A modified alkaline Comet assay for in vivo detection of oxidative DNA damage in Drosophila melanogaster

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ABSTRACT

Modifications to the alkaline Comet assay by using lesion-specific endonucleases, such as formamidopyrimidine-DNA glycosylase (FPG) and endonuclease III (ENDOIII, also known as Nth), can detect DNA bases with oxidative damage. This modified assay can be used to assess the genotoxic/carcinogenic potential of environmental chemicals. The goal of this study was to validate the ability of this modified assay to detect oxidative stress-induced genotoxicity in Drosophila melanogaster (Oregon R). In this study, we used three well known chemical oxidative stress inducers: hydrogen peroxide (H₂O₂), cadmium chloride (CdCl₂) and copper sulfate (CuSO₄). Third instar larvae of D. melanogaster were fed various concentrations of the test chemicals (50–200 µM) mixed with a standard Drosophila food for 24h. Alkaline Comet assays with and without the FPG and ENDOIII enzymes were performed with midgut cells that were isolated from the control and treated larvae. Our results show a concentration-dependent increase (p < 0.05–0.001) in the migration of DNA from the treated larvae. ENDOIII treatment detected more oxidative DNA damage (specifically pyrimidine damage) in the H₂O₂ exposed larvae compared to FPG or no enzyme treatment (buffer only). In contrast, FPG treatment detected more oxidative DNA damage (specifically purine damage) in CuSO₄ exposed larvae compared to ENDOIII. Although previously reported to be a potent genotoxic agent, CdCl₂ did not induce more oxidative DNA damage than the other test chemicals. Our results show that the modified alkaline Comet assay can be used to detect oxidative stress-induced DNA damage in D. melanogaster and thus may be applicable for in vivo genotoxic assessments of environmental chemicals.

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1. Introduction

Thousands of diverse chemicals, such as metals, pesticides, additives and dyes are regularly released into the environment because of human activities [1]. Previous studies have shown that exposure to environmental chemicals is associated with reactive oxygen species (ROS) generation, which causes oxidative stress [2] and can generate additional reactive species during cellular metabolism in vivo [3]. These reactive intermediates subsequently generate more free radicals as they interact with various cellular components [4,5]. Thus, an imbalance between ROS generation and the ability of a biological system to remove or detoxify these reactive intermediates results in oxidative stress [6,7].

Previous studies have suggested that oxidative stress can form reactive intermediates that damage proteins, lipids and nucleic acids [8,9]; such reactive intermediates can oxidize or modify the target molecule’s structure and function, which may ultimately lead to tissue damage and apoptosis [10,11].

DNA is considered to be the most biologically significant macro-molecular target of oxidative stress [4]. Damage to DNA by reactive intermediates can induce chemical or structural modifications in the nucleotides and can generate a number of different base oxidation states and modification products. Specifically, oxidation creates abasic (AP) sites in DNA that lead to single and double-strand breaks, which promote mutations [4,12–14]. The collective data suggest that reactive oxygen radicals induce oxidative DNA damage, which may impact disease progression (e.g., carcinogenesis and inflammation) and may affect cancer chemotherapy [15,16]. Steady state oxidative damage to DNA has been suggested to be a predictive marker for cancer development [17,18].

Oxidative DNA damage can be measured to assess the genotoxic/carcinogenic potential of environmental chemicals. A direct assessment of oxidative damage may provide important information about the molecular effects of oxidative stress on DNA [19].
Currently employed methods to measure oxidative DNA damage have certain limitations, such as artifactual DNA oxidation during sample isolation, DNA hydrolysis during high-performance liquid chromatography with electrochemical detection (HPLC–ECD), gas chromatography–mass spectrometry (GC–MS) or HPLC–tandem mass spectrometry analyses (HPLC–MS/MS), as well as DNA oxidation assays with antibodies are only semi-quantitative and thus may produce misleading results [4,20,21]. In contrast, the Comet assay is one of the most promising methods to detect the genotoxic potential of chemicals because it is simple, fast, specific and sensitive. Moreover, the Comet assay only requires small samples and can directly quantify the amount of oxidative DNA damage [22,23]. The Comet assay can be performed under alkaline or neutral conditions to detect and quantify a variety of DNA lesions at the single cell level with in vitro as well as in vivo systems [24–27]. Furthermore, lesion-specific endonucleases, such as formamidopyrimidine DNA-glycosylase (FPG) or endonuclease III (ENDOIII, also known as Nth) can recognize specific oxidatively damaged bases and create additional breaks to aid in the detection of oxidative DNA damage in a modified Comet assay [28]. FPG specifically recognizes the number of oxidized purine bases and other ring-opened purines [28], while ENDOIII recognizes oxidized pyrimidines [29,30]. Earlier in vitro studies suggested that FPG and ENDOIII could be used to assessed oxidative DNA damage induced by environmental chemicals, such as Na2Cr2O7, Pb(CH3COO)2, NiCl2, AlCl3 and SnCl2 [31–34]. Moreover, limited in vivo studies with rats reported that FPG and ENDOIII could detect oxidative DNA damage after exposure to environmental chemicals like permethrin and acrylonitrile [35,36]. Thus, these assays may eventually be applied to determine the detrimental effects of chemical exposure outside the laboratory [37].

