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Lymphocyte DNA damage in rats exposed to pyrethroids: effect of supplementation with Vitamins E and C

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Abstract

Pesticides have been considered potential chemical mutagens. In fact, some studies show that various agrochemical ingredients possess mutagenic properties inducing mutations, chromosomal alterations or DNA damage. Experimental evidence shows a marked correlation between mutagenicity and carcinogenicity and indicates that short-term mutagenicity tests are useful for predicting carcinogenicity. The present study on rat exposed to two pyrethroids, cypermethrin and permethrin, showed different lymphocyte DNA damage depending on the type of pyrethroid, the dose, and the period of treatment. Data obtained from comet assay showed that oral treatment with 150 mg/kg body weight/day of permethrin (corresponding to 1/10 of LD_{50}) for 60 days, induced a significant increase in all comet parameters. No lymphocyte DNA damage was measured after treatment with 25 mg/kg body weight/day of cypermethrin (corresponding to 1/10 of LD_{50}) for the same period. A higher dose of permethrin (300 mg/kg body weight/day), for a shorter period (22 days), did not induce lymphocyte DNA damage, while supplementation with 200 mg/kg of Vitamins E and C protected erythrocytes against plasma membrane lipids peroxidation. Moreover, treatment with Vitamins E and C maintained the activity of glutathione peroxidase, which was reduced in the presence of permethrin, and reduced the osmotic fragility, which had increased following permethrin treatment.
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Keywords: Cypermethrin; Permethrin; Lymphocyte; DNA damage; Comet; Vitamins

1. Introduction

With the recognition of molecular models of multistage carcinogenesis, it was clear that the induction of malignancies is much more complicated than originally thought, involving a series of genetic and epigenetic changes. Both the classical and molecular models, that have been used to describe the process of carcinogenesis, indicate that at least one, and possibly several, mutagenic events are required for malignant tumors to develop (Harris, 1991). Furthermore, the specific mutant cells that are involved in tumor development are themselves subject to many secondary influences (Ashby, 1992). Consequently, the specific mechanisms responsible for the induction and/or expression of these mutant cells are not always easily or well defined. Since epidemiological data show an increase in the number of cancers in

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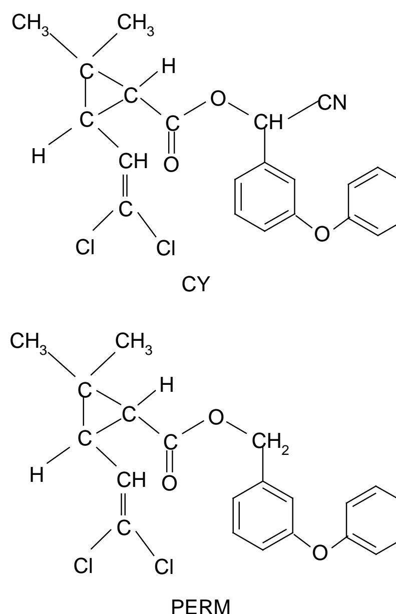
people involved in agricultural production with the use of pesticides (Bolognesi, 2003), the International Agency for Cancer Research (IARC) has reviewed the potential carcinogenicity of a wide range of insecticides, fungicides, herbicides and other similar compounds (IARC, 2002). Fifty-six pesticides have been classified as carcinogenic to animals. Moreover, the importance of cancer development after environmental contamination with chemicals has been observed (Bolognesi, 2003; Snyder and Green, 2001; Water et al., 1999). Many environmental pollutants are chemical carcinogens with the capacity of inducing DNA damage, which has therefore, been proposed as a useful parameter for assessing the genotoxic properties of environmental pollution.

The aim of the present study is to evaluate the effect of pyrethroids on rat lymphocyte DNA. The influence of low and high doses of type I (cypermethrin, CY) and type II (permethrin, PERM) pyrethroids on DNA, was assessed by comet assay. The influence of exposition to both PERM and Vitamins E and C was studied on lymphocytes and erythrocytes from rats. Data on lipid peroxidation, activity of antioxidant erythrocyte enzymes (glutathione peroxidase, catalase and superoxide dismutase) and osmotic fragility are presented.

