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

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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 midgut 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 at even 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 toxicity 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) Ca²⁺- and Mg²⁺-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

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Experiment 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 Poehl’s salt solution (PSS) (Lakhota 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 Na2EDTA and 300 mM Na2EDTA, pH 10) for 2 h. After lysis, the slides were scored using an image analysis system attached to a fluorescent microscope. The final magnification was ×400. 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 midgut (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

<table>
<thead>
<tr>
<th>Experimental condition</th>
<th>Conventional method</th>
<th>Modified method</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preparation of slides</td>
<td>1.0% LMA (final conc. 0.5%) is usually used</td>
<td>1.5% LMA (final conc. 0.75%) was used</td>
<td>Due to the relatively small cell size, the percentage of LMA was increased. This considerably increased the number of scorable cells</td>
</tr>
<tr>
<td>Lysing solution</td>
<td>Lysing solution containing 2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris pH 10, 10% DMSO and 1% Triton X-100 is usually used</td>
<td>DMSO was removed from the lysing solution; lysing time was restricted to 2 h</td>
<td>DMSO &gt; 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</td>
</tr>
<tr>
<td>Unwinding and electrophoresis</td>
<td>Composition: 1 mM EDTA and 300 mM NaOH, pH &gt; 13; unwinding and electrophoresis time is usually ≥20 min. Electrophoresis is performed at 0.7 V/cm</td>
<td>Composition unchanged; unwinding and electrophoresis time were reduced to 10 and 15 min. Electrophoresis was performed at 0.7 V/cm</td>
<td>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</td>
</tr>
<tr>
<td>Neutralization</td>
<td>0.4 M Tris; 3 changes of 5 min each 20 μg/ml EtBr</td>
<td>0.4 M Tris; 3 changes of 5 min each 20 μg/ml EtBr</td>
<td></td>
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<tr>
<td>Staining</td>
<td>Slides are scored using an image analysis system attached to a fluorescent microscope. The final magnification was ×400</td>
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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.
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

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.
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 (Nazar 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, 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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