Although rodent models are typically used for genetic toxicological studies, there has been a recent push in toxicological research to limit experiments with these higher organisms because of scientific, regulatory and ethical concerns. Therefore, recent biological research has emphasized alternative animal models that follow the principles of the 3R’S: reduction, replacement and refinement [38]. *Drosophila melanogaster* is a model organism for both genetic and developmental biology studies, as well as an established insect model for human disease and toxicological research [27,39,40]. Moreover, the European Centre for the Validation of Alternative Methods (ECVAM) for research and testing has advocated studies in this organism [41].

In this study we describe a modified alkaline Comet assay for in vivo assessment of oxidative DNA damage in *D. melanogaster*. Specifically, we used the DNA repair enzymes FPG and ENDOIII and assessed DNA damage after fly larvae exposure to three well known environmental chemicals: hydrogen peroxide (H2O2) [42], cadmium chloride (CdCl2) [43] and copper sulfate (CuSO4) [44].

2. Materials and methods

2.1. Fly strain

The *D. melanogaster* (Oregon R) flies and larvae were cultured at 24 ± 1 °C with 60% humidity on standard *Drosophila* food containing agar, corn meal, sugar and yeast.

2.2. Chemicals

The endonucleases formamidopyrimidine DNA-glycosylase (FPG, M02405) and endonuclease III (ENDOIII, Nth, M02685) were obtained from New England Biolabs (Hitchin, UK). Analytical grade H2O2 (CAS No. 7722-84-1) was purchased from Qualigens Fine Chemicals (Mumbai, India), CdCl2 (CAS No. 10108-64-2) was purchased from S.D. Fine Chemicals Limited (Mumbai, India) and CuSO4 (CAS No. 7758-99-8) was purchased from HiMedia Laboratories Pvt. Limited (Mumbai, India). The normal and benzo[a]pyrene point agrose, ethidium bromide and collagenase were purchased from Sigma Chemical Co. (St. Louis, MO, USA), and the Ca2+ and Mg2+ free phosphate-buffered saline (PBS) and trypan blue were purchased from HiMedia Laboratories Pvt. Limited (Mumbai, India).

2.3. Treatment schedule

The early third-instar larvae (70 ± 2 h) of Oregon R+ flies were allowed to feed on three different concentrations (50, 100 and 200 μM) of the test chemicals for 24 h. The control larvae received normal *Drosophila* food. All of the experiments were conducted in triplicate.

2.4. Single cell preparation

Single cells from the mid-guts of control and treated larvae were prepared according to the method of Siddique et al. [45]. The rationale for using these cells for the Comet assay was previously described by our lab [27–45]. Briefly, the dissected tissues were treated with collagenase in phosphate-buffered saline (PBS) for 15 min. The samples were subsequently filtered through a 60-μm nylon mesh to obtain single cells.

