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Evaluation of *in vivo* genotoxicity of cypermethrin in *Drosophila melanogaster* using the alkaline Comet assay

Indranil Mukhopadhyay[1], D.Kar Chowdhuri[1], Mahima Bajpayee[2] and Alok Dhawan[2,3]

[1]Embryotoxicology Section and [2]Developmental Toxicology Section, Industrial Toxicology Research Centre, PO Box 80, M.G. Marg, Lucknow, 226 001 Uttar Pradesh, India

The single cell gel electrophoresis (SCGE) assay, also known as the Comet assay, is one of the most promising genotoxicity tests developed in recent years to measure and analyse DNA damage in single cells. The present study was undertaken to assess the *in vivo* genotoxicity of the synthetic pyrethroid cypermethrin in brain ganglia and anterior mid gut of *Drosophila melanogaster*. Freshly emerged first instar larvae (22 ± 2 h) were placed in different concentrations of cypermethrin (0.0004, 0.0008, 0.002, 0.2 and 0.5 p.p.m.) mixed in standard *Drosophila* food and allowed to grow. At 96 ± 2 h, brain ganglia and anterior midgut from control and treated larvae were dissected out, single cell suspensions were prepared and a Comet assay was performed. Our results revealed a significant dose-dependent increase in DNA damage in the cells of brain ganglia and anterior midgut of *D.melanogaster* exposed to cypermethrin as compared with controls ($P < 0.05$ at 0.002 p.p.m.; $P < 0.001$ at 0.2 and 0.5 p.p.m.). The present study shows *in vivo* genotoxicity of cypermethrin even at very low concentrations, which proves *D.melanogaster* as a model for *in vivo* genotoxicity assessment using the Comet assay.

Introduction

The single cell gel electrophoresis (SCGE or Comet assay) is one of the most promising and upcoming genotoxicity tests. It is less resource intensive than the conventional genotoxic techniques and permits both qualitative and quantitative assessment of DNA damage in any eukaryotic cell population. The simplicity and sensitivity of the Comet assay has resulted in a rapid and widespread progression of this technique in many areas, e.g. environmental monitoring (Cavallo *et al*., 2002; Rajaguru *et al*., 2002), *in vivo* and *in vitro* genotoxicity testing (Anderson *et al*., 1996, 2001; Dhawan *et al*., 2002) and epidemiological and biomonitoring studies in human populations exposed occupationally, environmentally or clinically (Bajpayee *et al*., 2002; Marczynski *et al*., 2002; Mohankumar *et al*., 2002). This test procedure has been recommended in the Committee on Mutagenicity Guidelines of the UK Department of Health (COM) for determining mutagenicity of chemicals (Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment, 2000) as an *in vitro* genotoxicity assay using tissues other than haematopoietic tissue.

One of the advantages of the Comet assay for *in vivo* genetic toxicology studies is the ability to use any tissue (or different tissues) as the target for evaluation of chemical genotoxicity and some physiological conditions (Sasaki *et al*., 2000). Investigators have used the assay to monitor age-dependent DNA damage in hepatocytes of rats (Martelli *et al*., 2002) and the induction and persistence of DNA damage in somatic (Anderson *et al*., 1997) and germ cells (Anderson *et al*., 1997; Olsen *et al*., 2001) of chemically treated rodents. The Comet assay has now become an acceptable tool for environmental biomonitoring and has been successfully applied in assessing soil pollution using earthworms (Salagovic *et al*., 1996), in freshwater planaria (Guecheva *et al*., 2001), in various organs of medaka (*Oryzias latipes*) for evaluation of levels of DNA damage (Tice, 1995), in zebra mussels (Pavlica *et al*., 2001), in *Mytilus* (Pruski and Dixon, 2002), in freshwater *Hydra* (Devaux and Larno, 1999) for aquatic toxicology and also in yeast cells (Miloshev *et al*., 2002).

Over the past decade, issues of animal use and care in toxicology research and testing have become one of the fundamental concerns for both science and ethics. Emphasis has been given to the use of alternatives to mammals in testing, research and education. *Drosophila melanogaster* is the most widely used insect model because of its well-elucidated genetics and developmental biology. Moreover, the use of *Drosophila* has been recommended by the European Centre for the Validation of Alternative Methods (ECVAM), whose goal is to promote the scientific and regulatory acceptance of alternative methods which reduce, refine or replace the use of laboratory animals (Festing *et al*., 1998; Benford *et al*., 2000). In recent years, *Drosophila* has evolved into a model organism in toxicological studies (Gaivao *et al*., 1999; Kar Chowdhuri *et al*., 1999, 2001; Nazir *et al*., 2001, 2003a,b,c; Mukhopadhyay *et al*., 2002a,b, 2003). The present study was therefore undertaken to evaluate the usefulness of *D.melanogaster* as an *in vivo* model for assessment of genotoxicity of a synthetic pyrethroid insecticide, cypermethrin, using the alkaline Comet assay.