2. Materials and methods

2.1. Chemicals

Technical grade (62.8:37.2, *trans*:*cis*; 92.4% purity) (*R*,*S*)α-cyano-3-phenoxybenzyl(1*R*,*S*)-*cis*,*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate, CY, (NRDC 149) and technical grade (75:25, *trans*:*cis*; 94% purity) 3-phenoxybenzyl-(1*R*,*S*)-*cis*, *trans*, 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate, PERM, (NRDC 143) were generously donated by Dr. A. Stefanini of ACTIVA, Milan, Italy (Scheme 1). Corn oil, glutathione reductase, and NADPH, were obtained from Sigma St. Louis, MO, USA. Vitamins E and C were obtained from Roche S.p.A., Milano. Lymphoprep for separation of rat lymphocytes was obtained from Nycomed Pharma AS, Oslo, Norway, and Dulbecco's Modified Eagle from Life Technologies (Palsley, Scotland). Other reagents used in this study were of analytical grade.



Scheme 1. Chemical structure of (*R*,*S*)α-cyano-3-phenoxybenzyl (1*R*,*S*)-*cis*,*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate (CY) and 3-phenoxybenzyl-(1*R*,*S*)-*cis*,*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate (PERM).

2.2. Animals

Male Wistar rats ($n = 30$ for control (C), $n = 30$ for CY-treated and $n = 60$ for PERM-treated) (Charles River, Calco, LC, Italy) weighing 150–175 g were employed. 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.

2.3. Cypermethrin and permethrin administration

Cypermethrin was dissolved in corn oil and administered orally (5 ml/kg) ($n = 30$) for 60 days, at a high dose ($n = 15$) of 25 mg/kg body weight/day (1/10 of LD_{50}; Cantalamessa, 1993) and low dose ($n = 15$) of 2.5 mg/kg body weight/day (1/100 of LD_{50}, Cantalamessa, 1993) by intragastric tube. Permethrin was dissolved in corn oil and administered orally (5 ml/kg) ($n = 30$) for 60 days, at a high dose ($n = 15$) of 150 mg/kg body weight/day (1/10 of LD_{50}; Cantalamessa, 1993) and low dose (n

= 15) of 15 mg/kg body weight/day (1/100 of LD_{50}, Cantalamessa, 1993) by intragastric tube.

In order to evaluate the effect of PERM at the higher dose in presence and in absence of vitamins ($n = 30$) we dissolved 300 mg/kg body weight/day of PERM in corn oil and administered it orally for 22 days ($n = 15$). Another group ($n = 15$) was treated for 22 days with the same amount of PERM plus 200 mg/kg body weight/day Vitamin E and 200 mg/kg body weight/day Vitamin C. Rats serving as controls (group C; $n = 30$) received 5 ml/kg body weight of corn oil in the same manner.

The substances were administered daily in the morning to non-fasted rats. The actual volume administered was based on body weight taken daily during the dosing period.

The animals were observed daily and weighed at regular intervals; the behavior of the animals, treated with CY and PERM was compared with that of C, which received corn oil as treatment and were kept under the same conditions as the experimental groups.

2.4. Lymphocyte separation

Lymphocyte separation was performed by means of a Ficol density gradient. Whole blood was diluted (1:1) in PBS and stratified on a solution of lymphoprep and then centrifuged for 20 min at 3000 rpm. Peripheral blood lymphocytes (PBLs) were separated from erythrocytes and washed with PBS.

2.5. Comet assay

DNA single strand breaks or alkali-labile sites are induced by a great variety of genotoxic substances. The comet assay (Collins et al., 1997, 2001) used to measure the DNA strand breaks in individual cells was essentially the same as that described previously (Gabbianelli et al., 2002). Cells were examined with 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 specialised analysis system (Metasystem Altlussheim, Germany) to determine tail length, tail intensity and tail moment (TL, TI and TM)—all parameters correlated with the degree of DNA damage in the single cells.

2.6. Erythrocyte membrane preparation

After 60 days of treatment with pesticide, blood was drawn by cardiac puncture from all rats, collected in vials containing heparin (250 IU) and washed three times with physiological solution before the experiments. Plasma membranes were prepared by hypotonic hemolysis from 10 mM Tris, pH 7.4 to 2.5 mM Tris, pH 7.4, according to Bramley (Bramley et al., 1971).

Protein concentration was evaluated using Lowry's method (Lowry et al., 1951).

