Induction and repair of DNA strand breaks and oxidised bases in somatic and spermatogenic cells from the earthworm Eisenia fetida after exposure to ionising radiation

Turid Hertel-Aas*, Deborah Helen Oughton, Alicja Jaworska1 and Gunnar Brunborg2

Department of Plant and Environmental Sciences, Norwegian University of Life Sciences, PO BOX 5003, 1432 Aas, Norway, 1Department of Emergency Preparedness and Environmental Radioactivity, Norwegian Radiation Protection Authority, PO BOX 55, 1332 Østerås, Norway and 2Department of Chemical Toxicology, Division of Environmental Medicine, Norwegian Institute of Public Health, PO BOX 4404 Nydalen, 0403 Oslo, Norway.

To whom correspondence should be addressed. Department of Plant and Environmental Sciences, Norwegian University of Life Sciences, PO BOX 5003, 1432 Aas, Norway. Tel: +47 64 96 55 05; Fax: +47 64 96 60 07; Email: turid.hertel-aas@umb.no

Received on March 2, 2011; revised on June 15, 2011; accepted on July 2, 2011

Methods for analysing oxidised DNA lesions [formamidopyrimidine glycosylase (Fpg)-sensitive sites] in coelomocytes and spermatogenic cells from the earthworm Eisenia fetida using the Fpg-modified comet assay were established. The DNA integrity (SSBs = strand breaks plus alkali labile sites and Fpg-sensitive sites) in cells from E. fetida continuously exposed to 60Co gamma-radiation (dose rates 0.18–43 mGy/h) during two subsequent generations (F0 and F1) were measured and related to effects on reproduction end points which have already been reported. The data suggest a slight increase of Fpg-sensitive sites in spermatogenic cells from worms exposed at 11 mGy/h in the F0 generation but not in F1, whereas reduced reproduction had been observed at dose rates at or >4 mGy/h in F0 and at 11 mGy/h in F1. Using acute X-rays (41.9 Gy/h), dose–response relationships were established for SSBs in coelomocytes and spermatogenic cells exposed in vitro. In vivo DNA repair was studied by measuring the decrease in damage (SSBs and Fpg-sensitive sites) in coelomocytes and spermatogenic cells isolated from worms at different times (0–6 h) after acute X-ray exposure (4 Gy). SSBs were repaired in coelomocytes following biphasic kinetics, i.e. with a fast and a slow half-life (t1/2) of 36 min (95%) and 6.7 h (5%), respectively. Fpg-sensitive sites were repaired at considerably lower rates (t1/2 = 4–5 h). In spermatogenic cells, SSB repair during the first hour was observed but a half-life could not be estimated. Repair of Fpg-sensitive sites could not be determined. In general, a reduced repair of Fpg-sensitive sites suggests a potential for accumulation of oxidised lesions, compared to SSBs, in earthworms exposed to radiation and other environmental contaminants. This is the first study comparing DNA damage with reproduction in earthworms exposed to ionising radiation.

Introduction

Wildlife can be exposed to ionising radiation both from natural and anthropogenic sources. In the majority of cases associated with routine releases of radionuclides, the exposure is chronic and dose rates are generally low (≤100 μGy/h) (1), but after an accidental release, the levels can be significantly elevated. Radiological protection has historically focused on human protection. It is now, however, widely accepted that ecological risks need to be addressed, and the International Commission on Radiation Protection (ICRP) has proposed a new framework in which the concept of reference animals and plants (RAPS) are used to assess the impact of radiation (2). Based on their radioecological properties and their important role in the soil ecosystem, the earthworm has been selected as one of the RAPs. As part of the risk assessment, there is a need for information about the effects of chronic low dose exposure on environmentally relevant end points such as reproduction in many wildlife groups (3,4). Such studies are very costly and time consuming and standard test methods have not been established. As for other environmental stressors, monitoring would benefit from predictive biomarkers for reproduction effects. However, to date, the use of molecular markers in radioecology has not been the subject of careful validation, and it has been proposed that research in the area should include both end points that are relevant for population dynamics as well as molecular markers (5).

The obvious molecular biomarkers for early effects of ionising radiation are various types of DNA lesions. Ionising radiation interacts with DNA either directly by deposition of energy or indirectly through the generation of intermediate reactive oxygen species creating a spectrum of lesions. The initial levels of the lesions in different cells may vary, for example, in relation to the presence of low-molecular-weight scavengers, intracellular oxygen tension (reviewed in ref. 6) and physical protection due to chromatin packaging as with spermatozoa (7,8). Among the lesions induced, double-strand breaks (DSBs) are rare but considered to be of high significance since one to two unrepaired DSBs are lethal while they are mutagenic if misrepaired (9). In comparison with DSBs, single-strand breaks are produced at much higher frequencies but are generally repaired rapidly and mostly error-free. Ionising radiation also produces frequent base lesions of which oxidation of C8 in guanine is particularly important since 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG) is potentially mutagenic leading to G:C to T:A transversion mutations (reviewed in ref. 10). At least in some species and tissues, 8-oxo-dG—which also arises due to normal oxidative metabolism—is efficiently repaired via the base excision repair (BER) pathway. If 8-oxo-dG is induced by ionising radiation, it may be located within a clustered damage site (i.e. two or more elemental lesions formed within 10–20 base pairs); the rate of repair may then be reduced which in turn may increase the mutagenic potential of the lesion (11,12). Clustered damage sites may also lead to the formation of secondary DSBs during repair ((13), reviewed in ref. 14).

Repair of DNA lesions whether induced by ionising radiation or other agents varies between different tissues and
species and rates of repair are lesion specific. As an example, rodent somatic cells seem to repair oxidised lesions [formamidopyrimidine glycosylase (Fpg)-sensitive sites] more rapidly than human cells (15). We recently reported efficient repair of alkylated and other base lesions via BER in germ cells from both rats and humans (16), whereas repair of oxidised purine lesions was efficient in rodent but not in human male germ cells (17) and A.K. Olsen and G. Brunborg, unpublished results). Data for humans strongly support the involvement of oxidised DNA damage in the pathological process of male infertility, and sperm from infertile men contains higher levels of 8-oxo-dG than control individuals (reviewed in ref. 18). Taken together, these variations highlight the importance of understanding oxidised lesion induction and repair in the reproductive system.