2.5. Assay procedure

Cytotoxicity was measured with a trypan blue dye exclusion test according to Phillips [46]. The oxidized bases were detected according to the method of Collins et al. [29], while the modified alkaline Comet assay was performed according to Siddique et al. [45]. Three slides in triplicate were made for each treatment: one slide was treated with FPG, one with ENDOIII and one with the enzyme buffer only (control). Briefly, 40 μl of the cell suspension was mixed with 40 μl of 1.5% low melting point agarose (LMA; prepared in Ca2+ Mg2+ free PBS; final concentration 0.75%). For each treatment group, 75 μl of the above mixture was immediately layered on a base slide and covered with a cover slip. The slide was then placed on a chilled plate for 10 min to allow for agarose polymerization. After removing the cover slip, a layer of 0.75% LMA was added, and the mixture was immediately covered with a cover slip and allowed to solidify over a chilled plate. The cover slip was removed and the slide was immersed in freshly prepared, chilled lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris and 1.0% Triton X-100, pH 10) for 2 h. For the enzyme treatment, the slides were removed from the lysis buffer and incubated with enzyme reaction buffer (40 mM Heps, 0.1 M KCI, 0.5 mM EDTA and 0.2 mg/ml BSA, adjusted to pH 8 with KOH) for 5 min. Thereafter, either 75 μl of FPG (1:3000) or ENDOIII (1:3000) was added to the slide and the reaction was covered with a cover slip. The slides were incubated at 37 °C for 45 min (ENDOIII) or 30 min (FPG). The enzyme treatment control slide was incubated with enzyme reaction buffer only. After the enzyme treatment, the cover slips were removed and the slides were placed in a horizontal electrophoresis platform (Life Technologies, Gaithersburg, MD, USA) containing fresh chilled electrophoresis buffer (1 mM Na2EDTA and 300 mM NaOH, pH > 13). The slides were incubated in the platform for 10 min to allow the DNA to unwind. The samples were subsequently electrophoresed at 0.7 V/cm (300 mA/25 V) at 4 °C for 15 min using a power supply from Techno Source Pvt. Ltd. (Mumbai, India). The slides were then washed three times with 0.4 M Tris buffer (pH 7.5) at 4 °C to neutralize the reaction.

The slides were stained with ethidium bromide (20 μg/ml; 75 μl per slide) for 10 min in the dark. After staining, the slides were dipped once in chilled distilled water to remove the excess stain and cover slips were placed over the slides. All of the steps after the single cell preparation were performed under dimmed light to avoid any light-induced DNA damage.

The slides were examined on a Leica DMBL fluorescence microscope (Wetzlar, Germany) and the images were transferred to a computer with a charge coupled device (CCD) camera and analyzed with the Komet 5.0 software (Kinetik Imaging, Liverpool, UK). One hundred and fifty cells from each group (50 cells/slide; 3 slides/experiments; 3 experiments/group) were examined. The three Comet parameters that are generally used to indicate DNA damage are [47]: (i) the tail DNA (TD) (%), which is a measure of the pixel intensity, is expressed as a percentage and indicates the ratio of the DNA present in the tail to the total DNA content; (ii) the tail length (TL) (μm), which is the distance from the nuclear core to the end of DNA migration; and (iii) the tail moment (TM) (arbitrary unit), which is the percentage of tail DNA multiplied by the distance between the centre of mass of the tail and the centre of mass of the head. Because the tail moment parameter does not yield any information in addition to the TD and TL, the TM data are not included in the results section.

2.6. Statistical analysis

The data were compared with an analysis of variance (ANOVA) test with the GraphPad Prism 5 statistical software within and among the treatment groups. A probability (p) less than 0.05 (p < 0.05) was considered statistically significant.

3. Results and discussion

Oxidative damage to DNA, which may ultimately lead to cancer or cell death, is a major lesion that is induced by free radicals. Here we assessed the ability of a modified alkaline Comet assay with lesion-specific endonucleases (FPG and ENDOIII) to detect in vivo
oxidative DNA damage induced by three environmental chemicals. Third-instar larvae of Oregon R° D. melanogaster were assessed because they actively crawl and feed, which means that their midgut tissues are metabolically active [48]. The larvae did not show any overt signs of toxicity during the chemical exposure period.

During the past decade, the alkaline Comet assay has been modified for application to genetic toxicology [45,49,50]. Studies have shown that the procedure can be adjusted for the specific of tissue system studied, as well as the size and the complexity of individual cells [27]. The Drosophila Comet assay has been previously used to assess the in vivo genotoxicity potential of compounds in different cells, such as hemocytes [51,52], gut cells [40,53] and brain cells [45]. Because Drosophila cells are small, we increased the percentage of agarose from 1.0 to 1.5 to make the cell population more intact [45]. In addition, we used a lysis solution without DMSO. DMSO (10%) is usually added to scavenge radicals and prevent radical-induced DNA damage generated by haemoglobin iron release during the lysis of erythrocytes in blood and animal tissue samples [54,55]. However, previous reports have shown that DMSO is not required for the Drosophila Comet procedure and that DMSO is toxic to Drosophila cells at low concentrations [40,51,56,57]. We therefore incorporated these modifications to assess oxidative DNA damage in D. melanogaster larvae that were exposed to various chemicals. There were no indications of cytotoxicity resulting from exposure and the trypan blue exclusion test showed >95% of cells to be negative, in all cases.

