemotherapeutic agents such as cisplatin[14-17]. In this study, we scrutinized permethrin-induced programmed cell death leading to cytotoxicity in rat splenocytes. We measured the cellular GSH and MDA levels to find out permethrin -induced oxidative stress. Further, we investigated whether administration of N-acetylcysteine have any protective role against permethrin-induced apoptosis/cytotoxicity.

Materials and Methods

Chemicals

Permethrin (25:75 cis:trans isomer ratio, purity 99%, CAS No. 52645-53-1) was obtained from Sigma-Aldrich Limited, St.Louis, MO, USA. RPMI-1640 media, Histopaque-1077, N-acetyl cysteine (CAS No. 616-91-1) and required analytical grade chemicals brought from Sigma-Aldrich Limited, St.Louis, MO, USA.

Isolation and treatment of splenocytes

Male Wistar rats, 5-8 weeks old, weighing 200–250 g, were housed under standardized conditions of temperature (22-24°C) and 12 h dark/light cycles and received standard food and water according to guidelines of CPCSEA, New Delhi after approval of Institutional Animal Ethics Committee (IAEC), IFTM University, Moradabad. Naive wistar rats were euthanized by cervical dislocation and their spleens were removed. The spleen was then passed through a 100 mm nylon mesh and splenocytes were isolated using density gradient centrifugation. Briefly, Single cell suspensions were prepared by mincing and tapping spleen fragments on a 100 mm nylon mesh held in Hank's balance salt solution. Five mL tissue lysates was spread cautiously over equal volume of Histopaque 1077 and centrifuged for 30 min at 2,000 rpm. Splenocytes taken from the buffy layer formed at the lysate-Histopaque 1077 interface and were diluted in Hank's balance salt solution. Counting of cell was done in Neubauer chamber with the help of trypan blue and finally re-suspended in RPMI-1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U penicillin/mL and 100 mg streptomycin/mL to obtain a final media of concentration 2×10^6 cells/mL[18]. Cells were put in each well of 24-well plates and incubated in 5-95% CO_2-humid atmosphere at 37°C in CO_2 incubator. Cells were treated with increasing concentration of permethrin (0-39 μg/mL) for various time periods (6, 12 and 24 h). Cells were also treated with either NAC or (4.98 μg/mL), together with permethrin. The concentration of permethrin, and NAC selected in this study on the basis of our previous studies[14,19,20]. Control cells were exposed to 0.5% dimethyl sulfoxide (DMSO).

Cytotoxicity assay

Cell viability, being measure of cytotoxicity, was determined by estimating the capacity of the cells to reduce MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to intracellular purple formazan which was quantified at 540 nm with the help of a microplate reader (BGS-277, Biogen Microplate Reader). The result is presented as percentage of the control cells[21].

Apoptosis detection

AnnexinV-Cy3.18 binding assay

The method employed in this assay (Annexin V-Cy3ä Apoptosis Detection Kit - Catalog Number APOAC) to differentiate apoptotic cells from necrotic cells involves the use of two labels: Annexin-Cy3.18 (AnnCy3) binds to phosphatidylserine present in the outer leaflet of the plasma membrane of cells starting the apoptotic process. The binding is observed as red fluorescence. To measure viability, 6-carboxy-fluorescein diacetate (6-CFDA) was used. When this non-fluorescent compound enters living cells, esterases present hydrolyze it, producing the fluorescent compound, 6-carboxyfluorescein (6-CF). This appears as green fluorescence. Briefly, induced cells were washed twice with PBS (phosphate buffer saline) and were suspended at a concentration approx.1×10^6 cells/mL. A circle of 1.0 cm diameter was drawn on a polyprep poly-L-lysine coated slide. About 50 μL cell suspension was added to each circle and keep at room temperature i.e. 37°C for 10 min. Cells were washed two time with binding buffer and a double label staining solution (containing AnnCy3 and 6 CFDA) was used for staining. After washing with five aliquots of binding buffer each circle were covered by cover slip and visualized under fluorescent microscope (Nikon-HFx-Dx) with excitation and emission wavelength 490/515 nm. Live cells was labeled only with 6-CF (green), while necrotic cells was labeled with AnnCy3 (red) only. Cells in the early stage of apoptosis, was labeled with both AnnCy3 (red) and 6-CF (green). Based on staining, 300 cells were observed and categorized as necrotic (pink), apoptotic (green and pink) and non-apoptotic (green) and expressed as percentage of total cells[22,23].