2.7. Lipid peroxidation in erythrocytes

The "oxidation index" was measured on lipids extracted from plasma membrane erythrocytes, as previously described (Nasuti et al., 2003).

2.8. Antioxidant enzymes in erythrocytes

Glutathione peroxidase, catalase and superoxide dismutase activity were determined as previously described (Nasuti et al., 2003).

2.9. Osmotic fragility test

Osmotic fragility of erythrocytes was measured as previously described (Nasuti et al., 2003).

2.10. Statistical analysis

The experimental data are expressed as mean values ± S.E.M. of 12 samples ($n = 4$ rats for each group, three samples from each animal were used. For comet assay at least 150 scores/sample). Statistical analysis was carried out using the one-way analysis of variance (ANOVA) followed by the Newman–Keuls test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. General findings

Rats treated daily for 60 days with low and high doses of CY and PERM by intragastric tubing showed

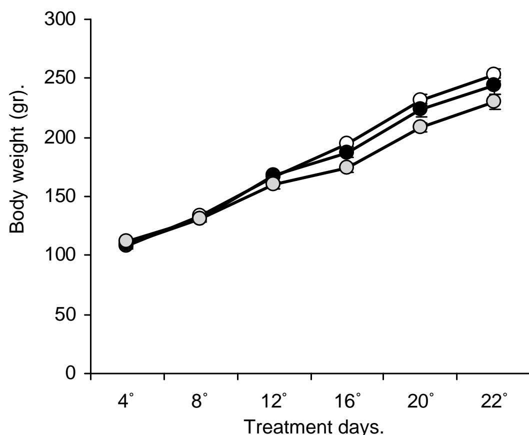


Fig. 1. Body weight in control (C) ($n = 15$) (○), PERM–treated ($n = 15$) (●) and PERM + vitamins-treated ($n = 15$) (◯) rats during exposure at 300 mg/kg of PERM ± Vitamins E and C.

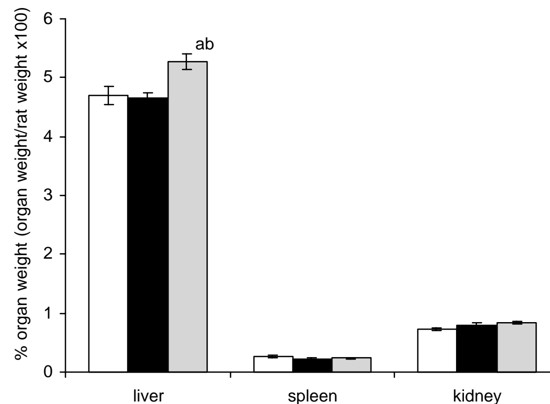


Fig. 2. Liver, kidney and spleen weight in the animal groups treated with 300 mg/kg of PERM ± Vitamins E and C from controls (□), PERM-treated (■) and PERM + vitamins-treated (▢). Data are expressed as percentage (%) calculated as grams of organ/100 g of animal weight. The results are indicated as mean values ($n = 5$ for every animal group) ± S.E.M. [a]$P < 0.05$ compared to control group [b]$P < 0.05$ compared to PERM group.

no signs of pyrethroid poisoning or gross behavioral abnormalities throughout the experimental period. Similar behaviour was observed in rats treated for 22 days with increased doses of PERM ± Vitamins E and C. Food and water consumption was not significantly affected (data not shown).

Data on body weight in rats treated with PERM and PERM + vitamins, and in untreated ones (C) are reported in Fig. 1. As shown, body weight values increased as a function of age and no significant differences were observed in the body weight of exposed versus control rats during treatment. The measurement of liver, kidney and spleen weight during treatment with low and high doses of PERM ± vitamins are summarized in Fig. 2. Treatment with PERM + Vitamins E and C induced an increase in liver weight ($P < 0.05$) compared with the other samples. No change in spleen and kidney weight was revealed.