Materials and methods

Fly strain

The fly and larvae of wild-type *D.melanogaster* (Oregon R^+) were cultured at 24 ± 1°C on standard *Drosophila* food containing agar, corn meal, brown sugar and yeast.

Chemicals

Agarose, low melting point agarose, ethidium bromide and collagenase were obtained from Sigma Chemical Co. (St Louis, MO). Phosphate-buffered saline (PBS) Ca^{2+}- and Mg^{2+}-free, Trypan blue and ethyl methanesulphonate (EMS) were procured from Hi-Media Pvt. Ltd (Mumbai, India). Technical grade cypermethrin (purity 98.5%) was a kind gift from Aimco Pesticides Ltd (Mumbai, India). All other chemicals were obtained locally and were of analytical reagent grade.

Pesticide

Cypermethrin dissolved in dimethyl sulphoxide (DMSO) (0.3% final concentration) was added to standard *Drosophila* food in five different concentrations: 0.5 (1/100 of recommended agricultural dose), 0.2 [maximum residue level

[3]To whom correspondence should be addressed. Tel: +91 522 2213618; Fax: +91 522 2228227; Email: dhawanalok@hotmail.com

(MRL) of cypermethrin in fruits; Chaddha, 1992], 0.002 (1/100 of MRL value), 0.0008 (1/250 of MRL value) and 0.0004 (1/500 of MRL value) p.p.m.

Experimental design

Freshly emerged first instar larvae (22 ± 2 h) were transferred to standard *Drosophila* food containing different concentrations of cypermethrin and were allowed to grow on it. Control larvae were grown on standard *Drosophila* food without cypermethrin. At 96 ± 2 h, the larvae were removed from the food and washed with 50 mM sodium phosphate buffer. Brain ganglia and the anterior region of the midgut from 50 larvae were explanted in Poels' salt solution (PSS) (Lakhotia and Mukherjee, 1980) and collected separately in 1.5 ml microcentrifuge tube. A single cell suspension of the tissues was then prepared by the method of Howell and Taylor (1968) with some modifications. PSS in the microcentrifuge tube was replaced with collagenase (0.5 mg/ml in PBS, pH 7.4) and kept for 15 min at 24°C. The cells were then passed through nylon mesh (60 µm). Collagenase was removed by washing the cell suspension three times with PBS. The cells were finally suspended in 80 µl of PBS.

Positive control

EMS, a well-known mutagen (Bilbao *et al*., 2002), was used as a positive control in the present study. Single cell suspensions were prepared as described above from brain ganglia and anterior midgut tissues of the larvae (96 ± 2 h stage) grown on normal food. After washing three times in PBS, the cells were suspended in 300 µl of PBS containing 5 mM EMS and incubated for 1 h at 24°C. The cells were then washed three times in PBS and processed for the Comet assay.

Viability assay

The cells were checked for viability before the start of the experiment using Trypan blue dye (Pool-Zobel *et al*., 1993).

Comet assay

Slides were prepared in duplicate according to the method of Bajpayee *et al*. (2002) with some modifications and were finally immersed in freshly prepared chilled lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris pH 10.0 and 1% Triton X-100, pH 10) for 2 h. After lysis, the slides were placed in a horizontal gel electrophoresis tank (Life Technologies, Gaithersburg, MD) filled with fresh, chilled electrophoresis solution (1 mM Na_2EDTA and 300 mM NaOH, pH > 13). The slides were left in this solution for 10 min to allow DNA unwinding. Electrophoresis was conducted for 15 min at 0.7 V/cm and 300 mA at 4°C using a power supply from Techno Source Pvt. Ltd (Mumbai, India). All the steps from slide preparation onwards were performed under dimmed light to avoid additional DNA damage. Following electrophoresis, Tris buffer (0.4 M Tris pH 7.5) was added dropwise to neutralize excess alkali and this was repeated three times. Slides were then stained with ethidium bromide (20 µg/ml, 75 µl/slide) for 10 min in the dark. They were dipped once in chilled distilled water to remove excess ethidium bromide and subsequently coverslips were placed over them. The slides were stored in a dark, humidified chamber and analysed within 3–4 h.