DNA fragmentation assay

It was done by electrophoresis of isolated DNA in agarose gel. Briefly as described by Ishikawa *et al.*[24]. Cultured cells were obtained after centrifugation (1500 rpm for 5 min). The cell pellets were washed twice with PBS (phosphate buffer saline), immediately lysed with 400 µL lysis buffer (1% TritonX, 50 mM Tris/HCL, pH-7.5, 20 mM EDTA) and centrifuged (4500 rpm for 5 min) in a microcentrifuge (Eppendorff). The supernatant containing DNA was separated and incubated at 50°C for 3 h with 20 µL of 10% SDS (sodium dodecyl sulphate) and 5 µL RNase A (10 mg/mL). After incubation 5 µL proteinase K (15.6 mg/mL) was added and the mixture was further incubated at 37°C for 3 h. DNA was precipitated by adding ethanol, removed through centrifugation and dissolved in TE buffer (Tris-EDTA buffer). Aliquots of DNA from different groups together with 100 bp marker were electrophoresed using 1% agarose gel holding 0.0001% ethidium bromide at 80V/18 Amp for 3 h. The bands were observed and photographed using a gel documentation system (UVP DigiDoc It LS)[24].

Caspase 3 level estimation

Caspase 3 levels were estimated by competitive enzyme immunoassay technique using Rat Caspase 3 ELISA kit (MyBiosource.Inc, San Diego, CA) utilizing a polyclonal anti-Caspase-3 antibody and Caspase-3-HRP conjugate as per the manufacturer protocols. Briefly, plate 10,000 cells/well in a 96-well plate with different concentration of permethrin and incubate plates for 24 h at 37°C in 5% CO_2. Cells lysateassay sample (100 µL) and buffer were incubated with 50 µL of caspase-3-HRP conjugate in pre-coated plate for 1 h. After the incubation period, the wells are decanted and washed five times. After incubation, well were washed and subsequently 50 µL of substrate was added to each well and incubated for 15 min at 37°C. The product of the enzyme-substrate reaction forms a blue colored complex. Finally, a stop solution was added to cease the reaction, which turns the solution yellow. The intensity of colour was measured spectrophotometrically at 450 nm in a microplate reader. The result is expressed as percentage of control cells[25].

Estimation of glutathione

The assay of glutathione (GSH) was done spectrophotometrically (UV-1800-Shimadzu UV spectrophotometer) in a reaction mixture comprising cell lysate, DTNB and NADPH. The reaction mixture (1.0 mL) comprised 25 µL of reduced glutathione in HCl, 50 µL of NADPH (0.2 µmoles/mL in 0.01M/0.005M phosphate EDTA buffer), 10 µL of glutathione reductase (1 unit) and 800 µL of phosphate EDTA buffer (pH 7.5). The test samples were made by adding 25 µL of cell lysate in lieu of glutathione reduced added in standard. The chromophoric product produced from reaction of reagent DTNB (0.6 µmol) with glutathione was measured spectrophotometrically at 412 nm. GSH concentration is shown as percentage of control cells[26].

Lipid peroxidation

Malondialdehyde levels in cell lysate were determined in accordance with the method narrated by Satoh, 1978 with little modification using thiobarbituric acid reagent (TBA). MDA–TBA adduct formation was determined spectrophotometrically at a wave length of 532 nm. The concentration of MDA was expressed as nmol/2 × 10^6 cells[27].

Statistical analysis

Samples were made in triplicate and experiments were carried at least four times. Data are shown as mean ± SD. Statistical analysis was done with one-way ANOVA using SPSS version 17 (Chicago, IL). The various treated groups were compared by Tukeys' multiple comparison test. P <0.05 was taken to be statistically significant.

Results and Discussion

Permethrin-induced cytotoxicity

The time and concentration-dependent cytotoxic influence of permethrin on splenocytes was estimated by the MTT assay (Fig. 1). The cells were treated with 0-39 µg/mL of permethrin for 6, 12 and 24 h. Permethrin at 0.3 µg/mL did not show a considerable decrease in number of living cells as compared to vehicle-treated control cells and 94-95% viability was examined during 24 h incubation. Above 7.8 µg/mL, 14-18% reduction in the number of surviving cells was examined. Above 0.3 µg/mL, 12-36% reduction in the number of living cells was recorded. However, for highest selected concentration, up to 55% decline in the number of living cells was observed.