3.2. Comet assay

Fig. 3 shows results of comet assays performed on rat lymphocytes after treatment with low (2.5 and 15 mg/kg body weight/day for CY and PERM, respectively) doses of pyrethroids. The tail length (A), tail intensity (B), and tail moment (C) are not changed following this treatment. When rats were exposed to high doses of CY (25 mg/kg body weight/day) and PERM (150 mg/kg body weight/day), a significant increase of all three comet parameters was measured only after treatment with PERM (Fig. 4A-C). Since only PERM induced DNA damage, we exposed rats to a more elevated dose of it (300 mg/kg body weight/day) for a shorter period (22 days). In addition, another rat group was treated, for the same period, with PERM (300 mg/kg body weight/day) and Vitamins E and C. The results in Fig. 5A-C, show that no changes in comet parameters can be revealed.

3.3. Effect on lipid peroxidation

The lipid peroxidation can be evaluated in plasma membrane by the measurement of conjugated dienes formation (Falcioni et al., 1998; Gabbianelli et al., 1997). In fact, in polyunsaturated fatty acids, the oxidative process starts with the formation of a carbon radical which stabilizes to form a conjugated dienes. In lipid membranes, this can be measured by the increase in the A_{233}/A_{215} absorbance ratio. The "oxidation index" was used as a relative measurement for conjugated dienes in lipid extracts. Fig. 6 shows the "oxidation index" measured in

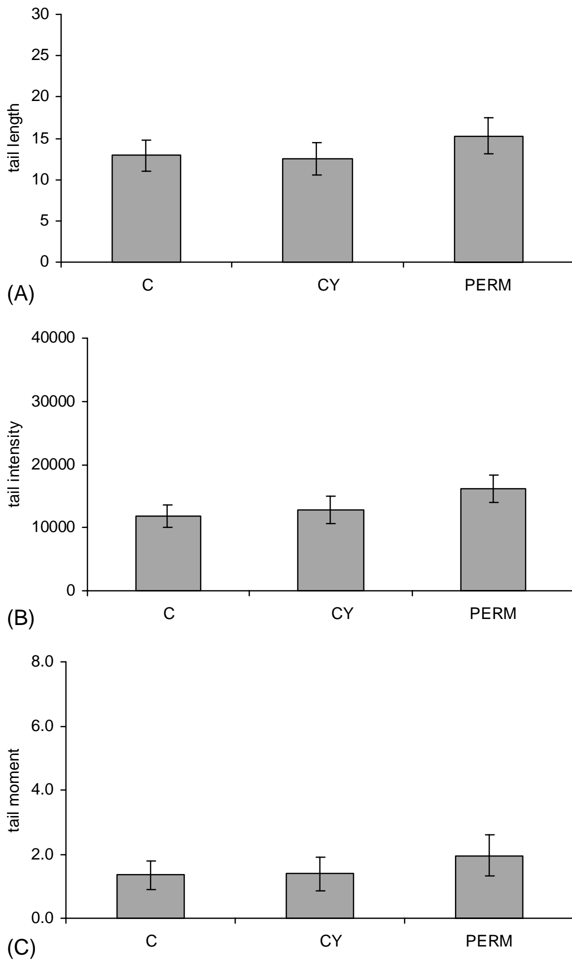


Fig. 3. Observed distributions of comet parameter tail length (A), tail intensity (B), and tail moment (C) in white blood cells from rat treated with low doses of pyrethroids (2.5 and 15 mg/kg body weight/day for CY and PERM, respectively). Data (at least 150 scores/sample) are mean values ± S.E.M. of 12 samples (n = 4 rats for each group, three samples from each animal were used).

