Research Article

Journal of Applied Toxicology

Received: 1 October 2008, Revised: 3 December 2008, Accepted: 3 December 2008, Published online 19 January 2009 in Wiley Interscience

(www.interscience.wiley.com) DOI 10.1002/jat.1412

Permethrin induces lymphocyte DNA lesions at both Endo III and Fpg sites and changes in monocyte respiratory burst in rats†

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ABSTRACT: Pyrethroids are widely used insecticides of low acute toxicity in mammals but the consequences of long-term exposure are of concern. Their insecticidal action is related to neurotoxicity and, in addition, there are indications of mammalian immunotoxicity. In this work the effect of 60 days permethrin (150 mg kg^{-1} body weight/day) exposure on two types of leukocytes (monocytes and lymphocytes) in adolescent rats was investigated. In particular, the monocyte respiratory burst response was first investigated, followed by studies on the degree and type of lymphocyte DNA damage induced by permethrin at this stage of life. Permethrin treatment reduces the monocyte respiratory burst response to phorbol myristate acetate, thereby decreasing superoxide anion (65%) and hydrogen peroxide (37%) production. Moreover an increase [correction made here after initial online publication] in monocyte plasma membrane fluidity in the hydrophilic–hydrophobic interface of the lipid bilayer was measured. Data obtained from the comet assay show that permethrin induces lymphocyte DNA lesions at both formamido pyrimidine glycosylase (Fpg) and endonuclease III (Endo III) sites in adolescent rats. Our results indicate the key role of permethrin in oxidative stress whose consequences lead to biochemical and functional changes. The reduced phagocyte respiratory burst induced by permethrin treatment and the type of DNA damage measured could represent new relevant aspects of pyrethroid toxicity which should be considered for human health. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: permethrin; adolescent rat; Endo III; Fpg; comet assay; respiratory burst

Introduction

Pyrethroid insecticides are a class of agents which mainly act on the central nervous system, although significant effects on peripheral nerves and muscle are also known. Their capacity to interact reversibly with a wide range of ion channels, possibly via their phosphorylation state, is a key property of pyrethroids, and sodium channels are their major target (Davies *et al*., 2007; Narahashi *et al*., 2007; Peterson *et al*., 2008). The widespread use of pyrethoids depends on their capacity to control pests. These insecticides are extensively employed throughout the world as wide-spectrum insecticides for numerous crops and for indoor pest control in the public health sector and housing (Costa *et al*., 2008; Bjørling-Poulsen *et al*., 2008). They are effective against ticks, mites and lice and consequently they are present in household sprays, flea preparations for pets and plant sprays for home and greenhouse use.

The consequent increased human exposure has led to an increase in studies on these insecticides and many papers indicate certain aspects that were not considered during the original evaluation of pyrethroid toxicity. Functional and pharmacological neurotoxicology mediated by an action on voltage-gated sodium channel was revealed in the first phase of studies on pyrethoids (Verschoyle and Aldridge, 1980; Ray and Cremer, 1979). Additional information has recently been obtained on neonatal and mammalian toxicity induced by pyrethroids and their metabolites (Nasuti *et al*., 2007; Abdel-Rahman *et al*., 2001; Pittman *et al*., 2003; Karen *et al*., 2001; Sogorb and Villanova 2002; Sevatdal *et al*., 2005; Young *et al*., 2005). An age-dependent effect was observed, indicating that neonatal rats are 4–17 times more vulnerable to the acute toxicity of pyrethroids than adult rats (Cantalamessa, 1993; Sheets *et al*., 1994). Although mammals are three orders of magnitude less sensitive at ion channel sites than insects to pyrethoids, recent studies show that these insecticides can induce oxidative damage not only in the brain but also in other targets such as liver, erythrocytes and lymphocytes (Song and Narahashi 1996; Vais *et al*., 2000; Dayal *et al*., 2003; Gabbianelli *et al*., 2002, 2004; Nasuti *et al*., 2003).

The aim of the present study was to investigate the damage induced by permethrin on two types of leukocytes (monocytes and lymphocytes), in adolescent rats, following oral treatment (60 days) with 1:10 solution of LD_{50} permethrin. Adolescent rats were chosen because it was of interest to evaluate if permethrin induces damage dependent on different life phases (pup, adolescent, adult) by comparison with our previous studies, where

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† This article was published online on January 19, 2009. An error was subsequently identified. This notice is included in the online and print versions to indicate that both have been corrected [April 17, 2009].

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permethrin treatment led to lymphocyte DNA damage and to decrease in monocyte respiratory burst in adult and pup rats (Gabbianelli *et al*., 2002, 2004; Nasuti *et al*., 2003, 2007). As known, permethrin toxicity is related also to oxidative processes involving biological macromolecules (Bjørling-Poulsen *et al*., 2008). Damage to macromolecules often leads to changes in structure, function and stability, such as altered folding, mistargeting, and altered epitope exposure (Rattan, 2008). Since the biological responses to the sources of damage and the repair pathways can change during the different life phases, the effect of permethrin could be different according to the age of organisms.