Effect of permethrin on endurance of splenocytes

In search of whether this toxicity at low dose exposure of permethrin is due to programmed cell death, three different approaches viz., Annexin-V-Cy3.18 binding assay, DNA fragmentation assay, and estimation of activated caspase 3 levels were employed in this study. The percentage of cells which bind to annexin-V (which were representative of cells either undergoing apoptosis or necrosis) are shown in Table 1. A clear trend can be noticed in the declining number of non-apoptotic splenocytes with increasing concentration of permethrin. At 0.3-7.8 µg/mL, the percentage of cells undergoing apoptosis risen significantly in comparison to control and was appeared to be in the range 7-13%, whereas cells undergoing necrosis varied between 2-10% (Table 1). All five concentrations of permethrin used exhibited significant variation in the number of cells undergoing apoptosis than vehicles control (24 h). Percentage of cells undergoing apoptosis decreased to some extent, at higher concentrations, however, number of cells exhibiting necrosis increased with increase in permethrin concentration.

In a separate experiment cells were treated with permethrin for DNA fragmentation assay. The electrophoretic patterns of DNA isolated from cells treated with different doses of pesticides for 24 h are depicted in Fig. 2. Distinct DNA ladder was noticed when cells were treated with 7.8 µg/mL concentration of permethrin. Smearing of DNA bands suggesting necrosis in case of high dose exposure. To further evaluate permethrin effect on apoptosis we have observed level of caspase 3 in treated cells. The level of caspase 3 was shown to be risen in a dose dependent manner (Fig. 3).

Table 1 — Effect of different concentrations of Permethrin on Induction of Apoptosis/Necrosis of rat splenocytes

Treatment	Nonapoptotic Cells	Apoptotic Cells	Necrotic Cells
Control	94.66±2.16	4.16±0.57	1.18±1.12
0.3 µg/mL	91.00±2.58[a]	7.33±1.84[a]	1.67±1.13
3 µg/mL	84.00±3.9[a]	8.58±1.50[a]	7.42±1.25[a]
7.8 µg/mL	76.89±2.63[a]	13.03±1.41[a]	10.08±4.11[a]
19 µg/mL	71.99±1.70[a]	12.09±1.63[a]	15.92±2.87[a]
39 µg/ml	62.01±2.64[a]	8.16±1.00[a]	29.83±2.08[a]
Positive control (30 µM NaAsO2)	57.00±2.44[a]	26.83±1.29[a]	16.17±2.44[a]

[Values show the percentage of cells that bind to annexin-V observed under fluorescent microscope, which were representative of cells either undergoing apoptosis or necrosis after 24 h incubation. [a] Significantly difference, $P < 0.05$ compared with vehicle control. Inorganic arsenic at concentrations of permethrin on induction of apoptosis/necrosis of normal rat splenocytes]

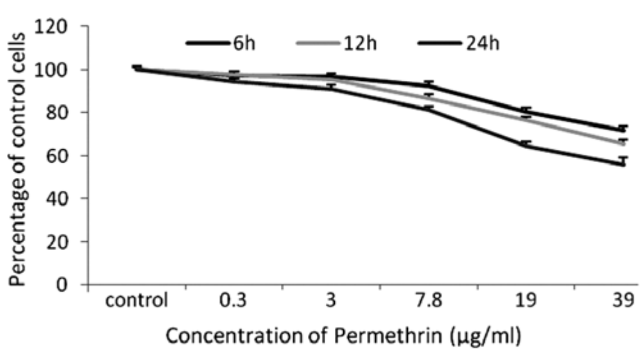

Fig. 1 — Effect of permethrin on the viability of Rat splenocytes. [Splenocytes were treated with permethrin (0–39 µg/mL) for 6, 12, and 24 h. Cell viability was determined by measuring the capacity of cells to reduce MTT (3-(4,5 dimethylthiazol-2yl)-2,5-diphenyltetrazoliumbromide) to intracellular purple formazan, which was quantified at 540 nm. The results are presented as percentage of control cells]

Effect of N-acetylcysteine (NAC)

To find out the attenuating effect of NAC in permethrin-mediated apoptosis, we incubated splenocytes up to 24 h with 7.8 µg/mL of permethrin along with NAC (4.98 µg/mL) in culture media and subsequently measured levels of lipid peroxidation and GSH, and the number of annexin V binding cells. Intracellular GSH level was measured in permethrin exposed cells using spectrophotometry. The levels of