The alkaline comet assay measures DNA damage (SSBs = mainly single-strand breaks and alkali labile sites) in single cells. In addition, oxidised purines (primarily 8-oxo-dG) can be detected by including treatment of the agarose embedded nucleoids with a bacterial DNA Fpg extract (19). This way of detecting oxidised DNA damage is considered to be the most reliable and sensitive method currently available (reviewed in ref. 20). Although the comet assay is widely used in ecotoxicology (especially aquatic), the assay has not yet been established as a robust tool for monitoring purposes; this is partly due to the lack of links between comet data and other ecologically relevant end points (reviewed in ref. 21). The alkaline comet assay has previously been applied to detect DNA damage in coelomocytes (immune cells) from earthworms exposed in the laboratory to artificially spiked soils (e.g. heavy metals and pesticides) [e.g. (22–24)] or soils collected from polluted sites (e.g. polycyclic aromatic hydrocarbons, heavy metals, uranium) [e.g. (25,26)]. Two studies compared genotoxic effects in coelomocytes with changes in earthworm reproduction (23,25). The end points had about the same sensitivity following exposure of Eisenia fetida to polluted soil from a former coking plant (25). Glyphosate (GLY) but not chlorpyrifos (CPF) resulted in reduced reproduction in E. fetida, whereas a significant increase in DNA damage only was observed after exposure to CPF (23). Bustos-Obregon and Goicochea (24) reported reduced reproduction and increased DNA damage in spermatogenic cells from E. fetida following exposure to parathion, and to our knowledge, this is the only study in which the comet assay has been applied to this cell type. Genotoxic damage in germ cells can be more directly linked to reproductive effects, and measuring DNA damage in spermatogenic cells could potentially be a better candidate as a biomarker for reproduction toxicity.

This study is part of a project investigating the effects of chronic 60Co γ-irradiation on survival, growth and reproduction end points in the earthworm E. fetida at environmentally relevant dose rates and during two generations (27). We have developed and validated methods for measuring SSBs and Fpg-sensitive sites using the comet assay in coelomocytes and spermatogenic cells, and we here present results from analysis of the chronically exposed worms. The persistence of induced lesions would be expected to depend largely on DNA repair. Little is known about efficiencies of DNA repair in earthworms, and we therefore compared DNA repair in coelomocytes and spermatogenic cells, following acute exposure to X-rays.

**Materials and methods**

**Chronic exposure to γ-irradiation**

*E. fetida* was continuously exposed during two successive generations (F0 and F1) to external γ-irradiation from a 60Co source at dose rates from 0.18 to 43 mGy/h as described in detail by Hertel-Aas *et al.* (27). The reproductive capacity of adult F0 worms (20–23 weeks of age) was registered at specified time intervals for 13 weeks. F1 offspring were irradiated from germ cell stage, and reproduction was registered between 11 and 24 weeks of age. At the termination of exposure at staggered intervals (1–3 days) for replicates, worms were transported from the Co-irradiation facility in small boxes of soil kept on ice to the ‘comet lab’ (35 km away) where isolation of cells for the comet assay was started 1–2 h later. Replicates were hence analysed on different days. If not stated otherwise, coelomocytes and spermatogenic cells were isolated from the same worm to investigate correlations between the levels of DNA damage in the two cell types; such differences could have been masked by inter-individual differences. The dose rates and numbers of F0 and F1 worms used are specified in Table 1. For F0, cells were isolated from worms irradiated at the four highest dose rates (1.7–43 mGy/h); spermatogenic cells from the 43 mGy/h samples were, however, not available since the testicles and seminal vesicles were totally atrophied. At this dose rate, the F0 worms stopped producing offspring after 4 weeks and no F1 individuals were available for analysis. Cells from the F1 4.2 mGy/h worms were not included for comet analysis since they were exposed for an additional 8 weeks to examine the effects of prolonged treatment (27). Not all samples from F0 were successfully analysed due to some comet gels, which could not be scored (see Results and legend to Figure 1).

**Acute exposure to X-rays**

The worms used were sexually mature, 13–15 weeks of age and weighed 0.361 ± 0.056 g obtained from the Norwegian Institute for Agricultural and Environmental Research (Bioforsk) and cultivated as previously described (27). Prior to exposure, worms were acclimatised in artificial Organisation for Economic Co-operation and Development (OECD) soil (pH 6.3, 27% water content) (28) for at least 4 days. For establishment of in vivo dose–response relationships cells from one worm were used, in each of three independent experiments. Cell aliquots were irradiated on ice with 0.5, 1, 2, 3, 6 or 10 Gy X-rays (260 KV, 0.5 mm Cu-filtering, dose rate: 41.9 Gy/h as estimated with Fricke’s solution), followed by comet analysis. In the in vivo repair experiments, worms were transferred to a 3-cm Petri dish containing a moist filter paper and placed on ice/water for 5 min followed by irradiation on ice with 4 Gy X-rays (dose rate 41.9 Gy/h). For determination of initial levels of DNA damage (SSBs), worms were kept on ice after irradiation until cell isolation, whereas the other worms were quickly transferred to 30 g OECD soil at 20.5–22°C to allow repair for 0.5, 1, 3 or 6 h. Irradiation was performed so that cells from all individuals were isolated and analysed for DNA damage at the same time (including a non-irradiated control worm). Three and five independent experiments were performed for coelomocytes and spermatogenic cells, respectively.

**Isolation of coelomocytes**

After chronic exposure to γ-irradiation, coelomocytes were harvested as described by Eyambe *et al.* (29) with minor modifications. Individual worms were rinsed in saline (0.85 g/100 ml deionised water), dried with a paper towel and weighed. Their posterior part was massaged to expel gut contents followed by incubation in 2 ml extrusion medium (98% saline, 2% ethanol, 2.5 mg/ml Na2EDTA and 10 mg/ml guaiacol glycerol ether (Sigma–Aldrich, St Louis, MO, USA); pH 7.3) for 1 h at room temperature (RT). Worms were rinsed in saline, transferred to OECD soil and kept at 9°C until isolation of spermatogenic cells. Extruded cells were transferred to 18 ml ice cold RPMI 1640 medium with 25 mM HEPES and t-glutamine (Lonza, Verviers, Belgium) (=cell medium) plus 10% foetal bovine serum (JBS; PAA laboratories GmbH, Pasching, Austria) and centrifuged for 10 min at 300 × g, 4°C. After one more washing/centrifugation, pelleted cells in ~0.5 ml supernatant were counted, diluted and centrifuged again and resuspended in cell medium.