In the present study, the objective was first to investigate the monocyte respiratory burst response and, second, the degree and type of lymphocyte DNA damage induced by permethrin in adolescent rat. Fluorescence measurements were performed on leukocytes in order to evaluate the involvement of plasma membrane fluidity changes in the respiratory burst response following permethrin treatment. Knowledge of these types of permethrin toxicity could be useful for underlining the life-related resistance of adolescent rats compared with pups and adults.

Materials and Methods

Materials

All reagents were of pure and analytical grade. Lucigenin, luminol and phorbol myristate acetate (PMA) were obtained from Sigma Chemical Co. 1,6-Diphenyl-1,3,5-hexatriene (DPH) and 6-lauroyl-2-dimethylaminonaphtalene (laurdan) were purchased from Molecular Probes (Eugene, OR, USA). Lymphoprep for separation of rat lymphocytes was obtained from Nycomed Pharma AS, Oslo, Norway, and Dulbecco's Modified Eagle from Life Technologies (Palsley, Scotland). Formamido pyrimidine glycosylase (Fpg) and Endonuclease III (Endo III) FLARE™ Assay Kit were purchased from Trevigen Inc. (Gaitherburg, MD, USA). Technical grade (75:25, *trans*:*cis*; 94% purity) 3-phenoxybenzyl-(1*R*,*S*)-*cis*,*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate (permethrin, PERM) (NRDC 143) were generously donated by Dr A. Stefanini of ACTIVA, Milan, Italy.

Animals

Male Wistar rats from Charles River (Calco, LC, Italy), weighing 120–130 g and about 5 weeks old were used. The animals were housed in plastic (Makrolon) cages (five rats/cage) in a temperature-controlled room (21 ± 5 °C) and maintained on a laboratory diet with water *ad libitum*. The light–dark cycle was from 7 a.m. to 7 p.m. Animal use in this study complied with the Italian government's guidelines for the care and use of laboratory animals (DL no. 116 of 27 January 1992).

Treatment

Permethrin was dissolved in corn oil and administered orally (5 ml kg^{-1}) for 60 days, at a dose of 150 mg kg^{-1} body weight/day (1:10 of LD_{50}) (Cantalamessa, 1993) by intragastric tubing. The animals were divided into two groups: one group was treated only with PERM ($n = 16$), and the other one serving as control ($n = 16$) received 5 ml kg^{-1} body weight of corn oil in the same manner as the PERM treated group. The substances were administered daily in the rats and the volume administered was based on body weight. After 60 days of treatment the rats were killed by CO_2 asphyxia for biochemical experiments. Blood was drawn by cardiac puncture from all rats, and collected in vials containing heparin (250 IU).

Respiratory Burst of Monocytes

Monocytes of the control and permethrin groups were separated from fresh blood using Ficol density gradient. After dilution with PBS (1:1), whole blood was stratified on a Lymphoprep solution and centrifuged for 15 min at 2500 rpm. Monocytes, obtained by Ficol density gradient, were washed three times with PBS and counted.

Chemiluminescence measurements can be usefully employed to evaluate the responses of monocytes following their activation, *in vitro*, with PMA. PMA can trigger monocyte respiratory burst *in vitro* similarly to another activating agent *in vivo*. In order to detect the levels of superoxide anion and hydrogen peroxide produced following monocyte respiratory burst, luminogenic probes (lucigenin and luminol) were employed. Lucigenin permits to detect the levels of superoxide anion, while luminol measures hydrogen peroxide production. A reaction mixture of 10^6 cells and 150 μM of lucigenin or 10 μM of luminol in 1 ml of Krebs–Ringer phosphate solution containing 1 mg ml^{-1} of glucose pH 7.4 was prepared and the chemiluminescence (CL) was measured soon after activation with 3×10^{-6} M PMA using an AutoLumat LB953. The CL reactions were monitored for 40 min and the values are expressed as counts per minutes (cpm). The measurements were performed three times in duplicate.

Fluorescence Measurements on Leukocytes

A Hitachi 4500 spectrofluorometer was used for fluorescence measurements on leukocytes. Fluorescent probes can be usefully employed to evaluate plasma membrane fluidity in different regions of the bilayer. The probe, localizing inside the plasma membrane, can give information on its physical state. These measurements are approximate evaluations of the state of phospholipids, because the fluorescence emission of the probe is correlated with the physical state of the lipids surrounding the probe. When the probe used is DPH, the fluorescence emission is used as described below, to evaluate the anisotropy value, while when laurdan is employed, the fluorescence emitted is used to calculate another parameter know as generalized polarization.

Steady-state fluorescence anisotropy (r) of DPH was calculated using excitation and emission wavelengths of 360 and 430 nm, respectively, according to the Shinitzky and Barenhold (1978) equation:

$$r = (I_{\parallel} - I_{\perp})g/(I_{\parallel} + 2I_{\perp})g$$

where g is an instrumental correction factor, and $I_{\parallel}$ and $I_{\perp}$ are the intensities measured with the polarization plane parallel and perpendicular to that of the exciting beam.