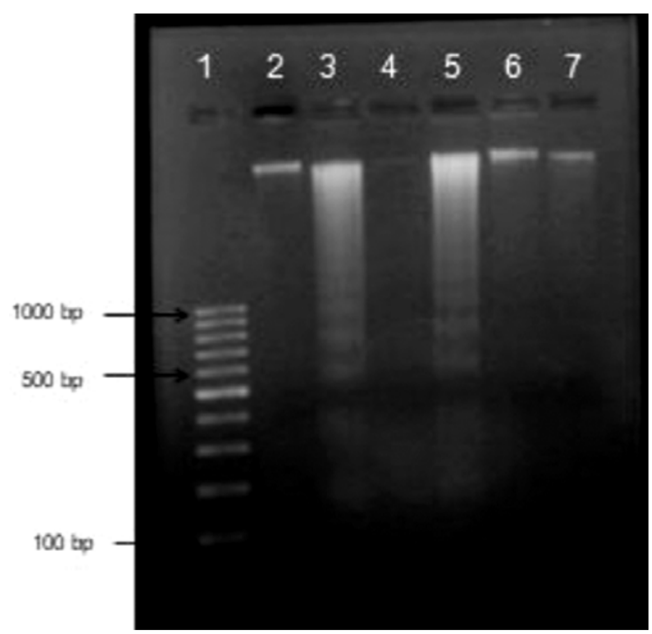

Fig. 2 — DNA fragmentation of splenocytes after permethrin treatment (24 h). [Lane 1, 100 bp marker; lane 2, control cells; lane 3, 19 µg/mL permethrin-treated cells; lane 4, negative control; lane 5, 7.8 µg/mL permethrin-treated cells, lanes 6 & 7, 7.8 µg/mL permethrin-treated cells along with 4.98 µg/mL NAC]

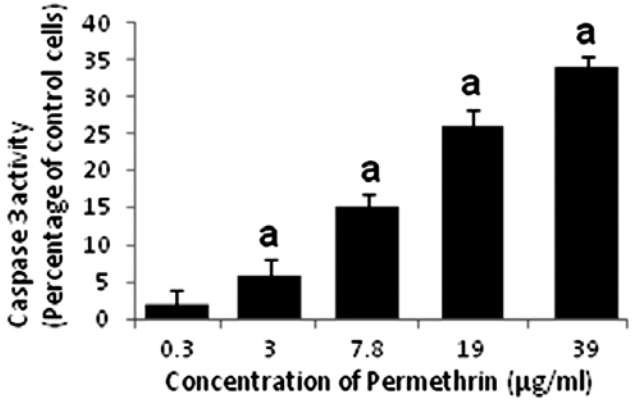

Fig. 3 — Levels of activated caspase 3 in cell lysate of permethrin treated cell at 24 h incubation. [Activated caspase 3 are expressed as percentage of control cells (mean±SD). [a]Significantly different compared with vehicle (0.05% DMSO) treated control cells ([a] P <0.05)]

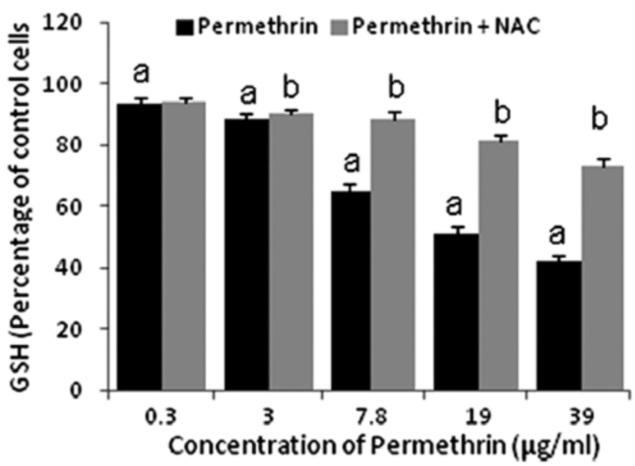

Fig. 4 — Effect of NAC on GSH level at 24 h incubation in permethrin-treated cells. [Bars with different letters are significantly different ([a,b]P <0.05)]

GSH depleted in a dose-dependent way in permethrin-treated cells (Fig. 4). Co-administration of NAC revived the GSH levels significantly as compared to permethrin-exposed cells (Fig. 4). Lipid peroxidation in permethrin treated cells was found to be significantly risen for 6, 12 and 24 h (Fig. 5). Co-treatment of NAC significantly reduced the levels of MDA (Fig. 5). Simultaneous treatment of NAC results a significant betterment in the number of surviving cells, in comparison with respective permethrin-treated groups (Fig. 6).