Previous studies have shown that H$_2$O$_2$ induces oxidative DNA damage via a Fenton-like reaction [58] that produces a hydroxyl radical (•OH) and results in oxidative DNA damage [42,59]. The class I carcinogen cadmium (Cd) [60] is a well known genotoxic agent that induces DNA strand breaks [61,62]. However, it has been hypothesized that Cd genotoxicity may be due to reactive oxygen free radical generation, and that the resulting oxidative stress is caused by the generation of 7,8-dihydro-8-oxoguanine (8-oxoGua), which is used as a marker of oxidative DNA damage [63,64]. Copper (Cu), which is an essential trace element for humans, induces oxidative damage to biological macromolecules such as DNA, proteins and lipids [65,66]. A few studies have reported that Cu-induced damage results from the reox potential of copper; in presence of the reducing agents, Cu catalyzes the production of ROS, such as superoxide ($O_2^-$), hydrogen peroxide (H$_2$O$_2$) and the hydroxyl radical (OH•) through Haber-Weiss, as well as Fenton reactions [65,67]. Previous studies have reported that •OH-induced DNA breaks are caused by a site-specific Cu-ion reaction both in vitro and in vivo [68,69].

In this study, we exposed Drosophila larvae to various concentrations of H$_2$O$_2$, CdCl$_2$ and CuSO$_4$; the data indicate that there is a concentration-dependent increase in the DNA migration, as is evident by a significant ($p<0.05-0.001$) increase in the Comet the tail length (μm) and tail DNA (%) under three different conditions (with FPG, with ENDOIII or without enzyme). In the H$_2$O$_2$ exposed larvae, there was a concentration-dependent significant ($p<0.05-0.001$) increase in DNA migration in the no enzyme buffer control, FPG and ENDOIII samples. However, a comparatively larger DNA migration was observed with ENDOIII than with FPG or buffer; at 200 μM H$_2$O$_2$, ENDOIII treatment increased the DNA migration 1.6-fold and 2.2-fold compared to FPG and buffer, respectively (Fig. 1A, Table 1). This is consistent with a previous study by Dutheie et al. [58], where increased levels of oxidized pyrimidines were detected in human lymphocyte DNA with the bacterial DNA repair enzyme ENDOIII. Although H$_2$O$_2$ is known to induce numerous strand breaks, the low number of strand breaks observed here with the buffer compared to the FPG and ENDOIII sensitive sites indicates that most of the breaks may have been repaired during the 24 h exposure, whereas the oxidized bases were not repaired during this period.

CdCl$_2$ exposure induced a significant ($p<0.01-0.001$) and concentration-dependent increase in DNA damage in the exposed larvae. FPG treatment yielded maximal DNA migration compared to ENDOIII or buffer. The DNA migration with FPG treatment was 1.6- and 1.9-fold higher in larvae that were exposed to 200 μM CdCl$_2$ compared to ENDOIII and buffer, respectively (Fig. 1B, Table 1). This suggests that CdCl$_2$ predominantly induces oxidation of purines in the exposed organism, because FPG detects oxidized purines, including 7,8-dihydro-8-oxoguanine (8-oxoGua), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FaPyGua) and 4,6-diamino-5-formamidopyrimidine (FaPyAde), as well as other ring-opened purines [70,71].

Similar results were observed with larvae that were exposed to CuSO$_4$, where the FPG DNA migration was 1.5-fold and 2.0-fold higher with 200 μM of CuSO$_4$ compared to ENDOIII and buffer, respectively (Fig. 1C, Table 1). Rodriguez et al. [72] reported that Cu-induced ROS damage primarily targets guanine bases; the increased amount of DNA damage that was observed in the presence of FPG (Fig. 1C) indicates that the metal oxidatively damaged the purines.