Generalized polarization of laurdan (GP_{340}) was calculated according to Parasassi *et al.*'s (1991) equation:

$$GP_{340} = (I_B - I_R)/(I_B + I_R)$$

where I_B and I_R are the intensities at the blue (440 nm) and red (490 nm) edges of the emission spectrum and correspond to the fluorescence emission maximum in the gel and liquid-crystalline phases of the bilayer, respectively (Parasassi *et al*., 1991).

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Leukocytes were normalized to the same protein concentration and incubated for 1 h when the probe was DPH, and for 5 min when laurdan was employed. The different incubation times are related to the time necessary for the two probes to reach their proper location within the plasma membrane. The reaction mixture contained leukocytes (final protein concentration was 0.4 mg ml^{-1}; Lowry *et al*., 1951) and 1 μl of fluorescent probe (DPH or laurdan) at a final concentration of 10^{-6} M in PBS.

Alkaline Single Cell Electrophoresis

The comet assay was used to analyze DNA damage in lymphocytes isolated by lymphoprep; 2.5×10^5 cells were mixed with 65 μl of 0.7% low-melting agarose (LMA) in Ca^{2+}- and Mg^{2+}-free PBS and rapidly spread over a microscope slide previously conditioned with 1% normal melting agarose (NMA) in Ca^{2+}- and Mg^{2+}-free PBS. After solidification of the LMA, a second layer of 75 μl of LMA was added to the slide. To lyse the embedded cells and to permit DNA unfolding, the slides were immersed in freshly prepared ice-cold lysis solution overnight. The lysis solution composition was 1% sodium *N*-lauroyl-sarcosinate, 2.5 M NaCl, 100 mM Na_2 EDTA, 10 mM Tris–HCl, pH 10 with 1% Triton X-100 and 10% DMSO added just before use.

Two different types of comet assays were performed: a classic comet assay (without enzymes) and one with enzymes. In the first case, soon after lysis, a 30 V electrophoresis was conducted for 20 min at room temperature. In the second case, the slides were washed three times with 1× FLARE buffer 1 and treated with DNA repair enzymes in order to examine the levels of oxidized purines (Fpg) and pyrimidines (Endo III). The 1× FLARE buffer 1 was obtained by diluting 25× FLARE buffer 1 (250 mM HEPES–KOH pH 7.4, 2.5 M KCl, 250 mM EDTA). A 100 μl aliquot of FLARE reaction buffer alone (control) or plus 1 U of Fpg or Endo III was added to each slide. FLARE reaction buffer was obtained by mixing 40 μl of 25× FLARE buffer 1 with 10 μl of 100× BSA and made up to a final volume of 1 ml in deionized water. All the slides were carefully placed in a humidity chamber and incubated at 37 °C for 1 h. After keeping the slides at 4 °C for 20 min to ensure the stability of the agarose film, they were placed in the electrophoresis box and electrophoresed (20 V, 200 mA) for 20 min at 4 °C.

After electrophoresis the slides were gently washed with a neutralization buffer (Tris–HCl 0.4 M, pH 7.5) to neutralize the excess alkali and remove detergents. Positive controls were performed by pre-incubating lymphocytes at 4 °C for 10 min with 0.25 mM H_2O_2 before suspending the cells in the LMA.

Slides were stained with ethidium bromide. In each experiment images of 150 randomly selected cells (50 cells from each of three replicate slides) were analyzed for each treatment using an Axioskop-2 plus microscope (Carl Zeiss, Germany) equipped with an excitation filter of 515–560 nm and a magnification of 20×. Imaging was performed using a specialized analysis system (Metasystem Altlussheim, Germany).

Two parameters were considered: tail moment (TM) and %tail DNA. TM was calculated as follows: TM = (TI/total comet intensity) × (tail center of gravity-head center) (Villarini *et al*., 2000), while the %tail DNA was obtained as TI/total comet intensity × 100 (where TI is the percentage of fluorescence in the comet tail). The %tail DNA is considered to be the most useful and meaningful because it gives a very clear indication of what the comets actually look like (Kumaravel and Jha 2006).

Statistical Analysis

The experimental data are expressed as mean values ± SD or SEM in the case of comet assay (at least 150 scores/sample for the comet assay). Statistical analysis was carried out using one-way ANOVA followed by the Student–Newman–Keuls test. A *P*-value <0.05 was considered statistically significant.

Results

General Findings

No significant differences were observed in the body weight of treated vs control rats as previously reported (Nasuti *et al*., 2008).

Respiratory Burst of Monocytes

Monocyte respiratory burst can be followed *in vitro* by activating monocytes with phorbol myristate acetate (PMA). The response of monocytes following PMA activation is similar to that observed *in vivo* and it allows monitoring of superoxide anion and hydrogen peroxide produced by these cells to kill bacteria (Dahlgren and Karlsson, 1999).

In this study, monocyte respiratory bust was measured following PMA activation in control and PERM rat groups. Lucigenin and luminol were employed to monitor superoxide and hydrogen peroxide production, respectively. Figure 1(A) shows superoxide anion production in the two groups following activation of the NADPH oxidase system by PMA. As can be observed, superoxide anion production was lower (65%) in the group treated with PERM compared with the control group. When monocytes were activated with PMA in the presence of luminol, the H_2O_2 level in the group treated with PERM was lower (37%) compared to the control one [Fig. 1(B)].