For rapid isolation of cells in the acute X-ray experiment, coelomocytes were collected using electrical stimulation, minimising the time from irradiation of worms to comet analysis. Slightly modified from (30), worms were placed in 2 ml cell medium, and 4.5 V DC was applied on the cuticle surface for 2 × 5 sec. Excreted coelomocytes were collected on ice, counted and diluted.

**Isolation of cells from seminal vesicles and testicles**

For isolation of spermatogenic cells following chronic γ-irradiation, we used an enzyme-based procedure (24) and Bustos-Obregon, personal communication) with modifications related to for example enzyme incubation temperature and duration and trypsin concentration. In the final procedure, testicles and seminal vesicles were dissected out and transferred to 30 ml cell medium w/pyruvate (0.1 mg/ml) and rt-lactate (0.05%) (both from Sigma–Aldrich), the tissue was minced with scissors, followed by incubation with collagenase (Type 2, final concentration: 100 U/ml, Worthington Biochemical Corporation, Lakehurst, NJ, USA) and hyaluronidase (Type 1-S, H3506, final concentration 300 U/ml; Sigma–Aldrich) for 30 min at 30°C with gentle shaking every 5 min. At 20
min, trypsin (T4665, final concentration 2325 U/ml; Sigma–Aldrich) was added. After 10 min, samples were mixed with cold cell medium plus 10% FBS 1:1 on ice, followed by centrifugation at 900 × g for 7 min and washing/centrifugation twice (900 × g 7 min) with cell medium plus 10% FBS. Finally, cells were resuspended in cell medium and filtered through a 120-μm nylon mesh. Samples were kept ice-cold if not stated otherwise. A fraction of each suspension was fixed in 0.2% paraformaldehyde, for flow cytometric analysis (Argus 100; Skatron Instruments AS, Lier, Norway). Fixed cells were stained with Hoechst 33258, and the distribution of cells in different stages of the spermatogenesis was estimated based on DNA content and light scatter. The

<table>
<thead>
<tr>
<th>Dose rate (mGy/h)</th>
<th>Controls</th>
<th>0.18</th>
<th>1.7</th>
<th>4.2</th>
<th>11</th>
<th>43</th>
</tr>
</thead>
<tbody>
<tr>
<td>F0 generation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absorbed dose (Gy)</td>
<td>—</td>
<td>—</td>
<td>3.6 ± 0.5</td>
<td>8.6 ± 1.3</td>
<td>23 ± 3</td>
<td>85 ± 13</td>
</tr>
<tr>
<td>Weight (g)</td>
<td>0.39 ± 0.06</td>
<td>—</td>
<td>0.38 ± 0.03</td>
<td>0.42 ± 0.03</td>
<td>0.38 ± 0.05</td>
<td>0.46 ± 0.03</td>
</tr>
<tr>
<td>Worms (n)</td>
<td>8</td>
<td>—</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Coelomocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Viability (%)</td>
<td>70 ± 7</td>
<td>—</td>
<td>57 ± 9</td>
<td>64 ± 16</td>
<td>65 ± 7</td>
<td>58 ± 9</td>
</tr>
<tr>
<td>Hedgehogs (%)</td>
<td>4 ± 3</td>
<td>—</td>
<td>2 ± 2</td>
<td>2 ± 2</td>
<td>2 ± 1</td>
<td>3 ± 3</td>
</tr>
<tr>
<td>Spermatogenic cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Viability (%)</td>
<td>76 ± 4</td>
<td>—</td>
<td>71 ± 4</td>
<td>82 ± 6</td>
<td>75 ± 7</td>
<td>—</td>
</tr>
<tr>
<td>Hedgehogs (%)</td>
<td>10 ± 3</td>
<td>—</td>
<td>11 ± 5</td>
<td>7 ± 3</td>
<td>10 ± 4</td>
<td>—</td>
</tr>
<tr>
<td>F1 generation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absorbed dose (Gy)</td>
<td>—</td>
<td>0.73 ± 0.11</td>
<td>7.0 ± 1.1</td>
<td>—</td>
<td>45 ± 7</td>
<td>—</td>
</tr>
<tr>
<td>Worms (n)</td>
<td>6</td>
<td>4</td>
<td>4</td>
<td>—</td>
<td>4</td>
<td>—</td>
</tr>
<tr>
<td>Weight (g)</td>
<td>0.60 ± 0.09</td>
<td>0.56 ± 0.06</td>
<td>0.61 ± 0.04</td>
<td>—</td>
<td>0.69 ± 0.03</td>
<td>—</td>
</tr>
<tr>
<td>Coelomocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Viability (%)</td>
<td>68 ± 7</td>
<td>71 ± 7</td>
<td>72 ± 10</td>
<td>—</td>
<td>76 ± 5</td>
<td>—</td>
</tr>
<tr>
<td>Hedgehogs (%)</td>
<td>3 ± 2</td>
<td>3 ± 2</td>
<td>4 ± 2</td>
<td>—</td>
<td>3 ± 2</td>
<td>—</td>
</tr>
<tr>
<td>Spermatogenic cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Viability (%)</td>
<td>79 ± 4</td>
<td>78 ± 9</td>
<td>82 ± 3</td>
<td>—</td>
<td>85 ± 6</td>
<td>—</td>
</tr>
<tr>
<td>Hedgehogs (%)</td>
<td>4 ± 2</td>
<td>5 ± 3</td>
<td>4 ± 2</td>
<td>—</td>
<td>7 ± 4</td>
<td>—</td>
</tr>
</tbody>
</table>

Table I. Total absorbed doses, numbers and weights of worms and characteristics of cells isolated from F0 and F1 worms continuously exposed to γ-radiation for 13 and 24 weeks, respectively.

Average values ± SD are shown.