Slides were analysed using an image analysis system (Kinetic Imaging, Liverpool, UK) attached to a fluorescent microscope (Leica, Germany). The images were captured by CCD camera and transferred to a computer and analysed using Komet 5.0 software. The parameters taken were tail DNA (%), tail length (estimated leading edge from the nucleus; µm) and tail moment (arbitrary units) (Olive *et al*., 1990, 1992). The tail moment is defined as the distance between the centre of mass of the tail and the centre of mass of the head, in micrometres, multiplied by the percentage of DNA in the tail. This number was then compared with the total DNA content. Images from 50 cells (25 from each slide) were analysed.

Statistical analysis

The values were compared using Student's *t*-test. Prior to analysis, homogeneity of variance and normality assumptions concerning the data were tested. Significance was ascribed at $P < 0.05$. Data for the tail moment were statistically analysed and are presented in the figure as box-and-whisker plots, which represent the range (minimum and maximum), medians and 75th percentile.

Results

Cell viability measured at the time of the experiment always exceeded 95% in all the treatment groups. The alterations incorporated in the present study from the conventional Comet assay are shown in Table I.

Drosophila larvae exposed to EMS and to different concentrations of cypermethrin showed a significant increase in DNA damage in the cells of brain ganglia and anterior mid gut (Tables II and III).

In brain ganglia, a dose-dependent increase was observed in all the comet parameters, i.e. tail moment (TM; arbitrary unit), tail length (µm) and tail DNA (%). As is evident from Table II, a statistically significant increase in all parameters was observed in these cells at the three higher concentrations of cypermethrin ($P < 0.001$ at 0.002, 0.2 and 0.5 p.p.m.). TM data are also presented as box-and-whisker plots (Figure 1A) and distribution of cells (%) (Figure 2B). Of the total cells observed, 64% were in the category <2.0 TM at 0.0004 p.p.m. cypermethrin concentration. However, in the highest treatment group (0.5 p.p.m.)

Table I. Comparison between conventional Comet assay technique and the modified version for assessment of *in vivo* genotoxicity in *D.melanogaster*

Experimental condition	Conventional method	Modified method	Comments
Preparation of slides	1.0% LMA (final conc. 0.5%) is usually used	1.5% LMA (final conc. 0.75%) was used	Due to the relatively small cell size, the percentage of LMA was increased. This considerably increased the number of scorable cells
Lysing solution	Lysing solution containing 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris pH 10.0, 10% DMSO and 1% Triton X-100 is usually used	DMSO was removed from the lysing solution; lysing time was restricted to 2 h	DMSO > 0.3% dietary concentration has been reported to be cytotoxic in *Drosophila* (Nazir *et al*., 2003a). Removal of DMSO resulted in the appearance of scorable comets
Unwinding and electrophoresis	Composition: 1 mM EDTA and 300 mM NaOH, pH > 13; unwinding and electrophoresis time is usually ⩾20 min. Electrophoresis is performed at 0.7 V/cm	Composition unchanged; unwinding and electrophoresis time were reduced to 10 and 15 min. Electrophoresis was performed at 0.7 V/cm	No scorable cells were observed when unwinding and electrophoresis times were maintained at 20 min each. When unwinding and electrophoresis time was reduced to 10 and 15 min, respectively, scorable cells with good comets were observed
Neutralization	0.4 M Tris; 3 changes of 5 min each	0.4 M Tris; 3 changes of 5 min each	
Staining	20 µg/ml EtBr	20 µg/ml EtBr	
Scoring	Slides are scored using an image analysis system attached to a fluorescent microscope. The final magnification is ×400	Slides were scored using an image analysis system attached to a fluorescent microscope. The final magnification was ×400	