Effect on Leukocyte Plasma Membrane Fluidity

Steady-state fluorescence anisotropy of DPH and generalized polarization of laurdan were employed to evaluate the fluidity changes in leukocyte plasma membranes from rats treated with pyrethroids. A decrease in GP_{340} value was measured in leukocytes treated with PERM (0.228 ± 0.076) compared with the control group (0.282 ± 0.074; Table 1). No changes were detected in the hydrophobic region tested by DPH (Table 1).

Table 1. Steady-state fluorescence anisotropy (*r*) of DPH and generalized polarization (GP_{340}) of laurdan in leukocytes from control and PERM-treated groups. Normalized samples were studied in PBS containing a final probe concentration of 10^{-6} M. Experiments were performed in triplicate. Data represent the means ±SD

	Anisotropy (*r*)	GP_{340}
Control	0.220 ± 0.0353	0.282 ± 0.0743
PERM	0.226 ± 0.0343	0.228 ± 0.0758*

*$P < 0.05$ vs control.

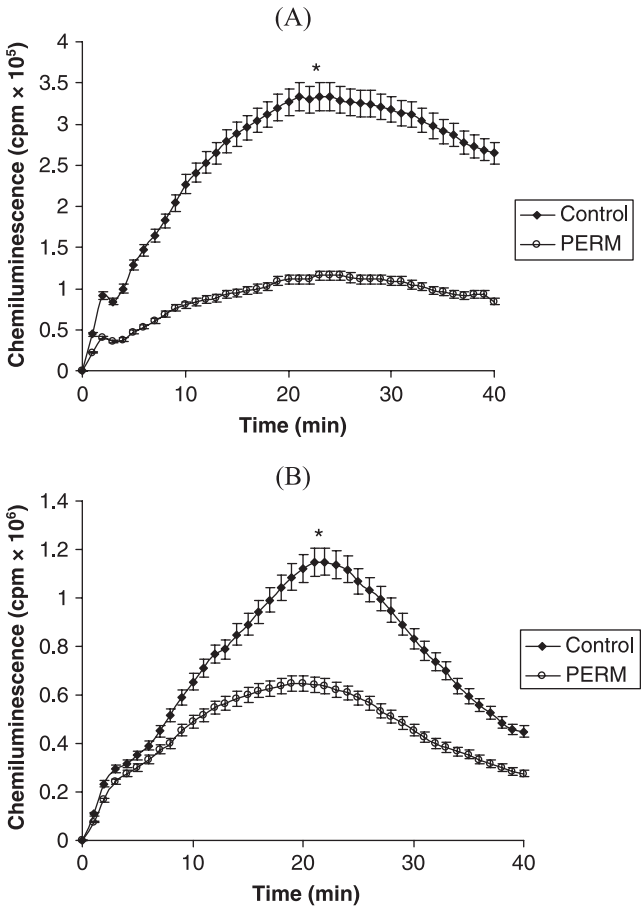

Figure 1. Time course of lucigenin (A) and luminol-amplified chemiluminescence (B) of rat monocytes from control and PERM groups. Monocytes were stimulated with 3×10^{-6} M phorbol myristate acetate. Chemiluminescence was measured as counts per minute (cpm). The results are indicated as mean values ± SD. *$P < 0.05$ vs control.

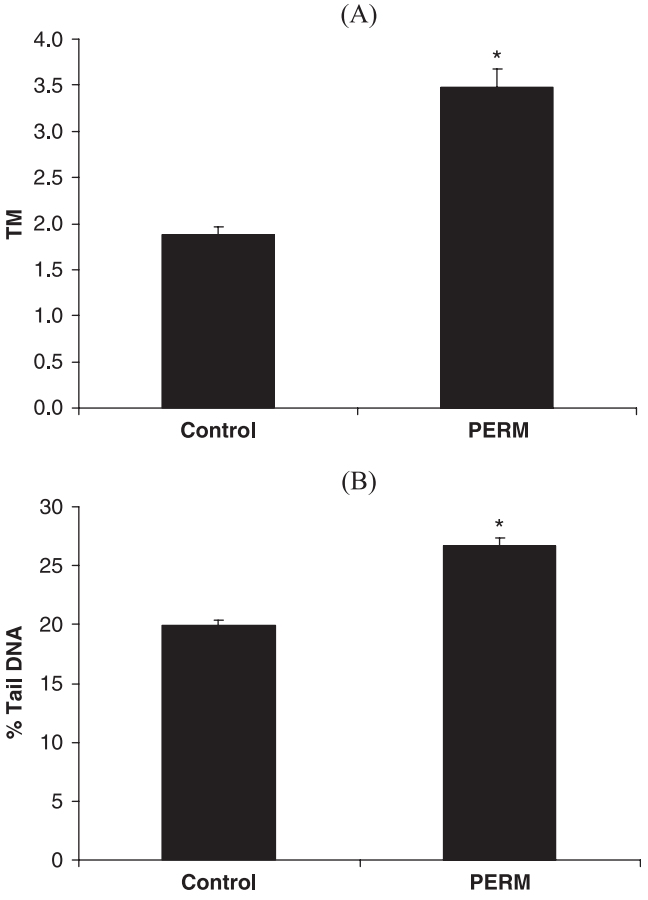

Figure 2. Observed distributions of the comet parameters tail moment (A) and %tail DNA tail (B) in lymphocytes from control and PERM groups. Data (at least 150 scores/sample) are mean values ± SEM of nine observations ($n = 4$ rats for each group, three samples from each animal were used). *$P < 0.05$ vs control.