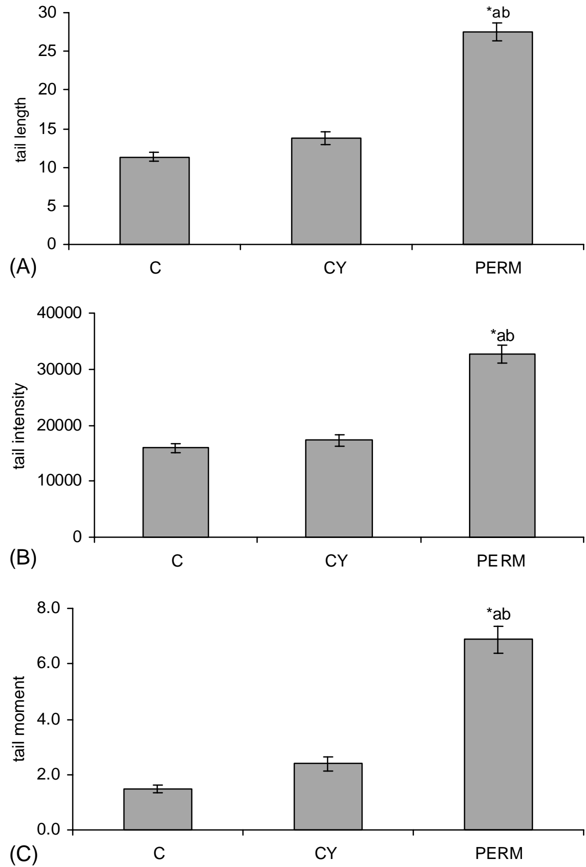


Fig. 4. Observed distributions of comet parameter tail length (A), tail intensity (B), and tail moment (C) in white blood cells from rat treated with high doses of pyrethroids (25 mg/kg body weight/day for CY and 150 mg/kg body weight/day for PERM). Data (at least 150 scores/sample) are mean values ± S.E.M. of 12 samples (n = 4 rats for each group, three samples from each animal were used). $^{a}P < 0.05$ compared to control group. $^{b}P < 0.05$ compared to CY group.

lipids extracted from erythrocyte membranes following the treatment (for 22 days) with a high dose (300 mg/kg body weight/day) of PERM ± Vitamins E and C. The rats treated with PERM showed a slight ($P < 0.05$) increase of lipid peroxidation (0.407 ± 0.013) compared with the C group rats (0.349 ± 0.020). When rats were exposed to PERM and Vitamins E and C a significant ($P < 0.05$) reduction (0.286 ± 0.200) of the oxidation index was measured compared to both the C and PERM groups.

3.4. Effect on antioxidant enzymes: GPx, CAT, SOD

The treatment with permethrin (300 mg/kg body weight/day) induced changes in the GPx activity. In particular, a slight ($P < 0.05$) reduction of antioxidant activity of this enzyme in rat erythrocytes treated with PERM (85.79 ± 7.788 U/mg Hb) was measured compared with the C (119.49 ± 7.915 U/mg

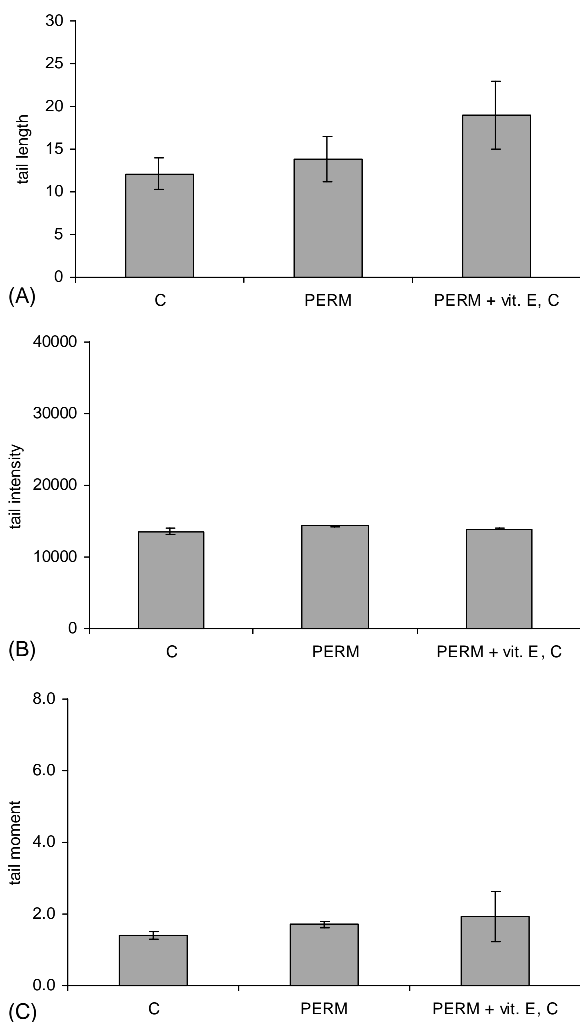


Fig. 5. Observed distributions of comet parameter tail length (A), tail intensity (B), and tail moment (C) in white blood cells from rats treated with PERM (300 mg/kg body weight/day) ± Vitamins E and C. Data (at least 150 scores/sample) are mean values ± S.E.M. of 12 samples ($n = 4$ rats for each group, three samples from each animal were used).