Fig. 1. DNA damage in cells from adult F0 worms exposed to γ-radiation for 13 weeks. Lesions were measured as SSBs (−Fpg) or as total damage (SSBs plus Fpg-sensitive sites; +Fpg), in coelomocytes (A) and spermatogenic cells (C) and are given as % tail DNA and number of lesions per 10⁶ bp (left and right axis, respectively). For coelomocytes, SSBs were also measured using the SACA. Each point represents the averaged medians of three technical replicates from one worm, and the curves are through group means. Fpg-sensitive sites (+Fpg minus −Fpg values) in coelomocytes (B) and spermatogenic cells (D) are derived from A and C, respectively. Coelomocytes (+Fpg): control, n = 7; 11 mGy/h, n = 2; 43 mGy/h, n = 5. Coelomocytes (SACA): control, n = 8; 1.8 and 4.2 mGy/h, n = 2; 11 mGy/h, n = 3; 43 mGy/h, n = 5. Spermatogenic cells (+Fpg): Control, n = 8 worms; 1.8 mGy/h, n = 3; 4.2 mGy/h, n = 2; 11 mGy/h, n = 4.
population, designated spermatogenic cells, contained ~7% 2C cells; some of these were vim-tenant staining from the reproductive organs as verified with anti-vimentin staining of the cytoskeleton and fluorescence microscopy.

For rapid isolation of spermatogenic cells in the acute X-ray experiments, a mechanical procedure was used (31). Seminal vesicles and testicles were dissected out and washed, ice-cold Merchant’s buffer (0.14 M NaCl, 1.47 mM KH2PO4, 2.7 mM KCl, 8.1 mM NaH2PO4, and 10 mM Na2EDTA; pH 7.4). The tissue was minced in the same buffer using a plastic piston in a 4.5-mm cylinder with a stainless-steel screen (0.4 mm) attached at the end. The suspension was filtered through two layers of gauze and a 100-μm nylon mesh, followed by centrifugation at 300 × g for 5 min at 4°C. The pellet containing a mixture of nuclei and intact cells was resuspended in Merchant’s buffer.

Viability of the cells, except spermatogenic cells isolated mechanically, was estimated with fluorescence microscopic examination of samples 5 min after staining with propidium iodide (5 mg/ml) and Hoechst 33342 (10 mg/ml).

Comet assay

SSBs and oxidised bases (≡Fpg-sensitive sites) were measured as described (32) with minor modifications. Aliquots of single cell suspensions (~10^6 cells/ml) were carefully mixed 1:10 with 0.75% low melting point agarose (NuSieve: Cambrex, Rockland, ME, USA) in phosphate-buffered saline (without Ca and Mg, 10 mM EDTA) at 34–35°C; 75 μl triplicates were applied to GelBond films (Cambrex) placed on a cold Aluminium plate, using a brass/Teflon mould giving 15-μm circular cells. The films were immediately transferred to lysis buffer (2.5 M NaCl, 10 mM Tris–base, 100 mM Na2EDTA, 1% N-lauroylsarcosine, 10% dimethyl sulfoxide (DMSO) and 1% Triton X-100; pH 10) at 4°C overnight.

For the standard alkaline comet assay (SACA), unwinding in electrophoresis solution (0.3 M NaOH and 1 mM EDTA; pH 13.2) was for 40 min at 4°C followed by electrophoresis (0.8 V/cm on platform) for 30 min at 4°C in fresh solution. After neutralisation in 0.4 M Tris buffer (pH 7.5) for 2–5 min and rinsing in water (1 min), gels were fixed in 96% ethanol for 2 h, dried and stored until scoring. For analysis of Fpg-sensitive sites, two additional steps were incorporated after lysis: (i) films were washed in buffer (40 mM HEPES, 0.1 M KCl and 0.5 mM Na2EDTA; pH 7.6) at 4°C for 1 h; (ii) parallel films were placed in this buffer with bovine serum albumin (0.2 mg/ml), pre-warmed to 37°C, either without or with Fpg-enzyme extract (final concentration, 0.71 μg/ml) for 1 h. For scoring, DNA was stained with SYBR Gold (Invitrogen, Carlsbad, CA, USA) in TE-buffer (10 mM Tris–HCl and 1 mM EDTA; pH 8) at 1:12 500 dilution for 20 min at RT followed by 1-min wash in distilled water. Seventy-five comets per replicate (225 per worm) were scored using a fluorescence microscope (Leica DMLB, ×40 magnification) with a CCD camera and the Komet 4.0 software (Andor Technology, Belfast, UK). The percentage of DNA in the comet-tail (% tail DNA) was chosen as the measure of DNA damage. Cells were selected randomly; ‘hedgehogs’ (cells with small or non-existent head and large diffuse tails containing >90% of the DNA) were not excluded. For the spermatogenic cells, elongated spermatids and spermatozoa, which are easily identified, were not analysed (both have highly compacted chromatin and specific procedures are needed for their analysis in the comet assay (5)). Based on the distribution of % tail DNA, the ploidy specific level of DNA damage could be estimated for the spermatogenic cells. In the Fpg-modified comet assay, Fpg-sensitive sites were calculated by subtracting the median % tail DNA values without Fpg from the median values with Fpg for the same sample. Net induction of DNA damage was calculated by subtracting the background values of control (unexposed) samples.

Comet assay of the acutely exposed samples was performed with some modifications. Electrophoresis was carried out in a different chamber with buffer circulation, 2 × 12.5 = 25 V car batteries as power supply (0.8 V/cm on the platform), and a different microscope (×20 magnification) and scoring system (Comet Assay IV; Perceptive Instruments Ltd, Suffolk, UK). One experiment was scored with both types of software for comparison and there was a good correlation in % tail DNA (slope = 0.97, R = 0.95).

The number of lesions per 10^6 base pair (bp) was calculated as described by Johansson et al. (34) using the slope (% tail DNA per Gy) of cell and experiment-specific linear calibration curves (0–6 Gy) and assuming that 1 Gy (X- or γ-irradiation) induces 0.31 strand breaks/10^6 Da of cellular DNA (35).