Alkaline Single Cell Electrophoresis

The Comet assay permits measurement of the level of DNA damage including DNA breaks and DNA base oxidations. The core procedure is that cells are embedded in agar, cell membranes are removed (lysis), the DNA allowed to unwind and electrophoresis performed at mildly or strongly alkaline pH (Collins *et al*., 1997; Collins, 2004). Loops of DNA around strand breaks are more relaxed, and are pulled toward the anode, giving the appearance of a comet tail. Undamaged DNA remains tightly wound in the nucleoid, or comet head (Collins *et al*., 2007; Collins, 2004). Figure 2 shows tail moment (A) and %tail DNA (B) in control and PERM groups. As can be observed, a significant increase in the two comet parameters was measured following PERM treatment (Fig. 2).

In order to identify the type of DNA damage observed after PERM treatment, two repair-specific enzymes (Fpg and Endo III) were employed. Table 2 shows tail moment results obtained following treatment with repair enzymes. As can be observed, PERM induced a significant increase in TM, but the addition of repair enzymes did not indicate an increase in values within the groups. Furthermore, if the data is presented as %DNA tail, as suggested by Kumaravel and Jha (2006), additional information (Table 2) can be obtained. First, the significant difference between

Table 2. Observed distributions of the comet parameters tail moment (TM) and %DNA tail in lymphocytes from rats treated with or without PERM. Data (at least 150 scores/sample) are mean values ± SEM of eight samples performed in triplicate

	TM	%DNA TAIL	(*n*)
Control[1]	3.08 ± 0.072	25.26 ± 0.385	1285
Control Endo III[2]	2.64 ± 0.070	22.94 ± 0.346	1314
Control Fpg[3]	3.10 ± 0.077	25.78 ± 0.382	1282
Perm[4]	4.58 ± 0.178	33.11 ± 0.806	480
Perm Endo III[5]	2.76 ± 0.143	24.69 ± 0.664	410
Perm Fpg[6]	3.33 ± 0.130	27.73 ± 0.622	565
H_2O_2[7]	12.88 ± 0.371	52.86 ± 0.839	688
H_2O_2 Endo III[8]	12.23 ± 0.373	51.41 ± 0.766	622
H_2O_2 Fpg[9]	11.42 ± 0.241	57.47 ± 0.706	666

$P < 0.05$: TM, 4,7,8,9 vs all samples, 6 vs 2; %DNA tail, 8 vs all samples except 7; 7 vs all samples except 8; 2,4,6,9 vs all samples.

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control (25.26 ± 0.385) and PERM (33.11 ± 0.806) groups is confirmed. Secondly, the presence of Endo III leads to higher %DNA tail damage in the PERM Endo III (24.69 ± 0.664) compared with the control Endo III (22.94 ± 0.346) groups. Furthermore, the presence of Fpg produces a significant difference between control Fpg (25.78 ± 0.382) and PERM Fpg (27.73 ± 0.622) groups.

Positive controls, with or without enzymes, and hydrogen peroxide showed significant DNA damage (Table 2), although only in the presence of Fpg did the %DNA tail increase significantly with respect to the sample without the enzyme, as previously reported by Wu *et al*. (2004).

Discussion

During the respiratory burst monocytes produce reactive oxygen species (superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$) and oxidizing compounds (H_2O_2, HOCl) that are useful for their immune response to pathogens. The production of $O_2^{\cdot-}$ depends on the NADPH oxidase complex, which utilizes electrons derived from intracellular NADPH to generate $O_2^{\cdot-}$, which subsequently dismutates to hydrogen peroxide (Dahlgren and Karlsson 1999). The inappropriate or excessive action of this system results in inflammation disorders. Our previous study on pup rats showed that PERM induced a reduction in superoxide production during monocyte respiratory burst (Nasuti *et al*., 2007). A similar effect was observed in adolescent rats: PERM treatment in adolescent rats reduced the monocyte respiratory burst response, decreasing superoxide anion and hydrogen peroxide production [Figure 1(A, B)]. This effect negatively influences the efficiency of phagocytosis, thus reducing the capacity to kill bacteria by monocytes. It might be suggested that PERM alters the signal transduction or interferes with the NADPH oxidase complex formation, thereby reducing the amount of superoxide anion produced and, consequently, hydrogen peroxide formed. The arrangement of the NADPH oxidase complex can be influenced by the physico-chemical state of monocyte plasma membrane. Our results on these cells show that the hydrophilic–hydrophobic region of the bilayer, measured by laurdan, was modified following PERM treatment. This fluorescent probe, localizing in the hydrophilic–hydrophobic interface of the lipid bilayer at the glycerol backbone level, is useful because of its spectral sensitivity to polarity changes produced by water penetration in the bilayer (Parasassi *et al*., 1991). Moreover it demonstrates a spectral sensitivity to the phospholipid phase state and the quantification of the two phases can be obtained using steady-state generalized polarization (GP) (Parasassi *et al*., 1991). The GP_{340} value measured in the PERM group was lower compared with the control one, indicating an increase in plasma membrane fluidity and polarity in the region tested by laurdan. The perturbation at the plasma membrane level could influence the translation of cytoplasmatic and membrane components of the NADPH complex and its subsequent activation that produces changes in oxidative burst. Moreover, other events could influence the respiratory burst in the PERM-treated group. In this respect, PERM could induce the release of pro-inflammatory cytokines that can activate monocytes. Following this pre-activation, monocytes cannot produce a normal response when activated again, but their responses are reduced, as observed previously (Knight, 2000; El-Benna *et al*., 2008). Our results are in line with previous studies where pyrethroid immunotoxicity was measured (Grosman and Diel, 2005; Madsen *et al*., 1996; Diel *et al*., 2003; Kote *et al*., 2006). In these studies, the effect of pyrethroids on cell membrane activities was measured, showing that pyrethroids can influence cytokines release and apoptosis of immunogenic cells (Grosman and Diel, 2005; Madsen *et al*., 1996; Diel *et al*., 2003; Kote *et al*., 2006; Emara and Draz, 2007).