Hb) and PERM + vitamins (116.10 ± 15.737 U/mg Hb) groups (Fig. 7). Also the CAT activity proved slightly ($P < 0.05$) decreased in the PERM (55.72 ± 3.643 U/mg Hb) and PERM + vitamins groups (47.11 ± 5.813 U/mg Hb) compared with the C group (88.50 ± 5.419 U/mg Hb) (Fig. 8). No significant modifications were revealed in SOD activity among the three groups (Fig. 9).

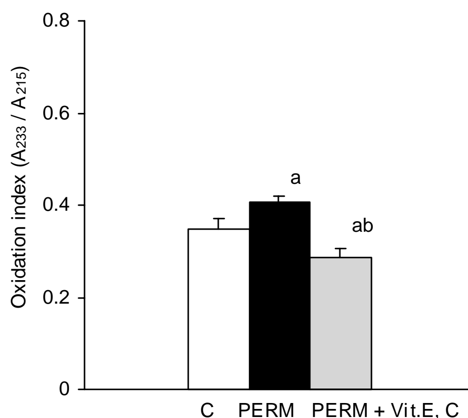


Fig. 6. Oxidation index obtained by the absorbance ratio A_{233}/A_{215} measured on lipids extracted from controls (□), PERM-treated (■) and PERM + vitamins-treated (▩) rats after treatment with a high dose (300 mg/kg) of PERM. Data represent the means ± S.E.M. 12 samples ($n = 4$ rats for each group, three samples from each animal were used). $^{a}P < 0.05$ compared to control group. $^{b}P < 0.05$ compared to PERM group.

3.5. Effect on osmotic fragility

Fig. 10 shows the effect of PERM (300 mg/kg body weight/day) and PERM (300 mg/kg body weight/day) + vitamins on osmotic fragility. The treatment with PERM induced a significant ($P < 0.05$) increase of

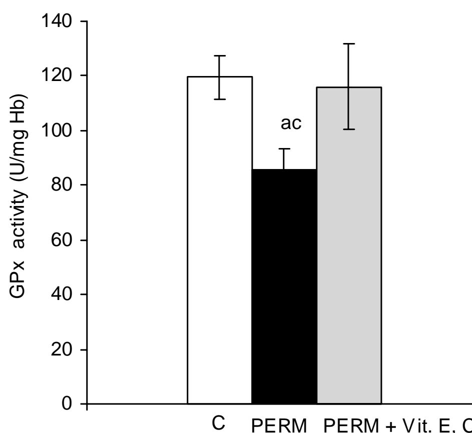


Fig. 7. Enzymatic activity of GPx (U/mg Hb) in controls (□), PERM-treated (■) and PERM+ vitamins-treated (▩) rats after treatment with a high dose (300 mg/kg) of PERM. Data represent the means ± S.E.M. of 12 samples ($n = 4$ rats for each group, three samples from each animal were used). $^{a}P < 0.05$ compared to control group. $^{c}P < 0.05$ compared to PERM + vitamins group.

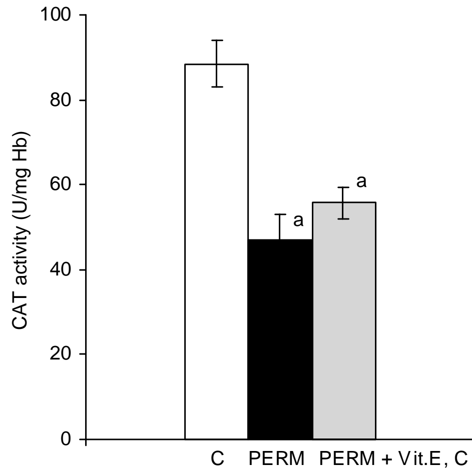


Fig. 8. Enzymatic activity of CAT (U/mg Hb) in controls (□), PERM-treated (■) and PERM+ vitamins-treated (▩) rats after treatment with a high dose (300 mg/kg) of PERM. Data represent the means ± S.E.M. of 12 samples ($n = 4$ rats for each group, three samples from each animal were used). [a]$P < 0.05$ compared to control group.