Discussion

The results obtained from the present study clearly revealed that permethrin induced cytotoxicity in Rat splenocytes. Apoptosis is an autonomous physiological process involved in development,

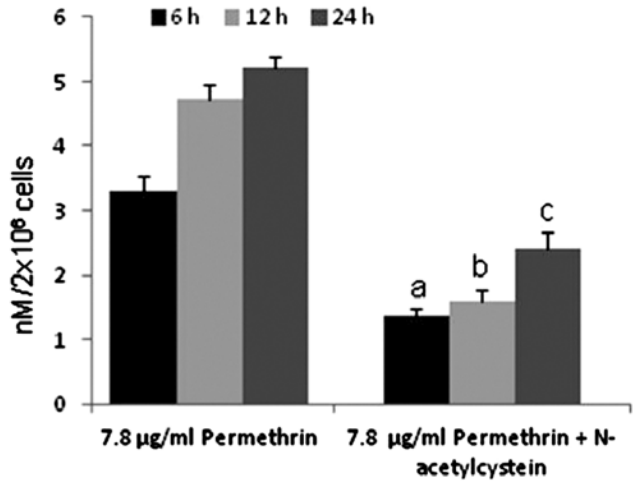

Fig. 5 — Effect of permethrin on the levels of MDA in rat splenocytes. [Splenocytes at a density of 2×10^6 were cultured with different concentration of permethrin ((0-39µg/mL) for 6, 12 and 24 h and then the levels of TBARS in cell lysates were determined by the TBA assay. [a,b,c] are significantly different compared to vehicle control (P <0.05)]

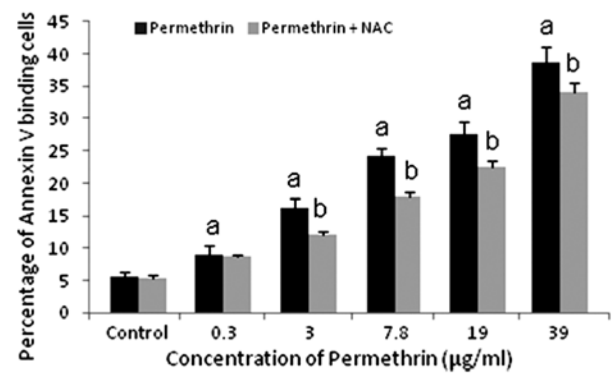

Fig. 6 — Effect of NAC on Annexin binding cells at 24 h incubation in permethrin-treated cells. [Bars with different letters are significantly different ([a,b]P <0.05)]

homeostasis and cellular defense of multicellular organism by omitting undesirable cells. It may be plausible that apoptosis of immunocyte's is one of the reason of permethrin-induced immunotoxicity, energizing caspases and apoptotic signaling pathways[28,29]. Wang *et al.*[28], have represented by schematic flowchart demonstrating that permethrin may directly produce oxidative stress that leads to modified expression of stress related genes such as Keap1/Nrf2/ARE, THFR1/TNF-α, along with NF-κB pathway[28]. The activation of caspase cascades depends on the apoptosome which is compose of Apaf-1 in the cytoplasm and the response to cellular protein cleavage, and eventually results in occurrence of apoptosis[30]. Thus, caspase 3 can serve as marker for apoptosis[25,31].

In the present study, we analyzed the cytotoxic effects of permethrin on rat splenocytes for different time periods. To determine the number of viable cells in permethrin treated groups, the MTT assay was used. A dose- and time-dependent decrease in the number of surviving cells was observed on addition of permethrin (Fig. 1). To further evaluate this cellular toxicity, we analyzed different parameters of apoptosis viz., annexin-V-Cy3.18 binding assay, DNA fragmentation assay and intracellular levels of caspase 3. At low concentration, number of cells showing apoptosis increases significantly, late apoptotic and necrotic cell death occurs when the cells were treated with higher concentration for 24 h. In the DNA fragmentation assay, clear DNA fragmentation was observed at 7.8 µg/mL concentration of permethrin (Fig. 2). However, exposing the cells to higher concentration results smearing effect in the gels indicating late apoptosis or necrosis.