Our previous study on adult rats showed that PERM can interact with DNA of lymphocytes inducing DNA damage (Gabbianelli *et al*., 2004). The same effect can be measured in adolescent rats as shown by the comet assay (Fig. 2 and Table 2). Since there are no data in the literature on the type of DNA damage induced by PERM, we studied which specific lesions are produced by PERM treatment on the DNA of rat leukocytes. The sensitivity and specificity of the comet assay can be increased by incubating the lysed cells with lesion-specific endonucleases that recognize particular damaged bases and create additional breaks. In theory, any lesion for which a specific endonuclease exists can be detected in this way. Therefore, Endo III and Fpg can be used to monitor oxidized pyrimidines and purines bases, respectively (Collins *et al*., 1997; Collins, 2004; Smith *et al*., 2006). In addition, Fpg is highly sensitive in detecting of 8-OH guanine, abasic sites (AP sites) and ring-opened N-7 guanine adducts (Tice *et al*., 2000; Epe *et al*., 1993; Tchou *et al*., 1994; Li *et al*., 1997; Tudek *et al*., 1998). Our results using these enzymes show that the presence of lesion-specific repair enzymes in PERM group increases DNA migration (%DNA tail) with respect to the control. It is worth noting that DNA migration (TM and %DNA tail) in PERM Endo III and PERM Fpg groups is reduced compared with the PERM group. A key question is how this decrease in DNA migration during electrophoresis should be interpreted. The results might be correlated with other types of DNA modifications induced by PERM. After Endo III and Fpg have hydrolyzed the DNA, possible DNA–DNA or DNA–protein cross-linking could occur. In this way, DNA cross-linking could be responsible for retardation in the degree of DNA migration during electrophoresis (Hartmann *et al*., 2003). Similar results in cross linkers between DNA and proteins were observed by other authors following chemical treatments (Tice *et al*., 2000; Anderson and Hellman, 2005).

In summary, our DNA data indicate that exposure to permethrin produces oxidation at the level of pyrimidine and purine bases, although subsesquent cross-linking cannot be excluded. The results obtained in this study are important because they demonstrate that the effect of PERM, at the concentration used in our experiments, on monocyte respiratory burst and DNA damage does not change with the age of rats, in accordance with our previous data on other life phases (Gabbianelli *et al*., 2004; Nasuti *et al*., 2007). In addition, this study increases the knowledge on the type of DNA damage induced by this insecticide and underlines the influence of permethrin on the imbalance of immune function. The relevance of this experiment is mainly focused on the relation between long-term exposure to this insecticide and the consequences for mammalian health during aging. Healthy aging in fact, depends on successful and dynamic interactions among biological and environmental factors. An imbalance of the immune function occurs during aging (Crighton and Puppione, 2006), therefore the additional effect of this pyrethroid, which can modify the immune response, should be considered in the light of accumulated low levels of exposure throughout the entire life.

Future investigations will be performed to study the involvement of cross-linking processes in PERM-induced DNA damage. Finally, it will be important to evaluate the mechanisms related to respiratory burst alterations (i.e. cytokines release) since a normal respiratory burst activity should normally be conserved for animal (and human) health.

References

Abdel-Rahman A, Shetty AK, Abou-Donia MB. 2001. Subchronic dermal application of *N,N*-diethyl *m*-toluamide (DEET) and permethrin to adult rats, alone or in combination, causes diffuse neuronal cell death and cytoskeletal abnormalities in the cerebral cortex and the hippocampus, and Purkinje neuron loss in the cerebellum. *Exp. Neurol*. **172**: 153–171.

Anderson MA, Hellman BE. 2005. Different roles of Fpg and Endo III on catechol-induced DNA damage in extended-term cultures og human lymphocytes and L5178Y mouse lymphoma cells. *Toxicol. Vitro* **19**: 779–786.