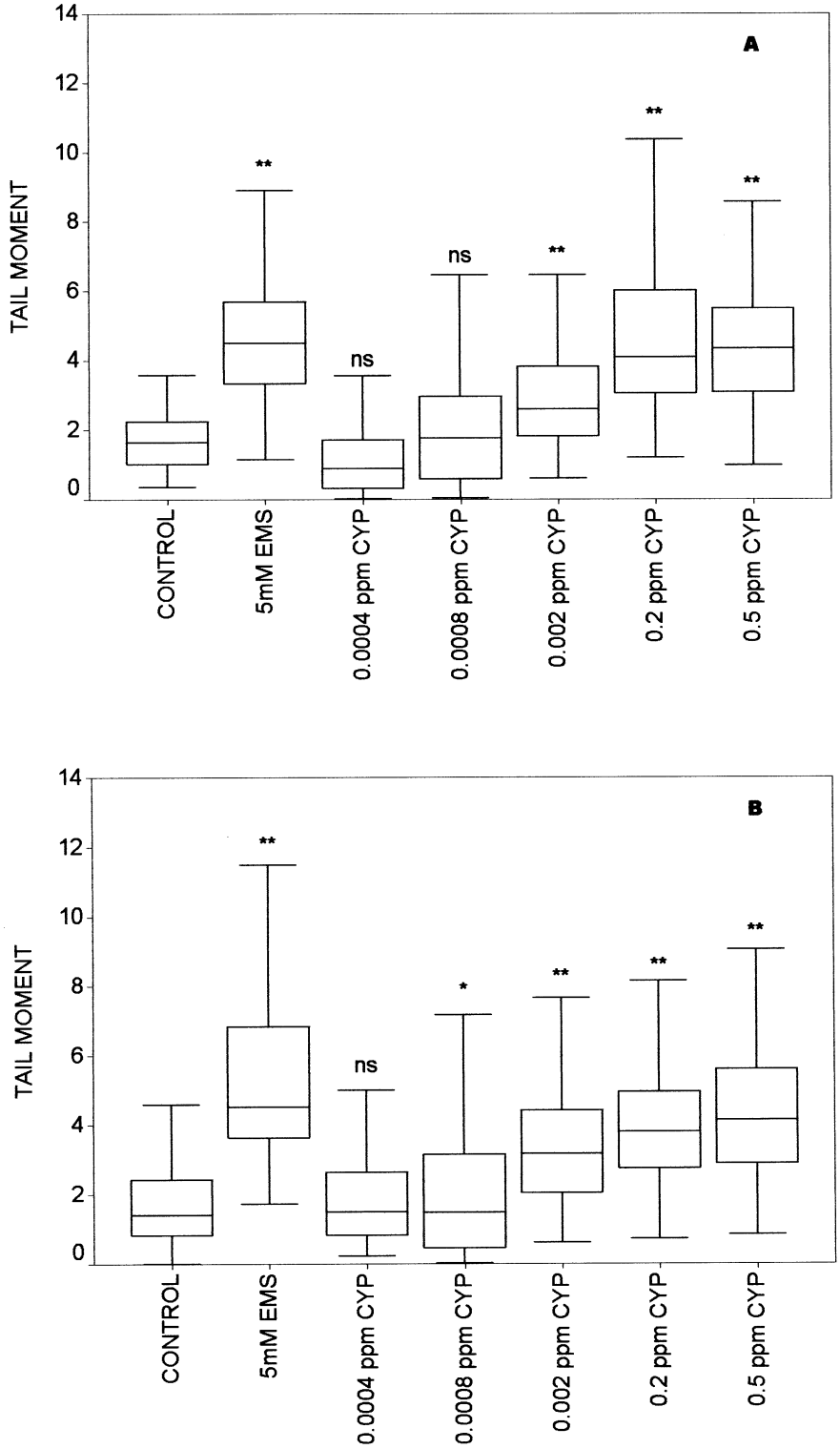

Fig. 1. Effect of cypermethrin (CYP) on the tail moment in brain ganglia (**A**) and anterior mid gut (**B**) of *D.melanogaster*. ns, non-significant; **P* < 0.01; ***P* < 0.001 in comparison with control. EMS, ethyl methanesulphonate.

only 4% cells were in the category <2.0 TM, while 32% were in the category <6.0 TM, reflecting the severity of DNA damage in this group.

A similar dose-dependent increase in DNA damage was also observed in cells of the anterior midgut of larvae exposed to cypermethrin (Table III and Figures 1B and 2B).

Discussion

Issues relating to the experimental procedure, data analysis and presentation of the results have been discussed (Tice *et al*., 2000). However, the Comet assay technique still requires some modification and standardization under different experimental conditions and/or using different experimental materials.