Samples of cells from control earthworms were exposed to acute X-irradiation and always included as positive controls for SSBs. To establish a positive control for induction of Fpg-sensitive sites, control cells were mixed with Ro 12-9786 (final concentration 3 μM in 0.25% DMSO; a gift from Dr Elmar Gocke, Roche Ltd, Basel, Switzerland) and after 1 min, the cells were exposed to visible cold light for various times (1.25, 2.5 or 5 min). After exposure, the suspension was diluted (1:1) with cold cell medium plus 10% FBS followed by centrifugation (300 × g; 4°C) and resuspension in cell medium (32). Lesions were induced linearly up to 2.5 min of light exposure with a net median damage level (i.e. minus DMSO vehicle control) of 55% tail DNA. As a routine positive control, light exposure for 1.25 min was used.

Statistical analysis

To evaluate whether there were any differences in weight of worms and viability of cells from the different exposure groups, the data were analysed with analysis of variance (ANOVA) in the General Linear model (GLM) mode of Mintab software (MINITAB® Release 14; Mintab Inc., State College, PA, USA). There is currently no consensus on a standard statistical method for the analysis of comet data (36). In the chronic exposure experiment and the acute repair study, each individual worm was considered as the experimental unit. Median % tail DNA values from each technical replicate were chosen since medians are less sensitive to outliers than means (37). In all experiments, the averaged medians from three technical replicates from each worm were subjected to statistical analysis. The effects of chronic γ-irradiation on DNA integrity were evaluated by comparing all groups using the ANOVA GLM followed by Tukey’s post hoc test. In addition, trend analysis in the form of linear regression was performed (Minitab) and a significant dose–response relationship was indicated by a slope significantly different (P < 0.05) from zero. In cases when assumptions of normality of residuals and/or homogeneity of variance were violated (Anderson Darling test, P < 0.01 and Levene’s test, P < 0.05, respectively), the data was log10-transformed.

To evaluate if there was any difference in the in vitro dose–response after acute X-irradiation between coelomocytes and spermatogenic cells, the slopes of the linear part of the polynomial regression curves from the three independent experiments were compared using the t-test (Minitab). In the repair experiments, one- or two-phase exponential regression lines were fitted to the data using GraphPad Prism (GraphPad Prism 5, GraphPad Software Inc., La Jolla, CA, USA).

Results

Chronic exposure to γ-irradiation

DNA damage (% tail DNA) was analysed in coelomocytes and spermatogenic cells, isolated from F0 worms exposed to γ-irradiation for 13 weeks and from their F1 offspring exposed until 24 weeks. During these exposures, no radiation-induced lethality was observed, but there was a time-dependent decrease in reproduction at the two highest dose rates (27).

The total absorbed doses at the time of sacrifice are shown in Table I. There was no significant difference in the weight of worms (P ≥ 0.082) or the viability of coelomocytes (P ≥ 0.054) and spermatogenic cells (P ≥ 0.24) between any of the groups in either F0 or F1 (Table I).

Flow cytometric analyses of spermatogenic cell suspensions showed mainly haploid cells (42 ± 8% and 41 ± 9% round and elongating spermatids, respectively) plus smaller fractions of diploid (spermatagonia, secondary spermatocytes and somatic cells) and tetraploid (primary spermatocytes) cells (7 ± 2% and 7 ± 3%, respectively) and cells in the S phase (2C–4C) (3 ± 1%). There was no significant difference (P ≥ 0.18) between control and irradiated worms or between the two generations. Although the cell preparation procedure should not be considered quantitative for the cell types, it is worth noting that, at 11 mGy/h, there was a higher fraction of tetraploid cells in one F0 and in two F1 worms. Without elongating spermatids, the flow cytometric distribution was 71 ± 7%, 12 ± 3% and 12 ± 4% for round spermatids, diploid and tetraploid cells, respectively. This was in good accordance with the ploidy of nucleoids as scored in the comet assay.

Levels of SSBs were measured using both the standard (SACA) and the modified (≡Fpg) comet assay of each coelomocyte cell sample from F0 and F1, and spermatogenic cells from F1 (Figures 1A, 2A and C). More SSBs were found with the modified (≡Fpg) assay compared with SACA. Such differences are also found for mammalian cells and probably reflect unspecific cleavage at the elevated incubation temperature (G. Brunborg, unpublished results).

F0 generation

The mean background levels of SSBs and Fpg-sensitive sites in control worms (Figure 1) were not significantly different in
coelomocytes and testicular cells ($P \geq 0.24$), but the inter-individual variation of Fpg-sensitive sites was much higher for the former, resulting in a lower precision of measuring these lesions in coelomocytes [coefficient of variance (CV) = 100%] compared to spermatogenic cells (CV = 40%).

After 13 weeks of adult F0 exposure, low but significant increases were observed for some of the end points (Figure 1). When significant dose–rate response trends were found using linear regression, the goodness of the fit ($R^2$ adj.) was generally low (all regression parameters are shown in Supplementary Table 1, available at Mutagenesis Online). For coelomocytes, SSBs and Fpg-sensitive sites were not significantly different between the groups ($P \geq 0.077$), but there was a weak dose–rate response relationship for SSBs (slope = 1.02, $P = 0.009$, $R^2$ adj. = 28.5%). The total damage level (SSBs + Fpg-sensitive sites) was significantly higher in coelomocytes from worms exposed at 43 mGy/h compared to controls ($P = 0.032$) and 11 mGy/h ($P = 0.029$) and a dose–rate response relationship was indicated (slope: 0.37, $P = 0.01$, $R^2$ adj. = 38%). It should be noted, however, that the ± Fpg coelomocyte samples were successfully analysed only for controls and the two highest dose rates (with $n = 2$ for 11 mGy/h).

With spermatogenic cells (Figure 1C and D), there was no effect of radiation (highest dose rate 11 mGy/h) on SSBs ($P = 0.48$ between groups, and 0.14 for regression). There was, however, a significant increase in Fpg-sensitive sites at 11 mGy/h compared to the controls ($P = 0.024$), and a dose–rate response relationship for these lesions was found (slope: 0.74, $P = 0.005$, $R^2$ adj. = 39%); the inter-individual variation at 11 mGy/h was relatively high (CV = 61%). For spermatogenic cells, the data set is somewhat more complete than for coelomocytes (see legend to Figure 1).