Bjørling-Poulsen M, Andersen HR, Grandjean P. 2008. Potential developmental neurotoxicity of pesticides used in Europe. *Environ Health*. **7**: 50–72.

Cantalamessa F. 1993. Acute toxicity of two pyrethroids, permethrin, and cypermethrin in neonatal and adult rats. *Arch. Toxicol*. **67**: 510–513.

Collins AR. 2004. The comet assay for DNA damage and repair. *Mol. Biotech*. **26**: 249–261.

Collins A, Dusinska M, Franklin M, Somorovska M, Petrovska H, Duthie S, Fillion L, Panayiotidis M, Raslova K, Vaughan N. 1997. Comet assay in human biomonitoring studies: reliability, validation, and applications. *Environ. Mol. Mutagen*. **30**: 139–146.

Costa LG, Giordano G, Guizzetti M, Vitalone A. 2008. Neurotoxicity of pesticides: a brief review. *Front Biosci*. **13**: 1240–1249.

Crighton MH, Puppione AA. 2006. Geriatric neutrophils: implications for older adults. *Semin Oncol Nurs*. **22**: 3–9.

Dahlgren C, Karlsson A. 1999. Respiratory burst in human neutrophils. *J. Immunol. Meth*. **232**: 3–14.

Davies TG, Field LM, Usherwood PN, Williamson MS. 2007. DDT, pyrethrins, pyrethroids and insect sodium channels. *IUBMB Life* **59**: 151–162.

Dayal M, Parmar D, Dhawan A, Ali M, Dwivedi UN, Seth PK. 2003. Effect of pretreatment of cytochrome P450 modifiers on neurobehavioral toxicity induced by deltamethrin. *Food Chem. Toxicol*. **41**: 431–437.

Diel F, Horr B, Borck H, Irman-Florjanc T. 2003. Pyrethroid insecticides influence the signal transduction in T helper lymphocytes from atopic and nonatopic subjects. *Inflamm Res*. **52**: 154–163.

El-Benna J, Dang PM, Gougerot-Pocidalo MA. 2008. Priming of the neutrophil NADPH oxidase activation: role of p47phox phosphorylation and NOX2 mobilization to the plasma membrane. *Semin. Immunopathol*. **30**: 279–89.

Emara AM, Draz EI. 2007. Immunotoxicological study of one of the most common over-the-counter pyrethroid insecticide products in Egypt. *Inhal. Toxicol*. **19**: 997–1009.

Epe B, Pflaum M, Haring M, Hegler J, Rudiger H. 1993. Use of repair endonucleases to characterize DNA damage induced by reactive oxygen species in cellular and cell-free systems. *Toxicol. Lett*. **67**: 57–72.

Gabbianelli R, Falcioni G, Nasuti C, Cantalamessa F. 2002. Cypermethrin-induced plasma membrane perturbation on erythrocytes from rats: reduction of fluidity in the hydrophobic core and in glutathione peroxidase activity. *Toxicology* **175**: 91–101.

Gabbianelli R, Nasuti C, Falcioni G, Cantalamessa F. 2004. Lymphocyte DNA damage in rats exposed to pyrethroids: effect of supplementation with Vitamins E and C. *Toxicology* **203**: 17–26.

Grosman N, Diel F. 2005. Influence of pyrethroids and piperonyl butoxide on the Ca(2+)-ATPase activity of rat brain synaptosomes and leukocyte membranes. *Int. Immunopharmacol*. **5**: 263–270.

Hartmann A, Agurell E, Beevers C, Brendler-schwaab S, Burlinson B, Clay P, Collins A, Smith A, Speit G, Thybaud V, Tice RR. 2003. Recomandations fro conducting the *in vivo* alkaline comet assay. *Mutagenesis* **18**: 45–51.

Karen DJ, Li W, Harp P, Gillette JS, Bloomquist JR. 2001. Striatal dopaminergic pathways as a target for the insecticides permethrin and chlorpyrifos. *Neurotoxicology* **22**: 811–817.

Knight JA. 2000. Review: Free radicals, antioxidants, and the immune system. *Ann. Clin. Lab. Sci*. **30**: 145–158.

Kote P, Ravindra V, Chauhan RS, Kote P, Girish V. 2006. Use of avian lymphocytes to detect toxicity: effects of a commonly utilized deltamethrin preparation. *J. Immunotoxicol*. **3**: 101–109.

Kumaravel TS and Jha AN. Reliable 2006. Comet assay measurements for detecting DNA damage induced by ionising radiation and chemicals. *Mutat. Res*. **605**: 7–16.

Li Q, Laval J, Ludlum DB. 1997. Fpg protein releases a ring-opened N-7 guanine adduct from DNA that has been modified by sulfur mustard. *Carcinogenesis* **18**: 1035–1038.

Lowry OH, Rosebrough NJ, Farr AL, Randall RL. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem*. **193**: 265–275.

Madsen C, Claesson MH, Röpke C. 1996. Immunotoxicity of the pyrethroid insecticides deltametrin and alpha-cypermetrin. *Toxicology* **107**: 219–227.