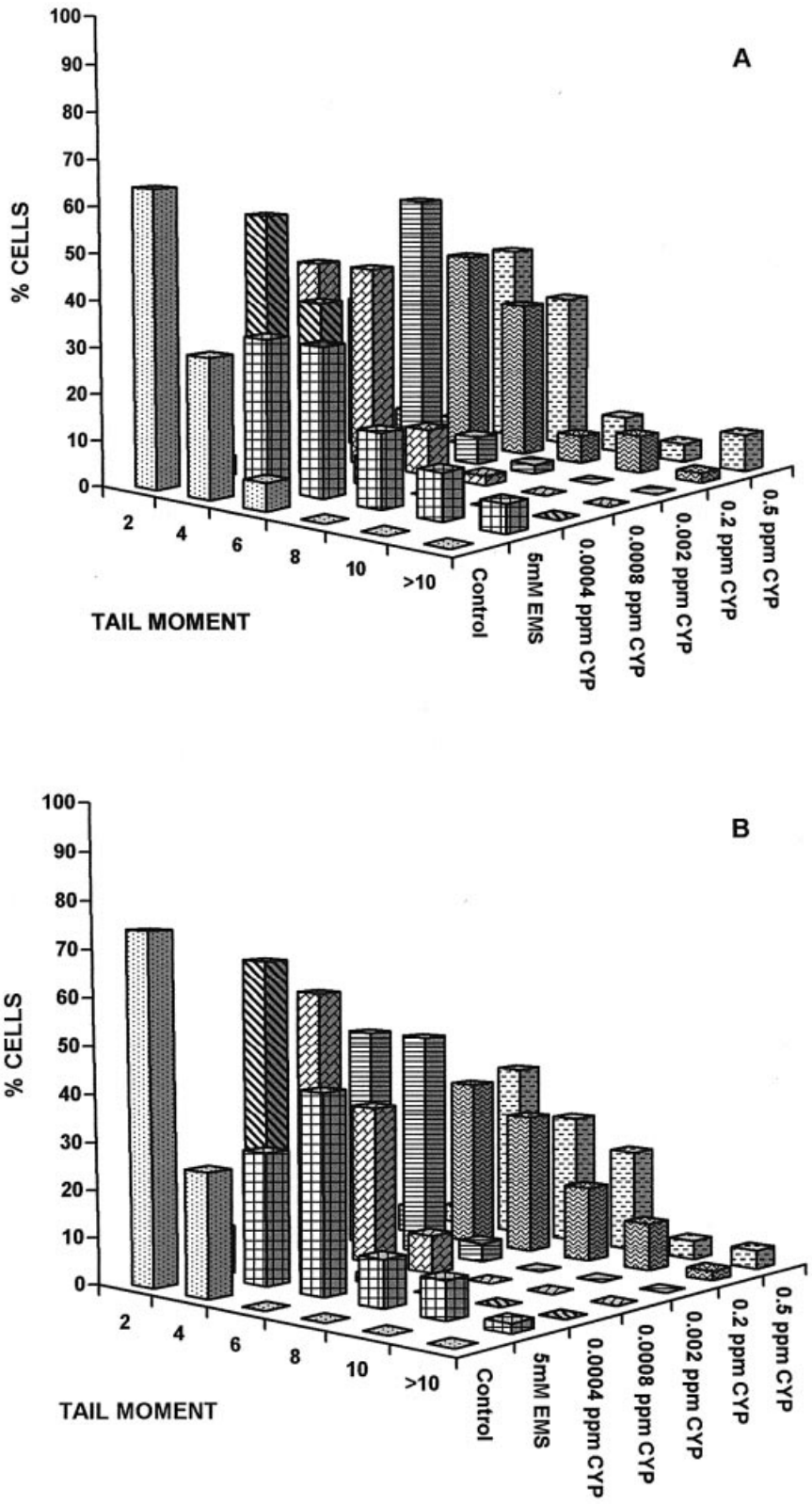

Fig. 2. Effect of cypermethrin (CYP) on the percentage distribution of cells with respect to the tail moment in (**A**) brain ganglia and (**B**) anterior mid gut of *D.melanogaster*. EMS, ethyl methanesulphonate.