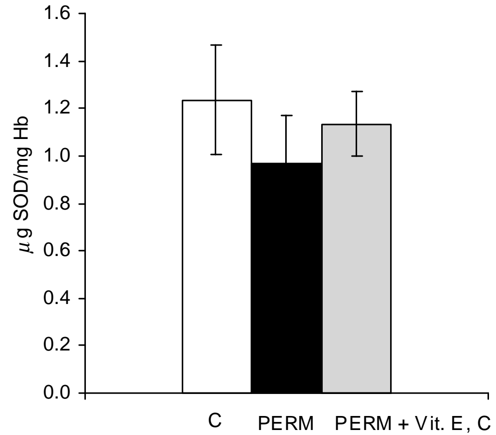


Fig. 9. Enzymatic activity of SOD (μ/mg Hb) in controls (□), PERM-treated (■) and PERM + vitamins-treated (▩) rats after treatment with a high dose (300 mg/kg) of PERM. Data represent the means ± S.E.M. of 12 samples ($n = 4$ rats for each group, three samples from each animal were used).

hemolysis at low (1 and 2 g/l) concentrations of NaCl compared with the control group. When the rats were exposed to PERM + Vitamins E and C, a significant ($P < 0.05$) reduction of hemolysis was measured at all concentrations of NaCl used, compared with the C and PERM groups (Fig. 10).

4. Discussion

Synthetic pyrethroids are used preferentially to organophosphates and organochlorines since they have high effectiveness for a wide range of insects, low toxicity to mammals and birds, and rapid biodegradibility (Aldridge, 1990; Vijverberg and Van Den

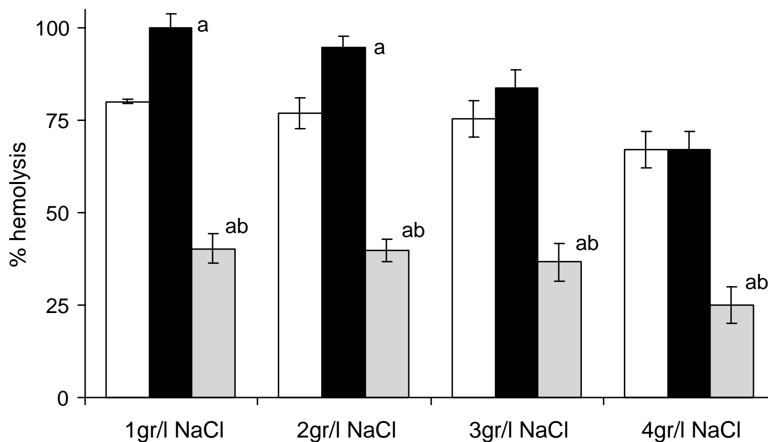


Fig. 10. Osmotic fragility in controls (□), PERM-treated (■) and PERM + vitamins-treated (▩) rats after treatment with a high dose (300 mg/kg) of PERM. Osmotic fragility was determined using the continuous dilution method described in Section 2. The extent of hemolysis is plotted for different salt concentrations in g/l. The test tube containing the lowest buffer salt concentration (1 g/l NaCl) gives the greatest percentage of hemolysis. Data represent means ± S.E.M. [a]$P < 0.05$ compared to control group. [b]$P < 0.05$ compared to PERM group.

Bercken, 1990; Ray, 1991). Nevertheless, the increased use of these pesticides increases the risk of environmental contamination and the ensuing intoxication of non-target organisms in different ecosystems (Bolognesi, 2003). Moreover, different studies have shown that many pesticides, used in agriculture, have the capacity to cause DNA damage (Bolognesi, 2003; Kornuta et al., 1996; Shuka and Taneja, 2002). Our previous studies, on rats orally treated with low and high concentrations of CY and PERM, show changes in the physicochemical properties of the erythrocyte bilayer and modifications in the antioxidant enzymatic activity (Nasuti et al., 2003). Since no investigations had been performed on DNA damage in these experimental conditions, we studied the influence of low and high doses of CY and PERM on lymphocyte DNA by comet assay.

The single cell gel test (SCG), or comet assay, is a rather new test with widespread potential applications in genotoxicity testing and biomonitoring (Fairbairn et al., 1995, Hartmann and Speit, 1997; Singh et al., 1988). Particularly, with this test, it has been possible to evaluate, using a very small sample of cells, the genotoxic effect of these pyrethroids by measuring three parameters: tail length, tail intensity, and tail moment (Gabbianelli et al., 2002).