**F1 generation**

For spermatogenic cells from control worms, a significantly lower level of SSBs was detected in F1 (Figure 2C) compared to F0 (Figure 1C), whereas the number of Fpg-sensitive sites (Figure 1D and 2D) was similar ($P = 0.005$ and $P = 0.21$, respectively). Exposure to γ-radiation for 24 weeks did not lead to significant changes in the DNA integrity in coelomocytes when groups were compared ($P \geq 0.126$) although a weak dose–response relationship was indicated for SSBs measured with SACA (slope: 0.21, $P = 0.035$, $R^2$ adj. = 20%). For the spermatogenic cells, no major radiation-induced differences were found, although statistical analysis was complicated by non-normality and heterogeneous variance (Supplementary Table 1 is available at Mutagenesis Online).

The response of the two cell types could not be thoroughly correlated in the same individual as intended, since (i) the male reproductive organs were totally atrophied in F0 worms exposed to 43 mGy/h and (ii) coelomocytes samples were not successfully analysed for all dose rates (Figure 1). In F1, lower damage levels were detected in spermatogenic cells; no consistent correlation patterns between levels in the two cell types were observed.

**Acute exposure to X-rays**

*DNA damage after in vitro exposure of cells.* The viability of coelomocytes prepared using electric simulation for *in vitro* exposure was 84 ± 3%. Figure 3 presents net DNA damage...
measured in the cells after X-irradiation. For both cell types, the dose-dependent increase in the induction of SSBs was linear up to 6 Gy, and the slopes of the linear part of the regressions curves were not significantly different ($P = 0.183$). The spermatogenic cell population comprised 68 ± 6% 1C, 21 ± 4% 2C and 11 ± 4% >2C as analysed in the comet assay. Ploidy specific dose–response curves did not indicate any difference in the induction of SSBs between the cell types (data not shown).

Fpg treatment gave only minor increases in the levels of damage compared to SSBs; no clear dose–response was observed for Fpg-sensitive sites in either coelomocytes or spermatogenic cells (Figure 3). This is in accordance with other observations that Fpg is unable to detect and quantitatively cleave oxidised base lesions in the presence of large numbers of SSBs and/or clustered lesions (discussed in refs 38,39).

### Induction and repair of DNA damage after in vivo exposure.

Worms were irradiated with 4 Gy on ice followed by incubation at RT. This approach was used since cells in their natural environment would be expected to have optimal conditions for repair. In the subsequent isolation of cells for comet analysis (which lasted for 67 or 85 min until lysis, for coelomocytes and spermatogenic cells, respectively), cells were kept ice-cold at all steps to prevent repair. In these experiments, the viability of coelomocytes was 90 ± 5% with no significant difference between controls and irradiated worms ($P \geq 0.81$).

Cell suspensions from control worms were irradiated with 4 Gy and the level of DNA damage was compared with the initial ($t = 0$ h) value as measured in cells from worms which were exposed to the same dose. The number of SSBs in the in vivo samples were ~20% lower for both cell types compared to in vitro, suggesting that some repair did occur during sample processing after irradiation. Frequency distributions of comets in groups with specified levels of DNA damage show clear differences between coelomocytes and spermatogenic cells at various times of repair (Figure 4).

For coelomocytes, curves containing one or two exponential terms could be fitted to the experimental data ($R^2 = 0.90$ for both models) describing the decline in SSBs with time. The half-life ($t_{1/2}$) of SSBs using monophasic repair kinetics was 40 min. For the biphasic model (Figure 5A), 95% of the initial damage underwent rapid repair with $t_{1/2} = 36$ min, whereas $t_{1/2}$ for the slow component was 6.7 h. The uncertainties associated with the calculated parameters were, however, considerable larger for the latter model (Supplementary Table II is available at Mutagenesis Online).

At time zero after in vivo irradiation—unlike in vitro irradiation—the Fpg treatment gave ~44% more DNA damage compared to SSBs alone (Figure 5A). The Fpg-sensitive sites increased slightly after 0.5-h repair incubation (Figure 5B); an increase at short repair times has also been observed for other cell types (15,39), probably reflecting that when a fraction of SSBs has been repaired, the Fpg enzyme is able to detect and cleave the base lesions more quantitatively (39). Curve fitting of the time-dependent data for Fpg-sensitive sites was attempted from time point 0.5 h (maximum level) using various regression models. Linear one- or two-phase exponential decay gave similar half-lives but limited fit ($R^2 \geq 0.50–0.55$). For the exponential models, $t_{1/2}$ was 3.7 h (one-phase) or 5.0 h (major fraction (77%), two-phase); again there are considerable uncertainties (Supplementary Table II available at Mutagenesis Online).

Figure 5C shows time-dependent repair of DNA damage in spermatogenic cells. The samples from different worms showed a relatively high variability in DNA damage initially and during repair. Within each of the five experiments, there was no clear time-dependent decrease in SSBs, and the damage levels did not consistently reach the control levels even after 6 h. A precise half-life could not be estimated by regression analysis using different models. Figure 4B suggests that some cells completely repaired the radiation-induced SSBs within the first hour after irradiation, but afterwards, there was almost no redistribution of cells between the major damage groups.

High inter-individual variation in the measured damage levels could be related to different repair capacities among spermatogenic cells of different ploidy combined with variation in the distributions of such cell types between independently isolated cell suspensions. Analysis of ploidy-specific comet data did, however, not reveal consistent cell type-specific differences in DNA repair, and no consistent reduction in the CVs for 1C, 2C and >2C ploidy-specific comet data was found, when comparing with CV for the mixed cell population (data not shown).

For the Fpg-sensitive sites (Figure 5D), a further analysis of the data gave no clear indication of repair.

---

**Fig. 3.** Dose–response relationship for DNA damage after acute X-ray irradiation of coelomocytes (A) and spermatogenic cells (B). Cells were derived from one worm, and aliquots were irradiated on ice followed by comet analysis. Lesions were measured as SSBs (−Fpg) or as total damage, i.e. SSBs plus Fpg-sensitive sites (+Fpg). Each point represents the averaged medians of three technical replicates in one independent experiment (n = 3). Background levels of SSBs (8 and 5% tail DNA) and total damage levels (15 and 9% tail DNA) in coelomocytes and spermatogenic cells, respectively, have been subtracted. Polynomial regression curves for SSBs (stipulated line) and total damage levels (solid line) are shown.
Discussion

There are few studies using the comet assay to monitor cellular DNA damage in non-human species environmentally exposed to ionising radiation, and none directly linking the measurement of DNA integrity with reproduction end points (26,40). In the present study, we used this assay to measure the DNA integrity of somatic and spermatogenic cells, from earthworms exposed to chronic or acute radiation. DNA lesions were compared to reproduction end points in chronically exposed worms, and Fpg-sensitive sites (oxidised purines) were measured for the first time in *E. fetida*. The background levels of Fpg-sensitive sites ranged from 0.13 to 0.33 and from 0.05 to 0.50.