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**Fig. 1.** Effects of (A) H$_2$O$_2$, (B) CdCl$_2$ and (C) CuSO$_4$ on tail DNA (%) in gut cells of D. melanogaster. Significance ascribed as *p < 0.05, **p < 0.01 and ***p < 0.001 vs. control as well as $p < 0.05$, $p < 0.01$ and $p < 0.001$ vs. buffer. FPG = formamidopyrimidine DNA-glycosylase; ENDOIII = endonuclease III.
Table 1

Effect of test chemicals on tail length (μm) of DNA in midgut cells of exposed Drosophila larvae after incubation with or without lesion-specific-endonucleases (FPG and ENDIII).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration (μM)</th>
<th>Buffer Median ± SD</th>
<th>+FPG Median ± SD</th>
<th>+ENDIII Median ± SD</th>
<th>F value Interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>7.1 ± 0.4</td>
<td>7.3 ± 0.6</td>
<td>7.5 ± 0.2</td>
<td>14.1</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>50</td>
<td>7.7 ± 0.3</td>
<td>8.9 ± 0.1*</td>
<td>12.5 ± 1.4**</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>8.2 ± 0.3*</td>
<td>10.6 ± 0.5**</td>
<td>13.9 ± 1.2**</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>9.8 ± 1.2</td>
<td>13.1 ± 0.6**</td>
<td>17.4 ± 0.3**</td>
<td></td>
</tr>
<tr>
<td>CdCl₂</td>
<td>50</td>
<td>7.8 ± 0.3</td>
<td>11.5 ± 0.3**</td>
<td>8.7 ± 0.7</td>
<td>8.18</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>8.1 ± 0.3</td>
<td>11.8 ± 0.6**</td>
<td>9.8 ± 0.5**</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>9.4 ± 0.5**</td>
<td>13.1 ± 0.5**</td>
<td>11.5 ± 0.5**</td>
<td></td>
</tr>
<tr>
<td>CuSO₄</td>
<td>50</td>
<td>7.5 ± 0.2</td>
<td>12.1 ± 0.6**</td>
<td>9.5 ± 0.9**</td>
<td>17.1</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>8.1 ± 0.3</td>
<td>13.0 ± 0.4**</td>
<td>10.1 ± 1.0**</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>10.0 ± 0.5</td>
<td>16.1 ± 0.5**</td>
<td>11.9 ± 0.6**</td>
<td></td>
</tr>
</tbody>
</table>

Data represent mean ± S.D. of three experiments (150 cells); Significance ascribed as *p < 0.05, **p < 0.01 and ***p < 0.001 vs. control as well as **p < 0.01 and ***p < 0.001 vs. buffer. FPG = formamidopyrimidine-DNA glycosylase; ENDIII = endonuclease III.

An overall comparison of the DNA damage that was elicited by the three different test chemicals indicates that maximal DNA damage in H₂O₂ exposed organisms was detected with ENDIII treatment, which detects oxidized pyrimidines. In contrast, maximal DNA damage after CuSO₄ exposure was detected with FPG treatment, which detects oxidized purines. Interestingly, although previous studies have reported that cadmium is a potent genotoxin agent, CdCl₂ did not generate a maximal amount of oxidatively damaged DNA, even at the high concentrations that were tested in this study. However, previous studies have suggested that although cadmium can generate DNA strand breaks, it may or may not induce oxidative base modification [73]. Furthermore, Potts et al. [74] reported that cadmium exposure is associated with inhibition of 8-oxoGua-DNA glycosylase and endonuclease III enzyme activity, which may decrease the detection of FPG and ENDIII sensitive sites.

Because FPG and ENDIII can detect apurinic sites (AP sites) and other types of base modifications [75], the use of these enzymes in Comet assays may increase the sensitivity of strand break detection. Furthermore, because most rodent in vivo genotoxicity testing with the Comet assay simply measures strand breaks, this modified Comet assay may be applied to genotoxicity testing to increase the sensitivity of detection in vivo.

Our data described here with Drosophila are comparable to the previously published data with higher organisms [76,77]. Thus, we recommend D. melanogaster as a sensitive and suitable in vivo model for genotoxicity testing in conjunction with a modified alkaline Comet assay that utilizes the FPG and ENDIII enzymes.

Conflict of interest

The authors have no conflicts of interest to declare.

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