In the present study, the Comet assay was applied to evaluate the *in vivo* genotoxic potential of cypermethrin in *D.melanogaster*. Due to the relatively small cell size, low melting point agarose was used at 1.5% (0.75% final concentration), in contrast to the 1% (0.5% final concentration) generally used and recommended (Tice *et al*., 2000; Bajpayee *et al*., 2002; Bilbao *et al*., 2002; Dhawan *et al*., 2002). A major modification made in the present study in the composition of

Table II. Effect of cypermethrin (CYP) on comet parameters in the cells of brain ganglia of *D.melanogaster*

Group	Tail DNA (%)	Tail length (μm)	Tail moment (arbitrary units)
Control	10.2 ± 0.55	11.0 ± 0.52	1.6 ± 0.07
5 mM EMS	23.7 ± 1.48[a]	29.5 ± 1.44[a]	4.5 ± 0.32[a]
0.0004 p.p.m. CYP	10.5 ± 0.58[b]	12.0 ± 0.67[b]	1.7 ± 0.08[b]
0.0008 p.p.m. CYP	11.3 ± 0.65[b]	13.5 ± 0.90[c]	1.8 ± 0.13[b]
0.002 p.p.m. CYP	14.9 ± 0.91[a]	22.5 ± 0.75[a]	2.6 ± 0.18[a]
0.2 p.p.m. CYP	20.2 ± 0.89[a]	33.0 ± 0.78[a]	4.1 ± 0.18[a]
0.5 p.p.m. CYP	21.3 ± 0.91[a]	36.0 ± 0.76[a]	4.3 ± 0.17[a]

Values are median ± SE of three experiments. EMS, ethyl methanesulphonate.
[a]$P < 0.001$ in comparison with control.
[b]Not significant in comparison with control.
[c]$P < 0.05$ in comparison with control.

Table III. Effect of cypermethrin (CYP) on comet parameters in the cells of anterior mid gut of *D.melanogaster*

Group	Tail DNA (%)	Tail length (μm)	Tail moment (arbitrary units)
Control	10.4 ± 0.85	12.5 ± 0.66	1.4 ± 0.12
5 mM EMS	24.7 ± 1.9[a]	31.0 ± 1.52[a]	4.5 ± 0.37[a]
0.0004 p.p.m. CYP	9.9 ± 0.92[b]	13.7 ± 1.06[b]	1.5 ± 0.12[b]
0.0008 p.p.m. CYP	11.5 ± 0.90[b]	14.4 ± 1.12[c]	1.7 ± 0.16[b]
0.002 p.p.m. CYP	17.1 ± 0.93[a]	24.0 ± 0.90[a]	3.2 ± 0.14[a]
0.2 p.p.m. CYP	19.0 ± 0.93[a]	31.0 ± 1.00[a]	3.8 ± 0.17[a]
0.5 p.p.m. CYP	19.6 ± 0.97[a]	35.0 ± 0.97[a]	4.1 ± 0.19[a]

Values are median ± SE of three experiments. EMS, ethyl methanesulphonate.
[a]$P < 0.001$ in comparison with control.
[b]Not significant in comparison with control.
[c]$P < 0.05$ in comparison with control.

the lysing solution as compared with that of Bilbao *et al*. (2002) for *Drosophila* was removing DMSO, which at 10% is usually added to scavenge radicals generated by the iron released from haemoglobin (Singh *et al*., 1988). No such heme groups are present in *Drosophila*. In the present study, no scorable cells could be detected when slides were placed in lysing solution containing DMSO as used conventionally. Our earlier study had shown that a dietary concentration of over 0.3% DMSO was cytotoxic to *D.melanogaster* (Nazir *et al*., 2003a). Although Bilbao *et al*. (2002) in their study used 2 h lysis and 20 min unwinding and electrophoresis of neuroblast cells of *Drosophila*, we optimized the experimental conditions and reduced the times of unwinding and electrophoresis to 10 and 15 min, respectively, resulting in an improvement in performance of the assay.

Cypermethrin, a pyrethroid insecticide, is a neurotoxicant, affecting the central nervous system (Smith and Soderlund, 1998), and has been shown to increase the cGMP content in mammalian brain (Abassy *et al*., 1983; Ruight, 1985). The anterior region of the midgut is one of the first tissues to come into contact with the chemical when the organism takes in the contaminated food. Cypermethrin has been reported to be genotoxic in mouse spleen and bone marrow (Amer *et al*., 1993). In the present study, DNA damage was observed in brain ganglia cells and the anterior midgut of *Drosophila*. Earlier, we reported the vulnerability of these tissues to cypermethrin as reflected by rapid heat shock protein 70 (*hsp70*) induction, an indicator of cytotoxicity (Mukhopadhyay *et al*., 2002b).

The present study shows the usefulness of the modified method for the Comet assay for the evaluation of *in vivo* genotoxicity in *D.melanogaster*. However, further validation of the modified method with further genotoxicants will be needed.

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