![Fig. 4. Frequency distribution of comets from coelomocytes (A) and spermatogenic cells (B) in groups with different levels of DNA damage (% tail DNA). Worms were irradiated on ice with 4 Gy X-rays and incubated at RT for 0.5–6 h to allow repair. Cells from all worms (including 0 h and controls) were isolated and subjected to comet analysis. Lesions were measured as SSBs (−Fpg) or as SSBs plus Fpg-sensitive sites (+Fpg). Means ± SD of independent experiments are shown. Coelomocytes (n = 3), spermatogenic cells (n = 5, except at t = 0 and 3 h for which n = 4); 225 cells were scored per worm.](https://academic.oup.com/mutage/article-abstract/26/6/783/1111261)
to 0.13 lesions/10⁶ bp (Figures 1–3 and 5) in control coelomocytes and spermatogenic cells, respectively. These levels are similar to what has been reported for human blood cells (0.68/10⁶ bp) (41) and rat and human testicular cells (0.14 and 0.3–0.4/10⁶ bp, respectively (17). A substantial increase above these levels is tolerated since we observed higher levels of Fpg-sensitive sites in the testis and liver of Ogg1−/− mice, which are fertile and apparently healthy ((42), and A.K. Olsen and G. Brunborg, unpublished results). However, in these studies, the level of oxidised purines in the fertilising sperm is not known.

**Exposure to chronic γ-radiation**

DNA SSBs induced by chronic exposure would be in dynamic equilibrium with their removal, and since their repair is rapid in most cell types, the steady-state level is expected to be very low at moderate dose rates. Correspondingly, we found no or very low increase in the level of SSBs by chronic irradiation in coelomocytes and spermatogenic cells (Figures 1A and C and 2A and C) which is also in accordance with the rapid repair in coelomocytes after acute X-ray exposure (Figure 5A). At the highest dose rate (43 mGy/h), the total level of DNA damage, i.e. SSBs plus Fpg-sensitive sites, was increased in coelomocytes from the F0 worms. These worms had stopped producing viable cocoons after 4 weeks of exposure and their testicles and seminal vesicles were totally atrophied at the time of cell isolation (27). A slight increase was also observed of oxidised lesions in spermatogenic cells from F0 at 11 mGy/h. This was the lowest dose rate causing a pronounced and significant reduction in the hatchability (to ~25%) of cocoons produced during the last 9–13 weeks of exposure (27) although there was an indication of reduced reproduction capacity also at 4.2 mGy/h. For the F0 generation, the comet-analysis should, however, be interpreted with some care—particularly for coelomocytes—since the data set is limited and does not represent all the dose rates employed.

In F1 worms, the reproduction capacity was severely reduced at 11 mGy/h during the whole registration period; the total dose absorbed by these worms was twice as high as in F0 (Table I). This exposure was, however, not accompanied by any apparent increase in oxidised DNA lesions in the spermatogenic cells. The data therefore do not suggest a consistent correlation between the reduced reproduction at 11 mGy/h and the level of oxidised DNA lesions. In general, oxidised lesions may be removed from the cell population not only by DNA repair but also via apoptosis, cell turnover and dilution via cell replication. The apparent different responses in F0 and F1 could reflect variations in such processes, which may be related to age and/or exposure history.

**Induction and repair of DNA damage after acute exposure to X-rays**

Induction of lesions and their post-irradiation removal were studied after exposure to high dose rate X-rays. The rationale

---

**Fig. 5.** Induction and repair of DNA damage in coelomocytes (upper panels) and spermatogenic cells (lower panels) from worms exposed to acute X-rays (4 Gy). Worms and cells are the same as described in legend to Figure 4. Lesions were measured as SSBs (–Fpg) and SSBs plus Fpg-sensitive sites (+Fpg). Net Fpg-sensitive sites (+Fpg minus –Fpg values) in coelomocytes (B) and spermatogenic cells (D) are derived from A and C, respectively. Each point represents the averaged median of three technical replicates from one worm in one experiment after subtraction of background levels (mean values: –Fpg, 1.5 and 3.5; +Fpg, 6.4 and 12% tail DNA for coelomocytes and spermatogenic cells, respectively). The two-phase exponential regression curve (solid) for SSBs is shown in (A); all other lines represent means of the damage levels. Coelomocytes: n = 3; spermatogenic cells: n = 5, except at t = 0 and 3 h for which n = 4. (At t = 0, one untypically low value was removed from data analysis; % tail DNA=14, versus 29–66 in the 4 other experiments (±1.7 standardised SD).
of these experiments was to provide a background for understanding the effects of chronic exposure and also to allow comparison of DNA repair in other species and cell types. Protocols involving rapid isolation of coelomocytes and male germ cells from *E. fetida* were used. The results suggested no significant difference in the *in vitro* induction of SSBs after acute X-ray irradiation in coelomocytes compared to spermatogenic cells or between spermatogenic cells of different ploidy. This is in accordance with the observations for spermatocytes, round spermatids and bone marrow cells from hamster (43).

Oxidised DNA lesions in the form of Fpg-sensitive sites are induced by ionising radiation at approximately the same frequencies as single-strand breaks, although the former lesions are not detected quantitatively directly after acute X-irradiation (Figure 3) as discussed in the Results section. During chronic irradiation SSBs are continuously repaired and it is therefore likely that Fpg-sensitive sites can be quantitatively detected in such exposure situations.