Our results show that only the treatment with a high dose (150 mg/kg body weight/day) of PERM for a long period (60 days) induces significant lymphocyte DNA damage (Fig. 4). This result is in agreement with our previous study where the PERM, being more permeable, filters through the membrane more easily than CY and so can more markedly produce oxidative damage in the erythrocyte (Nasuti et al., 2003). For this reason, we decided to take into consideration only the PERM treatment and to study simultaneously whether Vitamins E and C administration protects the cells against oxidative injury.

With regard to the capacity of Vitamins E and C to protect against oxidative stress, an important factor is the time of supplementation. In fact, long-term supplementation of diet with α-tocopherol has been shown to have only little effect on the incidence of disease or mean and maximum life span of mice (Meydani et al., 1998). Accordingly, we decided to use supplementation with Vitamins E and C for a short period (22 days) and then to increase the PERM dose since we had reduced the time of treatment. Given that the aim of our study was to evaluate the feasible protection of vitamins against the lymphocyte DNA damage produced by PERM treatment, the results that we obtained were unexpected, because the increased dose of PERM did not induce lymphocyte DNA damage (Fig. 5). Nevertheless, this treatment did induce plasma membrane and antioxidant enzymatic activity perturbation. In fact, the data obtained indicate that PERM increased the peroxidation of lipids extracted from rat erythrocyte plasma membrane and slightly reduced antioxidant activity of Gpx and CAT. This behaviour may be related to the hydrophobic character of PERM that limits its fast diffusion into the cell, causing oxidative damage particularly in the erythrocyte plasma membrane and after in the cytosol. On the basis of these first data, we could also suggest that the time of treatment is the most decisive parameter in inducing lymphocyte DNA damage by PERM.

With regard to Vitamins E and C supplementation, the data obtained indicate that the presence of vitamins reduces the peroxidation on lipids extracted from rat erythrocyte plasma membrane; moreover, the activity of GPx, reduced by the treatment with PERM, remained as the control when vitamins were added to the diet.

Moreover, several studies have indicated that reactive oxygen species have been implicated in the toxicology of pyrethroids (Kale et al., 1999), so the protective effect of vitamins, observed in our study, could be important for protecting the erythrocytes against the oxidative injury following on the use of this pesticide. Also the reduced osmotic fragility measured following the treatment with Vitamins E and C is an important parameter which exalts their role. Besides, since the treatment with a high dose of PERM (300 mg/kg body weight/day) induced a decrease of CAT activity, which was not avoided by vitamins, the maintenance of GPx activity could be important given that it protects the cells reacting with the hydrogen peroxide and lipid hydroperoxides. The unchanged GPx activity could be linked with the presence of Vitamin E, which is localized, preferentially, in the plasma membrane hydrophilic region, where it carries out antioxidant activity together with GPx. Since Vitamin E is not able to protect the hydrophilic cytosol region, the decreased CAT activity could be the consequence of a very small effect of Vitamin C against the damage induced by PERM.

In conclusion, with regard to the level of oxidative stress induced by PERM on rat erythrocytes, a longer period seems to bring about more damage than a higher dose for a shorter time. Moreover, a similar behaviour on lymphocyte DNA was observed; in fact, DNA damage was detected only after a long period of treatment (60 days, Fig. 4), but no effect was obtained with a higher dose, but for a shorter period (22 days, Fig. 5). Our results regarding the genotoxicity of PERM are in agreement with a previous in vitro study, where PERM induced DNA damage in human nasal mucosal cells (Tisch et al., 2002).

Since the literature has inadequate and limited human data, PERM could not be classified by the International Agency for Research on Cancer as a human carcinogen (IARC, 2002). For this reason, the present results could be useful for increasing information on the potential toxicity of this pyrethroid. Moreover, the present study could be useful also for distinguishing the genotoxic effect of PERM following short- and long-term subacute treatment. This point proves important for the biological monitoring to estimate the genetic risk deriving from integrated exposure to a complex mixture of pesticides. Further studies to evaluate a possible antioxidant effect of Vitamins E and C during the long period of treatment (60 days) with PERM will be the object of future investigations.

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