Narahashi T, Zhao X, Ikeda T, Nagata K, Yeh JZ. 2007. Differential actions of insecticides on target sites: basis for selective toxicity. *Hum. Exp. Toxicol*. **26**: 361–366.

Nasuti C, Cantalamessa F, Falcioni G, Gabbianelli R. 2003. Different effects of type I and type II pyrethroids on erythrocyte plasma membrane properties and enzymatic activity in rats. *Toxicology* **191**: 233–244.

Nasuti C, Gabbianelli R, Falcioni ML, Di Stefano A, Sozio P, Cantalamessa F. 2007. Dopaminergic system modulation, behavioral changes, and oxidative stress after neonatal administration of pyrethroids. *Toxicology* **229**: 194–205.

Nasuti C, Falcioni ML, Nwankwo IE, Cantalamessa F, Gabbianelli R. 2008. Effect of permethrin plus antioxidants on locomotor activity and striatum in adolescent rats. *Toxicology* **251**: 45–50.

Parasassi T, De Stasio G, Ravagnan G, Rusch RM, Gratton E. 1991. Quantitation of lipid phases in phospholipids vescicles by the generalized polarization of laurdan fluorescence. *Biophys. J*. **60**: 179–189.

Peterson RT, Nass R, Boyd WA, Freedman JH, Dong K, Narahashi T. 2008. Use of non-mammalian alternative models for neurotoxicological study. *Neurotoxicology* **29**: 546–555.

Pittman JT, Dodd CA, Klein BG. 2003. Immunohistochemical changes in the mouse striatum induced by the pyrethroid insecticide permethrin. *Int. J. Toxicol*. **22**: 359–370.

Rattan SI. 2008. Increased molecular damage and heterogeneity as the basis of aging. *Biol. Chem*. **389**: 267–272.

Ray DE, Cremer JE. 1979. The action of decamethrin (a synthetic pyrethroid) on the rat. *Pestic. Biochem. Physiol*. **10**: 333–340.

Sevatdal S, Allang A, Ingebrigtsen K, Horsberg TE. 2005. Monoxygenase mediated pyrethroid detoxification in sea lice (*Lepeophtheirus salmonis*). *Pest. Manag. Sci*. **61**: 772–778.

Sheets LP, Doherty JD, Law MW, Reiter LW, Crofton KM. 1994. Age-dependent dofferences in the susceptibility of rats to deltamethrin. *Toxicol. Appl. Pharmacol*. **126**: 186–190.

Shinitzky M, Barenholz Y. 1978. Fluidity parameters of lipid regions determined by fluorescence polarization. *Biochim. Biophys. Acta* **515**: 367–394.

Smith CC, O'Donovan MR, Martin EA. 2006. HOGG1 recognizes oxidative damage using the comet assay with greater specificity than FPG or ENDOIII. *Mutagenesis* **21**: 185–190.

Sogorb MA, Vilanova E. 2002. Enzymes involved in the detoxification of organophosphorus, carbamate and pyrethroid insecticides through hydrolysis. *Toxicol. Lett*. **128**: 215–228.

Song JH, Narahashi T. 1996. Modulation of sodium channels of rat cerebellar Purkinje neurons by the pyrethroid tetramethrin. *J. Pharmacol. Exp. Ther*. **277**: 445–453.

Tchou J, Bedepudi V, Shibutsni S, Antoshechkin I, Miller J, Grollman AP, Johnson F. 1994. Substrate specific of FPG protein: recognition and cleavage oxidatively damaged DNA. *J. Biol Chem*. **269**: 15318–15324.

Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF. 2000. Single cell gel/Comet Assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing. *Environ. Mol. Mutagen*. **35**: 206–221.

Tudek B, Van Zeeland AA, Kusmierek JT, Laval J. 1998. Activity of *Escherichia coli* DNA-glycosylates on DNA damaged by methylating and ethylating agents and influence of 3-substituted adenine derivatives. *Mutut. Res*. **407**: 169–176.

Vais H, Williamson MS, Devonshire AL, Usherwood PN. 2000. The molecular interactions of pyrethroid insecticides with insect and mammalian sodium channels. *Pest. Manag. Sci*. **57**: 877–888.

Verschoyle RD, Aldridge WN. 1980. Structure–activity relationships of some pyrethroids in rats. *Arch. Toxicol*. **45**: 325–329.

Villarini M, Scassellati-Sforzolini G, Moretti M, Pasquini R. 2000. *In vitro* genotoxicity of terbutryn evaluated by the alkaline single-cell microgel-electrophoresis 'comet' assay. *Cell Biol. Toxicol*. **16**: 285–292.

Wu LT, Chu CC, Chung JG, Chen CH, Hsu LS, Liu JK, Chen SC. 2004. Effects of tannic acid and its related compounds on food mutagens or hydrogen peroxide-induced DNA strands breaks in human lymphocytes. *Mutat. Res*. **556**: 75–82.

Young SJ, Robin Gunning RV, Moores GD. 2004. The effect of piperonyl butoxide on pyrethroid-resistance-associated esterases in Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae). *Pest. Mgmt Sci*. **61**: 397–401.