In general, there is little information of DNA repair including their time kinetics in wildlife species (discussed by (21)). To our knowledge, this is the first study of repair kinetics of SSBs and Fpg-sensitive sites in earthworms. Even with careful control of the worms’ temperature, some of the initial SSBs measured right after irradiation (*t* = 0 h) were apparently repaired during cell preparation suggesting that the reported half-lives (Figure 5A) might be longer than in reality. The underlying basis for a fast (*t*<sub>½</sub> = 36 min) and a slow (*t*<sub>½</sub> = 6.7 h) repair kinetics suggested for coelomocytes may be related to the localisation of the damage (in relaxed versus condensed chromatin) (44), or to various types of damage being repaired at different rates (separated single SSBs versus DSBs and/or clustered damage sites) [(11) discussed by (45)]. Similar two-component repair kinetics of radiation-induced SSBs have been observed in cells from a number of other species such as yeast (46), *Drosophila melanogaster* (47), rodents and humans (45). However, the *in vivo* rates of repair in *E. fetida* coelomocytes seem to be lower than in cells from these species [e.g. for *D. melanogaster*, *t*<sub>½</sub> fast/slow: ~5 min/~20 min (47); human peripheral blood mononuclear cells, *t*<sub>½</sub> fast: 6–12 min (45)].

The repair half-life was estimated as 20–30 min by Brash and Hart (48) in rats *in vivo*, at a high dose (50 GY) of X-rays using alkaline sucrose gradients. In plants, slow *in vivo* repair of SSBs was reported for tobacco seedlings and wood small-reed (*Calamagrostis epigejos*), with a half-life of 52 and 101 min, respectively (49,50).

Figure 5B suggests that Fpg-sensitive sites are repaired in coelomocytes, but this occurs at considerably lower rates than for SSBs, which is in accordance with observations for humans and rodents (15,39). Repair of radiation-induced (4 Gy X-rays) Fpg-sensitive sites was faster (*t*<sub>½</sub> = 25 ± 10 min) in various cultivated rodent cells compared to human cells (*t*<sub>½</sub> = 80 ± 20 min) (15), but both species seem to repair these lesions more rapidly than *E. fetida* (*t*<sub>½</sub> = 4–5 h). An accurate estimation of rates of repair of Fpg-sensitive sites is, however, complicated by the inefficient detection of oxidised lesions at time zero, and considerable uncertainties are associated with the given half-life. Biphasic repair kinetics were observed for the rodent and human cell lines (15), whereas in the present study, limited fits were achieved using exponential mono- or biphasic models.

It is known that the ability to repair certain DNA lesions decreases as the spermatogenesis proceeds with DNA chromatin becoming highly compacted together with reduced cytoplasm in the sperm [e.g. (43)]. In human and rodent spermatocytes and round spermatids, but not in mature elongated spermatids, SSBs are repaired at the same rates (*t*<sub>½</sub> = 10 min) as in several somatic cell types (43). Our study indicates repair of SSBs in crude spermatogenic cells during the first hour following acute X-ray exposure, but at later times, the average damage level seems to plateau (Figure 5C). High inter-individual variability was observed and no half-life could be estimated. Ploidy-specific comet data did not reveal significant differences in repair capacity among the different stages. The data gave no clear indication of repair of Fpg-sensitive sites in spermatogenic cells after acute exposure to X-rays (Figure 5D). As discussed above, there is probably a varying ability to repair oxidised lesions in spermatogenic cells between species (17), and a possible lack of such repair in the earthworm should therefore be considered; there is, however, no data available on activities or expression of DNA repair proteins in germ or somatic cells in these species. On the other hand, the low levels of Fpg-sensitive sites in spermatogenic cells arising spontaneously and after chronic irradiation would suggest considerable repair and/or apoptosis.

In general, the usefulness of the modified comet assay to detect DNA damage (SSBs and oxidised lesions) as environmental biomarkers will depend on various factors such as the background levels and the inter-individual variability in control animals, the sensitivity of the end point to exposure, and—most importantly—correlation with ecologically relevant end points. The comet assay as used in the present study seems to be less sensitive compared to reproduction end points since we were able to demonstrate reduced reproduction at dose rates which did not lead to detectable increases in DNA damage. The lowest dose rate used (0.18 mGy/h, F1) is within the same order of magnitude as dose rates estimated for soil invertebrates at sites heavily contaminated by uranium mining (51) or radioactive waste (52). The lowest dose rate inducing severe effects on reproduction (11 mGy/h) and significant increase in Fpg-sensitive sites in spermatogenic cells in FO is high compared to most environmental situations but might be encountered after serious accidents such as Chernobyl (53). The usefulness of the comet assay for earthworms in biomonitoring should not, however, be evaluated based on effects of ionising radiation alone. The lesions measured by the standard and Fpg-modified comet assay are not specific for ionising radiation and can be induced by a range of environmental contaminants. Significant increases in DNA damage (SSBs) in coelomocytes, as measured with the alkaline comet assay, have been reported for *Eisenia andrei* after exposure (1–56 days) to soil from an uranium mine highly contaminated with heavy metals and radionuclides (e.g. 234U, 234Th, 230Th, 226Ra) (26). Some metals are known to inhibit DNA repair enzymes (54), which also highlight possible synergy between effects of radiation and chemical contaminants. Further evaluations should involve combined exposures, whereas the quick cell isolation methods described (i.e. electrical stimulation and a refined mechanical procedure) could potentially increase assay sensitivity. The present results underline the need for investigation of the time-dependent change in the DNA damage response after increasing times of exposure, both within and between generations since the steady state levels of damage might change with time as protective mechanisms might be induced.

In conclusion, the exposure set-up which we used for chronic irradiation seems very useful for studying the effects of environmental exposures. Combined with the comet assay, it
allowed us to investigate possible accumulation of oxidised DNA lesions that are likely to accumulate in nature, in particular in species with low capacity for repair of such lesions. This is the first report of radiation-induced oxidised lesions in somatic and spermatogenic cells of earthworms. The persistence of DNA lesions in the worms could be directly compared with their reproductive capacities, which were described in our previous report (27).

Supplementary data
Supplementary Tables I and II are available at Mutagenesis Online.

Funding
ERICA project (Contract No: FI6R-CT-2004-508847); European Commission under the Euratom Research and Training Program on Nuclear Energy within the Sixth Framework Program (2002-2006); PROTECT project (Contract No: FI6R-2006-036425); Norwegian Radiation Protection Authority.

Acknowledgements
We thank Richard Wiger for characterisation of the spermatogenic cell suspensions by flow cytometry and anti-vimentin staining, and Dr Elmar Gocke (Roche, Switzerland) for supplying the Ro 12-9786 compound.

Conflict of interest statement: None declared.

References