We examined the role of apoptosis and oxidative stress in pesticide-induced cytotoxicity in rat splenocytes cell *in vitro*. The results of this study clearly displayed that apoptosis of immunocytes may be the underlying mechanism of permethrin-induced immunotoxicity, that is consistent with the previous studies on induction of apoptosis by pesticide and pesticide mixture *in vitro*[32-34]. A concentration dependent increase in the levels of caspase-3 further confirms activation of apoptotic process following permethrin exposure (Fig. 3).

Antiproliferative effect of permethrin on splenocytes may contribute to splenic atrophy and advocate a mechanism leading to hypocellularity supporting the hypothesis that splenocytes are metabolically less active[35]. It is debated that *in vitro* assays may not reflect potency in animal or human responses. Several studies have been undertaken with pesticides in an effort to understand potency determinants for genotoxicity, including binding affinity for P450 enzymes, metabolic intermediate stability, DNA adduct stability and DNA repair. These factors are likely to vary among systems and species[18]. Also, the risk of false negatives could be the main limitation of cell-based assays. However, development of *in vitro* models which are sensitive is of great importance that can be used for general assessment of xenobiotics induced toxicity and is likely to be applicable to all cell types[36] since maintenance of healthy immune system is fundamental requirement to an organism to resist disease. Effects of xenobiotics on various components of the immune system has become an area of research interest. Spleen, being the largest secondary lymphoid organ in mammals, lymphocytes in spleen gets encounter with antigen, undergoes clonal expansion, and regulates immune responses of the organisms. An increase in the degree of apoptotic cell death in lymphocytes could compromise immune regulation, because it could alter neural, endocrine or autoimmune response. Therefore, this study was designed to increase understanding of pesticide toxicity to a typical non target organ system *in vitro*, i.e, splenic mononuclear cells.

Pesticides are known as inducers of oxidative stress[14,17]. GSH is a major antioxidant system of the cells engaged in the maintenance of redox status of the cells. A shift in the cellular GSH-to-GSSG redox balance constitutes an important signal that could decide the fate of a cell. Once oxidative stress is induced, it activates mitochondrial-dependent apoptotic cascade[29]. The cellular GSH status also play an important role in the posttranslational modification of specific cysteine residues in a process termed S-glutathiolation, the formation of mixed disulphides between redox-sensitive cysteine and GSSG[38]. Reversible S-glutathiolation of caspases has been suggested as a sensitive mechanism for caspase activation in apoptotic signaling underscoring the importance of GSH/GSSG involvement in the redox-sensitive regulation of cell apoptosis through caspase-3[38,39]. As expected a correlation was found between the GSH and caspase 3, an apoptotic marker ($r = -0.986$; $P < 0.05$) establishing the link between oxidative stress and apoptosis in permethrin-induced cytotoxicity. MDA is an index of lipid peroxidation, the most profuse carbonyl products of this process[26,27]. In the present study, lipid peroxidation was found to be significantly increased following exposure with permethrin (Fig. 5). To further verify if oxidative stress plays a vital role in inducing apoptosis, we explored the thiol antioxidant NAC, which is a precursor of GSH, and examined the effect of NAC on cellular GSH level and apoptosis. The results suggest that greater the protection provided by NAC against GSH depletion, the lower is the number of necrotic/apoptotic splenocytes (Figs 4 & 6).

Cumulatively, this study reveals that the permethrin-induced cytotoxicity is due to induction of apoptosis of immunocytes at lower concentrations and oxidative stress may be the underline mechanism. Hence the results are consistent with our previous studies conducted with different other pesticides[9,14,17,30]. This study also shows that NAC may produce an attenuating effect via enhancing antioxidant capacity and inhibition of oxidative stress, suggesting its possible therapeutic role in case of permethrin exposure. It is important to note that *in vitro* mammalian cell assays detect classes of genotoxic agents not identified *in vivo* like bacterial assay (e.g., antibiotics and chromosomal damaging agents). Therefore, it should be taken in to consideration that some xenobiotics will be negative in bacterial assays and positive in *in vitro* mammalian assays. Therefore, *vis-a-vis* lowering the cytotoxicity or genotoxicity limits would likely eliminate detection of several xenobiotic compounds.

Conclusion

The present study is purely *in vitro* in nature and the results demonstrated the genotoxic risk of permethrin that is technically feasible. This in vitro study using rat splenocytes to reveal the molecular mechanisms behind pesticide poisoning and might suggest therapeutic approach for development of better remedy. Further studies are desirable to understand the effect of permethrin in an *in vivo* situation.

Conflict of interest

The authors declare no conflict of interest

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